Impaired Neuromuscular Transmission: Electrophysiological Investigations in Mouse and Human Muscles.

A Thesis Presented By

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October, 1989

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ABSTRACT

In this thesis, some aspects of impaired neuromuscular transmission have been investigated by electrophysiological means. Myasthenia gravis (MG) is an autoimmune muscle weakness disorder, caused by antibodies (Abs) directed against the acetylcholine receptor (AChR). However, in 10-15% of patients who show clinical symptoms of MG, there is no detectable anti-AChR antibody as shown by routine radioimmunoassays ("antibody-negative" patients). It is possible, however, that Abs may be present against sites other than the AChR in the latter patients. To investigate this, mice were injected with plasma (or the immunoglobulin G (IgG) fraction) from 7 patients, and intracellular recordings made from the mouse diaphragm muscles. Each plasma/IgG studied produced a significant reduction in miniature end-plate potential (mepp) amplitude. This did not appear to arise by a postsynaptic action since α-bungarotoxin binding and/or ACh sensitivity were unaffected. Also, analysis of voltage noise during ACh exposure showed no effect on single channel properties for those plasmas investigated. Furthermore, of the 7 plasmas studied, 4 gave significant reductions in end-plate potential (epp) quantal content. The results suggest an autoimmune basis of "antibody-negative" MG, with antibodies apparantly acting presynaptically, perhaps by interfering with vesicle packaging.

Experiments were also performed to investigate the actions of anti-AChR Abs present in 85-90% of patients with MG. The effects of acute applications of the Ig fraction on biopsied human muscle were studied. Mepp amplitude was significantly reduced as compared to controls by the MG Ig. Thus acute blocking actions of anti-AChR Abs may be important in the pathogenesis of normal MG.

Finally, possible modulation of neuromuscular transmission by the Ca^{2+} channel agonist CGP 28392 (2μM) was studied. This compound had no significant

effect on epp quantal content, on mepp frequency in high potassium (K^+) solutions or on mepp amplitude. Thus this agent had neither pre nor postsynaptic actions at this concentration. More specifically, since CGP 28392 is an agonist at L-type Ca^{2+} channels, L-type Ca^{2+} channels appear to play no major role in acetylcholine (ACh) release at the neuromuscular junction.

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ACKNOWLEDGEMENTS

I would like to express my sincere thanks to my supervisor, Dr. Dennis W.-Wray. I am indebted to him for all his helpful advice, constant teaching, encouragement and valuable support throughout the course of this project. I thank Professor Newsom-Davis and Dr. Angela Vincent for supplying the plasma and IgG from the myasthenia gravis patients, for essential clinical information concerning antibody-negative patients and for guidance in biochemical techniques.

I am grateful for the help and support from the other members of the Academic Department of Pharmacology and Neurology at the Royal Free Hospital School of Medicine.

I thank Action Research for the Crippled Child for support.

Finally, many thanks to Christine Beadle, for her careful and patient typing of this manuscript.

Chapter 1

GENERAL INTRODUCTION

Section I : Myasthenia Gravis and Antibody-Negative Myasthenia Gravis

Myasthenia gravis (MG) is a disorder of neuromuscular transmission in which patients show muscle weakness due to the loss of function of acetylcholine receptors (AChR) induced by anti-AChR antibodies (Vincent, 1980; Drachman, 1983). However in 10-15% of patients with the clinical symptoms of MG, anti-AChR antibody is undetectable ("antibody-negative" MG) (Vincent & Newsom-Davis, 1985; Mossman, Vincent & Newsom-Davis, 1986).

The initial symptoms, found on clinical examination, for most MG patients is referable to muscle weakness in the extraocular muscles: ptosis, diplopia and blurring of vision (Grob, Arsura, Brunner & Namba, 1987). In 13-15% of patients, the clinical symptoms of MG are restricted to the extraocular muscles throughout the course of the disease ("localized-ocular" MG) (Grob et al., 1987). Such patients with localised ocular MG often have abnormal neuromuscular transmission in peripheral muscles but impairment is not sufficient to lead to muscle weakness in these muscles. Most MG patients, however, have "generalized" MG in which facial muscle weakness is accompanied by development of muscle weakness in the proximal muscles of the limbs and trunk.

Anticholinesterase drugs enhance neuromuscular transmission by prolonging the action of acetylcholine (ACh) following inhibition of acetylcholinesterase. Edrophonium has a short duration of action and is thus used as a diagnostic test; a single test-dose causing a dramatic improvement in the muscle strength. Anticholinesterases with a more prolonged duration of action, such as pyridostigmine, have been used for longer term treatment (Genkins, Kornfeld, Papatestas, Bender & Matta, 1987). However their use is now generally restricted to the treatment of patients with mainly ocular MG or with mild, non-progressive ocular and extremity weakness (Johns, 1987).

Another common diagnostic test is to measure the compound muscle action potential (CMAP) produced either by single nerve stimulus or by regular repetitive nerve stimulation. Following a single maximal stimulus to the motor nerve, the evoked CMAP is reduced in amplitude (Lambert & Elmqvist, 1971). A decrement in CMAP occurs when repetitive stimulation is between 1 and 5Hz (Sanders, 1987). Such decrement occurs in most, but not all MG patients. In one study of 80 patients with clearly diagnosed MG, 95% of MG patients showed decrement, exceptions occurring in 2 "pure ocular" and 2 "moderate generalized" MG patients (5%) (Özdemir & Young, 1976).

Using intracellular microelectrode recording techniques from biopsied intercostal human MG muscle there is an observed reduction in the amplitude of miniature end-plate potentials (mepps) compared to normal muscle (Dahlbäck, Elmqvist, Johns, Radner & Thesleff, 1961; Elmqvist, Hofmann, Kugelberg & Quastel, 1964). The extent of this reduction is often to between 20 - 30% of the normal values (Elmqvist et al., 1964; Lindstrom & Lambert, 1978). The postsynaptic sensitivity to iontophoretically applied ACh is markedly reduced in MG (Albuquerque, Rash, Mayer & Satterfield, 1976b; Cull-Candy, Miledi & Trautmann, 1978; Ito, Miledi, Newsom-Davis, & Vincent, 1978b; Cull-Candy, Miledi & Trautmann, 1979). Further indication of the nature of this disorder comes from studies using α-bungarotoxin (α-BuTx) which binds irreversibly to the AChR. Binding of the toxin is reduced in MG indicating a postsynaptic disorder due to AChR loss (Fambrough, Drachman & Satyamurti, 1973; Engel, Lindstrom, Lambert & Lennon, 1977b). Furthermore, there is a correlation between the extent of mepp amplitude reduction and α-BuTx binding (Ito et al., 1978b).

It was initially thought that the impairment in MG was due to a reduction in quantal size (i.e. the number of ACh molecules in a packet; Elmqvist et al., 1964). However, the above studies clearly point to a postsynaptic reduction. Furthermore, electron microscope studies of the ultrastructure of the MG neuromuscular junction

have revealed a number of features suggesting an impairment in the postsynaptic membrane rather than a presynaptic defect (Albuquerque et al., 1976b; Santa, Engel & Lambert, 1972). The end-plate often has an immature appearance, the postsynaptic region is simplified and the average area of the postsynaptic folds and clefts per nerve terminal are reduced (Engel & Santa, 1971). Moreover, the abnormally widened synaptic clefts often contain globular and membranous material and the cross-sectional length of the postsynaptic membrane is reduced. However, the presynaptic locus is only slightly altered; there is a normal number of synaptic vesicles and their diameter is also unchanged. These abnormalities resemble those seen at developing neuromuscular junctions and may represent simultaneous processes of degeneration and repair. Sometimes the nerve terminal in juxtaposition to the postsynaptic region is reduced or absent, often causing spread of AChRs on the postsynaptic membrane (Bender, Ringel, Engel, Daniels & Vogel, 1975).

Light microscope investigations following cholinesterase staining show an elongation of the end-plate length, defined as the number of adjacent sarcomeres (Ito, Miledi, Molenaar, Newsom-Davis, Polak & Vincent, 1978a). From other studies (Cöers, 1975) using staining of the unmyelinated nerve terminal with methylene blue, two main classes of end-plates in MG have been suggested: (i) dysplastic or elongated end-plates, a primary impairment by the autoimmune mechanisms and (ii) end-plates with distal branching of motor axons resulting in expanded arborisations, possibly a secondary compensation for the transmission disorder. A further feature, usually seen in elderly MG patients, is an increase in the number of muscle fibres innervated by a given nerve which suggests a denervation process.

Further indications that MG is not due to an impairment in the presynaptic membrane is that the quantal content (i.e. the number of packets of acetylcholine released per nerve impulse) in MG muscle is the same as that for control muscle (Elmqvist et al., 1964; Lambert & Elmqvist, 1971; Lindstrom & Lambert, 1978)

although in some cases the quantal content has been seen to be slightly elevated (Cull-Candy et al., 1978: Cull-Candy, Miledi, Trautmann & Uchitel, 1980). This appears to correlate with the biochemical studies showing an increase in ACh content in MG muscles compared to controls (Ito, Miledi, Molenaar, Vincent, Polak, van Gelder & Newsom-Davis, 1976). The exact mechanisms underlying these observations are not clear although it is possible that this change in quantal content acts as a "compensatory" mechanism for the reduced postsynaptic sensitivity.

The postsynaptic reduction in sensitivity together with normal release leads to reduced end-plate potential (epp) amplitudes, which are often below threshold for triggering muscle action potentials (see Figure 3; Kim, 1982). One study reported that recordings from myasthenic patient's muscles resulted in epps large enough to trigger action potentials in about 25% of the surface fibers tested (Albuquerque et al., 1976b). Thus, action potentials are not triggered in many fibres, compound muscle action potentials are reduced and muscle weakness results. The disorder is at the neuromuscular junction and does not involve impaired nerve or muscle action potential conduction (Engel, Tsujihata, Lambert, Lindstrom & Lennon, 1976).

Repetitive nerve stimulation causes a progressive reduction in the number of packets of ACh released per nerve impulse for both normal and MG muscles. The epp amplitude therefore decreases throughout the course of the train of nerve stimuli falling to below threshold for triggering action potentials due to the postsynaptic impairment (Lambert & Elmqvist, 1971; Albuquerque et al., 1976b). Thus fewer fibres progressively fire action potentials leading to the observed decrement in compound muscle action potentials seen in electromyographic recordings (see above).

The postsynaptic impairment is caused, for most patients, by autoantibodies directed against the AChR (Appel, Almon & Levy, 1975; Bender et al., 1975; Lindstrom, Engel, Seybold, Lennon & Lambert, 1976; Lefvert, Bergström, Matell,

Osterman & Pirskanen, 1978). Such antibodies have been shown to be localized and bound at the postsynaptic membrane (Lindstrom et al., 1976; Engel, Lambert & Howard, 1977a). Indeed, for any one patient, during the course of their disease, there appears to be a correlation between the severity of the disorder and the anti-AChR antibody (Ab) titre (Newsom-Davis, Pinching, Vincent & Wilson, 1978). So, for instance, following thymectomy, a treatment often used for MG patients, the antibody titres fall and there is an improvement in the clinical status of the patient (Vincent, Newsom-Davis, Newton & Beck, 1983). Furthermore, when the disease is restricted to the ocular muscles throughout the course of the disease, the anti-AChR antibodies titres are significantly lower than when the disease becomes of a more generalized nature (Lindstrom et al., 1976, Compston, Vincent, Newsom-Davis & Batchelor, 1980; Vincent & Newsom-Davis, 1982).

However, between different patients, there is often a poor correlation between anti-AChR Ab titre and clinical severity of weakness (Lindstrom, Seybold, Lennon, Whittingham & Drake, 1976). It has also been investigated whether the clinical severity correlates with the presence of antibodies specifically blocking α-BuTx binding. However, there was also a poor correlation between blocking antibody titres and clinical changes in the degree of muscle weakness (Besinger, Toyka, Hömberg, Heininger, Hohlfeld & Fateh-Moghadam, 1983) suggesting that the estimation of antibodies directed against the α-BuTx binding site is of limited value when anti-AChR Abs do not reflect severity of symptoms. These observations are thought to occur because the anti-AChR antibodies in sera from different patients with MG have heterogeneous specificities (Lindstrom, Campbell & Nave, 1978). Thus antibody specificity appears to be the most important determinant of the effect on AChR activity rather than the anti-AChR titre.

Other evidence suggesting that MG has an autoimmune basis is also available. The most clear evidence is the passive transfer of the electrophysiological and biochemical features of MG from man to mouse by injection into mice of the MG

IgG fraction (Toyka, Drachman, Pestronk & Kao, 1975; Toyka, Drachman, Griffin, Pestronk, Winkelstein, Fischbeck & Kao, 1977; Toyka, Birnberger, Anzil, Schlegel, Besinger & Struppler, 1978). Thus mepp amplitudes and the number of AChRs at the mouse neuromuscular junction were reduced by anti-AChR antibody injection. Also in support of the autoimmune basis of the disorder, plasma exchange (Pinching, Peters & Newsom-Davis, 1976) has been shown to produce a marked, although temporary, improvement in many MG patients, associated with a fall in anti-AChR titres (Newsom-Davis et al., 1978). Furthermore, immunosuppressive drugs are also frequently successful in the treatment of MG (Dau, 1981). Drugs used include corticosteroids such as prednisolone and cytotoxic immunosuppressive agents such as azathioprine (Matell, 1987); the improvement with azathioprine is correlated with the reduction in the amount of anti-AChR antibody. Further, MG often associates with other autoimmune disorders such as Hashimoto's thyroiditis, lupus erythematosus, rheumatoid arthritis, etc (Simpson, 1960). Moreover, some babies, born to a mother with MG, have transient neonatal MG (Lindstrom et al., 1976; Keesey, Lindstrom, Cokely & Herrmann, 1977). This is presumably due to the transfer of an anti-AChR antibody from the mother to the child. Finally, evidence for an autoimmune basis may be provided by a possible involvement of the thymus gland. The thymus is thought to influence the immune response since it is the site of production and maturation of T-cells which control antibody production and are involved in the development of immunological tolerance. Many studies have reported the benefits of thymectomy in patients with myasthenia gravis and one study demonstrated that thymectomy as primary therapy provided improvement in more than 90% of patients (Olanow, Wechsler, Sirotkin-Roses, Stajich & Roses, 1987).

The main mechanism for loss of AChR by anti-AChR antibodies in most, though not all cases (Wilson, Vincent & Newsom-Davis, 1983), is by an increased rate of degradation of AChRs (Heinemann, Bevan, Kullberg, Lindstrom & Rice,

1977; Kao & Drachman, 1977). This depends on the ability of antibodies to cross-link receptors on the surface of the postsynaptic membrane due to the divalent structure of the Ab (Drachman, Angus, Adams, Michelson & Hoffman, 1978). By contrast, the monovalent fragment of the anti-AChR has no effect on the degradation of AChRs although it has been shown to bind to them. The cross-linking leads to clustering of the receptors (Lennon, 1978) which are then rapidly endocytosed and degraded. This effect of antibodies has been demonstrated both on human and rat muscle cell cultures and at the neuromuscular junction in mice passively injected with MG IgG (Appel, Anwyl, McAdams & Elias, 1977; Bevan, Kullberg & Heinemann, 1977; Kao & Drachman, 1977; Stanley & Drachman, 1978; Drachman, Adams, Josifek & Self, 1982). Furthermore, freeze-fracture electron-microscope studies of biopsied MG muscle shows tightly packed clusters of AChRs instead of the normal dispersed pattern over the muscle cell (Pumplin & Drachman, 1983).

Another process for loss of AChRs which can be important is antibody-dependent complement-mediated lysis of the postsynaptic membrane (Toyka et al., 1977). The third component of complement, C3, has been shown to be present on the postsynaptic membrane in acquired myasthenia gravis in man (Engel et al., 1977a) and in experimental autoimmune myasthenia gravis (EAMG) (Sahashi, Engel, Lindstrom, Lambert & Lennon, 1978; Engel, Sakakibara, Sahashi, Lindstrom, Lambert & Lennon, 1979). In the latter case, animals are immunised with highly purified acetylcholine receptor protein from, for example, eel electroplax together with complete Freund adjuvant and this results in an experimental model of MG in the animals. The localization of C3 at the MG end-plate is consistent with the assumption that antibodies fix complement. The ninth component of complement, C9, has been identified on the postsynaptic membrane in most MG patients and on degenerated folds and fragments released from the membrane into the synaptic cleft (Sahashi, Engel, Lambert & Howard, 1980). Complement components such as C9 are responsible for lysis of the postsynaptic membrane.

Another mechanism for antibody action is direct pharmacological block of receptor function by antibody. However, this mechanism does not seem to occur commonly since in vitro application of MG sera or IgG to adult end-plates does not usually impair AChR sensitivity or channel function (Albuquerque, Lebeda, Appel, Almon, Kauffman, Mayer, Narahashi & Yeh, 1976a; Shibuya, Mori & Nakazawa, 1978; Ito et al., 1978a, Peper, Sterz & Bradley, 1981; Lerrick, Wray, Vincent & Newsom-Davis, 1983; Dolly, Gwilt, Lacey, Newsom-Davis, Vincent, Whiting & W.-Wray, 1988). In contrast to a lack of effect at junctional/adult end-plate receptors, MG antibodies may have more widespread acute blocking actions at extrajunctional/embryonic AChRs (Anywl, Appel & Narahashi, 1977; Bevan et al., 1977; Schuetze, Vicini & Hall, 1985; Hall Pizzighella, Gu, Vicini & Schuetze, 1987).

However, although "antibody-negative" MG does not appear, at first sight, to be autoimmune initial evidence is accumulating which suggests that it does in fact have an autoimmune basis. Antibody-negative patients appear to fall into two broad categories. There are those patients with "short history" antibody-negative MG. In these patients anti-AChR Abs only become detectable in the serum after the first few months of the clinical illness (Newsom-Davis, Willcox, Schluep, Harcourt, Vincent, Mossman, Wray & Burges, 1987). It is likely, therefore, that in the early stages Ab is bound to the AChR and is not detectable in the plasma. For the other category of patients, serum anti-AChR Ab is persistently undetectable during the course of the disease although they respond to immunosuppressive treatment and plasma exchange. Antibody-negative MG patients appear to form a heterogeneous group and differences in conclusions about the underlying mechanism are probably explained by this fact.

Some MG patients appear, in fact, to have poorly detectable or undetectable (but nonetheless present) levels of anti-AChR Ab as assayed by standard immunoprecipitation and thus patients with antibody-negative MG may merely

represent one end of the spectrum of myasthenia gravis (Nicholson, 1986, Drachman, Silva, Ramsay & Pestronk 1987a). Muscle biopsies from 7 such patients showed AChR numbers that were significantly reduced compared to controls. Moreover daily intraperitoneal injections for 12-14 days of immunoglobulin (20mg Ig per day) from such antibody-negative patients to mice caused significant reductions in mepp amplitudes and significant reductions in the number of AChRs at the neuromuscular junction compared with control mice injected with normal sera (Drachman, Silva, Ramsay & Pestronk, 1987b). Thus the pathogenesis of this disorder for certain patients appears to be similar to MG and, although difficult to detect, immunoglobulin directed against the AChR is most likely to be the immune mechanism effector. However, Ig from the same patients did not accelerate AChR degradation nor block α-bungarotoxin binding of mammalian muscle cultures (Drachman et al., 1987b). This contrasts with observations using MG sera with detectable antibody where blockade of AChRs and accelerated degradation of receptors has been seen (Drachman et al., 1982). Thus the Ab present in antibody-negative MG patients must differ in some way from that of the majority of MG patients with anti-AChR Ab.

Alternative reasons for antibody-negative MG, assuming the presence of anti-AChR Abs, include: (i) Abs directed specifically against the α-BuTx binding site. Patients with such Abs would appear antibody-negative since the standard immunoprecipitation does not detect these Abs; (ii) Ab bound to the end-plate with an undetectable level of free circulating Ab; (iii) immune complexes formed between the Ab and the assay antigen so that the antigen binding site of the antibody was occluded. One or more of these possibilities may exist in antibody-negative MG.

In agreement with the experiment described by Drachman et al. above, mice injected with the IgG fraction from another group of antibody-negative patients, had impaired neuromuscular transmission (Mossman et al., 1986); there was an increased sensitivity of the mouse muscle to tubocurarine and a depression of twitch tension

during 3Hz trains of nerve stimulation. However, although there was a slight reduction in the number of AChRs in these mice, it would not be sufficient to account for the depression in twitch amplitude. Thus, in contrast to the above conclusion, the myasthenic disorder in antibody-negative patients may be due, for some cases at least, to antibodies directed against a determinant at the neuromuscular junction other than the AChR.

It appears that 5-25% of antibody-negative patients have generalized MG, with weakness in respiratory or bulbar muscles, and the remainder have ocular MG (Newsom-Davis et al., 1987; Solvien, Lange, Penn, Younger, Jaretzki, Lovelace & Rowland, 1988). Moreover, it is unlikely that such antibody-negative patients have congenital MG for a number of reasons; (i) the age of onset in antibody-negative MG is always more than 2 years in contrast to congenital MG which is seen usually before 2 years; (ii) there is a fluctuation in the severity of the disease in antibody-negative patients which contrasts with congenital MG where the severity tends to be stable; (iii) there is a degree of asymmetry in the muscle weakness of the eyelids in antibody-negative MG which is absent from the congenital form; (iv) the response to plasma exchange and immunosuppressive treatment seen in antibody-negative MG is not seen in cases of congenital MG; (v) there is no family history of cousin marriages in antibody-negative MG patients which usually explains congenital MG.

There are no significant differences in the clinical characteristics of patients with or without serum antibodies to AChR. There are conflicting reports concerning thymectomy: some report that the outcome of thymectomy in antibody-negative patients is the same as for MG patients (Solvien et al., 1988) whereas others have found no response to thymectomy (Newsom-Davis, personal communication). All antibody-negative patients have a positive response to edrophonium and a decremental response to repetitive nerve stimulation, although the results from one study showed a less frequent occurrence of decrement in antibody-negative patients

compared to MG patients with anti-AChR Ab (Solvien et al., 1988). No systematic morphological studies have been made in antibody-negative patients. Finally, 20% of antibody-negative MG patients have associated autoimmune diseases which compares well with 22% of MG patients with anti-AChR Ab (Solvien et al., 1988). Interestingly, moreover, one antibody-negative patient with diaphragm muscle weakness transferred a similar pattern of pronounced transient weakness in the diaphragm of her new born son, again suggesting a circulating factor is involved in this disorder (Newsom-Davis et al., 1987).

In this thesis, passive transfer studies were made of antibody-negative MG (Chapter 3). Plasma and/or IgG from 7 such patients was injected into mice; mepps, epps, action potentials and noise were recorded and α-BuTx binding studies were carried out. These experiments were designed to investigate whether there is an autoimmune basis for antibody-negative MG, and to study the site of action of autoantibodies, if involved, and whether they are directed against a site other than the AChR.

In Chapter 4, experiments were carried out using sera from MG patients known to have anti-AChR antibodies. The object was to investigate the importance and extent of acute pharmacological block of the AChR by anti-AChR antibodies. The MG plasmas were applied acutely to human muscles, and mepp amplitude and α-BuTx binding measured.

Previous reports of some of the data presented in this thesis have appeared in:- Newsom-Davis, Mossman, Vincent, Burges & Wray, 1986; Burges, Newsom-Davis, Vincent & W.-Wray, 1987a,b; Newsom-Davis, Willcox, Schluep, Harcourt, Vincent, Mossman, Wray & Burges, 1987).

Section II : Calcium Channel Agonists and Antagonists

Transmitter release at the neuromuscular junction depends on the entry of calcium (Ca^{2+}) into the nerve terminal. Although there are several types of neuronal Ca^{2+} channels (e.g. Nowycky, Fox & Tsien, 1985), those directly involved in the release of ACh at the neuromuscular junction have not yet been clearly characterised.

Organic agents such as the dihydropyridines (DHP's) act selectively at "L-type" Ca^{2+} channels in many tissues (see C. Peers, 1988 for summary). At least two other types of voltage-dependent Ca^{2+} channel, "N-type" and "T-type" have also been found to co-exist in the same neuronal (or other) membrane. Using whole cell and single channel patch clamp techniques (Hamill, Marty, Neher, Sakmann & Sigworth, 1981) the properties of these channels have been characterized and are summarized in Table 1.

From a weakly negative potential (for example, - 40mV), L-type channels can be activated by voltage steps (to for example 0mV) while N- and T-type channels remain inactivated. From a more negative holding potential (for example, - 80mV) all 3 types of channel are activated by large depolarizing steps (to for example, +10mV). From single channel studies, L-type channels continue to open and close throughout the depolarizing step, producing a sustained overall average current. N- and T-type channels open preferentially at the start of the depolarizing step, such that averaged single channel records show transient overall currents. L-type channels have a larger single channel conductance than N- and T-type channels when Ba^{2+} is the charge carrier. L- and N-type channels are sensitive to block by Cd^{2+} ions; L-type channels are selectively modulated by dihydropyrines while L- and N-type channels are sensitive to ω-Conotoxin (ω-CgTx).

Table 1

Ca^{2+} Channel Characteristics

Channel Type	Holding Potential Required for Removal of Inactivation	Size of Depolarizing Step for Activation (to test potential in brackets)	Whole Cell Current Characteristics	Single Channel Conductance (110mM Ba^{2+}) (ps)	Sensitivity to Block by Cd^{2+}	Responsiveness to Dihydro-pyridine	Sensitivity to W-CgTx
L	-40mV	Large (>-10mV)	Sustained	25	Strong	Yes	Yes
N	-80mV	Large (>+10mV)	Transient	13	Strong	No	Yes
T	-80mV	Small (>-50mV)	Transient	8	Weak	No	No

Legend for Table 1

This table shows the characteristics of Ca^{2+} channel types.

Dihydropyridines are a group of highly lipid soluble compounds and, as modifiers of L-type Ca^{2+} channels, can be divided into two groups; antagonists which reduce Ca^{2+} channel currents (e.g. nitrendipine, nimodipine; Lee & Tsien, 1983; Hess, Lansman & Tsien, 1984) or agonists which enhance Ca^{2+} channel currents (e.g. CGP 28392, BAY K 8644; Brown, Kunze & Yatani, 1984; Becham, Hebisch & Schramm, 1988). The development of Ca^{2+} antagonists has become important in clinical use for combating hypertension, arrhythmias and angina (Bailie & Kay, 1989).

The DHP's are thought to preferentially favour one or more states or "modes" of the Ca^{2+} channel rather than by blocking the channel (Reuter, Porzig, Kokubun & Prod'hom, 1985). Their high lipid solubility makes it likely that the drugs have access to the individual channels through the lipid face of the membrane. This class of drugs may be bound to the channel at or near to the interface between the channel and lipid, interfering with the channel gating (Kokubun & Reuter, 1984). Agonists interact with the channel to cause an increase in opening probability (Brown et al., 1984) without changing the single channel conductance or the open time distributions; "mode 2" state of the channel is favoured rather than the normal state of the channel, "mode 1" (Hess et al., 1984). Antagonists, by contrast prevent channel openings by stabilizing the channel in an inactivated state ("mode 0" state, Hess et al., 1984). Although the main action of nitrendipine is an antagonist it has been shown (Hess et al., 1984) that there are in fact both agonist and antagonist actions at low concentrations; thus there is a slight enhancement of mode 2 behaviour as well as the predominant mode 0. Furthermore, the agonists BAY K 8644 and CGP 28392 also show antagonist actions at higher concentrations (favouring mode 0) in addition to the predominant agonist action (favouring mode 2).

The effect of DHP's has been shown to be practically reversible, at least in the heart (Bean, 1984) and in addition, especially the effect of Ca^{2+} antagonist DHP's has been shown to be voltage-dependent (Bean, 1984; Reuter et al., 1985). Nitrendipine blocks cardiac calcium channels when the holding potential is held at a sufficiently depolarized level (around -10mV) to inactivate most of the channels. When the channels are not inactivated, i.e. for cells held at a more negative holding potential (-80mV), there is a decrease in the blocking potency of nitrendipine by as much as 1000 fold. Thus, nitrendipine appears to bind preferentially to the inactivated Ca^{2+} channel state rather than the resting state.

The selective nature of DHP's for L-type Ca^{2+} channels has led to them being a useful tool as high affinity radioligands for purifying and characterizing the L-type Ca^{2+} channels at the molecular level. The L-type Ca^{2+} channel ("DHP receptor protein") has been studied mainly from skeletal muscle T-tubules which are a rich source of such receptors (Borsotto, Barhanin, Norman & Lazdunski, 1984). However, L-type Ca^{2+} channels have also been purified from chick heart (Hosey, Chang, O'Callaham & Ptasienski, 1989) and the properties of this channel peptide compared with those purified from skeletal muscle. It has been shown that the two L-type Ca^{2+} channels are structurally and immunologically distinct even though there is a shared sensitivity to dihydropyridines (Rosenberg, Hess, Reeves, Smilowitz & Tsien, 1986).

The purified dihydropyridine-sensitive channel protein from rabbit transverse tubule membranes appears to have five subunits: α_1 (175kDa), α_2 (143kDa), β (54kDa), γ (30kDa) and δ (24- to 27- KDa) (Takahashi, Seagar, Jones, Reber & Catterall, 1987). The α_2 and δ subunits are glycosylated components but α_1 is not, implying that α_1 and the disulfide-linked $\alpha_2\delta$ complex are distinct, independent polypeptides. The α_1 subunit has those properties which identify it as the Ca^{2+} channel protein; these include a dihydropyridine binding site, at least one phosphorylation site which is cAMP-dependent and an extensive hydrophobic

domain. Evidence suggesting that the α_1 subunit is the channel has been derived from two lines of evidence. The primary structure of the α_1 subunit from rabbit skeletal muscle has been cloned, its hydropathicity profile determined and photoaffinity labelling with a DHP shown specific incorporation of the ligand into the α_1 subunit (Tanabe, Takeshima, Mikami, Flockerzi, Takahashi, Kangawa, Kojima, Matsuo, Hirose & Numa, 1987). Also following incorporation of the α_1 subunit into lipid bilayers the subunit is reconstituted to form a functional Ca^{2+} channel with a conductance of 20pS (Flockerzi, Oeken, Hofmann, Pelzer, Cavalié & Trautwein, 1986). Moreover, the channel has the pharmacological, biochemical and regulatory properties of the L-type Ca^{2+} channel seen in situ.

DHP's bind to a site on the α_1 subunit which is distinct from but interacts with two other sites which bind the other classes of Ca^{2+} antagonist, phenylalkylamines e.g. verapamil and benzothiazepines e.g. diltiazem (Freedman & Miller, 1984; Glossman, Ferry & Goll, 1984). Another agent which binds to the L-type Ca^{2+} channel and which has been used in the further characterisation of Ca^{2+} channels is ω-CgTx. This is a 27 amino acid polypeptide isolated from the venom of the marine snail Conus geographus and has also been chemically synthesised (Kerr & Yoshikami, 1984; Olivera, McIntosh, Cruz, Luque & Gray, 1984). Both N- and L-type Ca^{2+} channels are blocked by the toxin (McClesky, Fox, Feldmand, Cruz, Olivera, Tsien & Yoshikami, 1987). Transmitter release at the frog neuromuscular junction has been shown to be irreversibly blocked by ω-CgTx and its synthetic counterpart (Kerr & Yoshikami, 1984; Enomoto, Sano, Shibuya & Maeno, 1986; Koyano, Abe, Nishiuchi & Sakakibara, 1987).

Various attempts have been made to characterise presynaptic voltage-dependent Ca^{2+} channels involved in transmitter release at nerve terminals (for reviews see Tsien, 1983; Miller, 1987). Single channel patch recordings from nerve terminals are generally not possible, and so identification of the channels is dependent on less direct evidence. Organic Ca^{2+} channel antagonists have been

shown to be without effect on transmitter release at concentrations which usually block Ca^{2+} channels. Mepps, recorded from frog sartorius nerve-muscle preparations depolarized with high potassium (K^+) solutions, are unaffected with respect to frequency by either verapamil or D-600 (a methoxy derivative of verapamil) (Nachshen & Blaustein, 1979). Also, verapamil (100μM) and D600 (≅50μM) have no effect on the quantal content of the epp in frog skeletal muscle during repetitive nerve stimulation in the presence of tubocurarine (Gotgil'f & Magazanik, 1977). Higher concentrations of these drugs, however, do have non-specific intracellular effects, mepp amplitude being reduced and mepp frequency increased (Gotgil'f & Magazanik, 1977; Publicover & Duncan, 1979; Bregestovski, Miledi & Parker, 1980). At mouse motor nerve terminals in the presence of potassium channel blockers, however, a prolonged Ca^{2+}-dependent current can be recorded by an electrode placed inside the perineural sheath of nerve bundles and this is sensitive to block by verapamil and diltiazem (Penner & Dreyer, 1986). This indicates the presence of a presynaptic L-type Ca^{2+} channel. The origin of the current through this channel is, however, difficult to interpret and its physiological role remains to be elucidated. Moreover, such Ca^{2+} currents were not blocked by ω-CgTx as would have been expected if L-type channels had been involved (Anderson & Harvey, 1987).

The role of L-type Ca^{2+} channels in release at the neuromuscular junction therefore appears to be confused, and in this thesis experiments have been carried out using an agonist (CGP 28392) as well as an antagonist (nitrendipine) (Chapter 5). Effects of these DHP's on release evoked by high K^+ solutions and by nerve stimulation were studied. For this, mepps and epps were recorded from mouse diaphragm muscles during the application of these agents.

Previous reports of some of the data presented in this thesis have appeared in:- Burges & W.-Wray, 1989 .

Chapter 2

EXPERIMENTAL METHODS

Section I : Clinical Details

(a) Patients in Chapter 3

The clinical details of the patients whose plasmas and immunoglobulin G (IgG) were used in the studies in Chapter 3 are summarized in Table 2. All the patients showed the classical symptoms of normal myasthenia gravis and none were immunosuppressed at the time the plasma was taken. The patients ranged in clinical severity from grade IIB (moderately, generalized) through grade III (acute, severe generalized) to grade IV (chronic severe) according to the Osserman classification (Osserman & Genkins, 1971). In Table 2, the first seven patients with undetectable anti-AChR antibody titres are antibody-negative MG patients, while patient SP is an MG patient characteristic of those with anti-AChR antibody. Except for MA and MO for whom the thymic pathology was not determined, all the other antibody-negative MG patients had an involuted thymus. In other studies, patients with low anti-AChR Ab titres were found mainly to have an involuted thymus (Berrih-Aknin, Morel, Raimond, Safar, Gaud, Binet, Levasseur & Bach, 1987). Patient SP had a thymoma, defined as a neoplasia of the thymic epithelial cells which form a major portion of the thymus. Control plasma was pooled from patients without any neuromuscular disease.

(b) Patients in Chapter 4

Plasma was obtained from four myasthenia gravis patients undergoing plasma exchange (Table 3). The crude immunoglobulin (Ig) fraction extracted from plasma ML was prepared by Z. Hall using the ammonium sulphate method (Schuetze et al., 1985). ML Ig contained Ab against AChR and, more specifically, blocked the binding of α-BuTx (Table 3). The Ig from patient ML formed the major part of this study and the three other plasmas were used for comparison. Plasma CH and BQ were selected because they contained high titres of Abs directed against

Table 2

Clinical Details of Patients Used in Chapter 3

Patient	Sex	Age at Onset (years)	Osserman Grade	Thymic Pathology	Anti-AChR Ab Titre (nM)
KP	F	16	III	Involuted	<0.2
MA	M	30	IIB	Not Determined	<0.2
LW	F	19	III	Involuted	<0.2
KO	M	22	IIB	Involuted	<0.2
MO	F	25	IIB	Not Determined	<0.2
MI	M	44	IIB	Involuted	<0.2
CA	F	28	III	Involuted	<0.2
SP	F	30	IV	Thymoma	25.5

Legend for Table 2

This table shows clinical details of those antibody-negative MG patients used in this study and the one MG patient, with anti-AChR Ab. Patients were of both sexes, 5 female (F) and 3 male (M). The Osserman grade is defined in Osserman & Genkins, 1971. Antibody titres are in nMoles of ^{125}I-α-BuTx-AChR precipitated per litre of serum.

Table 3

Myasthenia Gravis Plasma Preparations Used in Chapter 4

Preparation	Sex of Patient From Which Preparation Taken	Anti-AChR Antibody Titre (nmoles/l) (a)	Anti-α-BuTx Binding Site Antibody Titre (nmoles/l) (b)
ML Ig	F	1.6	4.8
CH Plasma	F	13.5	<0.5
BQ Plasma	F	14.4	<0.5
MW Plasma	M	<0.2	<0.5
Control Plasma	Pooled	<0.2	<0.5

Legend for Table 3

This table shows values of antibody titre for the ML Ig and the other plasmas obtained by immunoprecipitation of ^{125}I-α-BuTx-AChR from pooled extracts of normal and denervated muscles (a) and by inhibition of ^{125}I-α-BuTx binding to pooled extracts (b).

sites on the AChR other than the α-BuTx site; thus they contained high titres of Abs against AChRs already labelled with α-BuTx, while not inhibiting toxin binding. Finally, for comparison, plasma MW was from an antibody-negative MG patient.

Section II : Extraction of IgG from Plasma

Specifically for studies in Chapter 3, 200-300mls of control plasma or patient KO's plasma (obtained at plasma exchange) was filtered and mixed for 2 hours at room temperature with a solution of Rivanol (3%) in distilled water and a few drops of octanol. Following filtration, NaCl was added to give 5% w/v of the total volume, left overnight at 4°C and filtered. Solid ammonium sulphate was added to give 50% w/v and left for 2 hours at room temperature. The mixture was centrifuged for 45 minutes at 15,000rpm using a "Europa 24" centrifuge, the supernatant discarded and the pellet re-dissolved in a volume of distilled water equivalent to the original volume of plasma. Following 2 hours at room temperature, the mixture was again spun for 45 mins at 15,000rpm, the supernatant discarded and the pellet re-dissolved in as small an amount of water as possible. This was then dialysed for 3 days against freshly made Hartmans solution (4 litres). The dialysate was then spun for 1 hour at 15,000rpm and then sterilised by millipore filtration. The IgG content of the final filtrate was measured by Giles Elrington by an ELISA method using β-galactosidase conjugated anti-human IgG. It was found to be 15mg/ml and 18mg/ml for patient KO and control plasma respectively. Control plasma was diluted with Hartmans solution to 15mg/ml making it the same concentration as that for the patient's IgG. The filtrates were then stored at -20°C until required for use.

When IgG was used for 3 day injections (Patients KP, LW and CA) extraction of IgG was performed by A. Vincent.

Section III : Treatments

(a) Of Mice

Mice (BKTO strain, 20-32g) were injected intraperitoneally (i.p.) with millipore sterilised plasma or IgG. Except when the mice were injected for only 3 days, mice were given cyclophosphamide (i.p. 300mg/kg) on day one to suppress their immune response to the injected plasma or IgG. The exact protocol for injection was varied depending on the duration of the injections. Mice were given either: (i) 3 times daily injections of 1ml plasma or IgG for 3 days; (ii) daily injections of 1.5ml plasma or 15mg IgG in 1.0ml for 7 days or (iii) daily injections of 1.5ml plasma for 15 days. For antibody-negative patients the IgG content of the plasma used in the injection of mice was assayed by the ELISA method (A. Vincent). Values (mg/ml) for the following patients were: KP, 5.8; MA, 4.7; LW, 2.7; KO, 7.3; MO, 7.5 and MI, 4.5. Unfortunately no assay was performed on SP plasma or control plasma although the normal range for the control plasma used in this thesis is 12-15mg IgG/ml of plasma (B. Lang, personal communication).

Mice were killed by cervical dislocation on day 4, 8 or 16 respectively and the hemi-diaphragm with its phrenic nerve dissected out.

(b) Of Human Muscle

Human intercostal muscles were removed from patients undergoing thoracotomy (often for carcinoma of the lung but without neuromuscular disease) and immediately placed in previously oxygenated Krebs solution. The muscles were dissected out in well oxygenated Krebs solution into at least four smaller bundles with intact fibres (i.e. tendon insertions at each end). One or two muscle bundles were pinned out via their tendons, into wells cut into petri dishes filled with dental wax. The muscles were then covered by 200µl of either control or MG plasma or ML Ig (prepared by Z. Hall) and the petri dish was placed in a moist, oxygenated

atmosphere at room temperature for three hours. This was achieved by standing the petri dish on top of a pad of absorbent paper in 1cm of distilled water in a beaker. The beaker was sealed with parafilm and the water bubbled with 95% O_2/5% CO_2.

Section IV : Solutions Used for Electrophysiological Recordings

In all experiments the phrenic nerve/hemidiaphragm preparation or human muscle bundles were continuously perfused with well oxygenated (95% O_2/5% CO_2) Krebs solution. This medium was normally of the composition (mM): NaCl, 118; KCl, 4.7; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $NaHCO_3$, 25.0; $CaCl_2$, 2.52; glucose 11.1.

Solutions were freshly made up for each experiment using deionized water, Analar salts and 1M stock solution $CaCl_2$ (BDH chemicals). Where stated, in the text, tubocurarine was present in normal Krebs solution while recording epps to prevent muscle contraction upon nerve stimulation. Muscles were exposed to increasing doses of tubocurarine until a dose was reached which was just sufficient to block contraction (0.86-7.00μM); this approach maximised the epp signal.

(a) Low Calcium Solutions

For some recordings of end-plate potential, instead of using tubocurarine the Ca^{2+} concentration was reduced to ≤ 0.24mM to prevent muscle contraction. No changes in Na^+ concentration were made to compensate for these small changes in Ca^{2+} concentration, since changing Na^+ concentration has been shown to affect action potentials (Colomo & Rahamimoff, 1968) and miniature end-plate potential frequency (Gage & Quastel, 1966).

(b) High Potassium Solutions

For some experiments, while recording mepps, the KCl concentration of the solution was increased to 13mM. The amount of NaCl was reduced by an equivalent molar amount to maintain iso-osmolarity (Elmqvist, 1965).

(c) Anticholinesterases

Where stated in the text, physostigmine (3μM) or the irreversible anticholinesterase, 3,3-Dimethyl-2-butylmethlyphosphonofluoridate (Soman, 0.05μM), was added to the normal perfusing solution to inhibit acetylcholinesterase. Soman changes its chemical character when stored over long periods (as the sample used in this thesis was) but retains its capacity to inhibit acetylcholinesterase. Muscles were perfused for at least 20 minutes before recording from these solutions.

(d) Calcium Agonists and Antagonists

Nitrendipine and CGP 28392 were each dissolved in ethanol at a concentration of 1mg/ml and added to the perfusing solution. For recordings made before drug addition, the appropriate volume of ethanol was added to the perfusing medium so that the ethanol concentration remained constant throughout the experiment. The final concentration of ethanol in the bathing solution was small (0.07% v/v) and not expected to affect neuromuscular transmission (Gage, 1965). Experiments were performed using a sodium vapour lamp as the light source since these agents are very light sensitive (Reuter et al., 1985).

Section V : Preparation of Muscles

Following dissection, the hemi-diaphragm/phrenic nerve preparation was placed into a small, perspex recording bath. The Sylgard coated base of the bath was

used to insert pins which held the muscle slightly stretched. At all times the bath was continually perfused using well oxygenated Krebs from a reservoir. A three-way tap was used to switch between perfusing solutions, enabling conversion between them without interuption to the flow through the system.

Experiments were carried out at room temperature and near 37°C. For the latter, a water bath was used which circulated heated water around the inflow pipe and underneath the recording bath. A thermistor probe was used to monitor the temperature in the bath throughout the experiments.

The hind limbs of the mice were removed, skinned and stored at -70°C for later α-bungarotoxin binding studies.

For the human studies, after exposure to the plasmas or IgG, the muscles were removed from their incubation wells and pinned in the bath via the tendons at either end of the bundles, and perfused with Krebs solution as above.

Section VI : Electrophysiological Recordings

Intracellular voltage recordings were made using 3M KCl filled microelectrodes (10-20 MΩ resistance). The bath was grounded using a Ag/AgCl 3M KCl agar filled bath electrode. The recording electrode was connected to an input probe (de Villiers & Fatt, 1972) with a gain of ten and then to two oscilloscope amplifiers (Tektronix 5A21N or 5A22N) in parallel. One amplifier was used to follow changes in the resting membrane potential or to monitor action potentials and was set to a low gain and D.C. coupled. The other amplifier was used to observe mepps, epps or voltage noise and was set to a high gain, A.C. coupled and low-pass filtered at 3kHz. For recording results, the amplifiers were connected to an FM tape

recorder (Racal 4DS) which ran at 7½"/sec (low-pass filtered at 2.5kHz). The low-gain amplifier was connected to a chart recorder (Unicam SP22) to visually monitor changes in resting potential during recordings.

A Zeiss microscope was used to position the electrode near nerve endings before impaling the cell. The phrenic nerve was stimulated using a Grass S48 stimulator with SIU5 isolator connected to platinum wire electrodes for both epp recordings (frequency 0.5Hz, 1Hz or 3Hz, pulse width 0.1ms, supramaximal voltage) and for action potential recordings (frequency 0.5Hz, pulse width 0.1ms, supramaximal voltage). Only mepps or epps with fast rise times were recorded to ensure proximity to end-plates. Epps were recorded only if the stimulus artefact recovered to the baseline before the onset of the epp. The nerve was stimulated for at least one minute before starting to record. For mouse muscles 35-88 mepps were recorded and analysed at each end-plate. For recording action potentials, the electrode was placed at an end-plate (applying the above criteria) or at a non-end-plate region (determined by an absence of mepps). Only one action potential was recorded per fibre since muscle contraction made the electrode move position. In all experiments in normal solutions, recordings were only made if the resting potential was more negative than -60mV at the beginning of the recording. However, for recordings made in raised K^+ concentration solutions, the end-plate was depolarized and recordings were made if the resting potential was around -55mV to -50mV at the beginning. If, during any recording, the potential changed by more than 3mV the recording was rejected.

For recording end-plate voltage noise, the muscle was perfused at a high flow rate (14-25mls/min) with a solution containing tetrodotoxin (TTX, 250nM) and physostigmine (3μM). Following a recording period of control noise for 1-2 minutes the solution was switched to one containing ACh (5μM) as well as TTX and physostigmine. TTX was present to prevent muscle contractions and physostigmine to inhibit acetylcholinesterase. Muscles were depolarized to a well maintained

plateau, and after at least 5 minutes at this depolarized level, the solution was switched back to the normal solution. Membrane potential and associated voltage noise were recorded throughout.

Section VII : Analysis of Data

(a) Mepps and Epps

The results were played back from magnetic tape and analysed by computer (Computer Automation/CED 701 interface). Data was digitized at 10kHz for mouse muscles and 5kHz for human muscles and then written onto floppy discs. The computer then searched through the data reading 256 points at a time. When a point was found which exceeded a previously set cursor level this array of points was displayed on an oscilloscope to allow for visual inspection and rejection of any artefacts.

(i) Mepps

The amplitude was calculated as the difference between the maximum point in the array and the average baseline noise (calculated using a short portion of noise just before the mepp). The rise time was calculated as the time during which the mepp rises from 20% to 80% of the maximum amplitude. The decay time was calculated using a least squares fit of the decay phase ($\exp(-t/T)$) between 80% to 20% of the maximum amplitude. The above parameters for each mepp were stored in the computer and used to calculate the average values per end-plate. If more than one mepp occurred simultaneously on the screen it was not possible to measure the amplitude of both of them correctly and both were rejected. This occurred rarely at low frequencies and, since it is a random event, should not affect calculation of other

mepp parameters. All responses were counted, however, and used in the calculation of mepp frequency. For recordings of mepps in low calcium solutions only values for amplitude and frequency were obtained as above.

Unless otherwise stated mepp amplitudes are given without and with correction for variations in membrane potential. In each case where the mean resting potential for the test muscles was significantly different from the control value, the mepp amplitudes were corrected to a standard resting potential by multiplying mepp amplitudes by the factor (Katz & Thesleff, 1957):-

$$(E_s\text{-}E_{rev})/(E_m\text{-}E_{rev})$$

E_s is the standard resting potential (taken here as -70mV for mouse muscles and -80mV for human muscles); E_{rev} is the reversal potential, assumed to be 0mV for mouse (Linder & Quastel, 1978) and human (Cull-Candy et al., 1979); E_m is the measured resting potential for the fibre.

(ii) Epps

Epp amplitudes were calculated as for mepps except that the average baseline was evaluated from the short section before the stimulus artefact. In solutions where tubocurarine was present to prevent muscle twitch, the quantal content was calculated from the variance method (Ginsborg & Jenkinson, 1976):

$$\text{Quantal content } (m) = \frac{(\text{mean epp amplitude})^2}{\text{variance epp amplitude} - \text{mean variance baseline noise}}$$

The variance of the baseline noise was calculated from the short section of baseline noise immediately before the stimulus artefact of each epp and the averaged value found. The variance in baseline noise becomes increasingly important as the signal to noise ratio (epp amplitude variance/baseline noise variance) falls. If this signal to noise ratio was less than 2.5, results were rejected as too inaccurate.

Efforts were made to eliminate trends in epp amplitudes that can occur if there is a systematic drift in the membrane potential. A least squares line was fitted through the points of epp amplitude plotted against time (represented as the number of the epp in the sequence). The gradient of this line reflects the trend in the amplitudes. If there are no trends, the variance of epp amplitudes, calculated as the sum of the squares of difference between observed amplitudes and the least squares fit predicted amplitudes, should be equal to the epp amplitude variance calculated in the standard way. A plot of the ratio of these variances against the gradient of the least squares fit, from epps recorded from every end-plate in mice treated for 15 days, is shown in Figure 1. It can be seen that when the slope was greater than around $\pm$ 0.0025 the above ratio becomes considerably greater than one (i.e. >1.25) and results for *m* were rejected as inaccurate. As can be seen from this figure only 12 recordings out of 224 were in fact rejected. For recordings in low calcium however, since the scatter of the epp amplitudes was large the recordings often had a gradient of greater than $\pm$ 0.0025. However from a similar plot for this data as that shown in Figure 1 (figure for low calcium data not shown) the deviation of the ratio from 1 was negligible in each case. The variance used in all quantal calculations was obtained by the standard method.

Two other statistical methods for determining quantal content were employed when low calcium solutions were used (Castillo & Katz, 1954). The failures method is based on counting the number of stimuli which fail to evoke an epp:

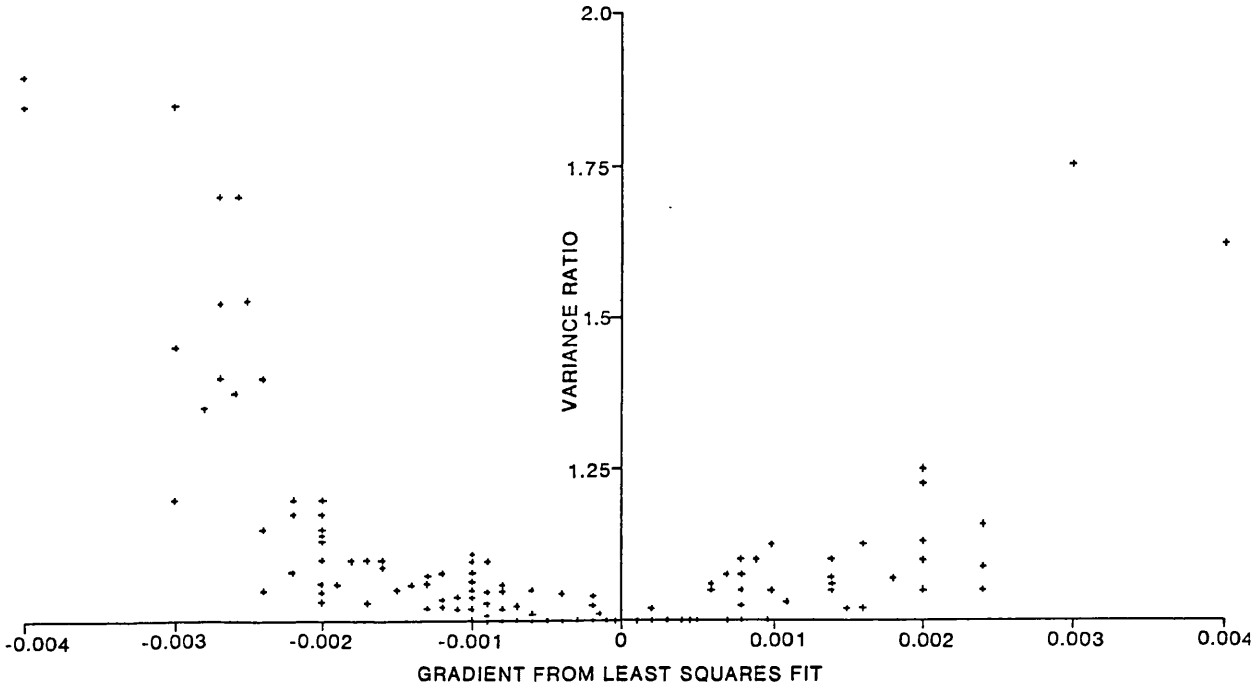
$$\text{Quantal content} = \log_e\left[\frac{\text{Number of nerve stimuli given}}{\text{Number of failures}}\right]$$

The direct method is based on the measurement of both mepps and epps simultaneously:

$$\text{Quantal content} = \frac{\text{mean epp amplitude}}{\text{mean mepp amplitude}}$$

Figure 1

Variance Ratio Plotted Against Gradient of Least Squares Fit for 15 Day Treated Animals



Legend for Figure 1

This figure shows a plot of the variance ratio (variance of epp amplitude: variance of epp amplitudes from the least squares fit) against the gradient of the least squares fit. Mice were injected with plasma from patients KP, MA, LW, KO, MO and MI or controls for 15 days. Each cross represents one end-plate; a total of 224 end-plates were recorded from in animals following the above treatments.

The above calculations using the failures and variance methods are based on Poisson statistics which assumes that the probability of release is small. This is true for low calcium solutions and leads to similar values for quantal content for the above three methods. When quantal content is higher, transmitter release deviates from Poisson statistics (Castillo & Katz, 1954). No empirical corrections for m obtained by the variance method are available for mouse muscle. However corrections have been studied for human and frog muscles (Cull-Candy et al., 1980; Miyamoto, 1975) and these corrections were applied in this thesis. We obtained a corrected value (m_{corr}) for the observed value of m (calculated by the variance method) as follows:

$$m \leq 8 \quad ; \quad m_{corr} = m$$

$$300 \geq m \geq 8 \quad ; \quad m_{corr} = 1.743 m^{0.7328}.$$

This was derived from the fit given for the data shown in Figure 1 of Cull-Candy et al., 1980. This latter empirical relationship for human muscle is similar to that seen in the frog (Miyamoto, 1975). As this correction may not be reliable in mouse muscle, values in this thesis are quoted both corrected and uncorrected.

To take into account variations in quantal size, a correction factor ($1+cv^2$) should be used (Martin, 1966), where cv is the coefficient of variation of mepp amplitude, defined as standard deviation(SD)/mean. However, this factor has been found to be small in mouse muscle (1.05; Lang, Newsom-Davis, Prior & Wray, 1983) and so has been neglected; except for low calcium solutions where mepps could be simultaneously measured.

A further correction to consider arises because of non-linear summation of epps (Martin, 1955). In low calcium solutions, where the quantal content is low, there was a large variation in epp amplitudes and each separate epp was corrected for non-linear summation using the formula :

$$\text{corrected epp amplitude} = \frac{\text{(measured epp amplitude)}}{(1-\frac{u}{v_0})}$$

where u is the measured individual epp amplitude (i.e. not the mean); v_0 is the difference between the muscle fibre membrane potential and the reversal potential (assumed to be 0mV for the mouse (Linder & Quastel, 1978)). In muscles with high quantal contents and tubocurarine present, the mean epp amplitudes were small (<5mV) and variation in epp amplitudes much less and so no correction was made.

(b) Action Potentials

Recordings were digitized at 10kHz and written onto floppy discs for computer analysis. The computer searched through the data until it found an action potential (AP). This was displayed so that artefacts could be removed if necessary. The computer then calculated: (i) the amplitude of the action potential, from the difference between the maximum point and the average baseline immediately preceeding the AP; (ii) the overshoot, knowing the resting potential of the fibre; (iii) the duration of the action potential at half maximum amplitude, by interpolating between digitized points as required; and (iv) the maximum rate of rise. This last value was obtained from the differentiated array by finding the difference between the maximum and the mean baseline of the differential array.

(c) Voltage Noise

The recordings of voltage noise and simultaneous DC recordings of membrane potential were played back from separate channels on the tape recorder into the computer and digitized at 1kHz. To avoid aliasing (Stevens, 1975) the high frequency components were filtered out using an 8th order low-pass Bezel filter set at 400Hz. An oscilloscope was used to display arrays of 256 points at a time for visual inspection and to avoid inclusion of any artefacts or mepps. The variance of each

array was then calculated if there were no artefacts or mepps. Fast fourier transform following cosine tapering yielded a power density spectrum for each array. Mean noise variance and power spectra were then obtained from fifty such arrays. The mean value of the resting potential was also obtained over the same period. Control values for the above parameters were obtained by averaging the period before ACh application and these were then subtracted from those values obtained during ACh addition. Spectra were corrected for the high-pass filter (2Hz) present in the A.C. coupling of the oscilloscope amplifier and plotted on an X-Y plotter as log of the spectral density (power) against log of the frequency. To avoid small artefacts due to mains interference the points around 50Hz and its multiples were substituted by interpolated values.

The depolarization of the membrane (V_m) and the noise variance ($\overline{E^2}$) are given by :

$$V_m = naT_m \quad \text{and} \quad \overline{E}^2 = na^2T_m$$

where n is the frequency of channel opening, a is the depolarization blip produced by a single channel and T_m is the decay time constant (exp $(-t/T_m)$, where t is time). These are based on the idea that voltage noise is caused by ACh molecules opening postsynaptic channels (Wray, 1980). The opening of these channels summates to give the overall depolarization V_m. A distribution in sizes of a (due to a distribution in channel open times) is also assumed.

The power spectral density can be fitted by a theoretical ("Lorentzian") curve given by:

$$S(f) = S(0) \quad / \quad \left(1 + \left(\frac{f}{fc}\right)^2\right)$$

$S(f)$ is the spectral density at frequency f; f_c is the corner frequency (i.e. the frequency at which the power spectrum density is reduced to half maximal value) and provides a value of T_m as follows:

$$T_m = \frac{1}{2\pi f_c}$$

Using a perspex template in the shape of a Lorentzian curve, with the corner frequency marked on it, the power spectra were fitted to it by eye. Then, in addition to the above direct method of deriving the variance from the data, measurement of the low frequency intercept, $S(0)$ of the fitted power spectra and T_m can also be used to calculate the noise variance since $\overline{E}^2 = S(0)/(4T_m)$. The value for the variance used in this thesis was derived by the direct method. Corrections were made for non-linear summation (Martin, 1955; Katz & Miledi, 1972). This was done by multiplying the observed values of V_m and $\overline{E}^2$ by the factors $(V_o/(V_o - V_m))$ and $(V_o/(V_o - V_m))^4$ respectively where $V_o = V_{rp} - V_{rev}$ (V_{rp} = resting potential, V_{rev} = reversal potential). For all the results quoted in this thesis V_{rev} was taken as 0mV.

Since the noise was low pass filtered at 400Hz, noise variance (measured directly) was corrected for this using the equation (Colquhoun, Large & Rang, 1977):

$$\text{true noise variance} = \frac{\text{measured noise variance} \quad x \quad \pi}{2 \text{ x } \tan^{-1} \quad (f_1 / f_c)}$$

where f_1 is the cut-off filter frequency.

For each end-plate, an average value of a was obtained over the period when V_m and n were steady, that is, when the ACh-induced depolarization reached an approximately steady plateau value ($a = \overline{E}^2/V_m$). This was to allow for the passive influx of Cl^- which distorts the early phase of the depolarization (Jenkinson & Terrar,

1973). Mean values of T_m for each end-plate were obtained from fits to spectra obtained for the fibre. Knowing a and T_m, values for n were then calculated from the variance using $n = \overline{E}^2/a^2T_m$.

Section VIII : α-BuTx Binding Studies

Mice were injected with MG plasma and used for electophysiological recordings (see above). The same mice were also used to obtain anti-AChR antibody titres and the number of receptors present in the hind limb muscles. The method used was similar to that described by Lang, Vincent & Newsom-Davis 1982 and Wilson et al., 1983. The hind limbs were removed from the mice at death and stored at -70°C until required. Muscles were then homogenised in an MSE atomix liquidizer using 5mls phosphate buffer (0.1M sodium phosphate, pH 7.3, containing 0.2% sodium azide and 0.1mM phenylmethylsulphonylfluoride (PMSF)). The PMSF was present throughout to prevent proteolysis of the AChR. After homogenisation to obtain a thick slurry, this was centrifuged for 30 minutes at 13,000rpm. The supernatant was discarded and the pellet re-suspended in phosphate buffer (0.02M PO_4, pH 7.3, 10^{-4}M PMSF) which contained the detergent Triton X-100 to solubilise the AChR (final concentration 1%). After 2 hours agitation at room temperature, followed by centrifugation at 13,000rpm, 100µl of supernatant was aliquoted into four tubes and 10µl of PMSF (0.1mM) added to each.

As controls, 2 of the tubes ("A" and "B") were incubated for 15 minutes with excess (10µl) unlabelled α-bungarotoxin (0.1µM) in phosphate buffer while the other tubes ("C" and "D") were incubated with 10µl phosphate buffer (0.02M PO_4, 0.1% Triton X-100, pH 7.3). All the tubes were then incubated with ^{125}I-α-bungarotoxin (3nM) for approximately 1-3 hours. The specific activity of α-bungarotoxin varied between 250-400 cpm/fmole.

To obtain a value for antibody bound, immunoprecipitation was performed by incubating tubes A and C with an excess of goat anti-human IgG (15µl), using 1µl normal human serum as a carrier to increase precipitation. The precipitates formed were centrifuged for 10 minutes, the supernatant removed and the pellet washed twice with phosphate buffer before the radioactivity was counted in a gamma counter. Counts for A were subtracted from C to obtain the counts due to receptor with antibody bound. Using the specific activity of α-BuTx results were expressed as fmol/g by the following method:

$$\left(\frac{\text{Counts}}{\text{specific activity of } \alpha\text{-Butx}} \times \frac{1000\mu l}{\text{volume of extract used in assay}(\mu l)} \right) / \text{ original weight of muscle used}$$

To obtain a value for total AChR number, immunoprecipitation was performed using incubations of tubes B and D with an excess of a mouse monoclonal antibody against human AChR (15µl) followed by an excess of anti-mouse IgG (15µl) and 1µl of normal mouse serum to help precipitation. The same procedure as before was carried out on the precipitates and the final value for total AChR content, obtained by subtracting the counts for B from D. Results were again expressed as fmol/g using the above method.

Section IX : Presentation of Data

The results from the same control treated animals are used throughout Chapter 3 but are repeated in each section for clarification. The data from mepp, epp and voltage noise recordings are expressed as means ± standard error of the mean (S.E.M.) using one value per end-plate unless stated otherwise. The biochemical data shown in Table 50 (Chapter 4) are expressed as means ± standard deviation. The two-tailed Student's t-test was used to compare the control and test (anti-AChR

Ab negative plasmas or MG preparations) parameters (Chapters 3 & 4). The paired Student's t-test was used to compare the effect before and after drug application at the same end-plate (Chapter 5). The level of significance was set at $P<0.05$.

Chapter 3

ELECTROPHYSIOLOGICAL AND BIOCHEMICAL EFFECTS OF PASSIVE TRANSFER OF ANTIBODY-NEGATIVE MG PLASMA OR IgG FROM MAN TO MOUSE

Section I : - Patient KP

I. Introduction

The purpose of this section was to examine the electrophysiological and biochemical features underlying the disorder seen in patient KP. This patient had antibody-negative myasthenia gravis from the age of 16 years (Table 2). The pattern of weakness in this patient was moderate respiratory, limb and ocular weakness and severe bulbar weakness and tendon reflexes were normal or brisk and the patient responded to edrophonium. Furthermore, improvement in muscle strength occurred upon plasma exchange, suggesting an autoimmune basis for the patient's disorder and this was investigated further here by passive transfer experiments. For this, mice were chronically treated for 3 - 15 days with either the plasma or IgG from patient KP or from controls. Following this treatment, electrophysiological recordings were made from the mouse phrenic nerve/diaphragm of mepps, epps and ACh-induced depolarization and the associated voltage noise. Also, α-BUTx binding studies using the hind limbs of the injected mice were made. It was therefore possible to look for features which may be transferred by the plasma or the IgG to the mice.

II. Spontaneous Release

(a) Spontaneous Release in Normal Calcium Concentration Solutions

Mepps (40 - 53 at each end-plate) were recorded from mice treated with IgG or plasma from patient KP and from respective controls at either room temperature (21.5-24.0°C) or at 35.0-37.0°C. A total of 17 mice were injected with KP plasma or IgG and 28 with control plasma or IgG. The mepp amplitudes were significantly reduced at all durations of treatment by KP IgG and plasma compared with controls (Table 4). At 3 days the mepp amplitude was reduced by 31% by KP IgG; at 7 days

Table 4

Effect of KP IgG and Plasma on Mepp Characteristics

Treatment	IgG Content of Daily Injection (mg)	Duration of Treatment (days)	Temp. (°C)	Resting Potential (mV)	MEPP CHARACTERISTICS						No. of End-plates, Mice
					Amplitude		Coefficient of Variation of Amplitude Distribution	Rise Time (ms)	Decay Time (ms)	Frequency (s^{-1})	
					Un-corrected for Resting Potential (mV)	Corrected to Resting Potential of -70mV (mV)					
KP IgG	60	3	22.0-22.6	-69.0 ±1.4	0.75 ±0.06*	0.77 ±0.07*	0.28 ±0.02	0.53 ±0.02#	2.23 ±0.08*	0.67 ±0.10	22,3
Control IgG	60	3	21.5-23.6	-66.1 ±1.1	1.09 ±0.06	1.16 ±0.07	0.25 ±0.01	0.62 ±0.03	2.83 ±0.10	0.78 ±0.06	27,4
KP Plasma	8.7	7	22.8-24.0	-67.0 ±0.9	0.37 ±0.02*	0.39 ±0.02*	0.23 ±0.01	0.68 ±0.06	2.08 ±0.10	2.43 ±0.47	46,6
Control Plasma	15.0-22.5	7	22.6-24.0	-65.9 ±0.9	0.72 ±0.03	0.77 ±0.04	0.22 ±0.01	0.61 ±0.10	2.17 ±0.10	2.57 ±0.49	48,6
KP Plasma	8.7	7	36.5-37.0	-71.8 ±1.2	0.40 ±0.03*	0.39 ±0.03*	0.25 ±0.01	0.35 ±0.02	1.35 ±0.07	10.3 ±1.3#	32,4
Control Plasma	15.0-22.5	7	35.0-36.5	-69.5 ±0.9	1.00 ±0.05	1.02 ±0.06	0.27 ±0.01	0.30 ±0.01	1.51 ±0.06	7.58 ±0.48	43,6
KP Plasma	8.7	15	36.0-37.0	-66.9 ±0.9	0.40 ±0.03*	0.42 ±0.28*	0.26 ±0.01+	0.44 ±0.03*	1.37 ±0.06	6.7 ±1.0	40,4
Control Plasma	15.0-22.5	15	36.0-37.0	-69.2 ±0.7	0.82 ±0.03	0.83 ±0.03	0.23 ±0.01	0.28 ±0.01	1.37 ±0.04	7.20 ±0.61	107,12

Legend for Table 4

This tables shows mepp characteristics measured from muscles pretreated with KP IgG and plasma and respective controls. Each value is the mean ± S.E.M. Mepp amplitudes have been corrected to a resting potential of -70mV. Coefficient of variation of amplitude distribution is defined as SD/Mean. The statistics were performed using 1 value per end-plate. If instead values were first averaged for each muscle and a single value used for each muscle in the statistical test, the values for mepp amplitudes were still significantly reduced. Significant differences compared to respective controls : *P<0.001; +P<0.02; #P<0.05.

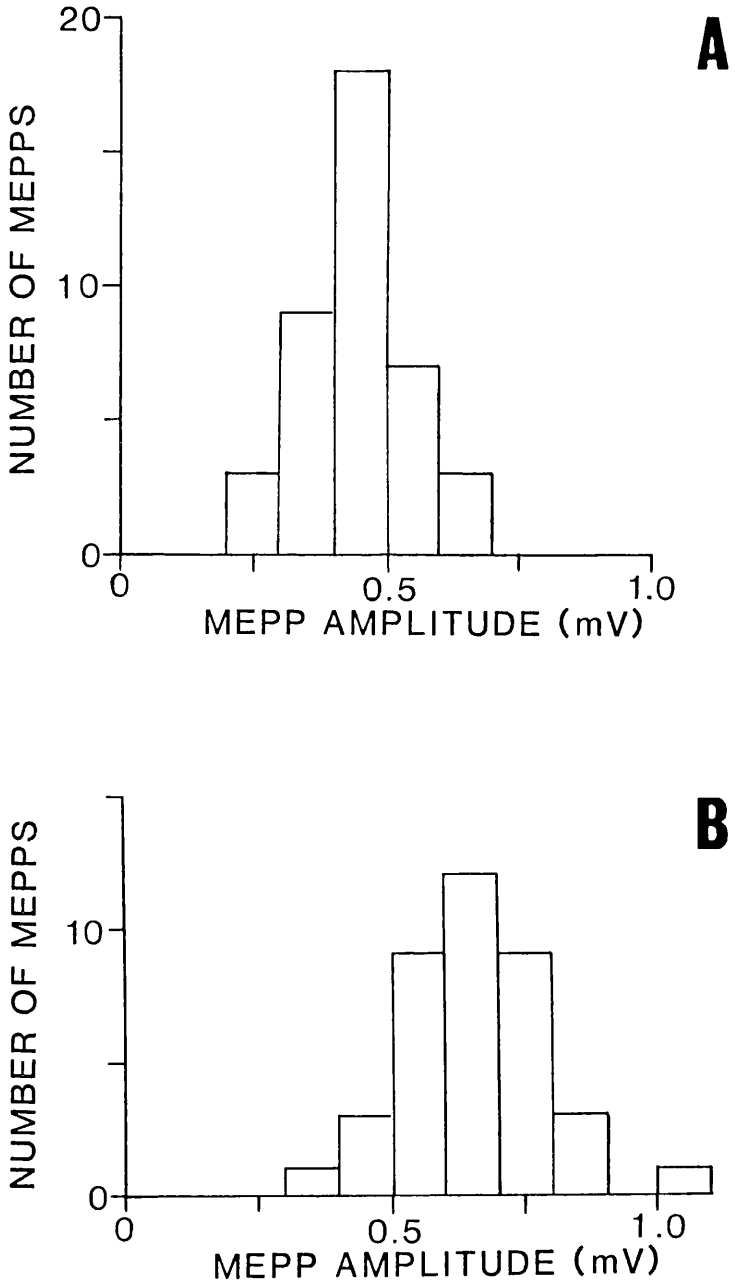
KP plasma caused greater reductions in the mepp amplitude: by 49% at 23°C and by 60% at 37°C; at 15 days there was no further reduction in mepp amplitude by KP plasma (reduced by 51% of control, see Table 4). Resting potentials were not significantly different in test and control muscles (Table 4) and indeed similar reductions in mepp amplitude by KP IgG and plasma were seen when the mepp amplitudes were corrected to a resting potential of -70mV (see Methods). There was no change in the coefficient of variation of mepp amplitude distributions by the KP IgG or plasma except at 15 days when there was a very small but significant increase in this value in KP plasma treated muscles. The general lack of effect on this parameter is seen when mepp amplitude distribution histograms are plotted. Figure 2 shows a histogram for a typical fibre from a test and control muscle; most fibres had mepps which were normally distributed about the mean.

Since the mepp amplitude was small for mice treated with KP IgG or plasma it is possible that some mepps may have been too small to be seen above the noise level, thus overestimating the mepp amplitude. To clarify the effect of KP plasma on mepp amplitude, at least for 7 day treated animals, the anticholinesterases physostigmine (3μM) or soman (0.05μM) were applied to the muscles in the bath. The mepp amplitudes were still significantly decreased in KP treated muscles compared with controls and there was again no change in the coefficient of variation of amplitude distributions (Table 5). Furthermore, it can be seen from Figure 3 that both control and KP treated muscles are normally distributed even in the presence of physostigmine.

The rise time of the mepps recorded in normal solutions (Table 4) appeared to be generally increased by KP IgG or plasma, although there was a slight but significant reduction at 3 days in rise time for KP treated animals. The apparent increase in rise time could arise if the microelectrode recordings were being made at sites more distant from the end-plate. This was specifically investigated in mice

Figure 2

Effect of KP Plasma on Mepp Amplitude Histogram Distributions



Legend for Figure 2

This figure shows mepp amplitude distributions from sample fibres recorded from animals treated for 7 days (35.0-37.0°C). Treatments were: (A) KP plasma (1.5ml containing 8.7mg IgG, per day), (B) control plasma. Vertical axis: number of mepps; Horizontal axis: mepp amplitude (mV). Each histogram represents 40 mepps.

Table 5

Effect of KP Plasma on Mepp Characteristics in the Presence of Anticholinesterases

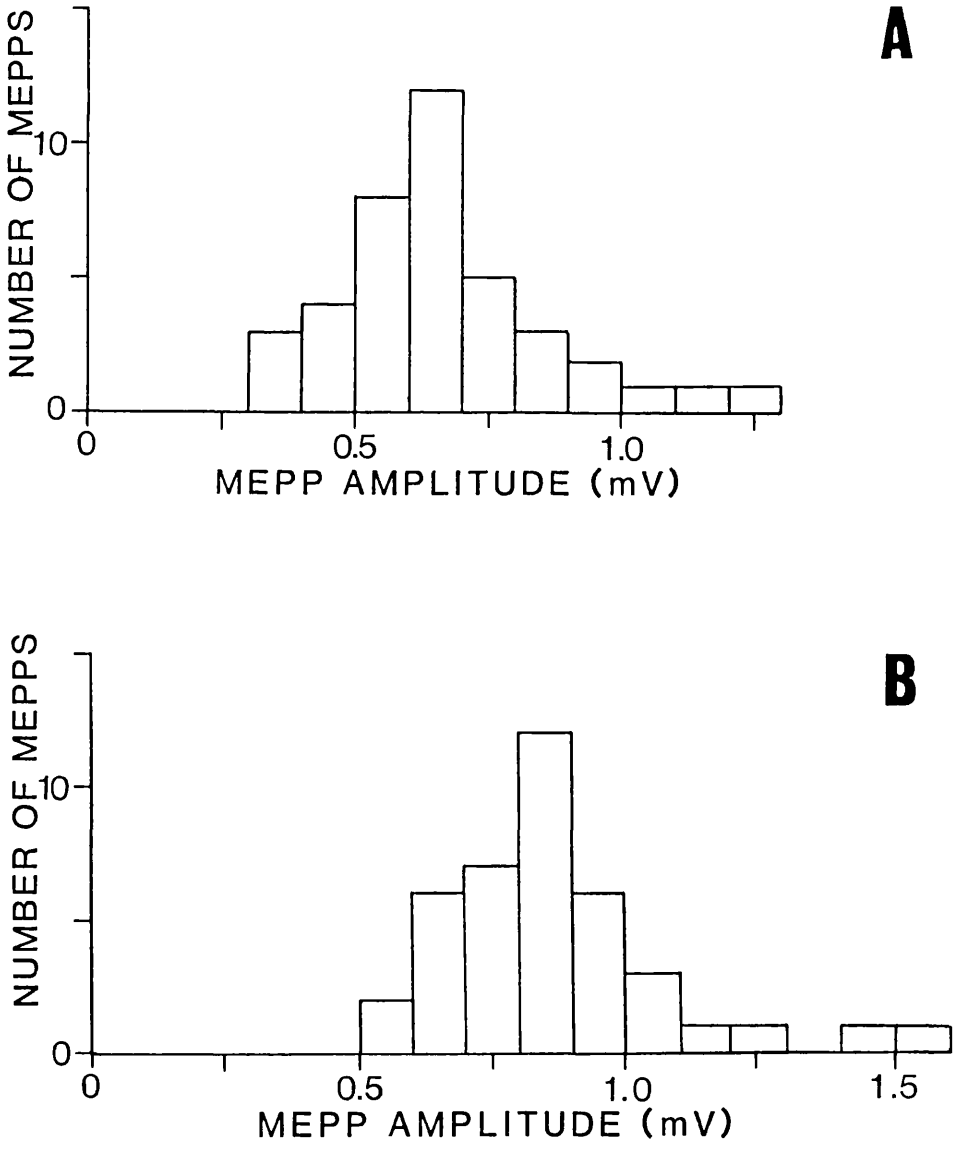
Treatment	Temp. (°C)	Resting Potential (mV)	MEPP CHARACTERISTICS										No. of End-plates, Mice
			Amplitude		Coefficient of Variation of Amplitude Distribution		Rise Time		Decay Time		Frequency		
			Physo-stigmine (mV)	Soman (mV)	Physo-stigmine (mV)	Soman (mV)	Physo-stigmine (ms)	Soman (ms)	Physo-stigmine (ms)	Soman (ms)	Physo-stigmine (s^{-1})	Soman (s^{-1})	
KP Plasma	22.8-24.0	-67.3 ±0.8	-	0.83 ±0.06*	-	0.29 ±0.01	-	1.24 ±0.08	-	4.44 ±0.13*	-	2.07 ±0.28	48,6
Control Plasma	22.6-24.0	-66.2 ±0.7	-	1.33 ±0.08	-	0.30 ±0.01	-	1.28 ±0.20	-	5.38 ±0.13	-	1.54 ±0.16	51,6
KP Plasma	36.5-37.0	-71.8 ±1.2	0.85 ±0.05*	-	0.30 ±0.01	-	0.46 ±0.04	-	2.79 ±0.10+	-	7.77 ±0.82	-	33,4
Control Plasma	35.0-36.5	-68.8 ±1.0	1.34 ±0.09	-	0.32 ±0.02	-	0.47 ±0.02	-	3.20 ±0.12	-	7.87 ±0.86	-	40,6

Legend for Table 5

This table shows the mepp characteristics recorded from muscles treated for 7 days with KP plasma (1.5ml containing 8.7mg IgG, per day) in the presence of an anticholinesterase, physostigmine (3μM) or an irreversible anticholinesterase, soman (0.05μM). Values shown are means ± S.E.M. averaged over the number of end-plates shown. Dashes indicate that experiments were not performed. All values for mepp amplitude are uncorrected for variations in resting potential since there was no significant difference in resting potential between control and test muscles at each temperature. Significant differences from control : *$P<0.001$; +$P<0.02$.

Figure 3

Effect of KP Plasma on Mepp Amplitude Histogram Distributions in Presence of Physostigmine



Legend for Figure 3

This figure shows mepp amplitude distributions recorded in the presence of physostigmine (3μM) in 7 day treated animals (35.0-37.0°C). Treatments were: (A) KP plasma (1.5ml containing 8.7mg IgG, per day), (B) control plasma (1.5ml containing 15.0-22.5mg IgG, per day). Vertical axis: number of mepps; Horizontal axis: mepp amplitude (mV). Each histogram represents 40 mepps.

treated for 15 days as follows. Mepp rise time was plotted against mepp amplitude for each end-plate recorded from, for controls (Figure 5) and for KP plasma treated muscles (Figure 4). These figures suggest that there was a correlation between rise times and amplitudes; mepps with smaller amplitudes appearing to have longer rise times. It is possible that the KP plasma itself caused these observations. For example, slowing the vesicle fusion could increase the rise time of the mepps and reduce the amplitude. Alternatively, and more likely, is that the effects could be observed if small mepps resulted from recordings made away from end-plates. The observations could then be attributed to the recording technique since with a recording temperature of around 37°C all mepps have a fast rise time and visual discrimination between mepps at or near to the end-plate region is more difficult especially in KP treated muscles where mepps are small. For control muscles, recorded at around 37°C, it appears that below a rise time of around 0.4ms there was little correlation between larger rise times and smaller amplitudes, suggesting that only fibres with a rise time of less than 0.4ms can be considered to be at an end-plate. Therefore, for both test and control muscles, only end-plates with rise times less than 0.4ms were selected (at 37°C). There was still a significant ($P<0.001$) reduction in mepp amplitude in KP muscles compared to controls (Table 6) confirming the effect of the plasma on mepp amplitudes at least for 15 days duration of treatment. After selecting mepps in this way the rise time was essentially unchanged by KP plasma; only small effects being seen (Table 6). Obviously mepps recorded at room temperature have slower rise times (Table 4) and so it was not appropriate to apply this selection procedure; rise times were not affected by KP plasma as compared with controls. Furthermore, in the presence of physostigmine or soman, when visual discrimination of mepps was improved due to the larger amplitudes, there was no change in the mepp rise time by KP plasma (Table 5), although in both cases there was a prolongation of the mepp rise time for both test and control muscles as compared to recordings in the absence of anticholinesterases (Table 4).

Legend for Figures 4 & 5

These figures show mepp amplitudes plotted against mepp rise time for mice treated with KP plasma (1.5ml containing 8.7mg IgG, per day) (Figure 4) and control plasma (1.5ml containing 15.0-22.5mg IgG, per day) (Figure 5). Animals were treated for 15 days and recordings were made at 36.0-37.0°C. Each cross represents the mean mepp amplitude and mepp rise time for each end-plate recorded from; KP treated muscles, 40 end-plates, 4 animals; control treated muscle, 107 end-plates, 12 animals.

Figure 4

Mepp Amplitude Versus Mepp Rise Time - KP Plasma Treated Muscles

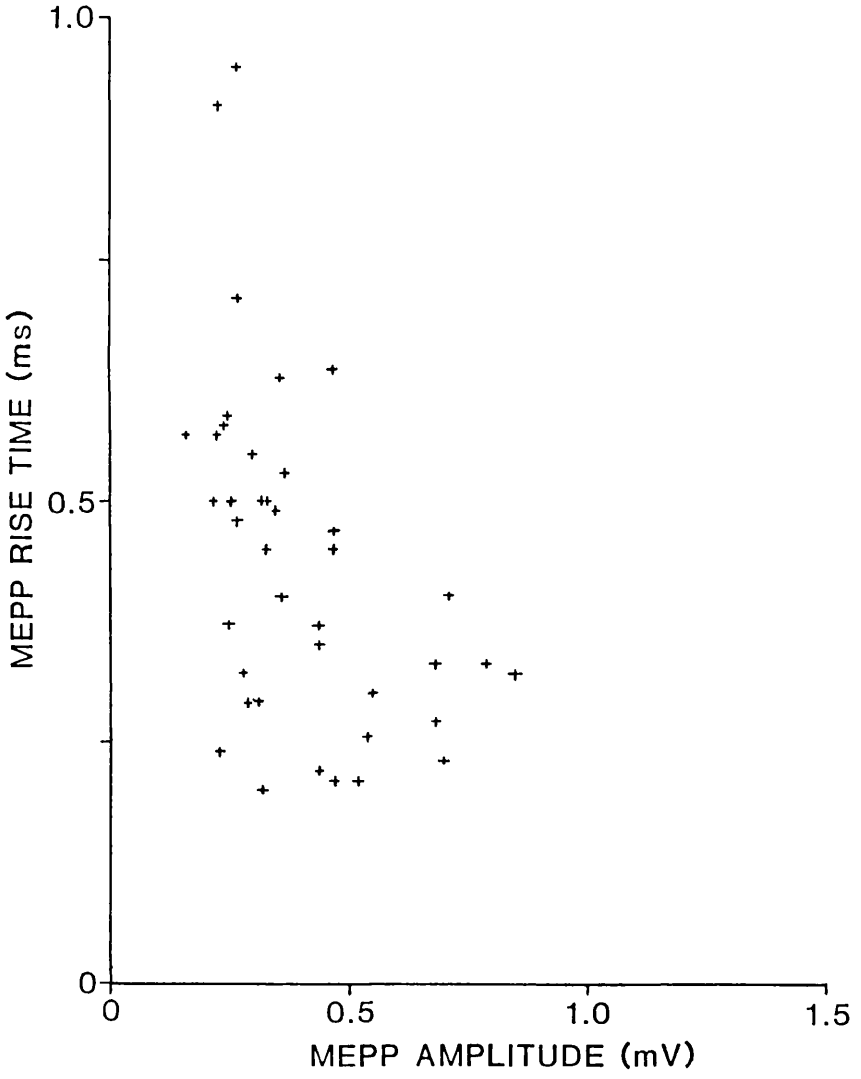


Figure 5

Mepp Amplitude Versus Mepp Rise Time - Control Plasma Treated Muscles

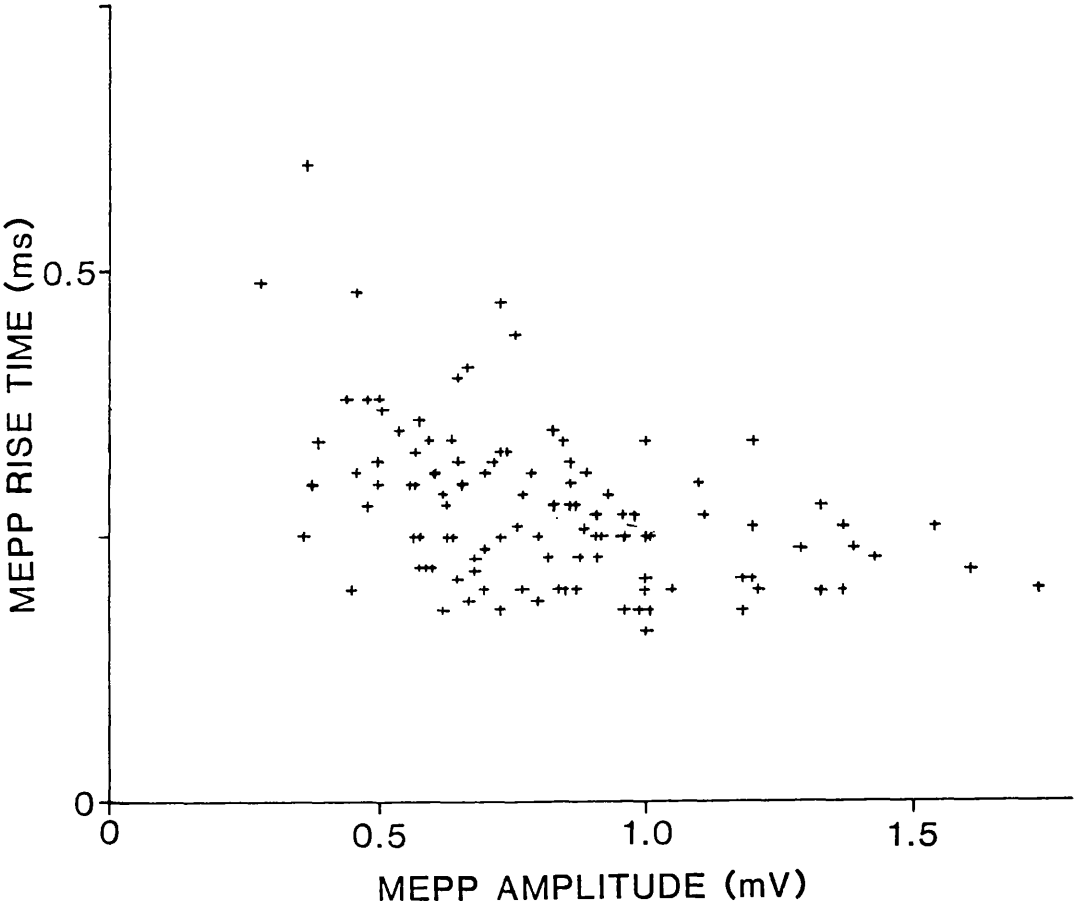


Table 6

Effect of KP Plasma on Mepp Characteristics Recorded at around 37°C.

Treatment	IgG Content of Daily Injection (mg)	Duration of Treatment (days)	Temperature (°C)	Resting Potential (mV)	MEPP CHARACTERISTICS		No. of End-plates, Mice
					Rise Time (Selecting mepps with rise time ≤ 0.4ms) (ms)	Amplitude (Selecting mepps with rise time ≤0.4ms) (mV)	
KP Plasma	8.7	7	36.5-37.0	-71.2 ±1.5	0.30 ±0.01	0.42 ±0.03*	23,4
Control Plasma	15.0-22.5	7	35.0-36.5	-69.1 ±0.9	0.29 ±0.01	1.02 ±0.06	40,6
KP Plasma	8.7	15	35.0-37.0	-66.7 ±1.2	0.29 ±0.02#	0.49 ±0.04*	20,4
Control Plasma	15.0-22.5	15	36.0-37.0	-68.8 ±0.9	0.27 ±0.01	0.84 ±0.03	101,12

Legend for Table 6

This table shows the effect of KP plasma on mepp rise time and amplitude after selecting mepps with a rise time ≤0.4ms at 37°C. Values shown are means ± S.E.M. Significant changes in KP treated muscles compared with controls: *P<0.001; #P<0.05.

Mepp decay time was little changed by treatment with KP plasma or IgG although there was a significant ($P<0.001$) decrease in decay time after 3 days duration of treatment with KP IgG (Table 4). This is probably due to non-specific effects of the IgG on passive membrane properties. This may also explain why there were reductions in the decay time of mepps by KP plasma recorded from muscles in the presence of physostigmine and soman (Table 5).

The mepp frequency reflects only presynaptic events and the effects on this parameter by KP IgG and plasma compared with controls are shown in Table 4. In all cases studied there was no change in mepp frequency except at 7 days (35.0-37.0°C), where there was a significant ($P<0.05$) increase in frequency, and even this difference was not significant when one value per muscle was used in the statistical analysis instead of one value per end-plate (KP plasma, $9.86 \pm 3.07 s^{-1}$, 4 muscles; control plasma, $7.56 \pm 0.64 s^{-1}$, 6 muscles). There was no change in mepp frequency by KP plasma when recorded in physostigmine or soman (Table 5). Finally, for both test and control muscles mepp frequency increased with increasing temperature.

(b) Spontaneous Release in Low Calcium Concentration Solutions

During the recordings of epps in low Ca^{2+} solutions (see part V), mepps (40-50 at each end-plate) were also recorded (35.0-37.0°C). Mepp amplitudes tended to be reduced in KP treated muscles but this only reached significance ($P<0.05$) at a Ca^{2+} concentration of 0.24mM (Table 7). This compares with the marked reductions in mepp amplitude recorded from the same muscles in higher Ca^{2+} concentration solutions of 2.52mM (Table 4, 5 & 6), suggesting that when release is low the defective mechanism is partially overcome. Measurements of mepp frequency

Table 7

Effect of KP Plasma on Mepp Characteristics Recorded in Low Ca^{2+} Concentrations

Treatment	Duration of Treatment (days)	Temperature (°C)	Ca^{2+} Concentration (mM)	Resting Potential (mV)	MEPP CHARACTERISTICS		No. of End-plates, Mice
					Amplitude Uncorrected for resting potential (mV)	Frequency (s^{-1})	
KP Plasma	7	36.5-37.0	0.20	-67.3 ±1.3	0.74 ±0.06	3.72 ±0.75#	29,4
Control Plasma	7	35.0-36.5	0.20	-67.0 ±1.0	0.83 ±0.05	2.20 ±0.28	42,6
KP Plasma	7	36.5-37.0	0.24	-68.9 ±1.3	0.70 ±0.05#	3.87 ±0.87	27,4
Control Plasma	7	35.0-36.5	0.24	-67.2 ±1.1	0.87 ±0.06	6.2 ±1.1	38,5

Legend for Table 7

This table shows mepp amplitude and frequency for mepps recorded in low Ca^{2+} concentration solutions from animals treated with KP plasma (1.5ml containing 8.7mg IgG, per day) and control plasma (1.5ml containing 15.0-22.5mg IgG, per day). Mepp amplitudes are uncorrected for resting potential since there is no significant difference between resting potentials. Values shown are means ± S.E.M. Significant changes from control: #P<0.05.

showed that, interestingly, there was no effect of KP plasma at 0.24mM Ca^{2+} but at 0.2mM Ca^{2+} there was an increase in mepp frequency that was just significant (Table 7).

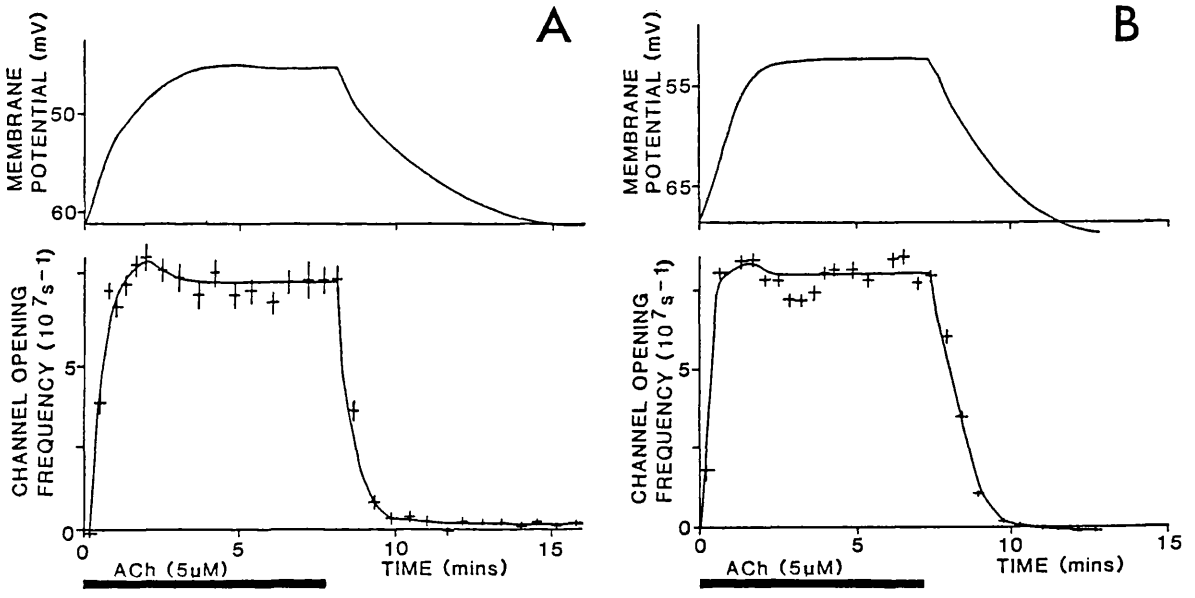
III. Noise Analysis

The results in the preceding two sections show that KP plasma and KP IgG reduced mepp amplitudes (especially in normal Ca^{2+} solutions) without any other clear changes in the mepp characteristics measured. This reduction in mepp amplitude could be the result of either a presynaptic decrease in the number of molecules of ACh released per packet or a postsynaptic decrease in the sensitivity of the membrane to ACh.

Postsynaptic effects of KP plasma were studied by applying ACh in the bath and recording the associated depolarization and noise in the mouse muscles previously treated for 15 days with KP or control plasma. Typical recordings from control and KP plasma treated muscles are shown in Figure 6. Acetylcholine caused a depolarization and an increase in noise variance, allowing calculation of channel opening frequency (lower traces, see Methods). It can be seen that there was little, if any, desensitization in channel opening frequency and depolarization, and this was found to be a typical response to ACh for all the other recordings. When the ACh was washed off, the channel opening frequency and the membrane potential returned to near initial values. Figure 7 shows power spectra of voltage noise for control and KP plasma treated muscles. The theoretical curve, a single Lorentzian curve, always fitted the experimental points well. Table 8 shows the mean values obtained from several such experiments for mice treated with control and KP plasma. There was no change in ACh-induced maximum depolarization (V_{max}), while values of single channel depolarization (a) and maximum frequency of channel opening (n_{max}) obtained from the noise were also unchanged in KP plasma treated muscles

Figure 6

Effect of KP Plasma on Time Course of Membrane Depolarization and Channel Opening Frequency

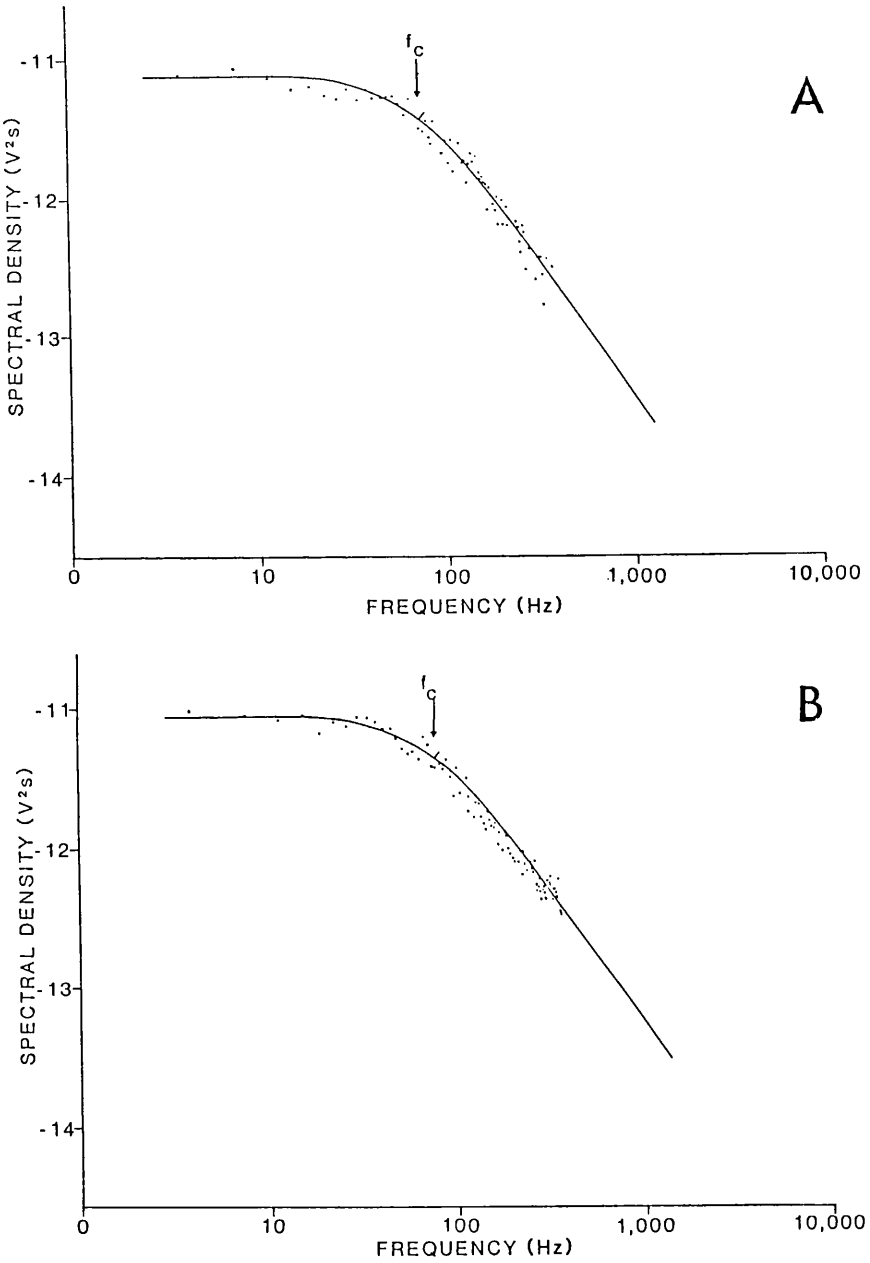


Legend for Figure 6

Time course of membrane potential and channel opening frequency in mouse diaphragm muscles (21.8-23.9°C), treated with control plasma (A) and KP plasma (B), during exposure to ACh (5μM) (shown as the bars). Channel opening frequency (lower trace) was derived from the noise variance and from mean values of a (0.122 ±0.003μV) and T_m (2.20 ±0.05ms) for control treated muscles and from a (0.139 ±0.003μV) and T_m (2.00 ±0.03ms) for KP treated muscles. Vertical lines are S.E.M. and horizontal lines represent the period of analysis.

Figure 7

Effect of KP Plasma on Power Spectrum of Voltage Noise



Legend for Figure 7

This figure shows power spectrum of voltage noise from mouse muscles (21.8-23.9°C), treated with control plasma (A) and KP plasma (B). For (A) spectra the value for f_c, corner frequency (see Methods), is 76.8Hz leading to a time constant, T_m of 2.22ms. For (B) the value for f_c is 80.0Hz leading to a time constant, T_m of 1.99ms for this spectra.

Table 8

Effect of KP Plasma on Channel Properties Measured by Noise Analysis

Treatment	Temperature (°C)	Resting Potential (mV)	V_{max} (mV)	a (μV)	n_{max} ($10^7 s^{-1}$)	T_m (ms)	No. of End-plates, Mice
KP Plasma	21.8-23.9	-69.0 ±1.7	15.2 ±1.8	0.34 ±0.08	5.4 ±1.1	1.92 ±0.10$^{\Delta}$	11,4
Control Plasma	22.8-23.8	-69.9 ±1.6	18.4 ±1.6	0.24 ±0.03	5.38 ±0.89	2.60 ±0.18	17,7

Legend for Table 8

This table shows the maximum depolarization produced by ACh (5μm) (Vmax) and results of voltage noise analysis (a, n_{max} and T_m). Recordings were made from animals treated for 15 days with KP plasma (1.5ml containing 8.7mg IgG, per day) or control plasma (1.5ml containing 15.0-22.5mg IgG, per day). Values shown are means ± S.E.M. using one value per end-plate. Significant difference from control: $^{\Delta}P<0.01$.

compared with controls. These results indicate a lack of postsynaptic action of KP plasma on end-plate channels. There was, however, a small but statistically significant ($P<0.01$) reduction in the noise time constant (T_m) derived from the spectra (Table 8). As expected for these passive membrane properties, the noise time constant was similar to the mepp decay time constants (see Table 4) at the same temperature, since both are determined by passive decay. As previously described, there was a tendency for mepp decay time constants to decrease which was also seen for the noise time constant. These small effects on T, as mentioned above, are probably due to non-specific effects of KP plasma on passive membrane properties.

IV. α-BuTx Binding Studies

Further investigation of any action of KP plasma on postsynaptic properties was carried out using α-BuTx binding studies on the hind limbs of mice treated for 15 days (see Methods). Table 9 shows results from such experiments and this data again suggests that KP plasma had no apparent postsynaptic actions. There was no reduction in the number of AChRs in KP plasma treated animals as compared with controls. This was as expected since there was also no detectable anti-AChR Ab bound to the acetylcholine receptor in either treated or control muscles. The negative value for anti-AChR Ab bound in KP treated muscles arises since radioactive counts from the "control" extracts were generally greater than those from the labelled extracts (see Methods). This may be explained if the incubation time with unlabelled α-bungarotoxin in the control tubes was too short to allow sufficient protection of the AChR against ^{125}I-α-bungarotoxin binding or if there had not been excess unlabelled α-bungarotoxin in the control tube before the labelled toxin was added.

Table 9

α-BuTx Binding Studies from Mice Treated with KP Plasma

Treatment	Duration of Treatment (days)	AChR number (f mol/g)	Anti-AChR Ab bound (f mol/g)	No. of muscles
KP Plasma	15	601 ± 84	-22.2 ± 7.8	3
Control Plasma	15	627 ± 57	24 ± 22	4

Legend for Table 9

This table shows the effect of KP plasma (1.5ml containing 8.7 mg IgG, per day) on AChR number and the amount of anti-AChR antibody bound to the receptors. Values shown are means ± S.E.M. averaged from the number of muscles shown. Values for each muscle were obtained from the mean of three separate assays on each muscle extract.

V. End-plate Potentials

Epp recordings (21.5-22.6°C or 35.0-37.0°C) were made at a nerve stimulation frequency of 0.5Hz from a total of 41 animals (63 - 118 epps at each end-plate). For all experiments at a Ca^{2+} concentration of 2.52 mM, the mean concentration of tubocurarine just large enough to prevent muscle twitch was less for KP treated muscles than for respective controls (significantly ($P<0.001$) lower than the respective control at 3 days, Table 10); this is itself indicative of some neuromuscular transmission defect by KP.

At each treatment time there was no significant difference between test and control quantal content values either before or after corrections were made for deviation from Poisson statistics (Table 10). As expected, quantal content values were higher at the raised temperatures, and reduced in the low Ca^{2+} concentration solutions, for both test and control muscles. For the low Ca^{2+} concentration solutions quantal content was calculated by the variance, failures or direct methods; there was also no significant difference between the values for quantal content obtained using these methods within either treatment group (Table 11). This suggests that the Poisson nature of quantal release of ACh was not affected by KP plasma.

In 7 day treated animals further evidence for a lack of effect of KP plasma on quantal content was derived as follows. The phrenic nerve was stimulated at a rate of 3Hz for a 30 minute period in the presence of tubocurarine ($7.7 \pm 1.0\mu M$) and soman ($0.05\mu M$) for both test and control muscles (2.52mM Ca^{2+} concentration solutions; 22.8-24°C). During this time at least 6 different end-plates were sampled from each muscle and epps (96-128 at each end-plate) were recorded. For some muscles, during the 30 minutes stimulation period, end-plates were recorded from for 3 minute periods and then 69-120 epps were analysed over a short period at the

Table 10

Effect of KP IgG and Plasma on Epp Quantal Content

Treatment	IgG Content of Daily Injection (mg)	Duration of Treatment (days)	Temp. (°C)	Calcium Concentration (mM)	Resting Potential (mV)	Tubocurarine Concentration Required to just Prevent Twitch (μM)	EPP QUANTAL CONTENT		No. of End-plates, Mice
							Uncorrected	Corrected for Deviation from Poisson Statistics	
KP IgG	60	3	21.6-22.2	2.52	-69.8 ±1.4	2.3 ±0.05*	136.4 ±9.9	63.3 ±3.5	19,3
Control IgG	60	3	21.5-22.6	2.52	-67.5 ±1.1	4.0 ±0.1	129 ±15	59.8 ±5.1	22,4
KP Plasma	8.7	7	36.5-37.0	0.20	-67.3 ±1.3	-	0.52 ±0.05	0.52 ±0.05	29,4
Control Plasma	15.0-22.5	7	35.0-36.5	0.20	-66.9 ±1.0	-	0.40 ±0.04	0.40 ±0.04	42,6
KP Plasma	8.7	7	36.5-37.0	0.24	-68.9 ±1.3	-	0.81 ±0.09	0.81 ±0.09	27,4
Control Plasma	15.0-22.5	7	35.0-36.5	0.24	-67.2 ±1.1	-	0.72 ±0.07	0.72 ±0.07	38,5
KP Plasma	8.7	15	36.0-37.0	2.52	-70.7 ±1.2	2.9 ±0.3	177 ±15	76.0 ±4.8	27,4
Control Plasma	15.0-22.5	15	36.0-37.0	2.52	-69.0 ±0.8	4.0 ±0.4	201 ±12	82.7 ±3.7	66,11

Legend for Table 10

This table shows values of epp quantal content measured for KP IgG or plasma and control treated animals. Values in normal Ca^{2+} concentrations were derived using the variance method in the presence of tubocurarine. Values in low Ca^{2+} concentrations were derived for each end-plate from the mean of the variance, failures and direct methods; this is because no tubocurarine was used and simultaneous recording of mepps and epps can be made. Epp quantal content was corrected as described in Methods. Values shown are mean values averaged over the number of end-plates shown (mean ± S.E.M.). Significant differences from controls : *P<0.001.

Table 11

Comparison of Quantal Contents Obtained in KP Plasma Treated Muscles in Low Ca^{2+} Concentration Solutions

Treatment	Duration of Treatment (days)	Calcium Concentration (mM)	QUANTAL CONTENT			No. of End-plates, Mice
			Variance	Failures	Direct Ratio	
KP Plasma	7	0.20	0.58 ±0.05#	0.52 ±0.05	0.47 ±0.05	30,4
Control Plasma	7	0.20	0.43 ±0.04	0.40 ±0.04	0.38 ±0.04	42,6
KP Plasma	7	0.24	0.89 ±0.10	0.81 ±0.08	0.73 ±0.09	27,4
Control Plasma	7	0.24	0.80 ±0.08	0.71 ±0.07	0.67 ±0.06	38,5

Legend for Table 11

This table shows quantal content values calculated by the variance, failures and direct ratio methods from mouse muscles treated with KP plasma (1.5ml containing 8.7mg IgG, per day) for 7 days (35.0-37.0°C). Values are means ± S.E.M. averaged from the number of end-plates shown. Significant difference from controls: #P<0.05. The small significant increase in quantal content using the variance method, at 0.2mM Ca^{2+} concentration, by KP plasma was not seen if, instead of using one value per end-plate, one value per muscle was used in the statistical test. In the latter case, when end-plates were averaged together for each muscle first, the values were KP, 0.55 ± 0.08, 4 animals; control 0.41 ± 0.07, 6 animals. There were no significant differences between values within each treatment group.

beginning and also at the end of this 3 minute period. From such recordings, to obtain one value of quantal content per end-plate, values for quantal content obtained at the beginning and end of the 3 minutes were averaged together. The data obtained from these experiments was analysed in several ways. Firstly, using one value for quantal content (as described above) per end-plate there was no significant difference in quantal content between test and control muscles (KP plasma 57.4 ± 3.7, 44 end-plates, 6 animals; control plasma 60.7 ± 4.2, 41 end-plates, 6 animals). Secondly, the value for quantal content recorded from the end-plate sampled at the beginning of each 30 minute period was subtracted from the value obtained from the end-plate sampled at the end of the 30 minute period. Although there was a large scatter between values obtained for each muscle there was no significant difference between these values (KP plasma -0.3 ± 16.4, 6 animals; control plasma -38.1 ± 15.2, 6 animals). This suggests that there was no difference in the continual run down in quantal content occurring during 30 minutes high frequency stimulation and, moreover, the run down in quantal content in KP plasma treated animals muscles was negligible. Thirdly, using only those fibres recorded from for 3 minutes, a value of quantal content for each end-plate was obtained by subtracting the value obtained at the beginning from the value at the end of the 3 minute stimulation period. It was seen that again there was no significant difference between test and control values (KP plasma -4.6 ± 3.5, 24 end-plates, 4 animals; control plasma -9.5 ± 5.5, 23 end-plates, 4 animals). This again suggest that there was no difference in epp quantal content run down over short time periods. Finally, the gradient of the least squares line fitted through the points of epp amplitude plotted against time (see Methods) was compared between KP plasma and control plasma treated muscles and there was no significant difference between them (KP plasma -0.00042 ± 0.00010, 44 end-plates, 6 animals; control plasma -0.00035 ± 0.00008, 41 end-plates, 6 animals). These results taken together suggest that not only was the quantal content unchanged by treatment with KP plasma but that the rate of run down of epp amplitudes, was also unchanged.

Finally it must be mentioned that for recordings of epps in tubocurarine solutions it was not possible to meaningfully compare epp amplitudes between test and control muscles. This is because the level of tubocurarine required to just prevent muscle twitch was not identical between test and controls. In low Ca^{2+} solutions, however, epp amplitudes could be directly compared between KP plasma and control plasma treated muscles. As can be seen from Table 12 there were no significant differences between epp amplitude in KP treated and control muscles in either Ca^{2+} concentration. This effect was expected since in low Ca^{2+} concentrations KP plasma caused less marked reductions in mepp amplitudes and no change in epp quantal content compared with controls.

VI. Discussion

Injections of the plasma or IgG fraction from patient KP into mice gave a reduction in mepp amplitude in normal solutions while the other mepp characteristics (rise time, decay time and frequency) were essentially unchanged. In low Ca^{2+} solutions there was a less marked reduction in mepp amplitude and again mepp frequency was essentially unchanged. There was no change in channel properties (single channel depolarization, channel opening frequency or depolarization produced by ACh) indicating no decrease in post-junctional membrane sensitivity to ACh. Underlying this, there was no decrease in the number of AChRs as measured by α-BuTx binding and no detectable Ab directed against AChR. The quantal content in these KP treated muscles was normal although there was an increased sensitivity to tubocurarine, less being required to just block muscle twitch. Since the effects on mepp amplitude were seen in mice treated with either KP plasma or KP IgG, this implies an autoimmune basis for the disorder in this patient.

Table 12

Effect of KP Plasma on Epp Amplitudes in Low Ca^{2+} Concentrations

Treatment	Duration of Treatment (days)	Temperature (°C)	Calcium Concentration (mM)	Resting Potential (mV)	Epp amplitude (mV)	No. of End-plates, Mice
KP Plasma	7	36.5-37.0	0.20	-67.3 ±1.3	0.31 ±0.03	29,4
Control Plasma	7	35.0-36.5	0.20	-66.9 ±1.0	0.31 ±0.03	42,6
KP Plasma	7	36.5-37.0	0.24	-68.9 ±1.3	0.47 ±0.04	27,4
Control Plasma	7	35.0-36.5	0.24	-67.2 ±1.1	0.55 ±0.05	38,5

Legend for Table 12

This table shows the effect of KP plasma (1.5ml containing 8.7mg IgG, per day) on epp amplitude measured in low Ca^{2+} solutions. Values shown are means ± S.E.M. There were no significant differences compared to the controls.

For MG with anti-AChR Abs, a large reduction in mepp amplitudes is observed following injection of mice with the IgG fraction from such patients (by 72% after 3 day injections and by 69% after 10-14 day injections, Toyka et al., 1975). For mice injected with KP plasma for greater than 3 days, there was a smaller, though appreciable, reduction in the mepp amplitude (by around 50% of the controls). However, it must be mentioned here that the reduction in mepp amplitude by KP plasma, compared with controls, was by a similar amount to that seen in muscles treated with plasma from MG patient SP (i.e. by 52% following 7 day injections, see Section VIII). The difference in this latter value compared to values obtained by Toyka et al. may be explained by the different preparations used for injections. Toyka et al. injected the immunoglobulin fraction of MG plasma into mice and the extracted antibody may have a higher affinity for the AChR than the plasma itself, which was used in this thesis. The smaller reduction seen in KP muscles would then be attributed to a poor cross-reactivity of any antibody at the mouse neuromuscular junction. It is noteworthy, however, that MG Ab directed against the AChR usually has a good cross-reactivity with the mouse neuromuscular junction (Toyka et al., 1975). Thus, whatever the reason for the somewhat smaller reduction in mepp amplitude, it suggests that perhaps the disease in patient KP had a different aetiology from MG. Measurement of mepp amplitudes in muscles from patient KP were not performed so that the extent of any reductions in mepp amplitude in the patient's muscle is unfortunately not known.

The results from α-BuTx binding studies are in accord with previous work using the IgG from patient KP; these showed a lack of anti-AChR antibody in mice pre-treated with KP IgG and no evidence for Abs directed against the α-BuTx binding site by measuring inhibition of α-BuTx binding by the IgG (Mossman et al., 1986). The observation of lack of reduction in the number of AChRs and the lack of reduction in AChR sensitivity contrasts with studies using intercostal muscle biopsied from MG patients who have anti-AChR antibodies (Albuquerque et al.,

1976b; Ito et al., 1978b, Cull-Candy et al., 1978; Cull-Candy et al., 1979). This again strongly suggests that the disorder for antibody-negative MG patient KP is not postsynaptic in origin and is a distinct disorder from MG.

The reduction in mepp amplitude by KP IgG or plasma, if not postsynaptic in origin, could arise from a defect of presynaptic origin. Direct measurements of the number of packets of ACh released either by nerve stimulation (indicated from values of quantal content) or spontaneously (estimated by mepp frequency) were unaffected by injecting mice with either KP IgG or plasma. However, it is possible that a reduction in mepp amplitude could arise by fewer ACh molecules reaching the postsynaptic membrane when a packet of ACh is released from the nerve terminal, which it turn could arise by a number of mechanisms. Firstly, there may be an excess of acetylcholinesterase, the enzyme responsible for ACh hydrolysis. In this case, the ACh might be destroyed before it reached the postsynaptic receptor. However, this is unlikely since a reduction in mepp amplitude in animals treated with KP plasma and IgG was seen even in the presence of acetylcholinesterase inhibitors. Secondly, the reduction in mepp amplitude could arise by an antibody-induced widening of the synaptic cleft. However, this would cause an increase in mepp rise time and, although KP plasma and IgG did slow the rise time, this was felt to be due to displacement of the microelectrode away from the end-plate during recording (see Part IIa). To clarify this point it would be very useful to carry out further studies of the morphology of end-plates in mouse muscles treated with KP plasma or IgG. Thirdly, the mechanism responsible for the observed reduction in mepp amplitude could be a reduction in the number of ACh molecules released from the nerve terminal in a single packet and this is probably the most likely mechanism.

There are a number of ways in which a decrease in the number of ACh molecules in a packet could arise. For instance, an antibody could interfere with choline uptake into the nerve terminal, ACh synthesis, storage of ACh or fusion with the nerve membrane and the subsequent exocytosis of the packet's contents. It is

probable that the mechanism of antibody action is associated with a process involving Ca^{2+} since in low calcium concentration solutions, mepp amplitude was reduced to a lesser extent than that recorded in normal calcium concentration solutions. This suggests that when release is low the packets can refill to a normal level of ACh and the resulting mepp has a normal amplitude.

If the antibody acted at a site in the nerve terminal membrane, as many antibodies do, then perhaps the most likely sites of action are either at the release site itself or at the choline uptake mechanism, a carrier-mediated uptake (Potter, 1970). A mechanism interfering with the uptake of choline would in turn influence ACh synthesis. Indeed the hemicholinium drug HC-3 blocks synthesis of ACh by this mechanism. Moreover, this drug causes a progressive run down in epp amplitudes during prolonged stimulation (Elmqvist, Quastel & Thesleff, 1963). KP plasma, however, did not affect epp amplitudes, as compared to controls, and furthermore, stimulation of the phrenic nerve for 30 minutes caused no run down in epp amplitudes. It is thus unlikely that the synthesis of ACh is affected in muscles treated with KP plasma. Any effect of the antibody in KP plasma on the availability of ACh packets for release is also improbable since there was no effect on epp quantal content. It seems possible, therefore, that the antibody in KP plasma may cause a defect in packaging of ACh or fusion with the membrane. Although no firm conclusions can be drawn from this data, labelling (e.g. by fluorescent labels) of the IgG from KP plasma could provide direct evidence of its sites of action.

In summary, injection of KP plasma or IgG into mice transfers a reduction in mepp amplitude and consequently, presumably a defect in epp amplitude in normal solutions. The latter defect was not studied directly at normal Ca^{2+} concentrations due to the presence of different concentrations of tubocurarine in test and controls, as already mentioned. In low Ca^{2+} solutions there was apparently little effect on epp amplitude probably due to the minor effect on mepp amplitude at low Ca^{2+} concentrations. The transferred defect of a reduction in mepp amplitude in normal

solutions indicates an autoimmune basis for this disorder. It is likely that the muscle weakness seen for patient KP was probably due to a reduction in both mepp and epp amplitudes.

Section II : - Patient MA

I. Introduction

In this section a possible role of autoantibodies in the disorder seen in the anti-AChR antibody-negative MG patient MA were investigated. The clinical weakness in this patient was similar to that seen in KP although it was less severe (Osserman grade 2B, Table 2). For this patient the bulbar weakness was most marked, with respiratory and limb weakness and little ocular weakness. A response was seen following plasma exchange.

Experiments described here used passive transfer of MA plasma to mice for 3 or 15 days. This was followed by electrophysiological recordings from the diaphragm muscle of mepps, epps, action potentials and ACh-induced depolarization and its associated voltage noise and by α-BuTx binding studies. Any impairment in neuromuscular transmission in the mice would point to the presence of circulatory hormonal factors such as autoantibodies.

II. Spontaneous Release

Mepps (35-88 at each end-plate) were recorded from mice treated with MA plasma or control plasma (35.0-37.0°C). Mepp amplitudes for MA treated muscles were significantly ($P<0.001$) reduced following 15 day injections but not after 3 days (Table 13). Moreover, there was still a significant ($P<0.001$) reduction at 15 days when mepps had been corrected for any resting potential variations as expected by the lack of difference between test and control resting potentials. This reduction at 15 days suggests the presence of a circulatory factor. The coefficient of variation of mepp amplitude distribution was unaltered in mouse diaphragms following treatment with MA plasma (Table 13). This suggests that MA plasma does not skew

Table 13

Effect of MA Plasma on Mepp Characteristics

Treatment	IgG Content of Daily Injection (mg)	Duration of Treatment (days)	Temp. (°C)	Resting Potential (mV)	MEPP CHARACTERISTICS						No. of End-plates, Mice
					Amplitude		Coefficient of Variation of Amplitude Distribution	Rise Time (ms)	Decay Time (ms)	Frequency (s^{-1})	
					Un-corrected for Resting Potential (mV)	Corrected to Resting Potential of -70mV (mV)					
MA Plasma	14.1	3	35.0-36.5	-64.5 ±0.9	0.81 ±0.07	0.88 ±0.07	0.23 ±0.01	0.24 ±0.01	1.42 ±0.10	4.19 ±0.49	24, 3
Control Plasma	30.0-45.0	3	36.5-37.0	-65.6 ±0.9	0.83 ±0.05	0.90 ±0.05	0.25 ±0.02	0.23 ±0.01	1.36 ±0.07	4.55 ±0.59	38, 5
MA Plasma	7.1	15	36.5-37.0	-66.9 ±0.9	0.56 ±0.06*	0.60 ±0.07*	0.22 ±0.01	0.37 ±0.02*	1.52 ±0.07	4.04 ±0.36+	28, 3
Control Plasma	15.0-22.5	15	36.0-37.0	-69.2 ±0.7	0.82 ±0.03	0.83 ±0.03	0.23 ±0.01	0.28 ±0.01	1.37 ±0.04	7.20 ±0.61	107, 12

Legend for Table 13

This table shows the effect of MA plasma on mepp characteristics. Each value is the mean ± S.E.M. Mepp amplitudes have been corrected to -70mV. The significance level of the difference in mepp amplitude between MA and control plasma was still seen when values for each end-plate were averaged first for each muscle and one value per muscle was used for significance test instead of one value per end-plate. Also, if the same procedure was applied to values of mepp frequency then in this way the mepp frequency in MA plasma treated muscles was no longer significantly different from controls. Significant changes from control: *$P<0.001$; +$P<0.02$.

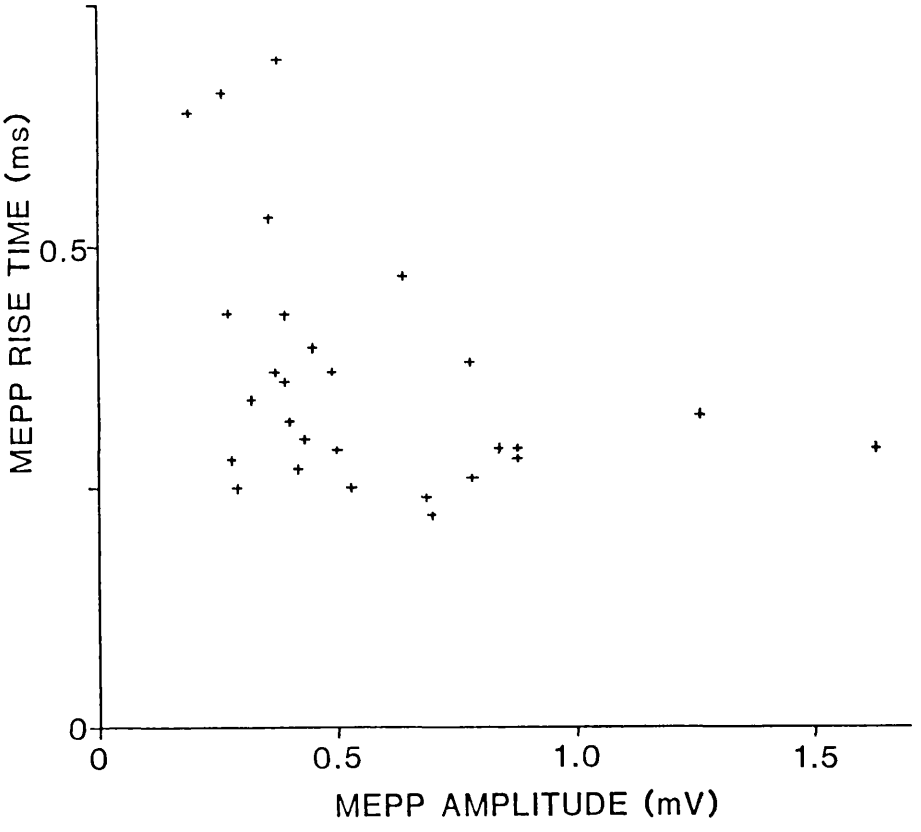
the amplitude distribution histograms. The rise times of the mepps was significantly ($P<0.001$) increased after 15 days injection with MA plasma but not after 3 days (Table 13). As previously described (Section I, part IIa), the source of the increase in rise time at 15 days was investigated, to determine whether it was due to the microelectrode position in relation to the end-plate. Figure 8 is plotted in a similar way to those shown for KP (for control see Section I, Figure 5). The smaller amplitude mepps tended to have larger rise times suggesting that some recordings were, in fact, not made at the end-plate. To minimise this effect, only mepps with a rise time of ≤ 0.4ms were again considered to be at an end-plate. After selecting in this way mepp amplitudes were still significantly ($P<0.01$) reduced, while the rise time was near, but not identical to, the control values (Table 14). There was no significant effect of MA plasma on mepp decay time constant (T) at either 3 or 15 days (Table 13). This suggests that MA plasma did not change the passive properties of the muscle membrane. Mepp frequency was not affected after 3 days treatment but was possibly reduced by MA plasma after 15 day injections (Table 13, see caption).

III. α-BuTx Binding Studies

From α-BuTx binding studies on the hind limbs of 15 day treated animals there was no apparent reduction in the total number of AChRs (Table 15). Moreover, there was no evidence of any anti-AChR Ab bound to the receptor. Such results imply no obvious postsynaptic changes to account for the observed reduction in mepp amplitude.

Figure 8

Mepp Amplitude Versus Mepp Rise Time - MA Plasma Treated Muscles



Legend for Figure 8

This figure shows mepp amplitude plotted against mepp rise time for mice treated with MA plasma for 15 days (36.5-37.0°C). Each cross represents the mean mepp amplitude and mepp rise time for each end-plate recorded from; 28 end-plates, 3 animals. For the respective control figure see Figure 5.

Table 14

Effect of MA Plasma on Selected Mepp Characteristics

Treatment	IgG Content of Daily Injection (mg)	Duration of Treatment (days)	Temperature (°C)	Resting Potential (mV)	MEPP CHARACTERISTICS		No. of End-plates, Mice
					Rise Time (Selecting mepps with rise time ≤0.4ms) (ms)	Amplitude (Selecting mepps with rise time ≤0.4ms) (mV)	
MA Plasma	14.1	3	35.0-36.5	-64.5 ±0.9	0.24 ±0.01	0.81 ±0.07	24, 3
Control Plasma	30.0-45.0	3	36.5-37.0	-65.6 ±0.9	0.23 ±0.01	0.83 ±0.05	38, 5
MA Plasma	7.1	15	36.5-37.0	-67.4 ±1.2	0.30 ±0.01[§]	0.63 ±0.07[Δ]	21, 3
Control Plasma	15.0-22.5	15	36.0-37.0	-68.8 ±0.9	0.27 ±0.01	0.84 ±0.03	101, 12

Legend for Table 14

This table shows mepp rise time and amplitude after selecting mepps with rise times ≤ 0.4ms. At 3 days these values are similar to those shown in Table 14 since no mepps had rise times > 0.4ms. Amplitudes are shown uncorrected for resting potential and all values are means ± S.E.M. Significant changes compared to controls: [§]$P<0.005$; [Δ]$P<0.01$.

Table 15

α-BuTx Binding Studies from Mice Treated with MA Plasma

Treatment	Duration of Treatment (days)	AChR Number (fmol/g)	Anti-AChR Ab Bound (fmol/g)	No. of Muscles
MA Plasma	15	620 ± 47	14.0 ± 7.6	3
Control Plasma	15	627 ± 57	24 ± 22	4

Legend for Table 15

This table shows the effect of MA plasma (1.5ml containing 7.1mg IgG, per day) on the number of AChRs in the postsynaptic membrane and the amount of anti-AChR antibody bound to the receptors. Values shown are means ± S.E.M. averaged from the number of muscles shown. Values for each muscle are the average of three separate assays performed on that muscle. There were no significant differences between MA and control plasma.

IV. End-plate Potentials

Epp recordings (65-111 at each MA plasma treated end-plate) were made at a phrenic nerve stimulation rate of 0.5Hz at 36.0-37.0°C. Tubocurarine was used in all cases at a concentration which just blocked muscle contraction. A lower tubocurarine concentration was used in MA treated muscles but this difference did not attain statistical significance (Table 16). Epp quantal content was not significantly affected by MA plasma at either 3 or 15 days (Table 16). After values for epp quantal content were corrected for deviations from Poisson statistics (see Methods) there was still no significant difference between values for quantal content in test and control muscles.

V. Muscle Action Potentials

Muscle action potentials were recorded from mouse diaphragms (35.0-36.5°C) to determine whether they were affected by MA plasma. Measurements of action potentials would indicate whether the voltage-dependent Na^+ and K^+ channels are functioning normally in the presence of MA plasma and whether the epp is above threshold for action potential production. For muscles treated with MA plasma, following 82% of nerve stimulations at end plates and 83% at the non-end-plates, an action potential resulted. This compared with 70% and 82%, respectively, for control treated muscles and thus results for test and control muscles were not significantly different (Table 17). There was no change in overshoot, duration at half maximal amplitude and maximum rate of rise by plasma MA as compared with controls (Table 17). This shows that the voltage-dependent Na^+ and K^+ channels were not affected by treatment with MA plasma and that the epps were generally, but not always, large enough to trigger action potentials. Hence, although it was not possible to compare epp amplitudes directly from epp recordings (because different tubocurarine concentrations were used in test and control muscles) presumably the

Table 16

Effect of MA Plasma on Epp Quantal Content

Treatment	IgG Content of Daily Injection (mg)	Duration of Treatment (days)	Temperature (°C)	Resting Potential (mV)	Tubocurarine Concentration Required to just Prevent Twitch (μM)	EPP QUANTAL CONTENT		No. of End-plates, Mice
						Uncorrected	Corrected for Deviation from Poisson Statistics	
MA Plasma	14.1	3	36.0-36.5	-65.3 ±0.8	2.7 ±0.5	184 ±17	78.4 ±5.2	24, 3
Control Plasma	30.0-45.0	3	36.5-37.0	-66.2 ±0.8	2.6 ±0.1	227 ±16	91.6 ±4.9	28, 5
MA Plasma	7.1	15	36.5-37.0	-66.7 ±1.1	3.3 ±0.3	242 ±29	95.3 ±8.0	19, 3
Control Plasma	15.0-22.5	15	36.0-37.0	-69.0 ±0.8	4.0 ±0.4	201 ±12	82.7 ±3.7	66, 11

Legend for Table 16

This table shows epp quantal content, derived using the variance method because all recordings were made in a solution of 2.52mM Ca^{2+}, measured from MA plasma and control plasma treated animals. Values have also been corrected to account for deviation from Poisson statistics. Resting potentials were not significantly different. Values shown are expressed as means ± S.E.M. There were no significant differences between the values for quantal content recorded from test and control muscles.

Table 17

Effect of MA Plasma on Muscle Action Potentials

Treatment	Temperature (°C)	Resting Potential (mV)	No. of Failures	ACTION POTENTIAL PARAMETERS			No. of Fibres, Mice
				Overshoot (mV)	Duration at Half Maximal Amplitude (ms)	Maximum Rate of Rise (Vs^{-1})	
END-PLATE							
MA Plasma	35.0-36.5	-69.6 ±1.2	5	8.2 ±2.2	0.50 ±0.02	363 ±22	23, 4
Control Plasma	36.5	-71.0 ±2.7	3	1.5 ±1.3	0.52 ±0.03	334 ±20	7, 1
NON-END-PLATE							
MA Plasma	35.0-36.5	-67.4 ±1.1	5	7.2 ±2.5	0.47 ±0.02	342 ±21	24, 4
Control Plasma	36.5	-71.6 ±2.8	1	4.6 ±1.6	0.53 ±0.04	362 ±35	7, 1

Legend for Table 17

This table shows values obtained for action potential parameters from mice treated with MA plasma (1.5ml containing 14.1mg IgG, per day) or control plasma (30.0-45.0mg IgG, per day) for 3 days. Values shown are means ± S.E.M. averaged from the number of fibres shown. Recordings were made at end-plate and non-end-plate regions. There were no significant differences between parameters recorded in MA treated muscles compared to controls or between values at end-plates and non-end-plates.

epps would have been reduced because the mepps were reduced, at least at 15 days. However, the extent of the reduction in mepp amplitude at 3 days was negligible in MA treated muscles compared with controls (Table 13) and thus presumably not below the safety factor which would usually be able to prevent action potential firing.

Interestingly, there were no significant differences between action potential parameters recorded at an end-plate (determined by the presence of fast rise time mepps) or away from the end-plate for both MA treated and control muscles (Table 17). At the end-plate region the amplitude of the action potential would be expected to be smaller because the end-plate potential has already driven the cell towards the ACh reversal potential. The lack of difference in amplitude thus suggests that the effect of the AChR channels has been swamped by the Na^+ and K^+ channels underlying the production of the action potential. The epp thus also appeared to be swamped by the action potential leaving measured parameters of the action potential essentially unaffected by the presence of the epp. Finally, the duration of the action potential at half maximal time was not prolonged at the end-plate region since presumably the ACh channels have closed during the half maximal time, so the action potential was not prolonged and the decay of the action potential was not affected.

VI. Discussion

Passive transfer of plasma from this patient to mice caused a reduction in mepp amplitude after 15 though not 3 days and a reduction in the amount of tubocurarine required to just block muscle twitch. Plasma MA had little or no effect on other mepp characteristics or on α-BuTx binding and there was no detectable anti-AChR Ab bound to the receptor. Furthermore, the epp quantal content and muscle action potential characteristics were unchanged. The significant reduction in mepp

amplitude and change in tubocurarine sensitivity in the mice treated with MA plasma were similar to those for patient KP and are again suggestive of a circulating plasma factor.

The slow onset of the reduction in mepp amplitude (between 3 and 15 days) could be attributed to two possible reasons: (i) a time lag before the antibody affects neuromuscular transmission once it has bound or (ii) a low concentration of the active circulating factor in the plasma so that the effects can not be seen until after more than 3 days injections when adequate binding is produced.

As for patient KP, the reduction in mepp amplitude was not accompanied by a change in the number of AChRs, indicating lack of postsynaptic action and distinguishing if from MG with anti-AChR antibody (Section I, Discussion). There was also no clear alteration in quantal content or mepp frequency showing that there were no changes in the number of packets of ACh released either following nerve stimulation or spontaneously. As discussed for patient KP the reduction in mepp amplitude appears to come about by a reduction in the number of molecules of ACh released per packet thus reducing the number which subsequently act on the AChRs.

Again, as for patient KP, the decreased mepp amplitude together with normal quantal content suggests that a decreased epp amplitude could underlie this muscle weakness disorder in this patient. A direct comparison of epp amplitudes was not possible because recordings were made in the presence of different concentrations of tubocurarine. However, action potentials were not always triggered in the mice suggesting that epps were probably reduced by MA plasma. It is likely that the muscle weakness in this patient himself was due to a greater reduction in epp amplitude; this could have been because of better cross-reactivity of the circulating factor, presumably antibody, in the patient compared to that in the mouse.

Section III : - Patient LW

I. Introduction

This patient was diagnosed as having MG by routine electromyography (EMG) and edrophonium tests. Moreover, anti-AChR antibodies were absent as shown by normal radioimmunoassay (Table 2) although the patient responded to plasma exchange.

In the experiments described in this section, electrophysiological recordings were made from the diaphragm muscle of mice treated with the plasma and IgG fraction of the plasma from patient LW. The purpose of this was to again investigate possible involvement of autoantibodies in this patient's disorder.

II. Spontaneous Release

Recordings of mepps (35-67 at each end-plate) were made from control and LW treated animals (21.5-24.0°C; 36.0-37.0°C). The mepp amplitude was significantly ($P<0.005$) reduced by LW IgG at 3 days but was not changed by LW plasma at 15 days (Table 18). The lack of reduction with plasma probably occurred because much smaller quantities of IgG were injected into the mice via plasma at 15 days than at 3 days (Table 18). The coefficient of variation of amplitude distribution gives an indication of the distribution of the mepp amplitudes around the mean. LW IgG and LW plasma had no effect on this variable (Table 18) suggesting that mepp amplitudes were still normally distributed about the mean after such treatment. There were no significant changes in rise time (Table 18) but there was a small but significant ($P<0.001$) reduction in T at 3 days but not 15 days (Table 18). This

Table 18

Effect of LW IgG and Plasma on Mepp Characteristics

Treatment	IgG Content of Daily Injection (mg)	Duration of Treatment (days)	Temp. (°C)	Resting Potential (mV)	MEPP CHARACTERISTICS						No. of End-plates, Mice
					Amplitude		Coefficient of Variation of Amplitude Distribution	Rise Time (ms)	Decay Time (ms)	Frequency (s^{-1})	
					Un-corrected for Resting Potential (mV)	Corrected to Resting Potential of -70mV (mV)					
LW IgG	60	3	23.0-24.0	-66.6 ±1.3	0.84 ±0.06[§]	0.87 ±0.05[¤]	0.26 ±0.01	0.56 ±0.03	2.27 ±0.08[*]	0.84 ±0.06	28, 4
Control IgG	60	3	21.5-23.6	-66.1 ±1.1	1.09 ±0.06	1.16 ±0.07	0.25 ±0.01	0.62 ±0.03	2.83 ±0.10	0.78 ±0.06	27, 4
LW Plasma	4.1	15	36.0-36.5	-67.7 ±1.0	0.77 ±0.06	0.80 ±0.06	0.23 ±0.01	0.28 ±0.01	1.23 ±0.04	8.8 ±1.3	30, 3
Control Plasma	15.0-22.5	15	36.0-37.0	-69.2 ±0.7	0.82 ±0.03	0.83 ±0.03	0.23 ±0.01	0.28 ±0.01	1.37 ±0.04	7.20 ±0.61	107, 12

Legend for Table 18

This table shows the effect of LW IgG and plasma on mepp characteristics. Each value is the mean ± S.E.M. Resting potentials were not significantly different. Values were averaged using one value per end-plate; if one value per muscle was first obtained and used in the statistical analysis, the significance level of the reduction of mepp amplitudes in LW treated muscles was unchanged. Significant changes from control: $^{*}P<0.001$; $^{¤}P<0.002$; $^{§}P<0.005$.

reduction probably comes about, as for KP plasma, by a non-specific effect of the IgG antibody on passive membrane properties. Finally, the mepp frequency was not altered by injection with either LW IgG or plasma as compared to respective controls (Table 18). Thus the number of packets of ACh released spontaneously was not affected by LW IgG or plasma. The increase in mepp frequencies at around 37°C compared to room temperature is expected for this parameter.

III. α-BuTx Binding Studies

The hind limbs of the mice treated for 15 days with LW plasma were used in α-BuTx binding studies. There was no change in the total number of AChRs in the muscles and no detectable anti-AChR antibody (Table 19). This suggests an absence of any action on the postsynaptic AChRs. However, there was no reduction in mepp amplitudes at 15 days; the origin of the observed reduction in mepp amplitudes at 3 days may also be attributed to a non-postsynaptic action, but unfortunately no further studies were carried out in mice treated with LW plasma for 3 days.

IV. End-plate Potentials

Epps (76-125 at each end-plate) were recorded from muscles treated with LW IgG or plasma and with respective control IgG or plasma (21.5-23.4°C; 36.0-37.0°C). Tubocurarine was present to just block muscle twitch.

There were reductions in the concentration of tubocurarine which was used to just block muscle contractions in LW treated muscles as compared to controls (Table 20). This reduction was significant ($P<0.001$) at 3 days, presumably due to the higher amount of IgG that was injected into the mice per day (see above). These

Table 19

α-BuTx Binding Studies from Mice Treated with LW Plasma

Treatment	Duration of Treatment (days)	AChR Number (fmol/g)	Anti-AChR Ab Bound (fmol/g)	No. of Muscles
LW Plasma	15	730 ± 116	49 ± 66	2
Control Plasma	15	627 ± 57	24 ± 22	4

Legend for Table 19

This table shows the effect of LW plasma (1.5ml containing 4.1mg IgG, per day) on the amount of anti-AChR Ab bound to AChRs and the total number of AChRs. Values shown are means ± S.E.M. averaged from the number of muscles shown. Values for each muscle were obtained from the mean of three separate assays on each muscle extract.

Table 20

Effect of LW IgG and Plasma on Epp Quantal Content

Treatment	IgG Content of Daily Injection (mg)	Duration of Treatment (days)	Temperature (°C)	Resting Potential (mV)	Tubocurarine Concentration Required to just Prevent Twitch (μM)	EPP QUANTAL CONTENT		No. of End-plates, Mice
						Uncorrected	Corrected for Deviation from Poisson Statistics	
LW IgG	60	3	22.6-23.4	-68.7 ±1.2	2.7 ±1.0*	123 ±11	58.2 ±3.9	21, 3
Control IgG	60	3	21.5-22.6	-67.5 ±1.2	4.0 ±0.1	129 ±15	59.8 ±5.1	22, 4
LW Plasma	4.1	15	36.0-36.5	-66.9 ±1.1	3.4 ±0.5	164 ±14	72.2 ±4.5	23, 3
Control Plasma	-	15	36.0-37.0	-69.0 ±0.8	4.0 ±0.4	201 ±12	82.7 ±3.7	66, 11

Legend for Table 20

This table shows the effect of LW IgG and plasma on epp quantal content. Values shown are means ± S.E.M. Epp quantal content was derived using the variance method; all recordings were made in a solution containing 2.52mM Ca^{2+}. Significant reductions compared to controls: *$P<0.001$.

reductions are themselves suggestive of a disorder in neuromuscular transmission in animals treated with LW IgG. Epp quantal content was not significantly affected by LW IgG or plasma at either 3 or 15 days (Table 20). These results suggest that LW IgG and plasma do not affect evoked quantal release. The raised temperature used at 15 days accounts for the increase in both test and control values of quantal content, compared to those values at 3 days.

Values for epp amplitudes were not compared because tubocurarine was increased to a level which just blocked muscle twitch and different concentrations were used in LW treated and control muscles (see Table 20).

V. Discussion

This chapter describes experiments which showed a change in the electrophysiological features in the mouse following passive transfer of LW IgG or LW plasma. There were decreases in mepp amplitude and increases in tubocurarine sensitivity at least for mice treated for 3 days with LW IgG. There was little change in any of the other measured mepp characteristics except perhaps for a non-specific effect on passive membrane properties at 3 days. There was also no change in the number of AChRs, no detectable Ab bound to the AChR and no change in the quantal content.

As already stated above, mepp amplitude and tubocurarine sensitivity were only altered in mice treated with LW IgG for 3 days. Longer treatment with much lower effective doses of IgG in the plasma had no effect. Plasma is obtained from patients undergoing plasma exchange; their plasma is continually removed and replaced by plasma substitute (Pinching et al., 1976). Successive plasma exchanges contain progressively less pathogenic antibody and since these experiments used LW plasma from the third plasma exchange there was little circulating antibody in it.

Moreover, since only a small amount of plasma can be injected into the mice the amount of IgG given daily to the 15 day treated animals was low. The general lack of effect on mepp characteristics for 15 day LW plasma treated animals is thus probably explained by the very low level of pathogenic antibody in the plasma.

The reduction in mepp amplitude is probably due to the same mechanism as has been suggested for patients KP and MA and can be attributed to a reduction in the number of molecules of ACh in a packet since there was no evidence of any postsynaptic change in AChRs or any Ab bound to them. Unfortunately, no recordings of ACh-induced depolarizations and the associated increase in voltage noise were made. This would have demonstrated any change in postsynaptic sensitivity to ACh or any change in ACh channel properties validating the above suggestion. The reduced mepp amplitude together with the normal quantal content implies that the epp amplitudes were also reduced and this might have led to the muscle weakness seen in this patient; epps being below the threshold for muscle contraction.

Section IV : - Patient KO

I. Introduction

The purpose of this section was to examine whether the plasma or the IgG fraction of the plasma from antibody-negative MG patient KO can impair neuromuscular transmission in the mice following passive transfer. This patient had those features of muscle weakness similar to that seen in the other patients of this Osserman grade, and also responded to plasma exchange (Table 2).

Mice were injected for 3 to 15 days with either the plasma or the IgG fraction of the plasma from patient KO and electrophysiological recordings and biochemical studies carried out. Furthermore, for this patient the effects of plasma and of IgG were directly compared when mice were injected for the same period of time (7 days).

II. Spontaneous Release

(a) Spontaneous Release in Normal Calcium Concentration Solutions

Mepps (36-88 at each end-plate) were record from mice treated with KO or control plasma or KO or control IgG (35.0-37°C). Mepp amplitudes were not reduced by KO plasma at 3 days, but they were significantly ($P<0.001$) reduced in KO plasma and IgG treated muscles as compared to the appropriate controls at 7 and 15 days (Table 21, Figure 9). It is important to note that the reduction in mepp amplitude in KO treated muscles at 7 days is similar when either plasma or the IgG fraction was used; there was no significant difference in mepp amplitude between KO plasma and KO IgG or between control plasma and control IgG. Moreover, the plasma contained a similar amount of IgG as given in the IgG fraction injection (Table 21). This suggests that the active factor of KO plasma was contained in the

Table 21

Effect of KO Plasma and IgG on Mepp Characteristics

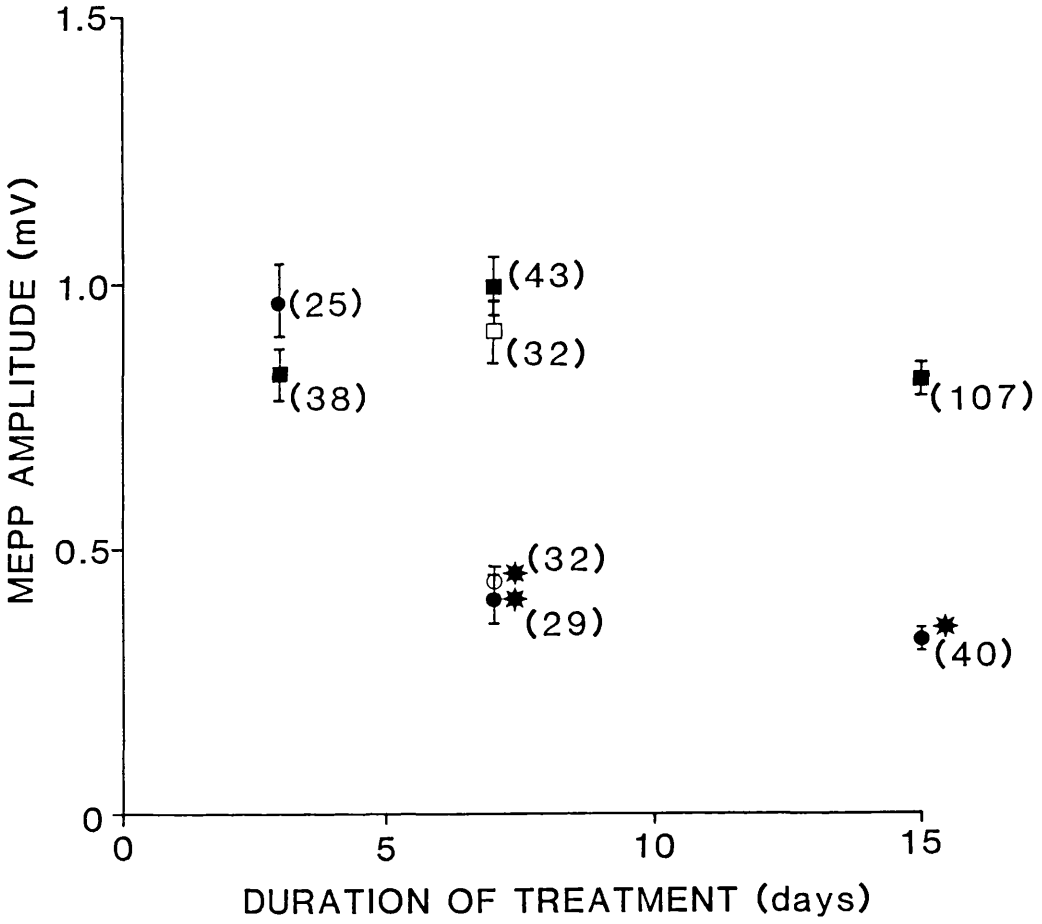
Treatment	IgG Content of Daily Injection (mg)	Duration of Treatment (days)	Temp. (°C)	Resting Potential (mV)	MEPP CHARACTERISTICS						No. of End-plates, Mice
					Amplitude		Coefficient of Variation of Amplitude Distribution	Rise Time (ms)	Decay Time (ms)	Frequency (s^{-1})	
					Un-corrected for Resting Potential (mV)	Corrected to Resting Potential of -70mV (mV)					
KO Plasma	21.90	3	36.0-37.0	-68.8 ±1.0$^{\#}$	0.97 ±0.07	0.99 ±0.08	0.24 ±0.01	0.29 ±0.02*	1.44 ±0.07	3.43 ±0.34	25, 3
Control Plasma	30.0-45.0	3	36.5-37.0	-65.6 ±0.9	0.83 ±0.05	0.90 ±0.05	0.25 ±0.02	0.23 ±0.01	1.36 ±0.07	4.55 ±0.59	38, 5
KO Plasma	12.0	7	36.0-37.0	-72.9 ±1.0^{+}	0.42 ±0.04*	0.39 ±0.03*	0.24 ±0.01	0.39 ±0.04$^{\Delta}$	1.52 ±0.06	6.42 ±0.73	29, 4
Control Plasma	15.0-22.5	7	35.0-36.5	-69.5 ±0.9	1.00 ±0.05	1.02 ±0.06	0.27 ±0.01	0.30 ±0.01	1.51 ±0.06	7.58 ±0.48	43, 6
KO IgG	15.0	7	35.5-37.0	-68.0 ±1.2	0.44 ±0.04*	0.45 ±0.03*	0.25 ±0.01	0.35 ±0.03$^{\#}$	1.41 ±0.06	6.03 ±0.59	32, 4
Control IgG	15.0	7	35.5-36.5	-65.6 ±1.1	0.91 ±0.06	0.99 ±0.07	0.26 ±0.02	0.27 ±0.10	1.30 ±0.07	5.41 ±0.43	32, 4
KO Plasma	12.0	15	35.0-37.0	-68.6 ±0.8	0.33 ±0.02*	0.34 ±0.02*	0.30 ±0.01*	0.39 ±0.02*	1.21 ±0.05$^{\#}$	5.18 ±0.67	40, 5
Control Plasma	15.0-22.5	15	36.0-37.0	-69.2 ±0.7	0.82 ±0.03	0.83 ±0.03	0.23 ±0.01	0.28 ±0.01	1.37 ±0.04	7.20 ±0.61	107, 12

Legend for Table 21

This table shows mepp characteristics for animals treated with KO plasma and IgG or appropriate controls. Values shown are means ± S.E.M. Mepp amplitudes have been corrected to a resting potential of -70mV. Statistics were carried out using one value per end-plate. When values were first averaged for each muscle and one value used per muscle for statistical analysis, mepp amplitudes were still significantly reduced. Significant changes from respective controls: $^{*}P<0.001$; $^{\#}P<0.05$; $^{+}P<0.02$; $^{\Delta}P<0.01$.

Figure 9

Effect of KO Plasma and IgG on Mepp Amplitude



Legend for Figure 9

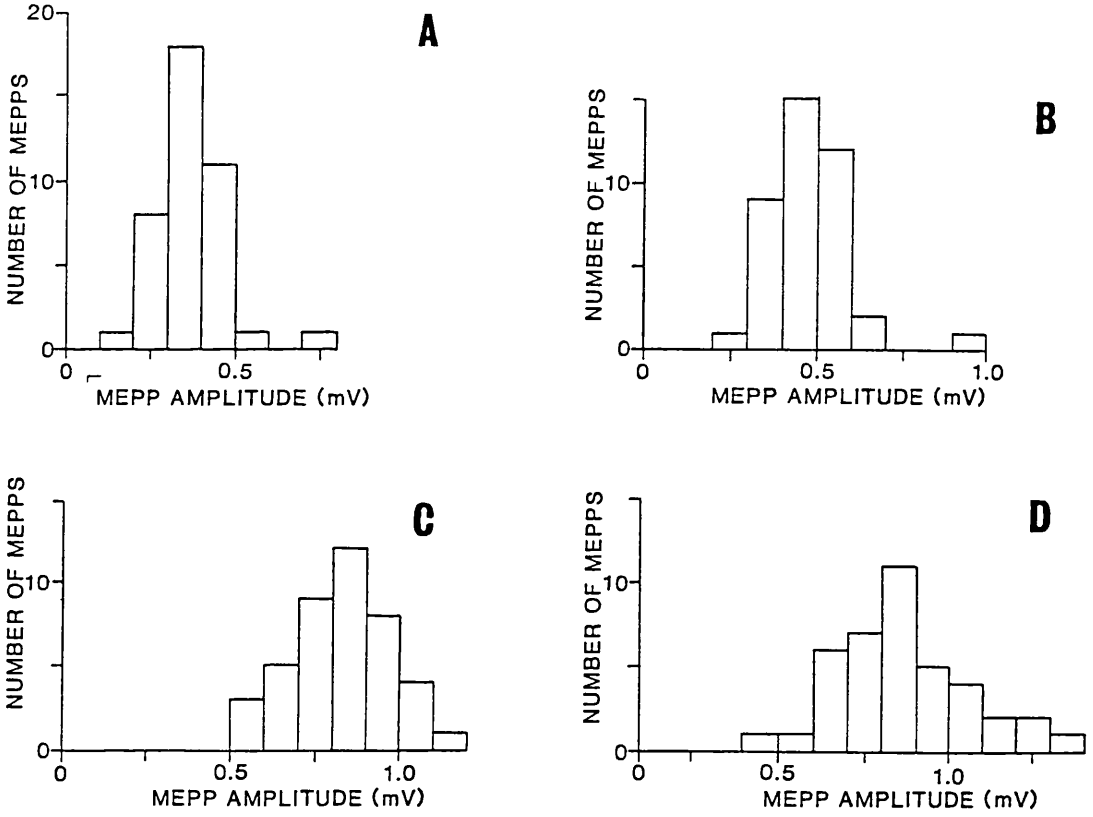
This figure shows a plot of mepp amplitude against duration of treatment recorded at 35.0-37.0 °C. Values shown are means ± S.E.M., averaged from the number of end-plates shown in brackets (data also shown in Table 21). Control plasma (■), KO plasma (●), control IgG (❑) and KO IgG (○) were injected into mice for 3, 7 or 15 days. Significant reductions compared to controls: *P<0.001.

IgG fraction of the plasma. The statistical significance of the reductions were unchanged following corrections of the mepp amplitude for any variations in resting potential (Table 21). Mepp amplitude histograms for both test and control muscles were studied to investigate any effect of the KO plasma or IgG on amplitude distributions. As is seen in Figure 10, which shows typical mepp amplitude histograms for KO plasma and IgG treated end-plates compared to respective controls, mepp amplitude distributions were bell-shaped. Moreover, there was no effect on the coefficient of variation of mepp amplitude distributions except at 15 days when there was a small but significant increase in the value in KO treated muscles compared to controls (Table 21). Thus KO IgG or plasma generally had little effect on mepp amplitude distributions and so it seems likely from these distributions that mepps were not usually being lost below the level of resolution of these experiments (around 0.1mV) which could cause skewing.

At all durations of treatment, KO IgG plasma produced significant increases in mepp rise time compared to controls (Table 21). To investigate whether this could have come about by sampling away from end-plates, a graph of mepp amplitudes versus rise time from 15 day treated animals was obtained as in previous sections (Figure 11). It can be seen that smaller amplitudes were associated with longer rise times. The reason that mepp amplitudes are small when rise times are slow is presumably due to recordings away from end-plates as described in previous sections. Recordings with longer rise times were probably made more frequently from KO treated muscles because the amplitudes were generally smaller. Hence, it is more difficult to visually select out fast rise time mepps when the mepps are small because the rise times of the mepps are often distorted by the background noise. However, selecting out mepps with rise times $\leq$0.4ms should help eliminate this problem (see previous sections). There were still significant reductions, after 7 and 15 days treatment with KO plasma, in mepp amplitude after selecting mepps in this way (Table 22). Furthermore, there was again no significant difference in values of

Figure 10

Effect of KO Plasma and IgG on Mepp Amplitude Histogram Distributions

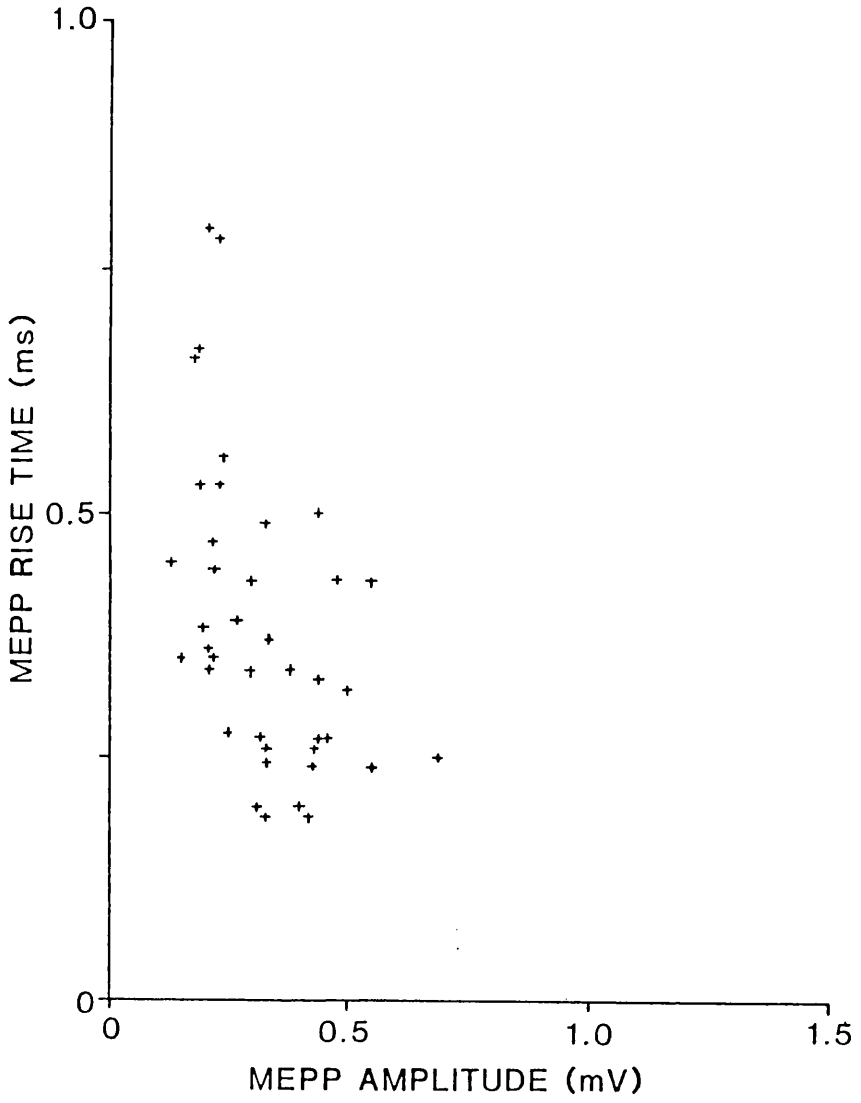


Legend for Figure 10

This figure shows mepp amplitude histogram distributions from sample fibres (35.0 - 37.0°C) recorded from 7 day treated animals. Treatments were: (A) KO plasma; (B) KO IgG; (C) Control plasma; (D) Control IgG. Vertical axis : Number of mepps; Horizontal axis : Mepp amplitude (mV). Each histogram represents 40 mepps.

Figure 11

Mepp Amplitude Versus Mepp Rise Time - KO Plasma Treated Muscles



Legend for Figure 11

This figure shows mepp amplitude plotted against rise time for mice treated with KO plasma for 15 days (35.0-37.0°C). Each cross represents the mean mepp amplitude and mepp rise time for each end-plate recorded from; 40 end-plates, 5 animals. For the respective control figure see Figure 5.

Table 22

Effect of KO Plasma and IgG on Selected Mepp Characteristics

Treatment	IgG Content of Daily Injection (mg)	Duration of Treatment (days)	Temperature (°C)	Resting Potential (mV)	MEPP CHARACTERISTICS		No. of End-plates, Mice
					Rise Time (Selecting mepps with rise time ≤0.4ms) (ms)	Amplitude (Selecting mepps with rise time ≤0.4ms) (mV)	
KO Plasma	21.9	3	36.0-37.0	-68.8 ±1.1[#]	0.27 ±0.01[□]	0.99 ±0.08	23, 3
Control Plasma	30.0-45.0	3	36.5-37.0	-65.6 ±0.9	0.23 ±0.01	0.83 ±0.05	38, 5
KO Plasma	12.0	7	36.0-37.0	-74.1 ±1.2[□]	0.28 ±0.01	0.48 ±0.04[*]	20, 4
Control Plasma	15.0-22.5	7	35.0-36.5	-69.1 ±0.9	0.29 ±0.01	1.02 ±0.06	40, 6
KO IgG	15.0	7	35.5-37.0	-68.1 ±1.4	0.25 ±0.01	0.50 ±0.03[*]	23, 4
Control IgG	15.0	7	35.5-36.5	-65.6 ±1.1	0.27 ±0.01	0.91 ±0.06	32, 4
KO Plasma	12.0	15	35.0-37.0	-68.1 ±1.2	0.29 ±0.01	0.36 ±0.03[*]	25, 5
Control Plasma	15.0-22.5	15	36.0-37.0	-68.8 ±0.9	0.27 ±0.01	0.84 ±0.03	101, 12

Legend for Table 22

This table shows the effect of KO IgG and plasma on mepp rise time and amplitude after selecting mepps with a rise time ≤0.4ms. Values shown are means ± S.E.M. Significant changes from control values: [*]P<0.001; [□]P<0.002; [#]P<0.05.

mepp amplitude recorded from muscles treated for 7 days with either KO plasma or KO IgG and both were reduced compared with the respective controls. However, rise times were not affected by KO plasma or IgG after selecting mepps (except for a small effect at 3 days), suggesting that the above effect on rise time was simply due to recording techniques. Mepp decay times were generally not significantly affected by KO plasma or IgG except, perhaps, for the small reduction at 15 days (Table 21). This suggests that after 15 days there may be a small effect of KO plasma on passive membrane properties. Finally, mepp frequency was not affected by treatment with KO plasma or IgG following any of the periods of injection studied (Table 21).

To further clarify the effect of KO plasma and IgG on mepp amplitude, physostigmine (3μM) was used to increase the amplitude and hence improve the resolution of mepps in 7 day treated animals. Surprisingly, under these conditions there was no reduction in mepp amplitude (Table 23). Mepp amplitude histograms in the presence of physostigmine were similar for test and control muscles (Figure 12) and there was no change in the coefficient of variation of mepp amplitude distributions (Table 23). Moreover, KO plasma and IgG had a similar action on this parameter. In the presence of physostigmine there was a small but significant decrease in rise time by KO IgG although there was no significant effect of KO plasma on rise time. Finally, in the presence of physostigmine there was no effect on decay time but there was a possible reduction in frequency in the KO plasma treated muscle compared to controls (Table 23).

<u>(b) Spontaneous Release in Low Calcium Concentration Solutions</u>

In low calcium concentrations (35.0-37.0°C), mepps (42-50 at each end-plate) were recorded simultaneously with recordings of epps since no tubocurarine was present. KO plasma and IgG reduced mepp amplitudes compared to controls (Table 24). However, the reductions in mepp amplitude were small and only statistically

Table 23

Effect of KO Plasma and IgG on Mepp Characteristics in the Presence of Physostigmine (3μM)

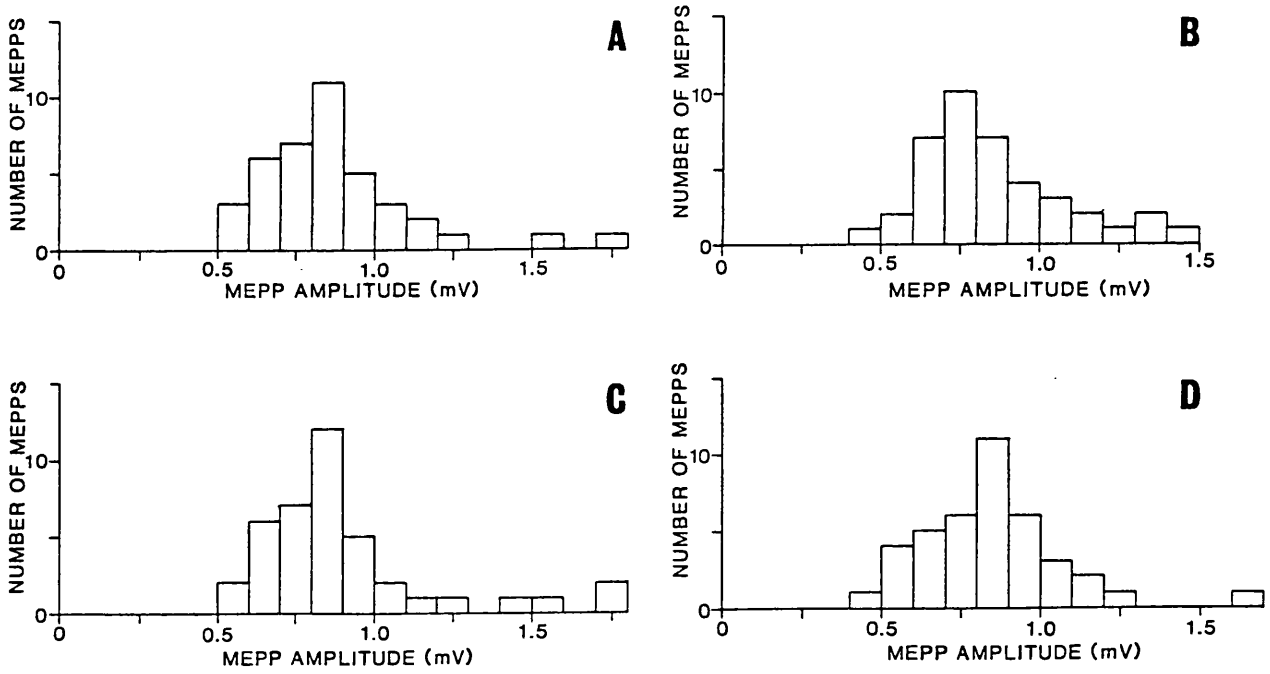
Treatment	IgG Content of Daily Injection (mg)	Duration of Treatment (days)	Temp. (°C)	Resting Potential (mV)	MEPP CHARACTERISTICS						No. of End-plates, Mice
					Amplitude		Coefficient of Variation of Amplitude Distribution	Rise Time (ms)	Decay Time (ms)	Frequency (s^{-1})	
					Un-corrected for Resting Potential (mV)	Corrected to Resting Potential of -70mV (mV)					
KO Plasma	12.0	7	36.0-37.0	-69.0 ±1.3	1.10 ±0.09	1.12 ±0.10	0.31 ±0.01	0.42 ±0.03	3.00 ±0.13	3.55 ±0.44*	25, 3
Control Plasma	15.0-22.5	7	35.0-36.5	-68.8 ±1.0	1.34 ±0.09	1.36 ±0.08	0.32 ±0.02	0.47 ±0.02	3.20 ±0.12	7.87 ±0.86	40, 6
KO IgG	15.0	7	35.5-37.0	-66.1 ±1.2	0.96 ±0.07	1.02 ±0.08	0.32 ±0.01	0.34 ±0.02+	2.64 ±0.08	6.71 ±0.60	31, 4
Control IgG	15.0	7	35.5-36.5	-66.5 ±1.2	1.08 ±0.06	1.15 ±0.07	0.31 ±0.02	0.45 ±0.03	2.79 ±0.10	7.16 ±0.62	31, 4

Legend for Table 23

This table shows the effect of KO plasma and IgG on mepp characteristics measured in the presence of physostigmine. The effect on amplitude, coefficient of variation of amplitude distributions, rise time, decay time and frequency are shown. If one average value is obtained for frequency for each muscle and used in the significance test (to account for scatter between end-plates) then there is no significant difference (KO plasma 3.85 ± 0.78s^{-1}, 3 muscles; control plasma 8.0 ± 1.5s^{-1}; 6 muscles). Values for mepp characteristics recorded in the absence of physostigmine but from the same end-plates as shown here, are given in Table 22. Values shown are means ± S.E.M. Significant difference compared to controls: *$P<0.001$; +$P<0.02$.

Figure 12

Effect of KO Plasma and IgG on Mepp Amplitude Histogram Distributions in Presence of Physostigmine



Legend for Figure 12

This figure shows mepp amplitude histogram distributions from sample fibres (35.0-37.0°C) recorded from 7 day treated animals in the presence of physostigmine. Treatments were: (A) KO plasma; (B) KO IgG; (C) Control plasma; (D) Control IgG. Vertical axis : Number of mepps; Horizontal axis : Mepp amplitude (mV). Each histogram represents 40 mepps.

Table 24

Effect of KO Plasma and IgG on Mepp Characteristics Recorded in Low Ca^{2+} Concentrations

Treatment	IgG Content of Daily Injection (mg)	Duration of Treatment (days)	Temperature (°C)	Ca^{2+} Concentration (mM)	Resting Potential (mV)	MEPP CHARACTERISTICS		No. of End-plates, Mice
						Amplitude Uncorrected for resting potential (mV)	Frequency (s^{-1})	
KO Plasma	12.0	7	35.5-37.0	0.20	-67.3 ±0.9	0.61 ±0.04*	2.66 ±0.42	29, 4
Control Plasma	15.0-22.5	7	35.0-36.5	0.20	-67.0 ±1.0	0.83 ±0.05	2.20 ±0.28	42, 6
KO IgG	15.0	7	35.5-37.0	0.20	-65.8 ±0.9	0.66 ±0.06	3.7 ±1.1	24, 3
Control IgG	15.0	7	35.5-36.5	0.20	-65.7 ±1.0	0.70 ±0.10	2.16 ±0.39	31, 4
KO Plasma	12.0	7	35.5-37.0	0.24	-68.1 ±1.6	0.65 ±0.05+	3.06 ±0.74	19, 3
Control Plasma	15.0-22.5	7	35.0-36.5	0.24	-67.2 ±1.1	0.87 ±0.06	6.2 ±1.1	38, 5
KO IgG	15.0	7	35.5-37.0	0.24	-62.2 ±0.6	0.65 ±0.05	3.88 ±0.97	20, 3
Control IgG	15.0	7	35.5-36.5	0.24	-62.6 ±0.6	0.71 ±0.05	5.24 ±0.87	29, 4

Legend for Table 24

This table shows mepp amplitude and frequency for mepps recorded in low Ca^{2+} concentration solutions. Mepp amplitudes are uncorrected for resting potential since there were no significant differences between resting potentials. Values shown are means ± S.E.M. Significant reductions compared to controls: *$P<0.001$; +$P<0.02$.

significantly reduced for KO plasma. As expected from recordings made in normal Ca^{2+} solutions, neither KO plasma nor IgG had an effect on mepp frequency in low Ca^{2+} concentration solutions (Table 24).

III. Noise Analysis

Possible effects of KO plasma (following 15 day injections) on the postsynaptic membrane were investigated by bath application of ACh (5μM). The ACh-induced depolarization of the membrane and associated increase in voltage noise were recorded as before. Power spectra of voltage noise for both test and control muscles were always well fitted by a single Lorentzian curve. The average values (Table 25) from such experiments show that KO plasma had no significant effects on either the maximum depolarization (V_{max}), the depolarization produced by a single channel opening (a), the maximum channel opening frequency (n_{max}) or the noise time constant (T_m). It is interesting to note, however, that V_{max} and n_{max} were not reduced; any slight reduction did not attain statistical significance. Further experiments would be necessary to clarify this. However, the present data indicates that KO plasma has no clear effect on postsynaptic sensitivity; the doses used being the same as those that gave reductions in mepp amplitude.

IV. α-BuTx Binding Studies

Using the hind limbs of mice treated for 15 days with KO plasma, possible changes in the postsynaptic membrane were investigated by α-BuTx binding studies. There was no significant effect of KO plasma on the number of AChRs and the negative value in KO plasma treated muscles may be explained as described previously for the same observation in KP plasma treated muscles. There was no

Table 25

Effect of KO plasma on Channel Properties Measured by Noise Analysis

Treatment	Temperature (°C)	Resting Potential (mV)	V_{max} (mV)	a (μV)	n_{max} ($10^7 s^{-1}$)	T_m (ms)	No. of End-plates, Mice
KO Plasma	22.8-23.0	-68.0 ±2.1	12.7 ±2.7	0.30 ±0.08	2.38 ±0.15	3.2 ±1.0	9, 4
Control Plasma	22.8-23.8	-69.9 ±1.6	18.4 ±1.6	0.24 ±0.03	5.38 ±0.89	2.60 ±0.18	17, 7

Legend for Table 25

This table shows the effect of KO plasma (1.5ml containing 12.0mg IgG, per day) on channel properties recorded from mice treated for 15 days. Values shown are means ± S.E.M. (using one value per end-plate) averaged from the number of end-plates shown. In all cases the concentration of ACh was 5μM, physostigmine 3μM and tetrodotoxin 250nM. There were no significant differences from control values.

evidence of any bound anti-AChR antibody (Table 26). However, there was considerable scatter in the results for receptor number and further experiments would have been desirable.

These data taken together do, however, lend further support to the above evidence for lack of postsynaptic action of KO plasma at similar doses to those which gave marked reductions in mepp amplitude.

V. End-plate Potentials

Epps (63-119 at each end-plate) were recorded from muscles treated with KO plasma or IgG or with control plasma or IgG, in solutions containing low or normal concentrations of Ca^{2+}. For those recordings with a normal Ca^{2+} concentration (2.52mM) solution, tubocurarine was present. The average concentration used to just prevent twitch in KO plasma treated muscles compared to controls was significantly ($P<0.001$) decreased at 15 days (Table 27). This difference is indicative of an impairment of neuromuscular transmission after 15 days. At 3 days, when mepps were not reduced by KO plasma compared with controls (Table 21) tubocurarine concentration was not decreased.

KO plasma and IgG caused reductions in quantal content at all durations of treatment and these were statistically significant in almost all cases, except those recorded at 3 days (Table 27). The lack of significant reduction in quantal content at 3 days suggests that there is a time lag in the effectiveness of the injection. The time lag could come about either by a time lag to build up an effective concentration of IgG or the time required to produce an effect once bound to the effector site. The significant ($P<0.001$) reduction in quantal content by KO plasma at 15 days was still seen after correcting for deviation from Poisson statistics (Table 27).

Table 26

α-BuTx Binding Studies from Mice Treated with KO Plasma

Treatment	Duration of Treatment (days)	AChR Number (fmol/g)	Anti-AChR Ab Bound (fmol/g)	No. of Muscles
KO Plasma	15	408 ± 69	-11.5 ± 4.1	3
Control Plasma	15	627 ± 57	24 ± 22	4

Legend for Table 26

This table shows the effect of KO plasma (1.5ml containing 12.0mg IgG, per day) on AChR number and the amount of anti-AChR Ab bound onto the receptors. Values shown are means ± S.E.M. averaged from the number of muscles shown. Values for each muscles were obtained from the mean of 3 separate assays on each muscle extract. There were no significant changes from controls in KO treated muscles.

Table 27

Effect of KO Plasma and IgG on Epp Quantal Content

Treatment	IgG Content of Daily Injection (mg)	Duration of Treatment (days)	Temp. (°C)	Calcium Concentration (mM)	Resting Potential (mV)	Tubocurarine Concentration Required to just Prevent Twitch (μM)	EPP QUANTAL CONTENT		No. of End-plates, Mice
							Uncorrected	Corrected for Deviation from Poisson Statistics	
KO Plasma	21.9	3	36.0-37.0	2.52	-70.5 ±1.4	3.1 ±0.1#	188 ±16	80.5 ±5.4	23, 3
Control Plasma	30.0-45.0	3	36.5-37.0	2.52	-66.2 ±0.8	2.6 ±0.1	227 ±16	91.6 ±4.9	28, 5
KO Plasma	12.0	7	35.5-37.0	0.20	-67.3 ±0.9	-	0.21 ±0.02*	0.21 ±0.02*	29, 4
Control Plasma	15.0-22.5	7	35.0-36.5	0.20	-66.9 ±1.0	-	0.40 ±0.04	0.40 ±0.04	42, 6
KO IgG	15.0	7	35.5-37.0	0.20	-65.8 ±0.9	-	0.27 ±0.04	0.27 ±0.04	24, 3
Control IgG	15.0	7	35.5-36.5	0.20	-65.7 ±1.0	-	0.36 ±0.03	0.36 ±0.03	31, 4
KO Plasma	12.0	7	35.5-37.0	0.24	-68.1 ±1.6	-	0.36 ±0.06+	0.36 ±0.06+	19, 3
Control Plasma	15.0-22.5	7	35.0-36.5	0.24	-67.2 ±1.1	-	0.72 ±0.07	0.72 ±0.07	38, 5
KO IgG	15.0	7	35.5-37.0	0.24	-62.2 ±0.6	-	0.43 ±0.06+	0.43 ±0.06+	20, 3
Control IgG	15.0	7	35.5-36.5	0.24	-62.6 ±0.6	-	0.83 ±0.09	0.83 ±0.09	29, 4
KO Plasma	12.0	15	35.0-37.0	2.52	-68.0 ±1.0	1.2 ±0.1*	99.2 ±9.3*	49.4 ±3.5*	30, 5
Control Plasma	15.0-22.5	15	36.0-37.0	2.52	-69.0 ±0.8	4.0 ±0.4	201 ±12	82.7 ±3.7	66, 11

Legend for Table 27

This table shows values of epp quantal content measured from KO IgG or plasma and control IgG or plasma treated muscles. Values at normal Ca^{2+} concentration (2.52mM) were derived using the variance method; values at low Ca^{2+} concentrations were derived from the three methods (variance, failures and direct ratio) and averaged for each fibre, since no tubocurarine was present and mepps were recorded simultaneously. In normal Ca^{2+} concentrations the values were corrected for deviation from Poisson statistics as shown; for recordings of epps in low Ca^{2+} solutions the corrected values are the same as the uncorrected ones. Values shown are means ± S.E.M. averaged over the number of end-plates shown. Significant differences compared to controls: *P<0.001; +P<0.02; #P<0.05.

For recordings in low Ca^{2+} concentration solutions (made following 7 day injections) it is worth stressing that the reduction in quantal content in KO plasma or IgG treated muscles compared with appropriate controls, was similar (Table 27). However, from Table 27 it is also clear that when the Ca^{2+} concentration was raised from 0.20mM to 0.24mM the quantal content was increased, as expected, by around 2 fold for both KO treated and control treated muscles although in every case the value for quantal control in KO treated muscles was less than for controls. This limited data suggests that KO plasma or IgG did not affect the Ca^{2+} sensitivity of release. Furthermore, for recordings made in low Ca^{2+} concentration solutions there were no significant differences between values for quantal content obtained by the three methods (direct, variance and failures) within each treatment group (Table 28). This suggests that at low quantal contents, the Poisson nature of ACh release is not affected.

Finally, for recordings made in normal Ca^{2+} solutions the end-plate potential amplitude depends on the tubocurarine concentration. Thus it is not possible to compare epp amplitude values obtained for test and control muscles since the tubocurarine concentration was never the same for test and control muscles; the criteria for tubocurarine concentration was that required to just prevent contraction of the muscle on nerve stimulation. However, in low Ca^{2+} concentrations it was possible to make a direct comparison of epp amplitude between test and control muscles. Epp amplitudes were reduced by KO plasma or IgG at each Ca^{2+} concentration used (Table 29) and there was no significant difference in values for IgG and plasma treated muscles (Table 29).

VI. Discussion

Passive transfer of plasma and the IgG fraction from patient KO to mice for 7 or 15 days gave significant reductions in mepp amplitude, epp amplitude and epp

Table 28

Comparison of Quantal Contents Obtained in KO Plasma or IgG Treated Muscles in Low Ca^{2+} Concentration Solutions.

Treatment	Duration of Treatment (days)	Calcium Concentration (mM)	QUANTAL CONTENT			No. of End-plates, Mice
			Variance	Failures	Direct Ratio	
KO Plasma	7	0.20	0.24 ±0.03*	0.21 ±0.02*	0.19 ±0.02*	29, 4
Control Plasma	7	0.20	0.43 ±0.04	0.40 ±0.04	0.38 ±0.04	42, 6
KO IgG	7	0.20	0.30 ±0.06	0.25 ±0.04	0.26 ±0.04	24, 3
Control IgG	7	0.20	0.39 ±0.04	0.34 ±0.03	0.32 ±0.03	31, 4
KO Plasma	7	0.24	0.38 ±0.07$^{\square}$	0.36 ±0.06$^{\square}$	0.34 ±0.06§	19, 3
Control Plasma	7	0.24	0.80 ±0.08§	0.71 ±0.07	0.67 ±0.06	38, 5
KO IgG	7	0.24	0.48 ±0.09	0.42 ±0.06$^{\square}$	0.40 ±0.05$^{\Delta}$	20, 3
Control IgG	7	0.24	0.92 ±0.10	0.80 ±0.08	0.72 ±0.08	29, 4

Legend for Table 28

This table shows quantal content values calculated by the variance, failures and direct ratio methods from mouse muscles treated with KO plasma (1.5ml containing 12.0mg IgG, per day); KO IgG (1.5ml containing 15.0mg IgG, per day); control plasma (1.5ml containing 15.0-22.5mg IgG, per day); control IgG (1.5ml containing 15.0mg IgG, per day) (35.0-37.0°C). Values shown are means ± S.E.M. Significant differences from respective controls: *$P<0.001$; $^{\square}P<0.002$; §$P<0.005$; $^{\Delta}P<0.01$. There were no significant differences between values within each treatment group.

Table 29

Effect of KO Plasma and IgG on Epp Amplitude in Low Ca^{2+} Concentrations

Treatment	Duration of Treatment (days)	Temperature (°C)	Calcium Concentration (mM)	Resting Potential (mV)	Epp Amplitude (mV)	No. of End-plates, Mice
KO Plasma	7	35.5-37.0	0.20	-67.3 ±0.9	0.11 ±0.01*	29, 4
Control Plasma	7	35.0-36.5	0.20	-66.9 ±1.0	0.31 ±0.03	42, 6
KO IgG	7	35.5-37.0	0.20	-65.8 ±0.9	0.16 ±0.03#	24, 3
Control IgG	7	35.5-36.5	0.20	-65.7 ±1.0	0.24 ±0.03	31, 4
KO Plasma	7	35.5-37.0	0.24	-68.1 ±1.6	0.25 ±0.06*	19, 3
Control Plasma	7	35.0-36.5	0.24	-67.2 ±1.1	0.55 ±0.05	38, 5
KP IgG	7	35.5-37.0	0.24	-62.2 ±0.6	0.25 ±0.03Δ	20, 3
Control IgG	7	35.5-36.5	0.24	-62.6 ±0.6	0.44 ±0.05	29, 4

Legend for Table 29

This table shows the effect of KO plasma (1.5ml containing 12.0mg IgG, per day) or IgG (1.5ml containing 15.0mg IgG, per day) on epp amplitude. Values are expressed as means ± S.E.M. averaged from the number of end plates shown. Significant reductions from respective controls: *$P<0.001$; Δ$P<0.01$; #$P<0.05$.

quantal content and increased the sensitivity of the muscle to tubocurarine. No effects were seen after 3 days treatment and KO plasma and IgG had little effect on the other mepp characteristics except for perhaps a small effect on passive membrane properties after prolonged injections, suggested by the decrease in decay time at 15 days by KO plasma. Data indicated no clear reduction in the α-BuTx binding or postsynaptic sensitivity to ACh, unaffected single channel properties and lack of antibody binding to the AChR. However, since data were limited we could not rule out a possible reduction in postsynaptic sensitivity. Furthermore, IgG injections produced fairly similar effects to the plasma. These results, taken together, again provide direct evidence for an IgG antibody underlying the observed disorder in this patient; the autoimmune nature is clear even though there was no evidence of antibodies present against the AChR.

At low Ca^{2+} concentrations, the effect of KO plasma or IgG on reducing mepp amplitudes was generally less. This suggest that a presynaptic mechanism for reducing the mepp amplitude, possibly a reduction in the number of ACh molecules in a packet as discussed in previous sections, is highly Ca^{2+} sensitive. At reduced Ca^{2+} concentrations it is again possible that the rate of release was reduced sufficiently to enable the packet to fill normally and so the mepp amplitude was not reduced. Other mepp characteristics were essentially unaffected by KO plasma of IgG in both normal and low Ca^{2+} concentration solutions. The apparent increase in rise time recorded in normal Ca^{2+} concentration solutions may suggest a real effect of the antibody; interference with vesicle fusion by the antibody would have reduced mepp amplitudes. However, following selection of mepps, to ensure proximity of recordings at an end-plate, the effect on rise time was removed but mepp amplitudes were still reduced. Hence it is unlikely that interference with vesicle fusion was responsible for the reduced mepp amplitude. It must not be overlooked, however,

that in the presence of physostigmine there were no reductions in mepp amplitude after treatment with either KO plasma or IgG. This may imply that there was an excess of anticholinesterase in the cleft.

As an alternative to the above explanation, it could be argued that the limited data on postsynaptic sensitivity and α-BuTx binding are consistent with a small decrease in postsynaptic sensitivity thus explaining the decreases in mepp amplitude by a postsynaptic mechanism. However, it is unlikely that this would have been sufficient to cause the marked reduction in mepp amplitude seen in normal Ca^{2+} concentrations. Moreover, unlike myasthenia gravis it would appear that if anti-AChR antibodies are indeed responsible for a reduction in the number of receptors, they were below the limits of detection using the biochemical method used in this thesis.

It is quite clear that KO plasma and IgG caused marked reductions in epp quantal content, compared with controls, suggesting a presynaptic disorder for the disease in this patient. The presynaptic action of tubocurarine (which was used in recordings of quantal content in normal Ca^{2+} concentrations) is the subject of controversy. It has been suggested, however, that it causes a depression of quantal content especially at high frequencies of stimulation (e.g. Glavinović, 1979). However, any effect of tubocurarine in these experiments would not alter the conclusions: the reduction in epp quantal content (KO muscles compared to controls) was in fact accompanied by less tubocurarine to block contraction, low (0.5Hz) frequencies of stimulation were used, and an effect on quantal release was seen even in the absence of tubocurarine in the presence of low calcium concentration.

Interestingly, there was a similar time lag (greater than 3 days treatment) in effect on both mepp amplitude and quantal content. Since the reduction in quantal content must be presynaptic it is difficult to envisage how one antibody could be responsible for both the reduction in mepp amplitude by a postsynaptic mechanism

and the reduction in quantal content. The reduction in quantal content may have been due to a down-regulation of presynaptic Ca^{2+} channels by antibodies directed against them, as is seen in the Lambert-Eaton myasthenic syndrome (LEMS) (Lang, Newsom-Davis, Peers, Prior & W.-Wray, 1987). As for LEMS, Ca^{2+} sensitivity of quantal content appeared not to be affected by KO plasma or IgG. However, it would have been interesting to study the effect of KO plasma and IgG on quantal content over a wider range of Ca^{2+} concentrations to verify this apparent lack of effect on Ca^{2+} sensitivity. Alternatively, one antibody in KO plasma could have blocked the fusion of a packet of ACh within the nerve terminal and prevented exocytosis. In this way both mepp amplitudes and epp quantal content would have been reduced. However, one would expect these effects to be accompanied by a reduction in mepp frequency and this was not observed.

Usually mice treated with plasma or IgG from MG patients with anti-AChR Ab are not weak although they have a marked decrease in mepp amplitudes (Toyka et al., 1975). This is due to the greater safety factor of neuromuscular transmission in rodents compared to humans; a larger number of ACh packets are released from the nerve terminal following nerve stimulation. None of the mice treated with antibody-negative plasma or IgG from patients, except KO were clinically weak. Mice treated with KO plasma for 15 days had marked muscle weakness, decreasing in weight over the duration of treatment and sometimes having respiratory difficulties. Thus finally, in the patient himself one could speculate that the muscle weakness comes about, as in the mice, by reductions in both mepp amplitude and quantal content, and hence epp amplitudes.

Section V : - Patient MO

I. Introduction

Patient MO had antibody-negative myasthenia gravis with similar clinical and electrophysiological features to the other patients (Table 2). However, this patient had an Osserman grade of IIB, her respiratory weakness had not required assisted ventilation and she had moderate bulbar, limb and ocular weakness as well. Again, the experimental plan and aims were the same as described for the other patients.

II. Spontaneous Release

Mepps (35-88 at each end-plate) were recorded (36.0-37.0°C) from mice after injections with MO plasma (containing 11.25-22.5mg IgG daily). Mepp amplitudes were significantly reduced after 15 days treatment with MO plasma (Table 30). The onset of this effect was greater than 3 days, since mepp amplitudes were only reduced after 3 days injection (Table 30). There was no difference between test and controls in the coefficient of variation of mepp amplitude distribution at 3 days although a small but significant ($P<0.001$) increase in MO treated muscles was seen at 15 days (Table 30). This may have occurred because MO plasma skewed the mepp amplitude distribution, some mepps being missed below the noise level. This in turn suggests that the mepp amplitude may have been slightly overestimated for MO treated muscles at 15 days. There was a significant ($P<0.001$) increase in mepp rise time after 15 days treatment (Table 30), though not at 3 days. As before, to investigate the reason for this increase, mepp amplitudes were plotted against mepp rise time for each fibre recorded from at 15 days (Figure 13). There appeared to be a strong correlation between amplitudes and rise times with most of the small amplitude mepps having the longer rise times; this was again felt to be due to microelectrode placement in relation to end-plates. After selecting mepps with a rise

Table 30

Effect of MO Plasma on Mepp Characteristics

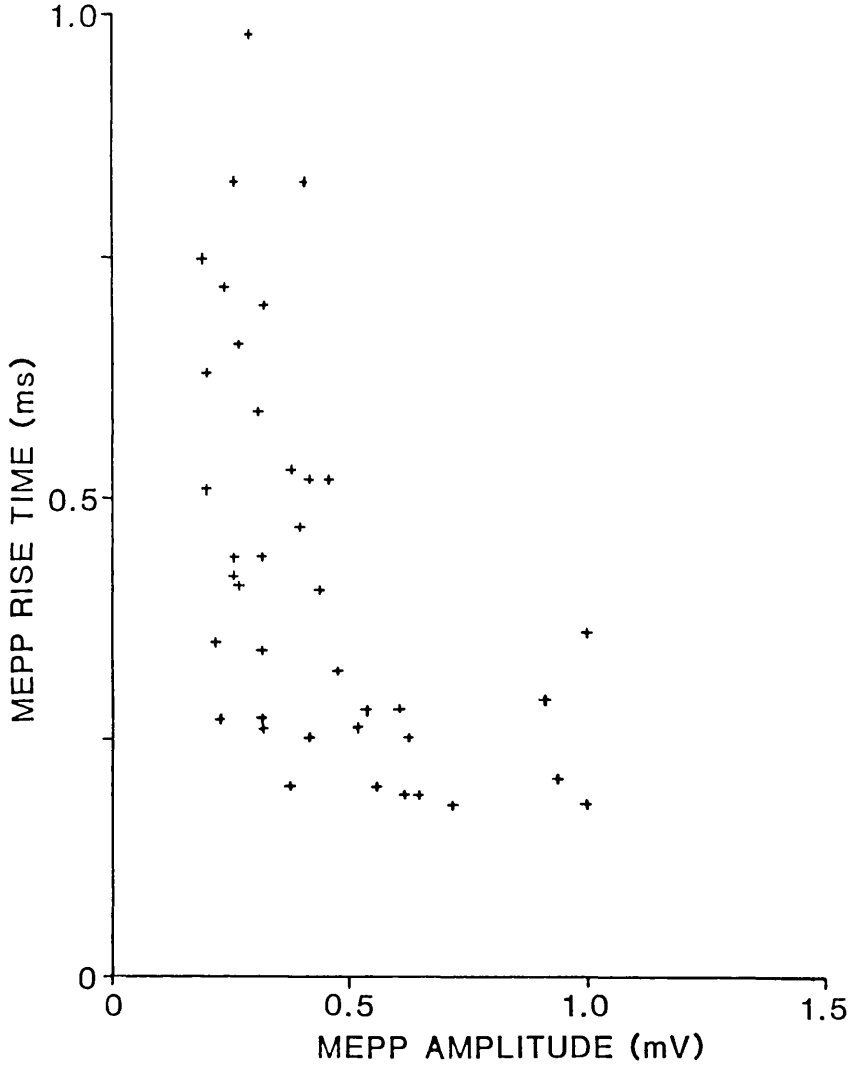
Treatment	IgG Content of Daily Injection (mg)	Duration of Treatment (days)	Temp. (°C)	Resting Potential (mV)	MEPP CHARACTERISTICS						No. of End-plates, Mice
					Amplitude		Coefficient of Variation of Amplitude Distribution	Rise Time (ms)	Decay Time (ms)	Frequency (s^{-1})	
					Un-corrected for Resting Potential (mV)	Corrected to Resting Potential of -70mV (mV)					
MO Plasma	22.5	3	36.5-37.0	-66.7 ±0.8	0.83 ±0.05	0.88 ±0.07	0.25 ±0.01	0.24 ±0.01	1.42 ±0.10	4.37 ±0.48	39, 5
Control Plasma	30.0-45.0	3	36.5-37.0	-65.6 ±0.9	0.83 ±0.05	0.90 ±0.05	0.25 ±0.02	0.23 ±0.01	1.36 ±0.07	4.55 ±0.59	38, 5
MO Plasma	11.25	15	36.0-37.0	-69.0 ±0.8	0.44 ±0.04*	0.46 ±0.04*	0.30 ±0.01*	0.42 ±0.03*	1.49 ±0.08	4.82 ±0.99#	39, 5
Control Plasma	15.0-22.5	15	36.0-37.0	-69.2 ±0.7	0.82 ±0.03	0.83 ±0.03	0.23 ±0.01	0.28 ±0.01	1.37 ±0.04	7.20 ±0.61	107, 12

Legend for Table 30

This table shows the effect of MO plasma on mepp characteristics as compared with appropriate controls (data previously shown). Values shown are means ± S.E.M. averaged over the number of end-plates shown. If instead, one value per muscle was obtained for use in statistical analysis, reductions in mepp amplitude were still significant ($P<0.001$) at 15 days but mepp frequency was not. Significant changes from controls: *$P<0.001$; #$P<0.05$.

Figure 13

Mepp Amplitude Versus Mepp Rise Time - MO Plasma Treated Muscles



Legend for Figure 13

This figure shows mepp amplitude plotted against mepp rise time for mice treated with MO plasma for 15 days (36.0-37.0°C). Each cross represents the mean mepp amplitude and mepp rise time for each end-plate recorded from; 39 end-plates, 5 animals. For the respective control figure see Figure 5.

time of $\leqslant$0.4ms, to ensure recordings were in the proximity of an end-plate, there was then no effect of MO plasma on the mepp rise time after 15 days injections, although the mepp amplitude was still significantly ($P<0.001$) reduced compared to controls (Table 31). There was no change in the mepp decay time constant at either 3 or 15 days, indicating no change in passive membrane properties by MO plasma (Table 30). Finally, there was no change in mepp frequency after 3 days treatment with MO plasma but a possible decrease after 15 days (Table 30 - see caption) indicating a presynaptic disorder.

III. Noise Analysis

To investigate any effects of MO plasma (after 15 days treatment) on postsynaptic sensitivity, ACh (5μM) was applied in the bath and depolarization and voltage noise recorded (see Methods). There were no significant changes in the maximum depolarization (V_{max}), single channel depolarization (a) and maximum channel opening frequency (n_{max}) induced by ACh in MO treated muscles compared with controls (Table 32). There was thus no change in the postsynaptic sensitivity to applied ACh and no change in single channel properties. There was a small decrease in noise time constant (Table 32); the reason for this small effect is unclear since mepp decay time constants were unchanged by MO plasma (Table 30).

IV. End-plate Potentials

Epp recordings (76-117 at each end-plate) were made while stimulating the phrenic nerve at a rate of 0.5Hz at 36.0-37.0°C. Using normal Ca^{2+} concentration solutions, the concentration of tubocurarine used in these experiments was just high enough to cause a block of muscle contraction. Although there was no difference at

Table 31

Effect of MO Plasma on Selected Mepp Characteristics

Treatment	IgG Content of Daily Injection (mg)	Duration of Treatment (days)	Temperature (°C)	Resting Potential (mV)	MEPP CHARACTERISTICS		No. of End-plates, Mice
					Rise Time (Selecting mepps with rise time ≤0.4ms) (ms)	Amplitude (Selecting mepps with rise time ≤0.4ms) (mV)	
MO Plasma	22.5	3	36.5-37.0	-66.9 ±0.8	0.21 ±0.01	0.83 ±0.06	35, 5
Control Plasma	30.0-45.0	3	36.5-37.0	-65.6 ±0.9	0.23 ±0.01	0.83 ±0.05	38, 5
MO Plasma	11.25	15	36.0-37.0	-69.9 ±1.3	0.26 ±0.01	0.57 ±0.06*	20, 5
Control Plasma	15.0-22.5	15	36.0-37.0	-68.8 ±0.9	0.27 ±0.01	0.84 ±0.03	101, 12

Legend for Table 31

This table shows the effect of MO plasma on mepp rise time and amplitude after selecting mepps with a rise time ≤0.4ms. Values shown are means ± S.E.M. averaged from the number of end-plates shown. Significant reduction compared to control value: *P<0.001.

Table 32

Effect of MO Plasma on Channel Properties Measured by Noise Analysis

Treatment	Temperature (°C)	Resting Potential (mV)	V_{max} (mV)	a (μV)	n_{max} ($10^7 s^{-1}$)	T_m (ms)	No. of End-plates, Mice
MO Plasma	22.2-24.4	-71.1 ±1.4	20.6 ±1.3	0.36 ±0.06	6.2 ±1.1	2.02 ±0.15#	12, 4
Control Plasma	22.8-23.8	-69.9 ±1.6	18.4 ±1.6	0.24 ±0.03	5.38 ±0.89	2.60 ±0.18	17, 7

Legend for Table 32

This table shows the effect of MO plasma (1.5ml containing 11.25mg IgG, per day) in 15 day treated animals on single channel properties. Values shown are means ± S.E.M. averaged from the number of end-plates. ACh (5μM) was bath applied in the presence of physostigmine (3μM) and tetrodotoxin (250nM). Significant reduction compared to control: #P<0.05.

3 days, the concentration of tubocurarine was significantly ($P<0.02$) less in MO plasma treated muscles after 15 days injection (Table 33). This is itself indicative of a disorder in neuromuscular transmission.

Epp quantal content followed a similar pattern; it was significantly reduced after the animals had been injected with MO plasma for 15 days though not after 3 days (Table 33). The significance ($P<0.001$) of the reduction in epp quantal content at 15 days was not affected by corrections for deviation from Poisson statistics (Table 33). Thus there is a presynaptic action of MO plasma in the mice. As for experiments in earlier sections, it was not possible to directly compare end-plate potential amplitudes in test and control muscles since different concentrations of tubocurarine were used to just block muscle contractions.

V. Muscle Action Potentials

Muscle action potentials were recorded from mouse diaphragms (36.5-37.0°C) in animals treated for 3 days with MO plasma. For MO treated muscles, following nerve stimulation (0.5Hz), action potentials were elicited in 95% of the muscle fibres at the end-plate region and 58% at the non-end-plate region. This compared with 70% and 83%, respectively, in control treated muscles. For those action potentials elicited, there was no significant effect of MO plasma on the overshoot, duration at half maximal amplitude and maximum rate of rise (Table 34). These results suggest that the voltage-dependent Na^+ and K^+ channels which underlie the muscle action potential are not affected by MO plasma and that the epps were generally, th ough not always, above threshold for triggering action potentials. It was perhaps surprising that action potential failures were observed since there was no effect of MO plasma on mepps or epps at 3 days. It would therefore have been more interesting to record action potentials in muscles treated by MO plasma for 15 days where an effect on the amplitude was seen. Again, as for MA, there was no

Table 33

Effect of MO Plasma on Epp Quantal Content

Treatment	IgG Content of Daily Injection (mg)	Duration of Treatment (days)	Temp. (°C)	Resting Potential (mV)	Tubocurarine Concentration Required to just Prevent Twitch (μM)	EPP QUANTAL CONTENT		No. of End-plates, Mice
						Uncorrected	Corrected for Deviation from Poisson Statistics	
MO Plasma	22.5	3	36.5-37.0	-65.0 ±0.8	2.5 ±0.2	221 ±16	89.3 ±4.9	34, 5
Control Plasma	30.0-45.0	3	36.5-37.0	-66.2 ±0.8	2.6 ±0.1	227 ±16	91.6 ±4.9	28, 5
MO Plasma	11.25	15	36.0-37.0	-68.7 ±1.0	2.3 ±0.3^{+}	117 ±10*	55.9 ±3.7*	27, 5
Control Plasma	15.0-22.5	15	36.0-37.0	-69.0 ±0.8	4.0 ±0.4	201 ±12	82.7 ±3.7	66, 11

Legend for Table 33

This table shows the effect of MO plasma on epp quantal content recorded in 2.52mM Ca^{2+} concentration solutions. Values for epp quantal content have also been corrected for deviation from Poisson statistics as shown. All values shown are means ± S.E.M. averaged over the number of end-plates shown. Significant differences compared to controls: $^{*}P<0.001$; $^{+}P<0.02$.

Table 34

Effect of MO Plasma on Muscle Action Potentials

Treatment	Temperature (°C)	Resting Potential (mV)	No. of Failures	ACTION POTENTIAL PARAMETERS			No. of Fibres, Mice
				Overshoot (mV)	Duration at Half Maximal Amplitude (ms)	Maximum Rate of Rise (Vs^{-1})	
END-PLATE							
MO Plasma	36.5-37.0	-68.6 ±1.2	1	2.3 ±2.2	0.57 ±0.02	301 ±18	21, 4
Control Plasma	36.5	-71.0 ±2.7	3	1.5 ±1.3	0.52 ±0.03	334 ±20	7, 1
NON-END-PLATE							
MO Plasma	36.5-37.0	-68.3 ±1.0	17	2.6 ±2.2	0.53 ±0.02	299 ±17	23, 4
Control Plasma	36.5	-71.6 ±2.8	1	4.6 ±1.6	0.53 ±0.04	362 ±35	7, 4

Legend for Table 34

This table shows characteristics of action potentials recorded from end-plate and non-end-plate regions in mice treated with MO plasma (1.0ml containing 22.5mg IgG, per day) or control plasma (1.0ml containing 30.0-45.0mg 1gG, per day) for 3 days. Values shown are means ± S.E.M. averaged from the number of fibres shown. There were no significant differences between parameters for test and control muscles or between parameters of action potentials recorded at or away from an end-plate.

difference between the parameters calculated from action potentials recorded at or away from the end-plate. As discussed before (see Section II above) this suggest that the effects of AChR channels at the end-plate region are swamped by the effects of voltage-dependent Na^+ and K^+ channels.

VI. Discussion

When injected into mice for 15 days, MO plasma reduced mepp amplitude, frequency and epp quantal content and increased tubocurarine sensitivity. This transfer of disorders in neuromuscular transmission to the mice is evidence that a circulating plasma factor is responsible for the disorder in this antibody-negative patient. Other mepp characteristics were not affected.

The reductions in mepp amplitude, frequency and epp quantal content were not seen at 3 days but required up to 15 days for the effect to be apparent. This slow onset of the effect on mepps was also seen for patient MA and the effect on both mepps and epps was seen for KO (see above), presumably for the same reasons as discussed earlier.

There was no change in ACh-induced depolarization and no change in single channel properties when analysed in mice which had been injected for 15 days with MO plasma. Thus there was no apparent postsynaptic impairment. On the other hand, there were obvious presynaptic abnormalities in the mice; a reduction in epp quantal content and mepp frequency after 15 day injections with MO plasma. Furthermore, since there were no changes in postsynaptic sensitivity, the reduction in mepp amplitude is probably also presynaptic.

The reduction in quantal content and mepp amplitude implies a reduction in epp amplitude which would probably underlie the disorder in the patient themselves. In the mouse, however, epps were not generally reduced in amplitude after 3 days

treatment with MO plasma to an extent great enough to prevent failure of action potentials. However, little effects were seen in neuromuscular transmission at this time anyway; action potentials were not studied at 15 days where more failures might have been expected. Also after 3 days treatment, action potential parameters were normal indicating lack of action on Na^+ and K^+ channels at this time.

Possible explanations for the observations have largely been discussed in previous sections. Briefly, the reduction in quantal content could be attributed to down-regulation of Ca^{2+} channels in the nerve terminal as previously shown for the Lambert Eaton myasthenic syndrome antibody (Lang et al., 1987). However, this would not explain the apparent reduction in mepp frequency. The reduction in mepp amplitude alone might be the result of an interruption of the choline uptake mechanism, in turn leading to synthesis disturbances and a decrease in the number of ACh molecules in a packet. Alternatively, release of ACh via ACh packet fusion with the presynaptic nerve terminal and exocytosis may be blocked by the antibody. This would lead to the observed decrease in mepp frequency as well as in quantal content and mepp amplitude.

Section VI : - Patient MI

I. Introduction

The experiments described in this section were designed to investigate a possible role of a circulating plasma factor underlying the disorder seen in antibody-negative patient MI. The clinical features for this patient were similar to the others described except that the age of onset was slightly later (44 years). Moreover the patient responded to plasma exchange suggesting an autoimmune basis for the disorder.

Animals were injected with MI plasma or control plasma for 3 and 15 days and electrophysiological recordings made of mepps, epps and action potentials and any impairment in neuromuscular transmission was studied.

II. Spontaneous Release

Mepps (40-88 at each end-plate) were recorded from animals treated with MI or control plasma (36.0-37.0°C). Mepp amplitudes were significantly reduced in MI treated muscles compared to controls at both 3 and 15 days (Table 35); the reduction was greater at 15 days. As can be seen from Table 35 there was no effect of MI plasma on the coefficient of variation of mepp amplitude distribution at 3 days but at 15 days there was a small but significant ($P<0.001$) increase in the value in MI treated muscles. Again this probably suggests a skewed distribution of mepp amplitudes; some mepps being below the discriminating level because of their small size and the amplitude for MI treated muscles being overestimated. Mepp rise times were unchanged or slightly reduced in MI treated animals (Table 35). At 15 days mepp rise times were always less than 0.4ms in MI treated muscles and there was little correlation with mepp amplitudes (Figure 14, compare previous sections)

Table 35

Effect of MI Plasma on Mepp Characteristics

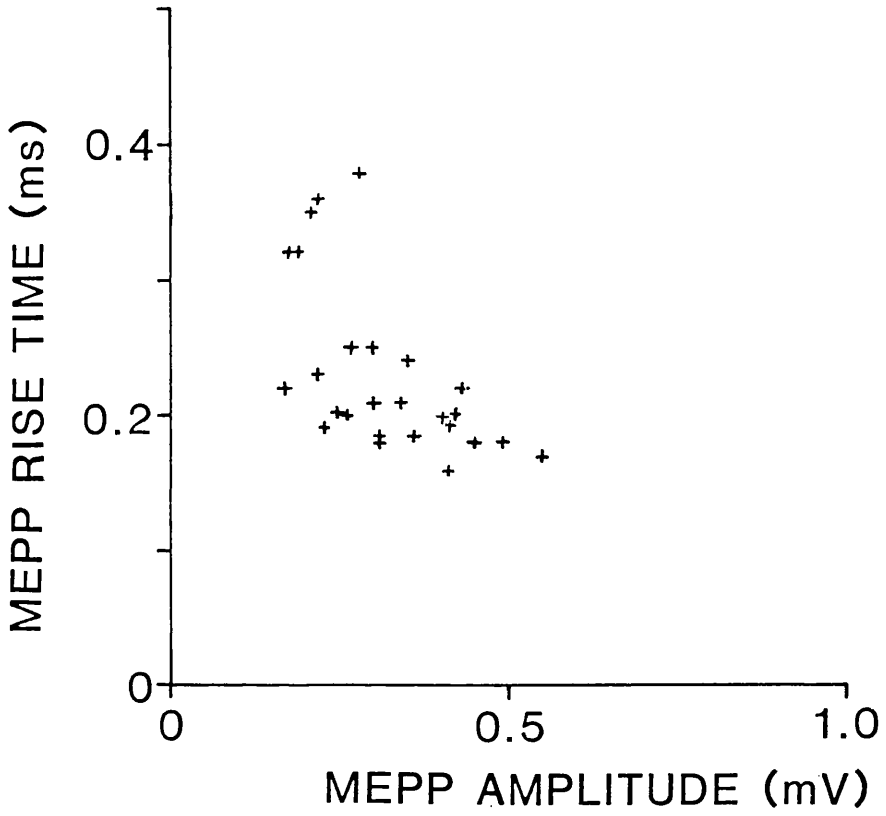
Treatment	IgG Content of Daily Injection (mg)	Duration of Treatment (days)	Temp. (°C)	Resting Potential (mV)	MEPP CHARACTERISTICS						No. of End-plates, Mice
					Amplitude		Coefficient of Variation of Amplitude Distribution	Rise Time (ms)	Decay Time (ms)	Frequency (s^{-1})	
					Un-corrected for Resting Potential (mV)	Corrected to Resting Potential of -70mV (mV)					
MI Plasma	13.5	3	36.5-37.0	-66.9 ±0.8	0.68 ±0.03$^{\Delta}$	0.71 ±0.03§	0.22 ±0.01	0.24 ±0.01	1.40 ±0.05	3.22 ±0.34	38, 5
Control Plasma	30.0-45.0	3	36.5-37.0	-65.6 ±0.9	0.83 ±0.05	0.90 ±0.05	0.25 ±0.02	0.23 ±0.01	1.36 ±0.07	4.55 ±0.59	38, 5
MI Plasma	6.75	15	36.0-36.75	-65.3 ±1.0$^{\Delta}$	0.32 ±0.02*	0.35 ±0.03*	0.30 ±0.01*	0.23 ±0.01§	0.86 ±0.05*	8.5 ±1.7	26, 3
Control Plasma	15.0-22.5	15	36.0-37.0	-69.2 ±0.7	0.82 ±0.03	0.83 ±0.03	0.23 ±0.01	0.28 ±0.01	1.37 ±0.04	7.20 ±0.61	107, 12

Legend for Table 35

This table shows values of mepp amplitude, coefficient of variation of mepp amplitude distribution, rise time, decay time and frequency. Values shown are means ± S.E.M. Instead of using one value per end-plate, each end-plate amplitude value for one muscle were averaged together and statistical tests performed using one value per muscle; there was no change in significance levels. Significant differences compared to controls: $^{*}P<0.001$; $^{\S}P<0.005$; $^{\Delta}P<0.01$.

Figure 14

Mepp Amplitude Versus Mepp Rise Time - MI Plasma Treated Muscles



Legend for Figure 14

This figure shows mepp amplitude plotted against mepp rise time for mice treated with MI plasma for 15 days (36.0-36.75°C). Each cross represents the mean mepp amplitude and mepp rise time for each end-plate recorded from; 26 end-plates, 3 animals. For the respective control figure see Figure 5.

indicating that all these recordings were probably made near to end-plates. The mepp decay time constant was not affected at 3 days but at 15 days there was a significant ($P<0.001$) reduction (Table 35). This suggests that continued injection of the plasma may have an effect on the passive membrane properties. Finally, mepp frequency was not affected by MI plasma at either duration of treatment (Table 35).

III. α-BuTx Binding Studies

Hind limbs of mice treated for 15 days with MI plasma were used for α-BuTx binding studies. As before, values were obtained for AChR number and the level of anti-AChR Ab bound to the receptors. There were no changes in these parameters in MI plasma treated muscles compared to controls (Table 36) indicating lack of postsynaptic action of MI plasma.

IV. End-plate Potentials

Epps (76-115 at each end-plate) were recorded from muscles treated with MI or control plasma at 0.5Hz phrenic nerve stimulation (36.0-37.0°C). There were reductions in the tubocurarine concentration required to just block muscle contraction on nerve stimulation (significant, $P<0.002$, at 15 days) (Table 37). At both 3 and 15 days there were significant reductions in quantal content (Table 37) and significant reductions were still observed following corrections for deviation from Poisson statistics (Table 37). As for experiments in earlier sections, a direct comparison of epp amplitudes between test and control muscles was not possible since different concentrations of tubocurarine were required to just block muscle twitch.

Table 36

α-BuTx Binding Studies from Mice Treated with MI Plasma

Treatment	Duration of Treatment (days)	AChR Number (fmol/g)	Anti-AChR Ab Bound (fmol/g)	No. of Muscles
MI Plasma	15	599 ± 60	25 ± 27	3
Control Plasma	15	627 ± 57	24 ± 22	4

Legend for Table 36

This table shows the effect of MI plasma (1.5ml containing 6.75mg IgG, per day) on AChR number and bound anti-AChR Ab. Values shown are means ± S.E.M. averaged from the number of muscles shown. Values for each muscle were obtained from the mean of 3 separate assays on each muscle extract. There were no significant differences between test and control values.

Table 37

Effect of MI Plasma on Epp Quantal Content

Treatment	IgG Content of Daily Injection (mg)	Duration of Treatment (days)	Temp. (°C)	Resting Potential (mV)	Tubocurarine Concentration Required to just Prevent Twitch (μM)	EPP QUANTAL CONTENT		No. of End-plates, Mice
						Uncorrected	Corrected for Deviation from Poisson Statistics	
MI Plasma	13.5	3	36.5-37.0	-66.1 ±0.8	2.2 ±0.1	166 ±14[§]	72.1 ±4.3[§]	35, 5
Control Plasma	30.0-45.0	3	36.5-37.0	-66.2 ±0.8	2.6 ±0.1	277 ±16	91.6 ±4.9	28, 5
MI Plasma	6.75	15	36.0-37.0	-69.6 ±1.3	1.1 ±0.3[□]	145 ±14[+]	65.9 ±4.7[#]	20, 3
Control Plasma	15.0-22.5	15	36.0-37.0	-69.0 ±0.8	4.0 ±0.4	201 ±12	82.7 ±3.7	66, 11

Legend for Table 37

This table shows values of epp quantal content measured from MI and control plasma treated muscles. All recordings were made in a solution containing 2.52mM Ca^{2+} and were analysed using the variance method. Values shown are means ± S.E.M. averaged over the number of end-plates shown. Significant reductions compared to controls: [□]P<0.002; [§]P<0.005; [+]P<0.02; [#]P<0.05.

V. Muscle Action Potentials

Recordings of muscle action potentials (36.5-37.0°C) were made, as before, to further clarify whether the voltage-dependent Na^+ and K^+ channels were functioning normally in 3 day MI plasma treated muscles as compared to controls. For MI treated muscles,action potentials were observed following 100% of nerve stimulations at end-plates, and 88% away from the end-plate. This compared with 70% and 87%, respectively, for control treated muscles, and the difference was not statistically significant. This indicates that the amplitude of the epp in the mice treated with MI plasma was nearly always above the critical level for action potential firing. At 3 days, however, the mepp amplitude and epp quantal content were only slightly reduced, indicating that epp amplitudes would generally not be reduced enough to fall below threshold. The overshoot, duration at half maximal amplitude and maximum rate of rise were calculated for each action potential. Values for these parameters were not affected by MI plasma after 3 days treatment compared with controls (Table 38) indicating that voltage-dependent Na^+ and K^+ channels were not affected at this time.

There were small differences between parameters recorded at or away from end-plates for MI treated muscles. The overshoot was significantly ($P<0.02$) less at the end-plate compared to that at the non-end-plate region. In addition, for MI plasma, the duration of the action potential at half maximal amplitude was slightly but significantly ($P<0.001$) longer at the end-plate compared to that at the non-end-plate region, while maximum rate of rise was not significantly different between the two recording sites. The small differences in action potential parameters between end-plate and non-end-plate regions for MI plasma treated muscles are probably due to the cell being driven towards the ACh reversal potential by the end-plate potential.

Table 38

Effect of MI Plasma on Muscle Action Potentials

Treatment	Temperature (°C)	Resting Potential (mV)	No. of Failures	ACTION POTENTIAL PARAMETERS			No. of Fibres, Mice
				Overshoot (mV)	Duration at Half Maximal Amplitude (ms)	Maximum Rate of Rise (Vs^{-1})	
END-PLATE							
MI Plasma	36.5-37.0	-68.5 ±0.9	0	0.1 ±1.4	0.54 ±0.02	347 ±16	35, 5
Control Plasma	36.5	-71.0 ±2.7	3	1.5 ±1.3	0.52 ±0.03	334 ±20	7, 1
NON-END-PLATE							
MI Plasma	36.5-37.0	-69.0 ±1.0	4	6.3 ±2.2	0.47 ±0.01	353 ±28	30, 5
Control Plasma	36.5	-71.6 ±2.8	1	4.6 ±1.6	0.53 ±0.04	362 ±35	7, 1

Legend for Table 38

This table shows values for action potential parameters from mice treated with MI plasma (1.0ml containing 13.5mg IgG, per day) or control plasma (1.0ml 30.0-45.0mg IgG, per day) for 3 days. Values shown are means ± S.E.M. averaged from the number of fibres shown. Recordings were made at end-plate and non-end-plate regions. There were no significant differences between parameters for test and control muscles. However, some parameters recorded at or away from the end-plate in MI treated muscles were significantly different: overshoot, $P<0.02$; duration at half maximal amplitude, $P<0.001$.

VI. Discussion

The observed electrophysiological features in the mice following passive transfer of MI plasma for 3 and 15 days were a reduction in mepp amplitude and epp quantal content and an increase in tubocurarine sensitivity at 15 days. This again suggests that a circulating factor (probably antibody) underlies the disorder in neuromuscular transmission for this patient. Other mepp characteristics were largely unaffected by MI plasma as compared to controls; the reduction in decay time at 15 days may suggest an effect on passive membrane properties following prolonged injection.

Mepp amplitudes and epp quantal content were reduced after both 3 and 15 days treatment, greater effects being seen after 15 days. This is reminiscent of the situation with patients KO and MO where reductions were only seen after greater than 3 day injections. In addition, epp amplitudes, although not directly measured, were probably reduced although not normally by an amount great enough to be below the critical level of action potential activation; hence action potentials were usually seen following nerve stimulation.

There was no evidence to suggest the presence of any anti-AChR antibody bound to the AChRs and no change in AChR number. Thus, there were no obvious postsynaptic abnormalities in mice treated with MI plasma to account for the reduced mepp amplitude but clear presynaptic abnormalities, demonstrated by the reduction in epp quantal content. Furthermore, from the absence of bound anti-AChR Ab or a change in receptor number, it seems likely that the reduction in mepp amplitude is probably also a defect which has a presynaptic origin. As for the other patients, these defects in mepp amplitude and quantal content imply a reduction in epp amplitude which is probably the factor responsible for the muscle weakness in this patient. Action potentials were usually triggered in muscles which had been treated with MI plasma for 3 days. This is perhaps not surprising in view

of the small implied reductions in epp amplitudes at this time. The parameters of the action potentials were normal, compared with controls, indicating lack of action of MI plasma on voltage-dependent Na^+ and K^+ channels.

Speculation as to the mechanism of action of the plasma factor in patient MI (presumably antibody) are similar to those discussed for other patients; loss of presynaptic Ca^{2+} channels reducing the quantal content and a decrease in the number of molecules of ACh in a packet reducing the mepp amplitude.

Section VII : - Patient CA

I. Introduction

This chapter describes experiments using the IgG fraction from antibody-negative patient CA passively transferred to the mouse. For this patient the age of onset of the disorder was 28 years and she had an Osserman grade of III (Table 2). The pattern of muscle weakness was the same as for patient KP; moderate weakness of limb, ocular and respiratory muscles, requiring assisted ventilation at some time, and severe weakness of bulbar muscles. The tendon reflexes were normal or brisk and this patient had a positive response to intravenous edrophonium. Furthermore, this patient responded to plasma exchange.

Mice were only injected with CA IgG for 3 days due to lack of availability of plasma from patient CA from which the IgG fraction was extracted.

II. Spontaneous Release

Mepps (40-53 at each end-plate) were recorded from CA IgG and control IgG treated animals (21.5-24.0°C). Mepp amplitudes were significantly ($P<0.005$) reduced in CA IgG treated muscles compared with respective control recordings (Table 39). This was also true after corrections had been made to a resting potential of -70mV (Table 39). The coefficient of variation of mepp amplitude distribution was unchanged following treatment with CA IgG as compared with controls (Table 39). This again shows that CA IgG did not skew mepp amplitude distribution. The rise time was also unchanged in CA IgG treated muscles compared to control values (Table 39). However, a possible change in passive membrane properties was suggested by the significant ($P<0.005$) but small decrease in the decay time constant of those mepps recorded from CA IgG treated muscles (Table 39). As for the other 4

Table 39

Effect of CA IgG on Mepp Characteristics

Treatment	IgG Content of Daily Injection (mg)	Duration of Treatment (days)	Temp. (°C)	Resting Potential (mV)	MEPP CHARACTERISTICS						No. of End-plates, Mice
					Amplitude		Coefficient of Variation of Amplitude Distribution	Rise Time (ms)	Decay Time (ms)	Frequency (s^{-1})	
					Un-corrected for Resting Potential (mV)	Corrected to Resting Potential of -70mV (mV)					
CA IgG	60	3	23.4-24.0	-66.2 ±1.3	0.81 ±0.06[§]	0.86 ±0.06[§]	0.24 ±0.01	0.54 ±0.03	2.30 ±0.13[§]	0.62 ±0.10	19, 3
Control IgG	60	3	21.5-23.6	-66.1 ±1.1	1.09 ±0.06	1.16 ±0.07	0.25 ±0.01	0.62 ±0.03	2.83 ±0.10	0.78 ±0.06	27, 4

Legend for Table 39

This table shows recordings made from mice treated with CA IgG or control IgG. Amplitudes have been corrected to a resting potential of -70mV. Each value shown in this table is the mean ± S.E.M. averaged over the number of end-plates shown. Significant differences compared to controls: [§]$P<0.005$.

patients in which there was also a decrease in mepp decay time, this effect on decay time is probably attributed to a non-specific effect of the IgG antibody on passive membrane properties. Finally, there was no effect of CA IgG on mepp frequency (Table 39).

III. End-plate Potentials

Epps (60-114 at each end-plate) were recorded from animals treated for 3 days with CA or control IgG (21.5-24.8°C). There was a significant ($P<0.001$) decrease in the concentration of tubocurarine required to just block muscle twitch in CA IgG treated muscles compared with controls (Table 40). This in itself suggests a neuromuscular defect. In addition, epp quantal content was also significantly ($P<0.001$) reduced in muscles treated with CA IgG compared to controls (Table 40). The reduction attained the same level of significance even after corrections had been made for deviation from Poisson statistics (Table 40).

IV. Discussion

Passive transfer of CA IgG to the mice produced alterations in their electrophysiological features compared to control IgG treated animals. This was seen as a decrease in mepp amplitude and epp quantal content and an increase in the sensitivity to tubocurarine. There were no marked changes in any of the other mepp characteristics measured except the decay time; the reduction in this value in CA IgG treated animals, compared to controls, being probably due to possible non-specific membrane effects of the IgG antibody. The observed defects in neuromuscular transmission again provide evidence to suggest that this disorder is caused by an antibody, directly implicating an autoimmune basis.

Table 40

Effect of CA IgG on Epp Quantal Content

Treatment	IgG Content of Daily Injection (mg)	Duration of Treatment (days)	Temp. (°C)	Resting Potential (mV)	Tubocurarine Concentration Required to just Prevent Twitch (μM)	EPP QUANTAL CONTENT		No. of End-plates, Mice
						Uncorrected	Corrected for Deviation from Poisson Statistics	
CA IgG	60	3	23.4-24.8	-66.4 ±1.1	2.4 ±0.1*	65 ±6.0*	36.7 ±2.5*	17, 3
Control IgG	60	3	21.5-22.6	-67.5 ±1.1	4.0 ±0.1	129 ±15	59.8 ±5.1	22, 4

Legend for Table 40

This table shows the effect of CA IgG on epp quantal content recorded in 2.52mM Ca^{2+} concentration solutions. Values shown are means ± S.E.M. and the epp quantal content was derived using the variance method. Significant reductions compared to controls: *P<0.001.

Although postsynaptic sensitivity was not studied, the disorder in patient CA clearly has at least a presynaptic component since the quantal content was reduced. The mechanism(s) underlying this may be similar to the patients already discussed.

Section VIII : - Patient SP

I. Introduction

This section examines the electrophysiological features produced following passive transfer of plasma from an anti-AChR antibody positive MG patient to mice. The disease in patient SP was characterised by the electrophysiological and biochemical features associated with MG; a reduced mepp amplitude, a decrease in the number of AChRs together with detectable anti-AChR antibodies (Ito et al., 1978b). SP had an anti-AChR Ab titre of 25.5 nmoles ^{125}I-α-BuTx precipitated per litre of serum (Table 2).

Mice were injected with SP plasma for 7 days as a positive control. Reproduction of the above electrophysiological features in the mice would be expected for this autoimmune plasma. It would also confirm that changes in electrophysiological features, following the same method of passive transfer, using antibody-negative MG IgG or plasma was a real effect rather than an injection artefact.

II. Spontaneous Release

(a) Spontaneous Release in Normal Calcium Concentration Solutions

Mepps (40-42 at each end-plate) were recorded from SP or control plasma treated animals (35.0-36.5°C). There was a highly significant ($P<0.001$) reduction in mepp amplitude as compared to controls (Table 41). This reduction was still significant ($P<0.001$) after corrections were made to a resting potential of -70mV (Table 41). There was no change in the coefficient of variation of mepp amplitude distribution (Table 41) showing that SP plasma did not skew the distribution of mepps; mepps were normally distributed about the mean. However, because the

Table 41

Effect of SP Plasma on Mepp Characteristics

Treatment	Duration of Treatment (days)	Temp. (°C)	Resting Potential (mV)	MEPP CHARACTERISTICS						No. of End-plates, Mice
				Amplitude		Coefficient of Variation of Amplitude Distribution	Rise Time (ms)	Decay Time (ms)	Frequency (s^{-1})	
				Un-corrected for Resting Potential (mV)	Corrected to Resting Potential of -70mV (mV)					
SP Plasma	7	35.5-36.5	-68.4 ±1.3	0.48 ±0.04*	0.50 ±0.05*	0.26 ±0.01	0.34 ±0.02	1.40 ±0.06	8.67 ±0.71	31, 4
Control Plasma	7	35.0-36.5	-69.5 ±0.9	1.00 ±0.05	1.02 ±0.06	0.27 ±0.01	0.30 ±0.01	1.51 ±0.06	7.58 ±0.48	43, 6

Legend for Table 41

This table shows the effect of SP plasma on mepp characteristics. Amplitudes have been corrected to a resting potential of -70mV. All values shown are means ± S.E.M. averaged from the number of end-plates shown. When one value per muscle was used in the statistical analysis of mepp amplitude, the significance level was the same. Significant reductions from controls: *$P<0.001$.

mepp amplitude was small for mice treated with SP plasma it is possible that some mepps may have been lost below the noise level. To confirm the effect of SP plasma, mepps (40 at each end-plate) were recorded in the presence of physostigmine (3μM). There was a significant ($P<0.05$) reduction in mepp amplitude (Table 42) which was less than that significance level attained in the absence of physostigmine. Again there was no effect of SP plasma on the coefficient of variation of mepp amplitude distribution (Table 42). In the presence of physostigmine SP plasma had no effect on decay time or frequency although there was a small but significant ($P<0.05$) reduction in rise time (Table 42). In the absence of physostigmine, however, SP plasma had no effect on any of the last three mepp characteristics; rise time, decay time or frequency (Table 41).

(b) Spontaneous Release in Low Calcium Solutions

During recordings of epps in low calcium solutions (see Part III) mepps (38-50 at each end-plate) were recorded (35.0-37.0°C). Mepp amplitudes were reduced by SP plasma but this only attained statistical significance ($P<0.005$) when the Ca^{2+} concentration was 0.24mM (Table 43). However, mepp amplitudes were also reduced in control muscles in low Ca^{2+} concentration solutions compared to recordings in normal Ca^{2+} concentrations. Furthermore, SP plasma appeared to cause an increase in mepp frequency when Ca^{2+} was 0.2mM, contrasting with the lack of effect on mepp frequency seen when recordings were made at 0.24mM and 2.52 mM Ca^{2+}.

III. End-plate Potentials

Epps (63-118 at each end-plate) were recorded from SP plasma treated muscles in low calcium solutions (35.0-37.0°C) at a nerve stimulation frequency of 0.5Hz. There was no significant difference in quantal content values obtained from SP

Table 42

Effect of SP Plasma on Mepp Amplitudes Recorded in the Presence of Physostigmine (3μM)

Treatment	Duration of Treatment (days)	Temp. (°C)	Resting Potential (mV)	MEPP CHARACTERISTICS						No. of End-plates, Mice
				Amplitude		Coefficient of Variation of Amplitude Distribution	Rise Time (ms)	Decay Time (ms)	Frequency (s^{-1})	
				Un-corrected for Resting Potential (mV)	Corrected to Resting Potential of -70mV (mV)					
SP Plasma	7	35.5-36.5	-68.4 ±1.3	1.04 ±0.09#	1.08 ±0.11#	0.29 ±0.01	0.40 ±0.03#	3.12 ±0.09	8.70 ±0.70	26, 3
Control Plasma	7	35.0-36.5	-68.8±1.0	1.34 ±0.09	1.36 ±0.08	0.32 ±0.02	0.47 ±0.02	3.20 ±0.12	7.87 ±0.86	40, 6

Legend for Table 42

This table shows the effect of SP plasma on mepp characteristics in the presence of physostigmine. When an average value for mepp amplitude was obtained from each muscle and used in the statistical test mepp amplitudes are not significantly reduced compared to controls. Values for mepp characteristics measured from the same end-plates as shown here but in the absence of physostigmine are seen in Table 41. Values shown are means ± S.E.M. Significant reductions compared to controls: #P<0.05.

Table 43

Effect of SP Plasma on Mepp Characteristics Recorded in Low Ca^{2+} Concentrations

Treatment	Duration of Treatment (days)	Temperature (°C)	Ca^{2+} Concentration (mM)	Resting Potential (mV)	MEPP CHARACTERISTICS		No. of End-plates, Mice
					Amplitude Uncorrected for resting potential (mV)	Frequency (s^{-1})	
SP Plasma	7	36.5-37.0	0.20	-66.7 ±0.9	0.70 ±0.06	4.79 ±0.77*	26, 4
Control Plasma	7	35.0-36.5	0.20	-67.0 ±1.0	0.83 ±0.05	2.20 ±0.28	42, 6
SP Plasma	7	36.0-37.0	0.24	-65.5 ±0.9	0.61 ±0.06§	4.81 ±0.75	26, 4
Control Plasma	7	35.0-36.5	0.24	-67.2 ±1.1	0.87 ±0.06	6.2 ±1.1	38, 5

Legend for Table 43

This table shows the effect of SP plasma on mepp characteristics recorded in low Ca^{2+} solutions. Since there were no significant differences in resting potential between test and control, amplitudes were uncorrected for resting potential. Values shown are means ± S.E.M using 1 value per end-plate. If instead values were first averaged for each muscle and a single value used for each muscle in the statistical test, the values for mepp frequency at 0.2mM Ca^{2+} were not significantly different (SP plasma, 4.13 ± 1.43s^{-1}; 4 end-plates; control plasma 2.10 ± 0.53 s^{-1}; 6 end-plates). Significant differences compared to controls: *$P<0.001$; §$P<0.005$.

treated muscles compared to controls at either Ca^{2+} concentration (Table 44). Furthermore, for the low Ca^{2+} concentration solutions there were no significant differences between the values for quantal content obtained using the three separate methods: variance, failures or direct ratio (Table 45). This suggest that low Ca^{2+} concentration solutions do not affect the Poisson nature of ACh release. Finally, in low Ca^{2+} concentration solutions epp amplitudes were directly compared between SP plasma treated and control plasma treated muscles. There were reductions in epp amplitude in SP treated muscles at both Ca^{2+} concentrations but this was only significant when the Ca^{2+} concentration was 0.24mM (Table 46).

IV. Discussion

Passive transfer of SP plasma to mice reproduced those electrophysiological features seen in autoimmune MG with antibodies against the AChR (Albuquerque et al., 1976b). The results from recordings of mepps show that SP plasma reduced mepp amplitude with little change in any of the other measured mepp characteristics except perhaps on mepp frequency at 0.2mM Ca^{2+}. Although the reduction in mepp amplitude by SP plasma was small when recorded in a solution containing 0.2mM Ca^{2+}, the possible increase in mepp frequency in 0.2mM Ca^{2+} may be a compensatory mechanism to overcome a decreased postsynaptic sensitivity to ACh. Recordings from MG intercostal muscle biopsies, however, show that mepp frequency is slightly reduced (Albuquerque et al., 1976b). This is presumably because mepp amplitudes are reduced to such an extent that they are lost within the noise of the recording system and so the frequency also appears reduced. SP plasma also reduced epp amplitudes but caused no marked change in quantal content. These experiments support other evidence which show that MG is an autoimmune disorder, and also act as a positive control for the other results reported in this thesis.

Table 44

Effect of SP Plasma on Epp Quantal Content

Treatment	Duration of Treatment (days)	Temperature (°C)	Calcium Concentration (mM)	Resting Potential (mV)	EPP QUANTAL CONTENT	No. of End-plates, Mice
					Uncorrected	
SP Plasma	7	36.5-37.0	0.20	-66.7 ±0.9	0.44 ±0.06	26, 4
Control Plasma	7	35.0-36.5	0.20	-66.9 ±1.0	0.40 ±0.04	42, 6
SP Plasma	7	36.0-37.0	0.24	-65.5 ±0.9	0.69 ±0.06	26, 4
Control Plasma	7	35.0-36.5	0.24	-67.2 ±1.1	0.72 ±0.07	38, 5

Legend for Table 44

This table shows the effect of SP plasma on epp quantal content. Values shown are the average of the three methods (direct ratio, variance and failures as described in Methods) recordings being made in low Ca^{2+} solutions. Values have not been corrected for deviation from Poisson statistics and no tubocurarine was present to prevent contraction due to the reduced quantal content. Values shown are means ± S.E.M. There were no significant differences between test and control.

Table 45

Comparison of Quantal Contents Obtained in SP Plasma Treated Muscles in Low Ca^{2+} Concentration Solutions.

Treatment	Duration of Treatment (days)	Calcium Concentration (mM)	QUANTAL CONTENT			No. of End-plates, Mice
			Variance	Failures	Direct Ratio	
SP Plasma	7	0.20	0.48 ±0.06	0.45 ±0.06	0.39 ±0.05	26, 4
Control Plasma	7	0.20	0.43 ±0.04	0.40 ±0.04	0.38 ±0.04	42, 6
SP Plasma	7	0.24	0.77 ±0.07	0.70 ±0.06	0.62 ±0.06	26, 4
Control Plasma	7	0.24	0.80 ±0.08	0.71 ±0.07	0.67 ±0.06	38, 5

Legend for Table 45

This table shows quantal content values derived by the variance, failures and direct ratio methods from mice treated with SP plasma for 7 days (35.0-37.0°C). Values shown are means ± S.E.M. There were no significant differences between the three methods for each treatment or between test and controls.

Table 46

Effect of SP Plasma on Epp Amplitudes in Low Ca^{2+} Concentrations

Treatment	Duration of Treatment (days)	Temperature (°C)	Calcium Concentration (mM)	Resting Potential (mV)	Epp Amplitude (mV)	No. of End-plates, Mice
SP Plasma	7	36.5-37.0	0.20	-66.7 ±0.9	0.27 ±0.05	26, 4
Control Plasma	7	35.0-36.5	0.20	-66.9 ±1.0	0.31 ±0.03	42, 6
SP Plasma	7	36.0-37.0	0.24	-65.5 ±0.9	0.34 ±0.04[§]	26, 4
Control Plasma	7	35.0-36.5	0.24	-67.2 ±1.1	0.55 ±0.05	38, 5

Legend for Table 46

This table shows the effect of SP plasma on epp amplitude. Values shown are means ± S.E.M. averaged from the number of end-plates shown. Significant reduction compared with control: [§]$P<0.005$.

It is widely accepted that the basic defect in anti-AChR antibody positive MG is a decrease in the number of junctional AChRs due to an antibody-mediated autoimmune response (see Introduction). Hence the reductions in mepp amplitudes and epp amplitudes seen in mice treated with SP plasma were most likely due to a postsynaptic impairment. This idea is also supported to a certain extent by the lack of reduction in epp quantal content and mepp frequency which are both indicative of a lack of change in presynaptic function. In other studies where mice have been treated with MG Ig the epp quantal content (stimulation at 3Hz) and the mepp frequency were not grossly altered in myasthenic animals compared to controls (Toyka et al., 1978).

For patient SP the decreased efficiency of neuromuscular transmission is presumably due to the loss of AChRs and results in reduced amplitudes of mepps and epps. The observed reduction in mepp amplitude by 52% in normal Ca^{2+} concentration solutions is, however, slightly less than that which has been found in other studies. Injection of the crude Ig fraction from MG patients into mice produced a reduction in mepp amplitude by 72% following 3 days injections and by 70% following 10-14 days (Toyka et al., 1975). The reduction by SP plasma of epp amplitude by 38% in the higher Ca^{2+} concentration is in agreement with other studies using the passive transfer of MG Ig from man to mouse (Toyka et al., 1978). In the latter study, the epp amplitude was reduced by 33% following up to 12 days injections. These discrepancies in reductions may be explained by poorer cross-reactivity of plasma (used in this thesis) with mouse muscles compared to IgG which was used in the studies just referred to. In those studies the mice were injected with the crude Ig fraction thus potentially maximising the effect on the AChR.

Finally, in patient SP herself presumably the mepps and epps are reduced to a greater extent than that seen in the mice, so that epps fail to trigger muscle action

potentials and contraction. Failure of transmission at many junctions would reduce the muscle power which is then clinically manifested as weakness and muscle fatigue.

Section IX : - General Discussion (see also addendum, p208)

From the results described in Sections 1-8 it appears that the disease seen in patients KP, MA, LW, KO, MO, MI and CA, patients with antibody-negative MG, is a distinct disease entity to anti-AChR Ab positive MG as seen in patient SP. Passive transfer of plasma or the IgG fraction from all of the above patients produced defects in neuromuscular transmission. This is strong evidence to suggest that the disorder in antibody-negative MG patients has an autoimmune basis with the pathological agent being most probably identified as an antibody in the IgG fraction of plasma.

Table 47 summarizes those results presented in Sections I-VIII. In all of the mice injected with plasma or IgG from antibody-negative patients there were marked reductions in mepp amplitude. For patients KP, MI and CA mepp amplitudes were always significantly reduced when recorded in normal Ca^{2+} concentration solutions although reductions were greater at 7 and 15 days in KP plasma treated muscles than after 3 days injections. Moreover, for patients MA, KO and MO there was a clear time lag before mepp amplitude was reduced; mepp amplitude was reduced after greater than 3 days injections. This time lag could be the consequence of either (i) the IgG concentration in the plasma being low and time being required to build up an effective concentration in the animals or (ii) the time delay of the IgG to display its effect on neuromuscular transmission once it has bound to the effector site. This contrasts with MG where, after 3 days of passive transfer of the IgG fraction from MG plasma, the neuromuscular disorder is transferred from the patient to the mouse (Toyka et al., 1975; Toyka et al., 1977). Strong evidence for an IgG antibody being the pathogenic agent was provided in Section IV; KO plasma was directly compared with KO IgG in animals treated for 7 days with the preparations. Both caused similar reductions in mepp amplitudes.

Table 47

Summary of Results

Patient	SPONTANEOUS RELEASE		POSTSYNAPTIC FEATURES			Epp Quantal Content	Action Potentials
	Mepp Amplitude	Mepp Frequency	ACh-Induced Depolarization	Single Channel Properties from Noise Analysis	α-BuTx Binding Studies		
KP (IgG/Plasma)	Reduced	Unchanged	Unchanged	Unchanged	No Anti-AChR Ab No change in AChR number	Unchanged	-
MA (Plasma)	Reduced	Unchanged/ Slightly reduced	-	-	No Anti-AChR Ab No change in AChR number	Unchanged	No effect but not always elicited
LW (IgG/Plasma)	Reduced	Unchanged	-	-	No Anti-AChR Ab No change in AChR number	Unchanged	-
KO (IgG/Plasma)	Reduced	Unchanged	Unchanged	Unchanged	No Anti-AChR Ab No change in AChR number	Reduced	-
MO (Plasma)	Reduced	Reduced at 15 days	Unchanged	Unchanged	-	Reduced	No effect but not always elicited
MI (Plasma)	Reduced	Unchanged	-	-	-	Reduced	No effect but not always elicited
CA (IgG)	Reduced	Unchanged	-	-	-	Reduced	-
SP (Plasma)	Reduced	Unchanged	-	-	(Anti- AChR Ab in plasma)	Unchanged	-

Legend for Table 47

This table shows a summary of results presented in Sections I-VIII. Only mepp amplitude and mepp frequency are shown under spontaneous release although more measurements were made. The ACh-induced depolarization was measured in the presence of ACh (5μM). A line indicates that the measurement was not made from muscles treated with the plasma or IgG fraction from that patient. Key: Ab = antibody; AChR = postsynaptic ACh receptor.

By contrast to the effect on mepp amplitude there were few marked effects on any of the other measured mepp characteristics. Coefficient of variation of mepp amplitude distributions were little changed except for increases after 15 days injection with MO and MI plasma suggesting a skewed distribution. Thus, there was hardly any skewing of mepp amplitudes about the mean suggesting that even though mepp amplitudes were reduced, few mepps would have been lost below the noise level. In antibody-negative MG patients themselves, however, it is likely that the mepps were reduced to a greater extent. In such a situation, the distribution of mepps would then be skewed, as for MG patients with anti-AChR Abs (Cull-Candy et al., 1979).

Mepp rise times were generally increased at 15 days when compared to controls but this was probably due to the recording technique rather than a real effect of the IgG. Selecting fast rise time mepps at or near to an end-plate removed the increase in rise time with any residual effect being attributed to microelectrode displacement from the end-plate; the IgG thus resulting in little or no change in the time course.

Mepp decay time was also little changed following treatment with any of the plasmas or IgG fractions; any small effect was probably due to a non-specific action on passive membrane properties and usually seen following prolonged injections as with KO and MI plasma. However, decay times were slightly reduced by CA plasma after only 3 days injections.

Finally, mepp frequency was also generally unchanged, although MA plasma and MO plasma did cause significant reductions in this measurement following 15 days treatment. Passive transfer of MG IgG to mice has been shown to slightly reduce mepp frequency by 21% compared to controls (Toyka et al., 1978). In recordings from myasthenia gravis patient's biopsied intercostal muscle, mepp frequency is either normal or reduced (Elmqvist et al., 1964, Lambert & Elmqvist,

1971; Albuquerque et al., 1976b). This possibly indicates either that many packets of ACh are released opposite sites where no receptors are or that there is an additional presynaptic effect involving a decrease in the number of releasable ACh packets. However, since there is also usually a large decrease in mepp amplitude, leading to the loss of very small mepps within the noise of the recording system, it is more likely that this underlies the apparent reduced mepp frequency. This may also explain the reduced mepp frequency seen in mice treated with MA and MO plasma for 15 days.

Lack of postsynaptic effects on mice treated with antibody-negative MG plasma generally identified antibody-negative MG as a distinct disease entity from MG with anti-AChR Ab. For patients KP and MO there was no change in the ACh-induced depolarization or the single channel properties assessed from noise analysis and for patients KP, MA and LW and KO there was no detectable anti-AChR antibody and no change in AChR number. Patient KO, however, may be in a distinct class of antibody-negative MG patients from those other patients in this study, since a considerable scatter in the results was seen and there could well have been reductions in ACh-induced depolarization and in AChR number. The general lack of obvious postsynaptic effects suggests that the reduction in mepp amplitude is due to a presynaptic abnormality. As discussed before in detail, this is probably explained by a reduced number of molecules of ACh released from the nerve terminal and consequently, a decrease in the amount of ACh reaching the postsynaptic membrane. However, further work would need to be done to elucidate the exact site of impairment using, for example, fluorescent labelling of the antibody before injection into the animals. Also, from electron microscope analysis of the presynaptic nerve terminal membrane, using freeze fracture techniques, it would be possible to determine histometrically whether the size of the synaptic vesicles were in fact reduced (Santa et al., 1972).

Interestingly, it was originally thought, from investigations on biopsied human intercostal muscle from MG patients, that the underlying mechanism responsible for MG was a reduction in the number of ACh molecules in each packet (Elmqvist et al., 1964). However, labelling of human MG muscle established that the impairment was not due to a reduction in the number of ACh molecules in a packet, but a reduction in AChR number (Fambrough et al., 1973; Vincent, 1980). Furthermore, histometric studies of MG neuromuscular junction ultrastructure showed that the average diameter (and hence volume) of synaptic vesicles was unchanged compared to controls (Santa, et al., 1972).

In the majority of the mice treated with plasma or IgG from antibody-negative MG patients, there was an increase in tubocurarine sensitivity compared with respective controls. This in itself is indicative of a disorder in neuromuscular transmission. Measures of epp quantal content showed that there were 2 distinct classes of antibody-negative patient. For mice injected with plasma or IgG from patients KP, MA and LW there was no change in epp quantal content. On the other hand, the plasma or IgG from patients KO, MO, MI and CA generally caused large significant reductions in mouse epp quantal content. The mechanisms for this were not investigated; one possibility could be an action of the IgG antibody on presynaptic Ca^{2+} channels as for LEMS patients (Lang et al., 1987).

For mice treated with KP plasma, KO plasma or KO IgG a measure of epp amplitude was possible in low Ca^{2+} solutions. KP plasma did not cause a measurable reduction compared to controls possibly due to poor cross-reactivity in mice, but KO did. Some indirect indication of epp amplitude was, however, obtained from recordings of muscle action potentials in muscles treated with plasma from patients MA, MO and MI. Action potentials were sometimes not initiated implying that epps were sometimes below the threshold for action potential firing. When muscle action potentials were triggered they had normal properties, indicating lack of action of plasma factors on voltage-dependent Na^+ and K^+ channels.

For patients KP, MA and LW it is likely that the reduction in mepp amplitudes and hence epp amplitudes are responsible for their observed muscle weakness. For patients KO, MO, MI and CA, in addition to the reduction in mepp amplitude there was a reduction in epp quantal content, both effects leading to a decreased epp amplitude and hence muscle weakness.

Plasma from patient SP, the anti-AChR Ab positive MG patient, affected mepp characteristics recorded in mouse muscles. Mepp amplitude was reduced but the coefficient of variation of mepp amplitude distribution, the rise time, the decay time and the frequency were generally unchanged in the mice after treatment with SP plasma for 7 days. The effects on mepps by SP plasma were generally similar to those obtained in muscles treated with the plasma or IgG fraction from antibody-negative MG patients; mepp amplitudes were reduced while the other characteristics were largely unaffected except in the cases noted above.

More specifically, comparison of mepp amplitude reductions (as a percentage of controls) between antibody-negative MG plasma treated muscles and SP plasma treated muscles showed that reductions were generally of a similar magnitude. SP plasma reduced mepp amplitudes, in normal Ca^{2+} concentration solutions, by 52% which compared well with KP, KO, MO, and MI plasma which reduced mepp amplitudes by 61-46%. Reductions by MA or LW plasma and CA IgG were less, ranging between reductions in mepp amplitude by 32-23%. SP plasma also reduced mepp amplitudes in the presence of physostigmine suggesting that the reduction was not due to an excess of acetylcholinesterase in the cleft. For KP plasma treated muscles reductions were also seen in the presence of anticholinesterases. However, neither KO plasma nor KO IgG reduced mepp amplitudes, compared to respective controls, in the presence of physostigmine. Thus the possibility of an excess acetylcholinesterase in the cleft being responsible for the small mepp amplitudes in patient KO cannot be excluded. For mepp recordings in low Ca^{2+} concentration solutions following treatment with SP, KP and KO plasma and KO IgG the

reductions were smaller as compared to controls. However, again reductions were of a similar magnitude when compared between SP plasma treated muscles and antibody-negative muscles. Moreover, the extent of the reduction in mepp amplitude for these patients in low Ca^{2+} concentration solutions was, as expected, similar to the extent of reduction in epp amplitudes recorded in the same solutions.

Thus although there are similarities in the effects on mepps between the two classes of MG patients there were distinct differences in certain patients. SP plasma had no effect on quantal content when recorded in low Ca^{2+} concentrations. Likewise KP plasma or IgG, MA plasma and LW plasma had no effect on quantal content. However KO plasma or IgG, MO plasma, MI plasma and CA IgG did reduce quantal content both in normal Ca^{2+} concentration solutions and also, where recorded, in low Ca^{2+} concentration solutions. Unfortunately neither α-BuTx binding studies nor noise analysis were carried out in SP plasma treated animals. However, since SP plasma has been shown to have a high titre of anti-AChR Abs (Table 2) it seems reasonable to predict that a decrease in AChRs accounts for the reduction in mepp amplitudes and decrease in postsynaptic sensitivity to ACh (Ito et al., 1978b).

In summary then, it would appear that antibody-negative MG is a distinct disease entity to MG which is a postsynaptic disorder. Although both are autoimmune diseases, the antibody is most probably directed against different antigenic neuromuscular junction determinants in the two diseases. Finally, antibody-negative MG encompasses a heterogeneous population; one class has muscle weakness due to a reduced mepp amplitude alone while the other class has an additional reduction in epp quantal content; both classes seem to occur by presynaptically acting antibodies.

Chapter 4

ELECTROPHYSIOLOGICAL RECORDINGS OF MEPPS FROM HUMAN MUSCLE FOLLOWING ACUTE INCUBATION WITH MG PREPARATIONS

Section I : Introduction

The experiments reported in this chapter were designed to investigate the action of anti-AChR antibodies from myasthenia gravis patients on human muscle AChR. The main preparation studied in this chapter was the Ig fraction from patient ML; the clinical information of this MG patient is shown in Table 3. The plasma from patient ML contains anti-AChR antibody and has previously been shown to block one of the two α-BuTx binding sites on the rat or mouse embryonic AChR and to inhibit the function of AChR in the same preparations. It does not, however, affect α-BuTx binding or function of the adult form (>14 days after birth) in these species as shown, for example, by a lack of reduction in mepp amplitudes, lack of effect on ACh channels measured by noise analysis and lack of block of toxin binding (Maricq, Gu, Hestrin & Hall, 1985; Hall, Gorin, Silberstein & Bennett, 1985; Schuetze et al., 1985). Furthermore, the Ig fraction from plasma ML has also been shown to block α-BuTx binding to solubilized AChRs from human denervated and normal muscle and to intact adult muscle AChR (Burges, Pizzighella, Vincent, W.-Wray & Hall, 1989).

The aim of these experiments was to elucidate whether this MG preparation had an acute functional effect on human adult end-plate AChRs. To do this, adult human muscles were incubated in vitro with ML Ig and electrophysiological recordings made of mepps. In addition, MG plasmas BQ and CH (Table 3) were chosen as negative controls having high anti-AChR Ab titres in routine testing but having no antibodies directed against the α-BuTx binding site. Results were compared with plasma from patient MW which had no detectable anti-AChR Ab in routine testing (Table 3).

Section II : Acute effects of Myasthenia Gravis Preparations on Mepps

Biopsied human muscle bundles were incubated with the MG preparations or control plasma for 3 hours and prepared for electrophysiological recordings (see Methods).

Mepps (5-42 at each end-plate) were recorded from muscles acutely treated with MG preparations, at a temperature of 21.0-24.0°C. Mepp amplitudes were significantly ($P<0.001$) reduced by ML Ig but the other MG preparations had no effect on this parameter as compared to controls (Table 48). MG preparation ML Ig had a noticeable effect on recordings and in muscles treated with ML Ig it was generally harder to find end-plates with recordable mepps. Mepp rise times were significantly ($P<0.001$) increased by ML Ig compared with controls but were not affected by treatment with the other plasmas (Table 48). To investigate whether any increase in rise time was due to the microelectrode not being located at end-plates, mean mepp rise time was plotted against mean mepp amplitude for each end-plate studied (Figure 15). There appears to be a strong correlation between the rise time and the amplitude; small mepp amplitudes tend to have longer rise times. This is the effect that would be expected if the microelectrode was distant from the end-plate during recording. However, mepps with a rise time less than 1.95ms show little correlation between rise time and amplitude (Figure 15). When mepps were selected with rise times less than 1.95ms there was still a significant ($P<0.001$) reduction in the mepp amplitude by 52% by ML Ig but the rise time was not affected (Table 49). This suggeststhat the reduction in mepp amplitude was due to the action of ML Ig and not due to errors in the recording technique. Moreover, corrections of amplitude to a resting potential of -80mV did not affect the statistical significance of the

Table 48

Effect of MG Preparations on Mepp Characteristics

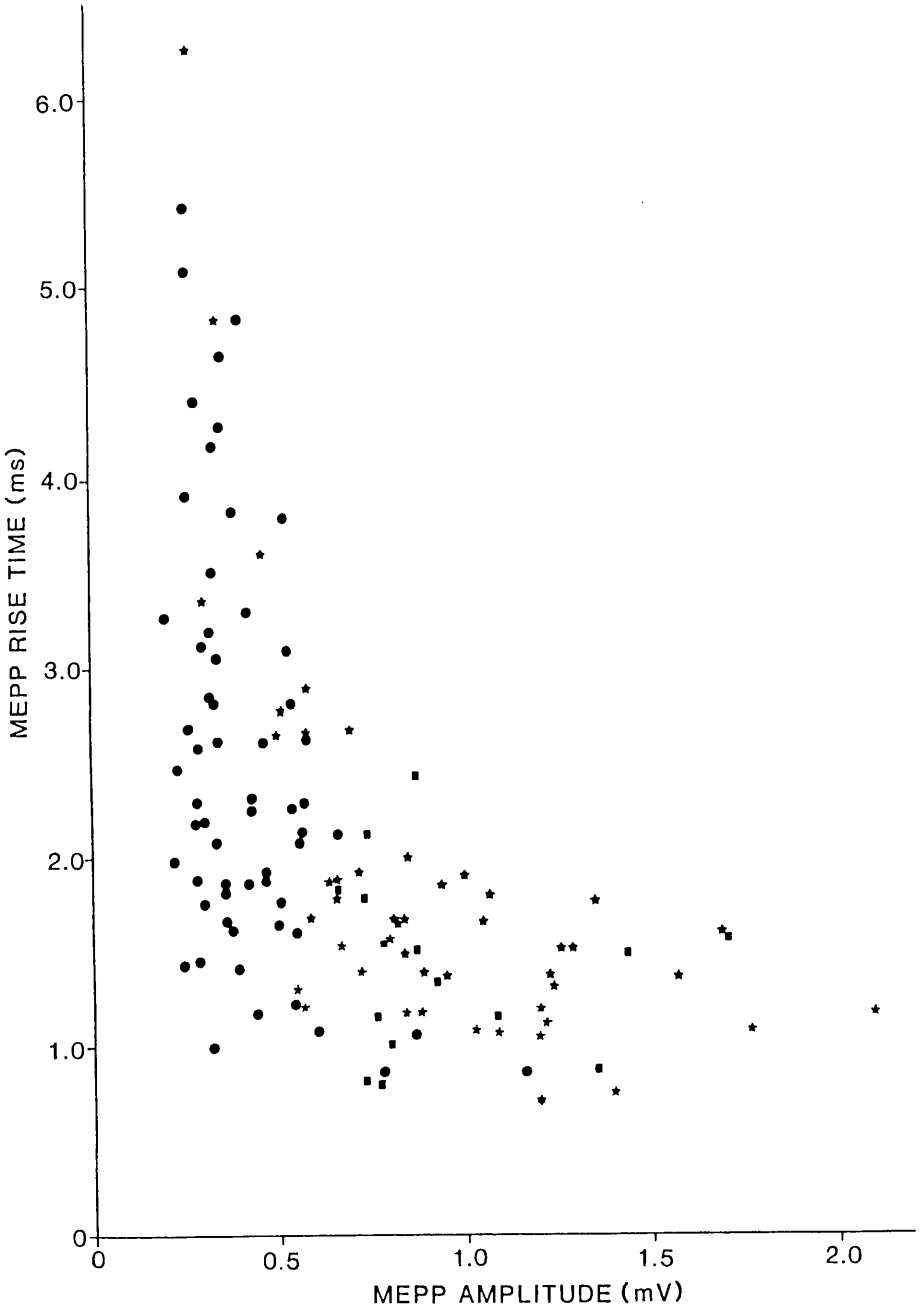
Treatment	Temperature (°C)	Resting Potential (mV)	MEPP CHARACTERISTICS		No. of End-plates, Mice
			Amplitude (mV)	Rise Time (ms)	
ML Ig	21.0-24.0	-69.3 ±0.82	0.41 ±0.02*	2.23 ±0.11*	66, 10
CH Plasma	22.9	-69.7 ±3.2	1.06 ±0.16	1.38 ±0.13	6, 1
BQ Plasma	22.8	-79.6 ±1.7¤	0.99 ±0.12	1.39 ±0.29	5, 1
MW Plasma	23.0	-71.9 ±2.2	0.81 ±0.09	1.46 ±0.20	6, 1
Control Plasma	21.8-24.0	-68.9 ±0.90	0.91 ±0.06	1.60 ±0.13	49, 9

Legend for Table 48

This table shows mepp amplitude and rise time for all mepps recorded from muscles treated with the MG preparations and control plasma. Values shown are means ± S.E.M. Significant differences from controls: *P<0.001; ¤P<0.002.

Figure 15

Mepp Amplitude Versus Mepp Rise Time - Acute Treatment with MG Preparations



Legend for Figure 15

This figure shows a plot of mepp amplitude against mepp rise time for each end-plate recorded from. Muscles have been acutely treated with the MG preparations and control plasma. This figure represents a total of 132 end-plates: ● ML Ig (66 end-plates); ■ CH plasma (6 end-plates), BQ plasma (5 end-plates) and MW plasma (6 end-plates); ★ Control plasma (49 end-plates).

Table 49

Effect of MG Preparations on Mepp Characteristics with a Rise Time Less than 1.95ms

Treatment	Temp. (°C)	Resting Potential (mV)	MEPP CHARACTERISTICS						No. of End-plates, Mice
			Amplitude		Coefficient of Variation of Amplitude Distribution	Rise Time (ms)	Decay Time (ms)	Frequency (s^{-1})	
			Un-corrected for Resting Potential (mV)	Corrected to Resting Potential of -80mV (mV)					
ML Ig	21.8-24.0	-67.7 ±1.4	0.49 ±0.04*	0.58 ±0.05*	0.47 ±0.04*	1.51 ±0.07	6.56 ±0.32	0.08 ±0.02	24, 9
CH Plasma	22.9	-69.7 ±3.2	1.06 ±0.16	1.21 ±0.16	0.28 ±0.05	1.38 ±0.13	8.11 ±0.98	0.07 ±0.01	6, 1
BQ Plasma	22.8	-79.9 ±2.1[¤]	1.02 ±0.15	1.01 ±0.14	0.41 ±0.06[§]	1.12 ±0.15	7.05 ±0.85	0.05 ±0.01	4, 1
MW Plasma	23.0	-70.2 ±1.8	0.83 ±0.11	0.95 ±0.13	0.34 ±0.11	1.33 ±0.18	7.6 ±1.1	0.06 ±0.01	5, 1
Control Plasma	21.8-24.0	-68.6 ±1.0	1.04 ±0.06	1.20 ±0.06	0.25 ±0.03	1.44 ±0.05	7.09 ±0.27	0.08 ±0.01	38, 8

Legend for Table 49

This table shows the effect of MG preparations on mepp characteristics. All values shown in this table are for end-plates with a mean rise time less than 1.95ms. Mepp amplitudes have been corrected to a resting potential of -80mV to account for variations in resting potentials. Values shown are means ± S.E.M. averaged over the number of end-plates shown. Significant differences from controls: *$P<0.001$; [¤]$P<0.002$; [§]$P<0.005$.

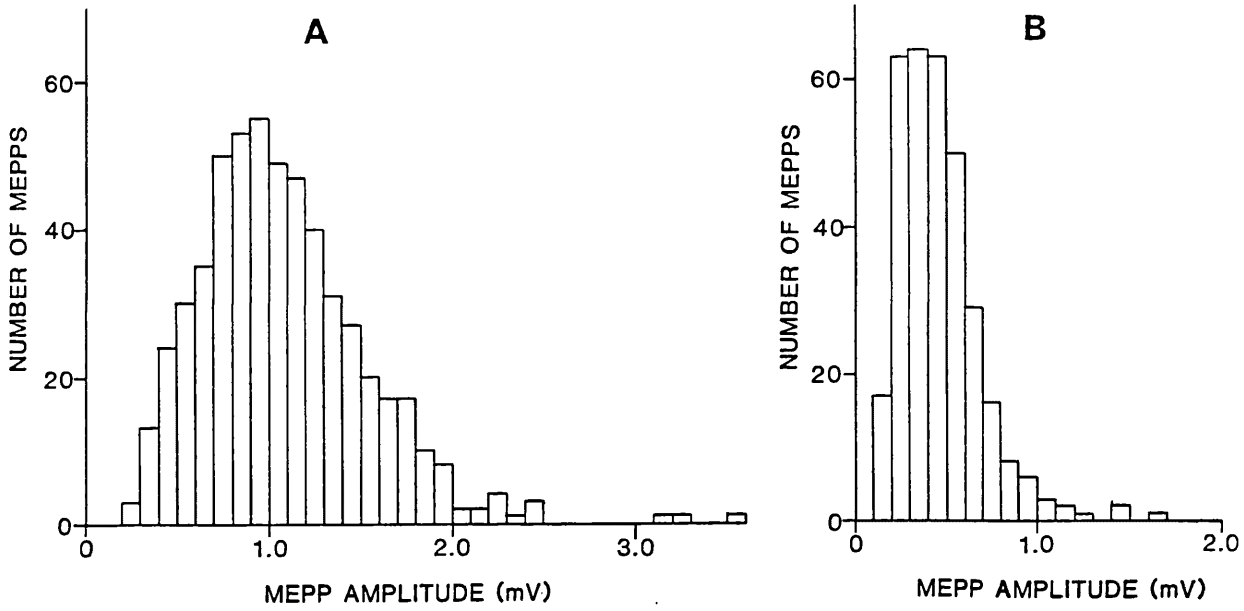
reduction in mepp amplitude by ML Ig (Table 49). After selecting for rise time less than 1.95ms, the other plasma preparations continued to show no effect on amplitude and rise times (Table 49).

Plasma CH and MW did not affect the coefficient of variation of mepp amplitude distributions (Table 49) but for BQ plasma and ML Ig treated muscles there was a significant increase in this parameter (Table 49). To clarify the effect of ML Ig, mepp amplitude histograms were studied (Figure 16). It can be seen from this figure that ML Ig skewed the distribution of the mepps towards smaller amplitudes. Figure 16 also shows the smaller mepps disappearing into the background noise level, i.e. <0.1mV. This suggests that the mean mepp amplitude for the ML Ig treated muscle was overestimated. In contrast, mepps recorded from the control end-plates showed a typical bell-shaped distribution, the smallest mepps were clearly above the background noise level. For BQ plasma treated muscles, the increase in coefficient of variation of mepp amplitude distribution is largely attributed to the wide spread of mepp amplitude size within one fibre.

None of the MG preparations had any effect on mepp decay time constants (Table 49). Thus, none of them had any non-specific effect on passive membrane properties. Finally, it can be seen that the mepp frequency was not changed by any of the MG preparations as compared to controls (Table 49). This suggests that none of these preparations had any effect on the presynaptic factors involved in spontaneous release. Although a reduction in frequency may have been expected for ML Ig (because of loss of small mepps into the noise) this was not detected, probably because of the large standard errors (about 25%) in the mean frequency for ML Ig.

Figure 16

Effect of ML Ig on Mepp Amplitude Histogram Distributions



Legend for Figure 16

This figure shows mepp amplitude histogram distributions. Data was pooled for muscles incubated in (A) control pooled human plasma (38 end-plate, 8 muscles) and (B) ML Ig (24 end-plates, 9 muscles). Mepps were selected with rise time less than 1.95ms.

Section III : Discussion

ML Ig caused reductions in mepp amplitude with little change in the other mepp characteristics measured; the effect on rise time was attributed to recording technique and not ML Ig. Thus ML Ig had an acute effect on function of AChRs at adult end-plates. By contrast, none of the other three plasmas had any clear acute effects on mepp characteristics suggesting lack of acute functional effects.

In addition, α-BuTx binding studies were performed on one of the two muscle bundles incubated with the MG preparations (A. Vincent). Thus muscle bundles used for electrophysiological recordings and α-BuTx binding studies had been incubated under identical conditions. In this way the electrophysiological and biochemical results could be readily compared. The results are shown in Table 50 (from Burges et al., 1989) and it can be seen that ML Ig caused significant ($P<0.001$) reductions by 68% in the number of ^{125}I-α-BuTx sites per end-plate. The other plasmas had no affect on α-BuTx binding (Table 50).

It has previously been shown (Hall et al., 1985; Schuetze et al., 1985) that ML Ig blocks function of embryonic rat end-plates, by binding to one of the α-BuTx binding sites on the AChR (as suggested by a 50% decrease in α-BuTx binding),but does not affect adult rat end-plates. In contrast, the reduction in mepp amplitude found in this chapter shows that ML Ig acutely interacted with adult human end-plates. Thus the effect at adult human end-plates differs from that at adult rat end-plates.

The ML Ig had a clear functional effect on mepp amplitudes and hence presumably on postsynaptic ACh sensitivity at adult human end-plates. The increased difficulty in finding mepps is suggested by the skewed distribution seen in the mepp amplitude histogram; mepps were reduced at some end-plates to such an extent that they were masked by the background noise. Such skewed distributions

Table 50

Effect of MG preparations on α-BuTx Binding Studies

	Number of ^{125}I-α-BuTx Sites Per End-plate (10^7)	Number of Assays, Muscles
ML Ig	$0.35 \pm 0.21^*$	15, 3
CH Plasma	1.8 ± 0.3	3, 1
BQ Plasma	1.6 ± 0.6	6, 1
MW Plasma	1.3 ± 0.3	6, 1
Control Plasma	1.1 ± 0.2	22, 4

Legend for Table 50

This table shows the number of ^{125}I-α-BuTx sites per end-plate obtained from muscles incubated with ^{125}I-α-BuTx (125nM) after exposure to the MG preparations. The method used is described in detail in Ito, Miledi, Vincent & Newsom-Davis, 1978b. Following overnight washing the muscle was fixed, stained with acetylcholinesterase to locate the end-plates, and small segments containing either end-plate or non-end-plate regions separated before gamma counting. Values shown are means ± S.D. of the number of ^{125}I-α-BuTx binding sites per end-plate after subtraction of non-end-plate binding. Significant difference compared with control: $^*P<0.001$.

have previously been observed in recordings from MG patients muscles (Albuquerque et al., 1976b). At end-plates in ML Ig treated muscles where mepps were detectable, they were reduced in amplitude by 52%, although because of the skewed distribution the true reduction must be more than this. Acute effects of MG Ig on channel function, as seen with ML Ig, have rarely been observed (Albuquerque et al., 1976a; Ito et al., 1978b; Shibuya et al., 1978; Howard & Sanders, 1980; Peper et al., 1981; Dolly et al., 1988). This is in contrast to the widely reported acute effects of antibodies on embryonic, denervated, neonatal or cultured AChR channel function (for list see Dolly et al., 1988; Gu, Silberstein & Hall, 1985; Maricq et al., 1985).

The ML Ig also reduced α-BuTx binding at adult human end-plates by 68% (see above). However, this is within experimental scatter of a value of 50% expected if the Ab binds to one of the toxin sites on the AChR as has been shown using a mouse muscle cell line, C2 (Gu et al., 1985). Such binding by ML Ig may also be expected to block one of the two binding sites on the AChR. In this case, mepps would be expected to be reduced much more than 50%, since two ACh molecules are required for channel opening. Indeed, the reduction in mepp amplitude is probably much greater than 50% (see above). Moreover, a correlation between functional block and inhibition of toxin binding by ML Ig has been suggested following experiments using the cell line, C2 (Hall et al., 1987).

Antibodies that block α-BuTx, without effect on function, are common in myasthenic sera (Almon, Andrew & Appel, 1974; Bender et al., 1975, Lefvert, Cuénoud & Fulpius, 1981; Drachman et al., 1982; Vernet-der Garabedian, Morel & Bach, 1986). For patient ML, since the antibodies bind to a toxin site and simultaneously affect function, they fall into category I as defined by Dolly et al., 1988. Such antibodies that can block both function and toxin binding are indeed rarely found and are not usually the predominant species in a myasthenic serum (Dolly, Mehraban, Gwilt & Wray, 1983; Goldberg, Mochly-Rosen, Fuchs & Lass,

1983; Donnelly, Mihovilovic, Gonzalez-Ros, Ferragut, Richman & Martinez-Carrion, 1984; Wan & Lindstrom, 1985; Blatt, Montal, Lindstrom & Montal, 1986; Fels, Plümer-Wilk, Schreiber & Maelicke, 1986). Alternatively, it is possible that since ML IgG is polyclonal, it may contain antibody clones that separately affect function and the binding of α-BuTx to the AChR.

That ML Ig affects human adult AChR differently from rat adult AChR strongly suggests that there are antigenic differences between AChRs in different species. Thus it is possible that there are a class of epitopes on adult human receptors not found on adult rodent receptors, and using rodent end-plates, therefore, may not be useful for assessing the pathophysiological effect of myasthenia gravis antibodies in humans. Moreover, antigenic differences may explain the difference in binding of ML Ig to the embryonic and adult AChRs within the rat and human (Burges et al., 1989).

Although plasmas CH and BQ had high apparent anti-AChR Ab titres they did not affect mepps. This may be due either to a low affinity of the Ab for the AChR, such that Abs did not bind during the short exposure time and reduce toxin binding, or to Ab binding at a site not involved in channel function. Plasma MW had no detectable anti-AChR Ab in routine testing. It is possible that anti-AChR Abs were present but at levels too low to be detectable (see Introduction). In such a case the lack of effect of MW plasma on function or α-BuTx binding may be explained as for CH and BQ plasma (see above). Alternatively, Abs in MW plasma may be directed at a site other than the AChR (as for those antibody-negative patients described in Chapter 3). In this case Abs would not be expected to affect toxin binding but short exposure times may have prevented any functional effect being recorded.

In summary, the acute functional effect of ML Ig against adult human end-plate AChRs suggests that these antibodies have a pathophysiological role in myasthenia gravis. The short incubation periods exclude the possibility that the observed effect

is due to cross-linking or complement-mediated lysis leading to receptor degredation (Tzartos, Sophianos & Efthimiadis, 1985). How frequently anti-AChR antibodies which both block function and inhibit AChR toxin binding are found in myasthenia gravis sera from other patients, is unknown. However, for at least patient ML, acute pharmacological block of AChRs by Abs could produce the observed symptoms of myasthenia gravis instead of, or in addition to, any degredation of receptors by cross-linking or via complement.

Chapter 5

NEUROMUSCULAR TRANSMISSION:

EFFECT OF CALCIUM CHANNEL AGONISTS AND

ANTAGONISTS

Section I : Introduction

Entry of Ca^{2+} into the nerve terminal is essential for transmitter release. Previous work has shown that L-type Ca^{2+} channels have a dihydropyridine binding site (Reuter et al., 1985). Although there are known to be Ca^{2+} antagonist sensitive channels in the presynaptic motor nerve terminal (Penner & Dreyer, 1986) such channels are not usually involved in transmitter release since release is not blocked by these organic antagonists. However, Ca^{2+} antagonist sensitive channels may play a role in transmitter release when their effect has been enhanced by the presence of Ca^{2+} agonists. Accordingly, a dihydropyridine agonist, CGP 28392 was applied in vitro to mouse diaphragms and a dihydropyridine antagonist, nitrendipine was also studied by way of comparison. The time course of the effects of these drugs on nerve stimulated quantal and high K^+ evoked release were continuously followed in single end-plates. This provided information on the possible involvement of dihydropyridine sensitive (L-type) Ca^{2+} channels in transmitter release.

Section II : Action of Dihydropyridines in Low Ca^{2+} Concentration Solutions

(a) Nerve Stimulated Quantal Release

Epps were recorded (22.0-23.6°C) in 0.24mM Ca^{2+} concentration solutions, at a stimulation frequency of 1Hz, before and during addition of the drugs to the end-plate. Since quantal release was low in these solutions, quantitative analysis of epp quantal content (i.e. the number of packets of ACh released per nerve stimulation) was obtained using the direct, failures and variance methods described in Methods. There were no significant differences between the values for quantal content obtained by three methods, and epp quantal content was calculated as the average of these three values. Recordings were analysed in blocks of at least one

minute (average 50 epps). Table 51 shows the results for epp quantal content and amplitude continuously recorded from muscles in the absence and presence of CGP 28392 (2μM). There was no significant change in the average resting potential during the recording and at each end-plate the resting potential changed by less than 3mV during the continuous recording. There was no significant effect of the agonist, CGP 28392, on the nerve stimulated quantal release (Table 51). This information is also shown in Figure 17 which illustrates the average time course of CGP 28392 on epp quantal content for 6 end-plates. Moreover there was no significant action of the drug on epp amplitude (Table 51).

Table 52 shows results obtained during continuous recordings of epps, during the addition of nitrendipine (2μM) to mouse diaphragms. The average resting potential did not vary significantly during this time; the resting potential for individual fibres drifted by less than 3mV. The antagonist, nitrendipine, had no significant effect on either epp quantal content (Table 52, Figure 18) or on epp amplitude (Table 52).

These results for epps suggest that neither the calcium channel agonist, CGP 28392, nor the antagonist, nitrendipine (in the conditions and at the concentration used here) have any presynaptic action on transmitter release at the nerve terminal as shown by the lack of effect on epp quantal content.

(b) Spontaneous Release

During recordings of epps in 0.24mM Ca^{2+} solutions (see above), simultaneous recordings of mepps (15-30 at each end-plate) were made (22.0-23.6°C). For recordings made in CGP 28392 containing solutions, mepp amplitudes were unaffected (Table 51). For mepp frequency, there was a hint of an increase in the presence of CGP 28392 but there were large standard errors in the mean frequency and so this increase did not attain statistical significance (Table 51).

Table 51

Effect of CGP 28392 on Nerve Stimulated Quantal Content and Spontaneous Release in 0.24mM Ca^{2+} Concentration Solutions

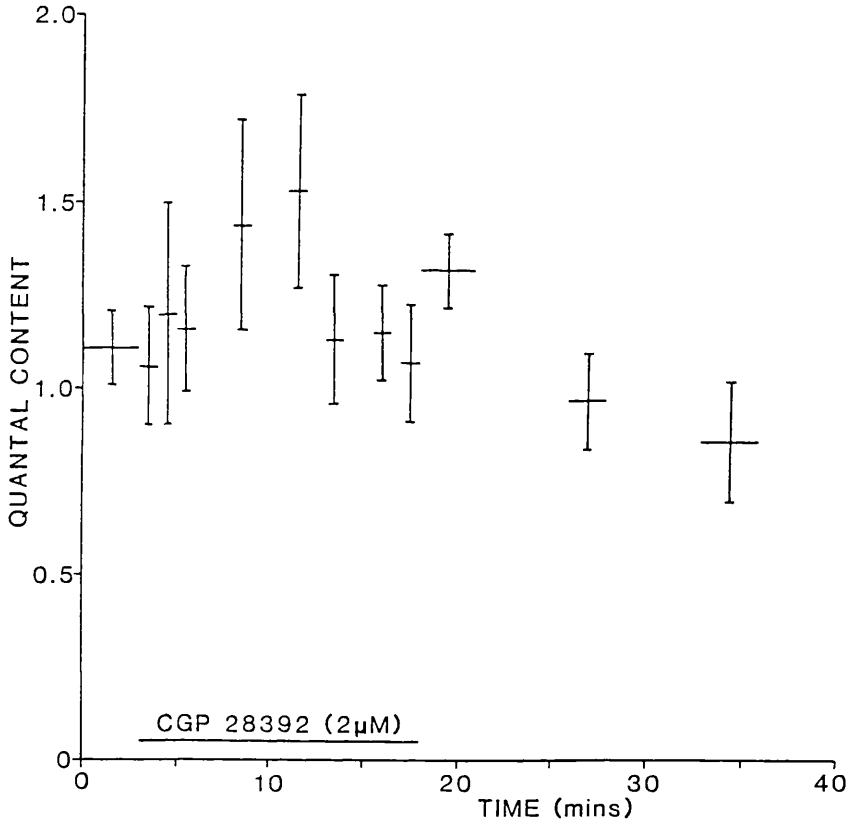
Recording Solution	Time (mins)	Resting Potential (mV)	EPP CHARACTERISTICS		MEPP CHARACTERISTICS		No. of End-plates, Mice
			Quantal Content	Amplitude (mV)	Amplitude (mV)	Frequency (s^{-1})	
Pre-Drug	0-3	-61.8 ±0.7	1.11 ±0.10	0.98 ±0.08	1.29 ±0.18	0.22 ±0.03	6, 6
CGP 28392 (2μM)	3-4	-62.8 ±0.6	1.06 ±0.16	0.98 ±0.16	1.24 ±0.30	0.32 ±0.09	6, 6
	4-5	-62.8 ±0.6	1.20 ±0.25	1.01 ±0.19	1.25 ±0.33	0.24 ±0.11	6, 6
	5-6	-62.8 ±0.6	1.16 ±0.17	1.01 ±0.18	1.26 ±0.32	0.26 ±0.08	6, 6
	8-9	-62.8 ±0.6	1.44 ±0.28	1.19 ±0.24	1.34 ±0.35	0.31 ±0.13	6, 6
	11-12	-62.8 ±0.6	1.53 ±0.26	1.16 ±0.20	1.26 ±0.32	0.42 ±0.20	6, 6
	13-14	-62.6 ±0.4	1.13 ±0.18	1.01 ±0.19	1.26 ±0.31	0.45 ±0.26	6, 6
	15.5-16.5	-62.6 ±0.4	1.15 ±0.13	1.11 ±0.21	1.26 ±0.36	0.69 ±0.50	6, 6
	17-18	-62.1 ±0.5	1.07 ±0.16	0.99 ±0.20	1.26 ±0.35	0.62 ±0.43	6, 6
Washout	18-21	-62.0 ±0.6	1.32 ±0.10	1.17 ±0.26	1.24 ±0.24	0.62 ±0.36	6, 6
	26-28	-61.2 ±0.7	0.97 ±0.13	0.56 ±0.05	1.00 ±0.33	0.22 ±0.03	6, 6
	33-36	-60.9 ±0.5	0.86 ±0.16	0.66 ±0.03	1.13 ±0.21	0.20 ±0.03	6, 6

Legend for Table 51

This table shows the effect of CGP 28392 (2μM) on epp quantal content and epp amplitude. Recordings were made in a 0.24mM Ca^{2+} concentration solution (22.0-23.2°C) for 3 minutes before addition of CGP 28392, for 15 minutes in the presence of CGP 28392 and during a washout period of 18 minutes. Values shown are means ± S.E.M. averaged over the number of end-plates shown. There were no significant differences between the values measured during the presence of CGP 28392 or washout as compared to pre-drug values.

Figure 17

Effect of CGP 28392 on Epp Quantal Content



Legend for Figure 17

This figure shows the average time course of CGP 28392 (2μM) on epp quantal content. A baseline value was obtained for 3 minutes and then CGP 28392 was applied for 15 minutes before a washout period. Values shown are means ± S.E.M. averaged from 6 end-plates; data as for Table 51.

Table 52

Effect of Nitrendipine on Nerve Stimulated Quantal Release and Spontaneous Release in 0.24mM Ca^{2+} Concentration Solutions

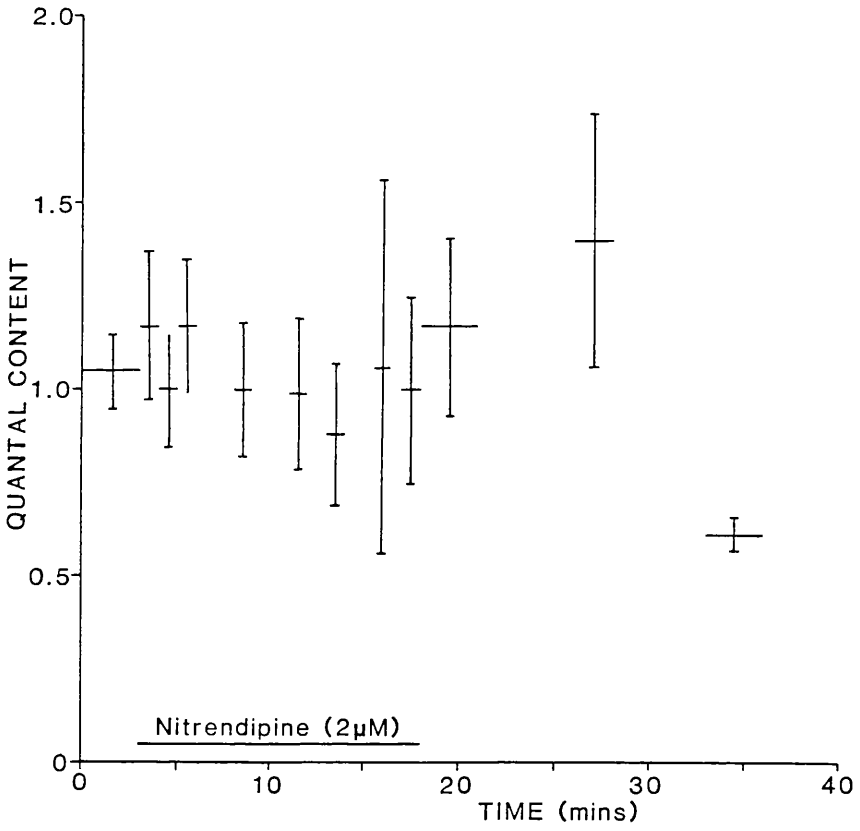
Recording Solution	Time (mins)	Resting Potential (mV)	EPP CHARACTERISTICS		MEPP CHARACTERISTICS		No. of End-plates, Mice
			Quantal Content	Amplitude (mV)	Amplitude (mV)	Frequency (s^{-1})	
Pre-Drug	0-3	-67.8 ±3.6	1.05 ±0.10	0.99 ±0.18	0.92 ±0.04	0.52 ±0.16	6, 6
Nitrendipine (2μM)	3-4	-67.1 ±3.4	1.17 ±0.20	1.12 ±0.38	0.96 ±0.09	0.72 ±0.46	6, 6
	4-5	-67.1 ±3.4	1.01 ±0.15	1.04 ±0.35	0.96 ±0.06	0.83 ±0.65	6, 6
	5-6	-67.1 ±3.4	1.17 ±0.18	0.98 ±0.25	0.95 ±0.07	0.82 ±0.68	6, 6
	8-9	-67.0 ±3.6	1.01 ±0.18	0.96 ±0.31	0.94 ±0.08	0.83 ±0.60	6, 6
	11-12	-67.0 ±3.6	0.99 ±0.20	0.90 ±0.30	0.93 ±0.07	0.71 ±0.49	6, 6
	13-14	-66.9 ±3.2	0.88 ±0.19	0.75 ±0.29	0.97 ±0.11	0.72 ±0.51	6, 6
	15.5-16.5	-66.9 ±3.2	1.27 ±0.32	0.89 ±0.30	0.89 ±0.05	0.69 ±0.40	6, 6
	17-18	-66.5 ±3.4	1.22 ±0.20	0.94 ±0.27	0.91 ±0.04	0.70 ±0.48	6, 6
Washout	18-21	-66.3 ±3.2	1.17 ±0.24	0.81 ±0.17	0.84 ±0.09	0.72 ±0.33	6, 6
	26-28	-66.2 ±3.3	1.40 ±0.34	1.01 ±0.37	0.86 ±0.02	0.63 ±0.52	6, 6
	33-36	-66.2 ±3.3	0.61 ±0.05	0.54 ±0.13	0.86 ±0.08	0.75 ±0.63	6, 6

Legend for Table 52

This table shows the effect of nitrendipine (2μM) on epp quantal content and epp amplitude. Recordings were made in a 0.24mM Ca^{2+} concentration solution (23.2-23.6°C) for 3 minutes before addition of nitrendipine, for 15 minutes in the presence of nitrendipine and during a washout period of 18 minutes. Values shown are means ± S.E.M. averaged over the number of end-plates shown. There were no significant differences, compared to pre-drug values, for values recorded in the presence of nitrendipine or during washout.

Figure 18

Effect of Nitrendipine on Epp Quantal Content



Legend for Figure 18

This figure shows the average time course of nitrendipine (2μM) on epp quantal content. A baseline value was obtained for 3 minutes and then nitrendipine was applied for 15 minutes before a washout period. Values shown are means ± S.E.M. averaged from 6 end-plates; data as for Table 52.

For mepp characteristics measured in the presence of nitrendipine, mepp amplitudes were also unchanged (Table 52) and there was no alteration in mepp frequency (Table 52).

It seems unlikely, therefore, that either CGP 28392 or nitrendipine had any postsynaptic action since both the mepp amplitudes, and hence epp amplitudes, were unaffected by these agents. Although nitrendipine did not affect mepp frequency, CGP 28392 may have had a slight presynaptic action on spontaneous release.

Section III : Action of Dihydropyridines on High K^+ Evoked Release

Using high K^+ solutions, mepp frequency is raised compared to normal K^+ solutions (Elmqvist, 1965). Depolarization by the high K^+ solutions, increases entry of Ca^{2+} into nerve terminals due to the opening of voltage-dependent Ca^{2+} channels and increases the rate of ACh release. Mepp frequency thus reflects a presynaptic phenomenon (Cooke & Quastel, 1973). Mepps (100-305 per 30 second analysis period) were recorded from muscles depolarized with high K^+ (13mM) solutions (22.8-24.0°C). Drugs were applied to the muscles for 15 minutes, after a baseline recording period of 3 minutes, and the effects on spontaneous release followed continuously in single end-plates.

Table 53 shows that the average resting potential was not significantly affected by CGP 28392 application and no fibre showed a drift of more than 3mV in resting potential during recordings. From the values of mepp frequency it can be seen that there was a slight increase in this parameter during drug application which approached statistical significance (Table 53, Figure 19). Moreover, there was no change in mepp amplitude in the presence of CGP 28392 (Table 53), as expected from the lack of effect on mepp amplitude and epp amplitude in low Ca^{2+} concentration solutions. In summary this data suggests that at a concentration of

Table 53

Effect of CGP 28392 on Mepp Characteristics

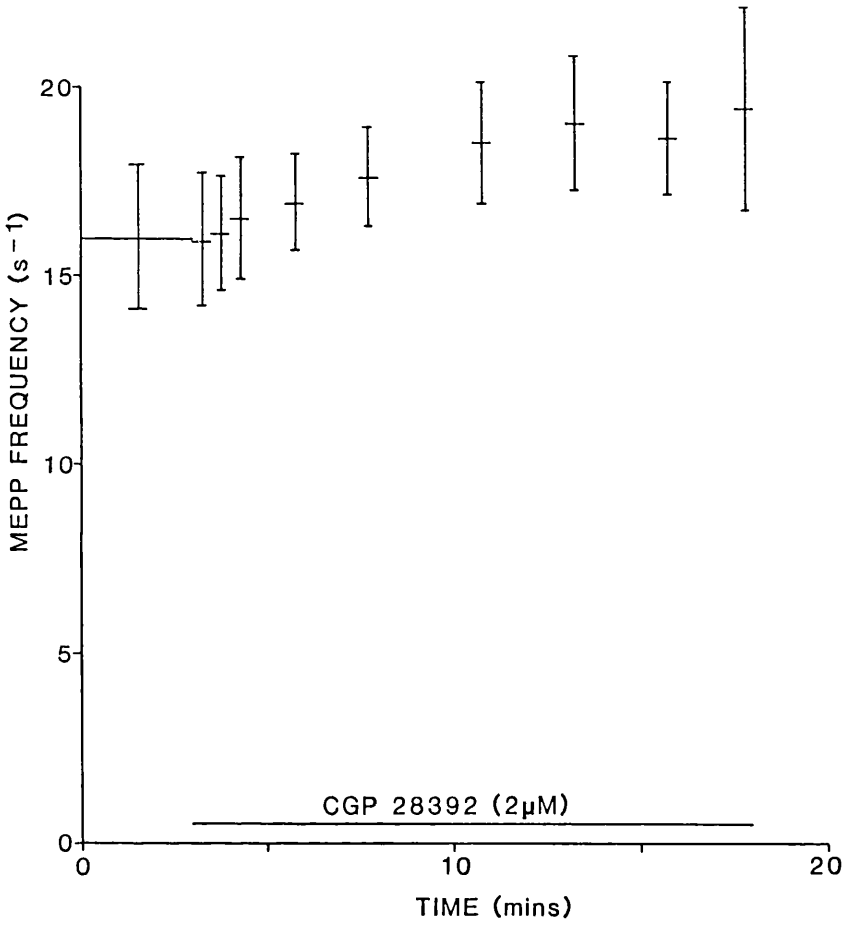
Recording Solution	Time (mins)	Resting Potential (mV)	MEPP CHARACTERISTICS		No. of End-plates, Mice
			Frequency (s^{-1})	Amplitude (mV)	
Pre-Drug	0-3	-54.2 ±1.0	16.0 ±1.9	1.20 ±0.22	7, 7
CGP 28392 (2μM)	3-3.5	-54.2 ±1.1	15.9 ±1.8	1.20 ±0.22	7, 7
	3.5-4	-54.2 ±1.1	16.1 ±1.5	1.20 ±0.23	7, 7
	4-4.5	-54.2 ±1.1	16.5 ±1.6	1.20 ±0.23	7, 7
	5.5-6	-54.2 ±1.1	16.9 ±1.3	1.20 ±0.24	7, 7
	7.5-8	-54.2 ±1.1	17.6 ±1.3	1.24 ±0.25	7, 7
	10.5-11	-54.2 ±1.1	18.5 ±1.6	1.25 ±0.25	7, 7
	13-13.5	-54.1 ±1.1	19.0 ±1.8	1.26 ±0.25	7, 7
	15.5-16	-54.1 ±1.1	18.6 ±1.5	1.29 ±0.27	7, 7
	17.5-18	-54.1 ±1.1	19.4 ±1.7	1.28 ±0.26	7, 7

Legend for Table 53

This table shows the effect of exposure to CGP 28392 (2μM) for 15 minutes on mepp frequency and amplitude recorded from muscles depolarized with a recording solution containing 13mM K^+ (22.8-24.0°C). Values shown are means ± S.E.M. There were no significant differences between the values recorded before the drug was added and at each time interval during the presence of CGP 28392.

Figure 19

Effect of CGP 28392 on Mepp Frequency



Legend for Figure 19

This figure shows the effect of CGP 28392 (2μM) on the average time course of mepp frequency. A baseline value was obtained for 3 minutes and muscles were exposed to CGP 28392 for 15 minutes. Values shown are means ± S.E.M. averaged from 6 end-plates; data as for Table 53.

2μM, CGP 28392 has no postsynaptic actions, although there may be a very slight presynaptic action under depolarized conditions as seen from the values for mepp frequency.

For continuous recordings made in the presence of nitrendipine (2μM) there was again no change in the average resting potential, as compared to values obtained before drug addition (Table 54). Moreover at each end-plate the resting potential always changed by less than 3mV. The presence of nitrendipine in the recording solution perhaps gave a hint of a reduction in mepp frequency but this was again not statistically significant (Table 54, Figure 20). There was also no significant effect on mepp amplitude (Table 54). This data thus shows that under these recording conditions nitrendipine (2μM) had no postsynaptic effect and probably no presynaptic action.

Finally, experiments were carried out to investigate any effect on mepp frequency by nitrendipine (2μM) at depolarized terminals (13mM K^+) already exposed to CGP 28392 (2μM). As expected, Table 55 shows that CGP 28392 alone had little effect on mepp frequency. Furthermore, the addition of the antagonist nitrendipine did not reduce the mepp frequency suggesting no antagonistic action on transmitter release. Interestingly, the general trend when both agents were present was an increase in the mepp frequency but this did not attain statistical significance.

Section IV : Discussion

This chapter describes experiments designed to study the time course of any modulatory effects of a dihydropyridine agonist on transmitter release at the mouse neuromuscular junction. This was carried out by making continuous recordings from single end-plates. By comparison, the action of an antagonist directed against Ca^{2+} channels was also studied. Neither CGP 28392 nor nitrendipine had any effect

Table 54

Effect of Nitrendipine on Mepp Characteristics

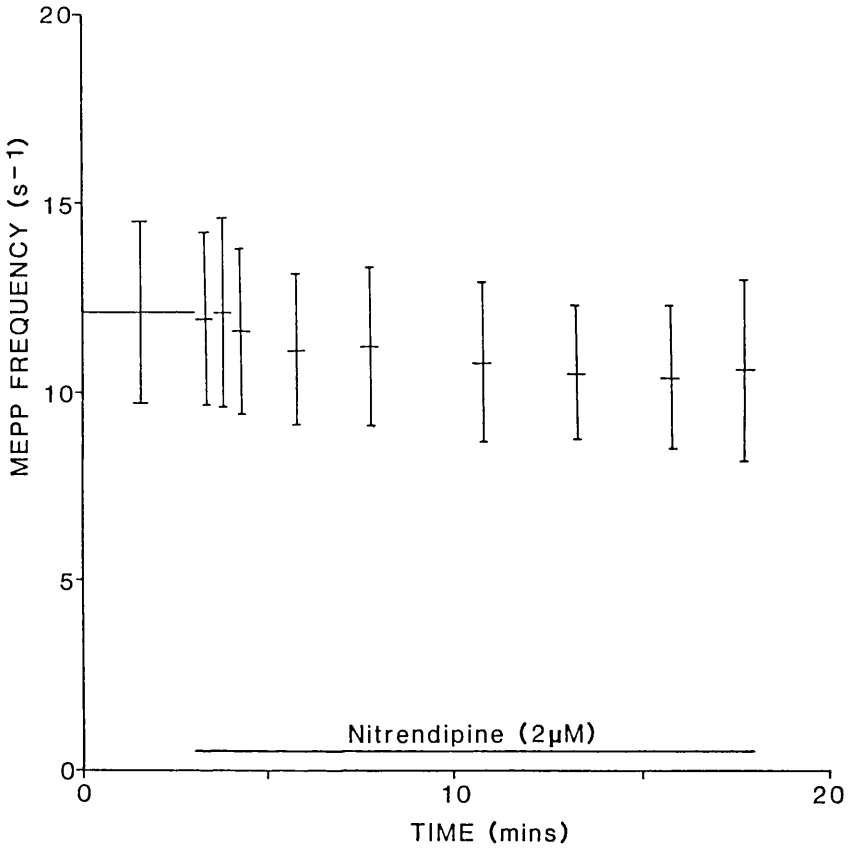
Recording Solution	Time (mins)	Resting Potential (mV)	MEPP CHARACTERISTICS		No. of End-plates, Mice
			Frequency (s^{-1})	Amplitude (mV)	
Pre-Drug	0-3	-55.3 ±1.3	12.1 ±2.5	0.85 ±0.13	6, 6
Nitrendipine (2μM)	3-3.5	-55.6 ±1.6	11.9 ±2.3	0.84 ±0.13	6, 6
	3.5-4	-55.4 ±2.0	12.1 ±2.5	0.84 ±0.13	6, 6
	4-4.5	-55.4 ±2.0	11.6 ±2.2	0.84 ±0.14	6, 6
	5.5-6	-55.8 ±1.6	11.1 ±2.0	0.84 ±0.16	6, 6
	7.5-8	-55.8 ±1.6	11.2 ±2.1	0.87 ±0.14	6, 6
	10.5-11	-55.6 ±1.8	10.8 ±2.1	0.89 ±0.14	6, 6
	13-13.5	-55.6 ±1.8	10.5 ±1.8	0.90 ±0.14	6, 6
	15.5-16	-55.5 ±1.6	10.4 ±1.9	0.93 ±0.16	6, 6
	17.5-18	-55.5 ±1.6	11.1 ±1.9	0.94 ±0.17	6, 6

Legend for Table 54

This table shows the effect of exposure to nitrendipine (2μM) on mepp frequency and amplitude recorded from muscles depolarized with a recording solution containing 13mM K^+ (24.0°C). Values shown are means ± S.E.M. There were no significant differences between values recorded before the addition of drug and at each time interval following addition of drug.

Figure 20

Effect of Nitrendipine on Mepp Frequency



Legend for Figure 20

This figure shows the effect of nitrendipine (2μM) on the average time course of mepp frequency. A baseline value was obtained for 3 minutes and muscles were exposed to nitrendipine for 15 minutes. Values shown are means ± S.E.M. averaged from 6 end-plates; data as for Table 54.

Table 55

Effect of CGP 28392 and Nitrendipine on Mepp Frequency

Recording Solution	Time (mins)	Resting Potential (mV)	Mepp Frequency (s^{-1})	No. of End-plates, Mice
Pre-Drug	0-3	-55.1 ±1.4	13.0 ±1.9	6, 6
CGP 28392 (2μM)	3-3.5	-55.3 ±1.4	13.1 ±2.0	6, 6
	3.5-4	-55.3 ±1.4	12.9 ±1.7	6, 6
	7.5-8	-55.3 ±1.4	13.5 ±1.9	6, 6
	10.5-11	-55.6 ±1.3	13.6 ±1.7	6, 6
	13-13.5	-55.8 ±1.5	13.1 ±1.8	6, 6
	17.5-18	-55.8 ±1.5	14.6 ±2.0	6, 6
CGP 28392 (2μM) +	18-18.5	-55.3 ±1.6	15.0 ±2.0	6, 6
Nitrendipine (2μM)	18.5-19	-55.3 ±1.6	15.7 ±2.1	6, 6
	22.5-23	-55.3 ±1.6	16.1 ±2.1	6, 6
	25.5-26	-54.7 ±1.8	15.8 ±2.3	6, 6
	28-28.5	-54.8 ±1.9	16.5 ±2.4	6, 6
	32.5-33	-55.2 ±1.8	15.9 ±2.1	6, 6

Legend for Table 55

This table shows the effect of CGP 28392 and a combination of CGP 28392 and nitrendipine on mepp frequency. Recordings (22.8°C) were made in the absence of drug, for 15 minutes during exposure of muscles to CGP 28392 (2μM) and for a further 15 minutes during exposure of muscles to CGP 28392 (2μM) and nitrendipine (2μM). Values shown are means ± S.E.M. averaged from 6 end-plates. There were no significant differences compared to pre-drug values or between values in the presence of CGP 28392 compared with those in the presence of CGP 28392 and nitrendipine.

on mepp amplitudes when recordings were made in reduced Ca^{2+} solutions or raised K^+ solutions. Moreover, as expected there was no effect of either agent on epp amplitude. This suggest that dihydropyridine agonists and antagonists have no postsynaptic action. Nerve stimulated quantal release (measured as epp quantal content) was not affected by either the agonist, CGP 28392 or the antagonist, nitrendipine. In the presence of high K^+ (13mM) neither the agonist nor the antagonists caused any marked change in mepp frequency although the data were consistent with a small increase in mepp frequency in the presence of CGP 28392. These effects were also seen in low Ca^{2+} concentration solutions. Thus at the concentration used in these experiments neither the agonist nor antagonist had any marked presynaptic effects. This suggests that L-type Ca^{2+} channels are not of major importance in the release of ACh at the neuromuscular junction. As discussed previously (see Introduction) any presynaptic action of such agents at the neuromuscular junction which have been reported are probably due to non-specific intracellular effects of the agents.

Antagonist-sensitive Ca^{2+} channels, however, are reported to be present at the mouse motor nerve terminals (Penner & Dreyer, 1986). It has been suggested for sympathetic neurones cultured from the rat superior cervical ganglion (SCG) that L-type channels are located at the soma, away from the active zone and the channels at the release site are N-type channels (Miller, 1987). This has also been suggested for release from rat brain synaptosomes (Reynolds, Wagner, Snyder, Thayer, Olivera & Miller, 1986). If this were also true at the mouse neuromuscular junction then this would explain why L-type Ca^{2+} channels do not appear to be directly involved in ACh release.

Another possible reason for the lack of involvement of L-type Ca^{2+} channels could have been the recording conditions. L-type Ca^{2+} channels are known to be voltage-dependent (Reuter, 1983) and dihydropyridines (such as CGP 28392 and nitrendipine) have been shown to preferentially bind to inactivated channels present

under depolarized conditions (Reuter et al., 1985). The action of dihydropyridines is thus more likely to be seen under conditions of prolonged depolarization due to exposure to high K^+ solutions (when there will be an increase in the Ca^{2+} influx) rather than during brief depolarizations that occur following phrenic nerve stimulation. Indeed, other reports have shown that in a K^+ concentration of 12mM, verapamil had no effect on release from neuromuscular junctions (Nachshen & Blaustein, 1979) as shown by lack of effect on mepp frequency. Moreover, in situations where an effect of the dihydropyridine BAY K 8644 has been shown on transmitter release (Miller, 1987) the K^+ concentration was very high. Release of noradrenaline from cultures of rat SCG neurons was only elevated by BAY K 8644 (10^{-6}M) by around 2.5 fold when the K^+ concentration was 70mM. However, at very high K^+ concentrations the mepp frequency is raised to such an extent that it is not easy to obtain an accurate measure of this parameter. It is possible, therefore, that if the K^+ concentration was much higher than could be used here an effect of dihydropyridine agonists may then have been seen.

If the model suggested for the rat SCG cells did fit the release process involved at the mouse neuromuscular junction, then the agonist would be expected to further enhance the entry of calcium into the nerve terminal via the distant L-type Ca^{2+} channels under prolonged depolarization. Since so much calcium might then enter, there may be enough Ca^{2+} to spill over and influence the mechanisms involved in release of ACh (Miller, 1987). The results presented in this chapter support this idea since there was a slight increase in the mepp frequency caused by CGP 28392 in 13mM K^+ solution although not statistically significant. Also it may be expected that the antagonist (nitrendipine) alone, would have no effect on mepp frequency; in support of this the extent of the reduction in mepp frequency by nitrendipine appeared to be negligible. Nitrendipine, acting as an antagonist, would reduce Ca^{2+} entry at the distant channels. This would not be expected to influence the release of the transmitter since such release is only determined by Ca^{2+} entry via channels at the

active zone release sites (unless there is an excess Ca^{2+} in the terminal due to the presence of an agonist). In this latter situation, the nitrendipine would then by expected to have an effect on transmitter release (Miller, 1987). Further experiments were carried out to investigate the validity of the proposed model. The lack of effect of nitrendipine on mepp frequency in the presence of CGP 28392 in these experiments may perhaps be attributed to a concentration effect. For example, the nitrendipine should perhaps have been present at a concentration higher than the CGP 28392. However, the effect of CGP 28392 alone before the addition of nitrendipine was only slight anyway. Hence CGP 28392 had not sufficiently enhanced the entry of Ca^{2+} into the nerve terminal for the antagonist to produce an effect.

If we assume that N-type Ca^{2+} channels are at the active zone regions it is perhaps surprising that ω-CgTx has been shown to be ineffective in blocking the release of ACh at mouse neuromuscular junctions in concentrations that are effective at the frog neuromuscular junction (Anderson & Harvey, 1987). Since the toxin blocks N- and L-type Ca^{2+} channels it is therefore likely that the Ca^{2+} channels present at active zones and hence involved in transmitter release from mouse motor nerve terminals are neither N- nor L-type, and thus has characteristics which distinguish it from previously defined neuronal Ca^{2+} channels (Anderson & Harvey, 1987).

It is possible, that the channel type at the mouse motor nerve terminal has antigenic differences from L-type channels in other tissues, e.g. cultured cardiac cells (Kokubun & Reuter, 1984) and neuroblastoma-glioma (NG108-15) cells (Freedman & Miller, 1984) making it less sensitive to dihydropyridines while still displaying some of the kinetic behaviours of those channels. Obviously more experiments need to be done to clarify the situation.

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ADDENDUM

In addition to the possible actions of a circulating plasma factor in antibody-negative MG patients, discussed in Section VIII, there may be alternative actions which need stressing.

For mice injected with KP, MA, KO and MO plasma and KO IgG the observed decrease in mepp amplitude was accompanied by a significant increase in mepp rise time. Although by applying a cut off of $\leqslant$0.4ms only very fast rise time mepps were being compared, it is possible that the action on rise time, before correcting, reflects a real effect of any antibody in these patients. Indeed, an antibody-induced widening of the synaptic cleft could produce such observations. Alternatively, any interposition of a diffusion barrier to ACh in the synaptic cleft would produce the same electrophysiological features. Morphological studies of the diaphragm muscles from the mice treated with the plasmas or IgG from antibody-negative patients would help resolve whether there were any Ab-induced structural changes. Results could then be compared with those obtained in SP plasma treated mice.

A measure of the input resistance of the muscle could have helped elucidate the mechanism underlying a reduction in mepp amplitude. Although normal in MG muscle (Elmqvist et al., 1964; Cull-Candy et al., 1979) there may have been an Ab-induced alteration of muscle membrane electrical properties in the antibody-negative patients.

It would also have been interesting to compare voltage noise recordings in SP plasma treated muscles with those obtained in KP, KO and MO plasma treated muscles. In this way, any postsynaptic action by the plasma from antibody-negative patients on ACh-induced depolarization and open channel properties could have been directly compared with the actions of an anti-AChR Ab. Interestingly, acute application to mouse diaphragm of an anti-human AChR monoclonal antibody caused a reduction in V_{max}, under the same conditions as used in this thesis (Dolly et al., 1988).

Finally, use of the voltage clamp method would have given the number of ionic channels opened by a single vesicle of ACh; comparing this with the results from the α-BuTx binding studies would have provided further insight into any postsynaptic action of an Ab in antibody-negative patients.