An investigation of the effects of potassium channel opening drugs in an in vitro innervated preparation of the guinea-pig trachea.

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ABSTRACT

The effects of potassium channel opening drugs (KCOs) were investigated in an in vitro vagally innervated tube preparation of the guinea-pig trachea. It has previously been reported that cromakalim inhibits transmission in pulmonary cholinergic nerves, (Hall & Maclagan, 1988) . This thesis further investigates the action of KCOs on pulmonary nerves using cromakalim (racemate), lemakalim ((-) isomer), BRL38226 ((+) isomer) and SDZPC0400 (an unrelated (-) isomer).

Cromakalim, lemakalim and SDZPC0400 did not significantly affect the dose related rise in intraluminal pressure elicited by the spasmogens acetylcholine, histamine or the thromboxane mimetic U46619. Therefore the KCOs do not appear to have any significant antispasmodic action nor do they inhibit postjunctional responses elicited by muscarinic receptor activation.

Contractions of the trachea evoked by preganglionic stimulation of the cholinergic vagus nerve (PGS) or transmural stimulation in the presence of hexamethonium (TMS) were both inhibited by cumulative doses of cromakalim, lemakalim or SDZPC0400. The KCOs act prejunctionally on pulmonary parasympathetic cholinergic nerves since they have no action on responses to exogenous acetylcholine which contracts the trachea by acting on postjunctional muscarinic receptors. As the inhibitory effect of cromakalim was greater on responses to PGS than TMS, this suggests that cromakalim has an inhibitory action at the ganglia as well as at the final nerve terminals.

The specific blocker of ATP-operated potassium channels, glibenclamide significantly reduced the inhibitory action of cromakalim, lemakalim and SDZPC0400 on contractions of the guinea-pig trachea elicited by PGS and TMS suggesting that ATP operated K^+ channels are opened by these KCOs in the pulmonary nerves.

The (+) isomer of cromakalim, BRL38226 caused only a slight inhibition of responses to nerve stimulation, but its presence significantly reduced the effect of lemakalim on responses to both PGS and TMS. It is possible that BRL38226 is blocking K^+ channels opened by lemakalim in the pulmonary nerves.

Transmitter release in the pulmonary cholinergic nerves could be inhibited by prejunctional muscarinic M_2 autoreceptors. An investigation

was made into a possible interaction of muscarinic autoreceptors and potassium channels using methoctramine and pilocarpine. However results obtained did not support this theory. Inhibition of nerve-induced contractions (PGS and TMS) by cromakalim was unaffected by the presence of the M_2 muscarinic antagonist, methoctramine. The M_2 receptor agonist, pilocarpine caused an inhibition of contractions of the trachea to PGS and TMS which was unaffected by the presence of glibenclamide

When tracheal tone was raised by the spasmogen histamine, NANC mediated relaxations were seen to transmural stimulation (in the presence of atropine, l-propranolol and hexamethonium). Cromakalim and lemakalim facilitated these NANC mediated relaxations of the guinea-pig trachea. Experiments were performed to see if this facilitatory effect was due to a potentiation of the effect of the inhibitory NANC transmitter (possibly VIP) on the trachealis muscle. Relaxations of the trachea to VIP were facilitated by cromakalim and lemakalim to the same extent as NANCergic relaxations indicating a postjunctional action of KCOs on facilitation of NANCergic transmission.

In summary, in the isolated guinea-pig trachea, opening of potassium channels inhibits cholinergic neurotransmission but facilitates NANC mediated relaxations. The in vivo bronchodilator effect of cromakalim and lemakalim in vivo is probably due to a combination of inhibition of cholinergic bronchoconstriction, a facilitation of NANC mediated relaxation and a direct effect on airway smooth muscle. These properties suggest that potassium channel opening drugs should have potential for clinical use in asthma therapy.

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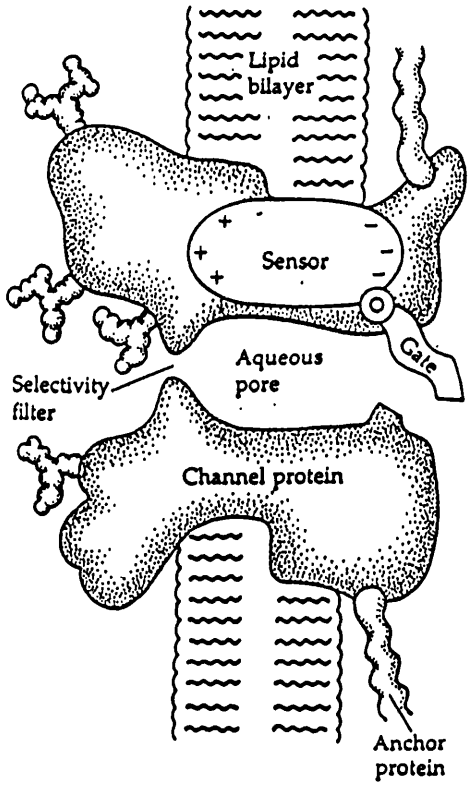


WORKING HYPOTHESIS FOR A CHANNEL

The channel is drawn as a transmembrane macromolecule with a hole through the center. The external surface of the molecule is glycosylated. The functional regions, selectivity filter, gate, and sensor are deduced from voltage-clamp experiments but have not yet been charted by structural studies. We have yet to learn how they actually look.

Diagram 1

A INTRODUCTION

A1. A working hypothesis for the structure of an ion channel.

A view of a hypothetical ion channel is shown in Diagram 1. The channel is a transmembrane protein with a hole in the centre sitting in the lipid bilayer of the membrane, but anchored in many cases to other membrane proteins or to elements in the intracellular cytoskeleton. The macromolecule is large, consisting of several thousand amino acids arranged in one or several polypeptide chains with some hundreds of sugar residues covalently linked as oligosaccharide chains to amino acids on the outer surface.

When open, the channel forms a water-filled pore extending fully across the membrane. The pore is much wider than the average ionic diameter over most of its length and may narrow to atomic dimensions only in a short stretch, the selectivity filter, where ionic selectivity is established. Hydrophilic amino acids would line the pore wall and hydrophobic aminoacids would interface with the lipid bilayer. Opening and closing of the ion channel is affected by a process called gating. Gating requires a conformational change of the pore that moves a gate into and out of an occluding position. The probabilities of opening and closing are controlled by a gate sensor. In the case of a voltage-sensitive channel, the sensor includes many charged groups that move in the membrane electrified field during gating. The open-shut nature of gating in single channels can be seen directly with patchclamp recording techniques, (Hille, 1984).

A2 Electrophysiology of the different types of potassium channel

Potassium channels are the most diverse group of ion channel so far investigated. The diversity seems to lie in the varied gating properties of the channels, their opening and closing response to changes in potential, ion concentration or chemical stimuli. It is not possible to formulate a definitive classification of potassium channels. In some cells only data on potassium currents are available and it is unclear whether these represent ion flow through a single channel or through a group of ion channels.

The objective of this section is to overview the electrophysical characteristics of the different potassium channels and their possible functions.

A2.1 Voltage dependant potassium channel

The delayed rectifier (I_K)

This channel has been widely described in neurones and skeletal muscle. On depolarisation, it activates after a short delay and shows rectification (increased conductance) the greater the membrane depolarisation. The channel is slow to inactivate and carries the K^+ ion during action potential repolarisation and it influences the refractory period. Its very slow activation suggests involvement in the repolarisation of agonist induced depolarisation which effectively terminates the action of the agonist.

In mammalian smooth muscle a similar current has been described in several tissues; urinary bladder (Klochner & Isenberg, 1985), intestine (Ohya et al, 1986) and pulmonary artery (Okabe et al, 1987). Delayed rectifier potassium channels have a typical conductance range of 10 to 50 pS and can be blocked by tetraethylammonium (TEA) applied to the inner or outer cell membrane surface or by Ba^{2+}.

A2.2 The 'A' current (I_A)

In neurones, this transient potassium current is activated by depolarisation after a period of hyperpolarisation. It also shows outward rectification on depolarisation, but activates and deactivates more quickly than I_K (Hille et al, 1984). A similar current has been described in pulmonary artery smooth muscle (Okabe et al, 1987). In general, the channels carrying I_A have a conductance of 15 to 20 pS and can be blocked by 4 aminopyridine (4AP). In comparison to the inhibitory action on I_K, TEA is inactive when applied to the inner or outer cell membrane. The function of this channel in smooth muscle is unknown.

A2.3 The inward rectifer

Channels which carry this current have been described in frog skeletal muscle, starfish eggs (Weston, 1988) and in cardiac muscle (Carmeliet, et al, 1988). In smooth muscle, currents with inward

rectifying properties have been described in intestine (Benham et al, 1987) and in cerebral arterioles, (Edwards et al, 1988). These inward rectifier currents are blocked by TEA, Cs^+ and Ba^{2+}.
The channels may be open at resting potentials giving rise to resting K currents. They close during depolarisation but show rectification (increased conductance) at membrane potentials more negative than E_K, potassium flows into the cell hence the description 'inward'. By remaining closed under depolarising conditions, inward rectifiers allow the membrane to remain depolarised for long periods resulting in plateau type action potentials. The inward rectifying channels will allow potassium flux at resting potentials and have a conductance of of approximately 20 pS. The exact function of the inward rectifier is unknown.

A2.4 Calcium dependant potassium channels.

The Maxi K-channel (BK or KL)

The opening of this channel is increased as intracellular calcium concentration ($[Ca]_i$) increases and also during membrane depolarisation. The channel is widely distributed in neurones, skeletal muscle and secretory cells. In these cell types it is involved in action potential repolarisation, modulation of action potential frequency and after hyperpolarisation. In mammalian smooth muscle this channel has been described in a variety of tissues arterial and intestinal muscle (Benham et al, 1985) in portal vein and airways (McCann & Welsh, (1986). It is probably important during spike repolarisation and is activated by any procedure which raises intracellular calcium concentration. It may be responsible for the rapid hyperpolarisation seen in airways smooth muscle on exposure to acetylcholine and histamine (Ahmed et al, 1984). The channel has a typical conductance of 100 to 300 pS and is blocked by charybdotoxin, TEA applied to the outer surface of the cell membrane and barium (Ba^{2+}).

A2.5 The small conductance K channel (SK or Ks).

The SK channels were first described in skeletal muscle (Blatz and Macleby, 1986) in which they were associated with long after hyperpolarisations in cultured cells. A key factor in the identification

of these channels is their selective blockade by apamin, a toxin which has no effect on BK channels. Typically apamin sensitive SK channels exhibit a conductance of 10 to 14 pS. They are voltage independant over a wide range of potential and are more calcium sensitive than BK channels. The ability of apamin to block these channels has led to the use of apamin to identify SK type channels in different tissues. There is no direct evidence of apamin sensitive SK channels in smooth muscle, however currents that might be supported by an SK channel have been reported in trachea and urinary bladder (Isenberg & Klockner, 1986). The Ca sensitivity of this channel suggests that if it is present within a cell it will be activated during any procedure which increases intracellular calcium concentration. Thus SK channels probably serve to regulate calcium entry, may modify action potential frequency patterns and be involved in membrane hyperpolarisation.

A2.6 The medium conductance K channel (IK or K_M).

Several calcium dependent potassium channels with a conductance intermediate between BK and SK have been described. One example is in red blood cells in which a -calcium sensitive channel of conductance 20 pS has been described (Hamil et al, 1981) The function of this channel is unknown.

A2.7 Receptor operated channels.

These are channels which are modulated by agonist receptor interactions. This modulation does not imply that the receptor and the channel are closely linked since second messenger channels may be involved. In guinea-pig taenia caeci the relaxant actions of noradrenaline and nonadrenergic noncholinergic (NANC) nerve stimulation are selectively inhibited by apamin suggesting the involvement of the SK channel (Banks et al, 1979; Shuma and Vladimirova 1980).

A2.71 The M and S potassium channels

The properties of the M channel has been described by Brown (1988) and the S channel by Sieglebaum (1987). Both channels are probably open at resting membrane potential and so contribute to resting membrane currents. In neurones, acetylcholine closes the M channel to mediate an

excitatory response. In aplysia sensory neurones, serotonin closes the S channel resulting in slow membrane depolarisation. Neither channel has been described in mammalian smooth muscle.

A2.72 The ATP sensitive potassium channel.

These are found in pancreatic ß cells. Like many other endocrine secretory cells, the secretion of insulin from the pancreatic ß cells is a calcium dependant phenomenon. Glucose is the trigger which initiates this event by depolarising the cell membrane and thereby allowing the entry of Ca^{2+} through voltage sensitive calcium channels. The link between plasma glucose level and the changes it induces in ß cell membranes involves a class of potassium channels that are sensitive to intracellular ATP concentration. In the quiescent ß cell at plasma glucose levels less than 5mM, it appears that sufficient of these ATP dependant K+ channels are open to keep the resting potential at around -50 to -70 mV. An increase in the the ATP concentration at the inner surface of the ß cell membrane decreases the number of channels in the open state. It is understood that for glucose to exert its effects on the pancreatic ß cells it must first be metabolised within the cells, accounting for the slight delay in the onset of actions. The glucose induced depolarisation of pancreatic ß cells results from its intracellular metabolism to ATP which in turn blocks the ATP dependant K^+ channels (Ashcroft et al, 1984, Cook 1988). To summarize, high intracellular ATP inhibits the opening of K_{ATP} channels and this depolarises the ß cell causing the activation of voltage sensitive calcium channels, the influx of calcium and the triggering of insulin release.

A3.1 The history of the potassium channel opening drugs.

The modulation of K^+ channel opening is not a novel physiological mechanism. Inhibition of contraction of the heart by acetylcholine, (Sakman et al, 1983) and relaxation of intestinal smooth muscle by noradrenaline, (Bulbring and Tomita, 1987) are examples of the inhibitory effects resulting from potassium channel opening. The pharmacological exploitation of this mechanism is however recent and novel developments result from work with cromakalim, nicorandil, and pinacidil, compounds synthesised with potassium channel opening in mind

(Hamilton & Weston, 1988). The novel benzopyran, cromakalim, lowers blood pressure in animal models by relaxing blood vessels (Buckingham et al 1986). Mechanistic studies have shown that cromakalim hyperpolarises vascular smooth muscle by opening K^+ channels and increasing K^+ conductance (Hamilton et al, 1986b). These results showed similarities between the electrophysiological findings in the heart and the vascular smooth muscle suggesting an enhancement of outward potassium conductance by cromakalim, (Cain & Metzler, 1985).

Subsequently, nicorandil (a nicotinamide ester) was shown to open potassium channels in the coronary circulation of the dog, (Uchida et al, 1978). Nicorandil was shown to increase membrane potassium conductance in the vascular smooth muscle (Furukawa, et al, 1981; Itoh et al, 1981). However nicorandil has mixed actions and has also been shown to activate guanylate cyclase (Holzman et al, 1983). This effect is linked to its nitro group and the possible production of nitric oxide.

Recently examination of the vasorelaxant properties of the cyanoguanidine derivative, pinacidil, has revealed that it opens potassium channels as it hyperpolarises vascular smooth muscle, (Bray et al, 1987).

In vivo, the predominant effect of nicorandil is to increase cardiac output with only a small transient effect on blood pressure. However cromakalim and pinacidil caused a dose dependant vasodilation resulting in reductions in blood pressure in vivo, cromakalim being 10 times more potent than pinacidil orally (Longman et al 1988).

The electrophysiological changes produced by cromakalim, pinacidil and nicorandil are indicative of opening of potassium channels (Hamilton and Weston, 1989).

This has led onto measurements of potassium flux using rubidium as a marker. In rat portal vein, and guineapig trachea and taenia caeci, nicorandil produced an increase of ^{86}Rb efflux. (Allen et al, 1986 Weir and Weston, 1986 a & b). Similar changes in $^{86}Rb^+$ efflux were produced by cromakalim and pinacidil in the portal vein, taenia caeci and trachea of the guinea-pig; in the aorta and portal vein of the rat; pulmonary, ear and mesenteric artery of the rabbit (Allen et al, 1986; Hamilton et al, 1986; Bray et al, 1987; Cook et al 1988; Southerton et al, 1987). To

summarize, the effects of cromakalim, nicorandil and pinacidil on smooth muscle have been demonstrated to be due to potassium channel opening.

A3.2 Nature of the channel opened by potassium channel opening drugs.

Identification of the target channels opened by potassium channel opening drugs and an understanding of the mechanism by which they open these channels would help in designing more specific agents and would help in identifying the therapeutic situations in which these drugs could be useful. The search for the site of action of potassium channel opening drugs has so far yielded results that vary according to the compound investigated, tissue studied and experimental method used.

Single channel analysis has shown that cromakalim and diazoxide can open large conductance calcium dependant potassium channels (BK_{Ca}) in vascular smooth muscle cells. However the vasorelaxation and $^{86}Rb^{+}$ efflux (used as a marker of K^{+} permeability) induced by potassium channel openers are not sensitive to charybdotoxin, a potent blocker of BK_{Ca}. In addition tetraethylammonium TEA which blocks BK_{Ca} in submillimolar concentrations inhibits effects of of potassium channel openers only in a higher concentration range. Although there is evidence that the high concentrations of potassium channel openers may open calcium dependant potassium channels, there is stronger evidence that the ATP sensitive potassium channels are responsible for the mechanoinhibitory effects of potassium channel opening drugs (Cook & Quast, 1989).

A4 The family of ATP sensitive K^{+} channels.

The ATP sensitive K^{+} channels provide a link between the metabolic state of a cell and its excitability. First described in cardiac muscle (Sakman et al, 1983), these channels have subsequently been found in pancreatic ß cells (Ashcroft et al 1984) and skeletal muscle (Quast & Cook, 1989) and also in cortical neurones (Ashford et al, 1988). Diabetics have been treated over the years with sulfonylurea drugs like tolbutamide. The ATP sensitive potassium channels in the pancreatic ß cells are the target of tolbutamide and glibenclamide which act by blocking the channel. The effect of blocking the ATP operated channel is analogous to the effect of increasing intracellular ATP to elicit

insulin secretion, (Ashcroft et al, 1984; Kakei et al, 1986). In addiition, it has recently been shown that hormones like galanin and somatostatin can open the ATP sensitive potassium channel in pancreatic ß cells via a pertussis toxin sensitive G-protein and the channel is further modulated by protein kinase C (Petersen & Dunne, 1989). The hyperglycaemic drug, diazoxide was found to produce similar effects to galanin and somatostatin by opening the K_{ATP} channels, hyperpolarising the ß cell and inhibiting insulin release.

In heart and skeletal muscle, ATP sensitive potassium channels are proposed to open following an ischaemic insult. Under these conditions of a fall in ATP:ADP ratio, changes in pH and perhaps metabolic factors may modulate channel opening. The ensuing hyperpolarisation would prevent calcium entry through voltage operated calcium channels thereby reducing calcium overload. The opening of K_{ATP} channels would provide an important safety mechanism in cases of ischaemic muscle. A hyperpolarisation would oppose contractile activity thereby preserving intracellular ATP concentration, (Miller, 1990).

The sulfonylurea receptor and the associated channel has now been purified from pig brain and affinity labelled, both procedures yield a single polypeptide chain of 150 Kilo Daltons, (Bernardi et al, 1988). Furthermore the mRNA coding for K_{ATP} in the insulin secreting cell line, HITTIS, has been functionally expressed in xenopus laevis oocytes (Miller et al, 1990). It is also interesting that a peptide endogenous ligand for the sulfonylurea receptor was recently obtained (Virsolvy-Vergine et al, 1988). Cloning, sequencing and experiments on pure subtypes in reconstituted systems will be extremely valuable in understanding the function and modulation of this group of channels and their possible alteration in disease states.

A5 The action of potassium channel openers on ATP sensitive potassium channels.

Evidence has been found in support of the hypothesis that potassium channel openers act on K_{ATP} channels. Firstly the sulfonylurea glibenclamide, a potent selective blocker of K_{ATP} channels (Malaisse et al, 1983; Gylfe, 1984) blocks in a competitive manner both the in vitro vasorelaxation, increase in $^{86}Rb^{+}$ efflux and the invivo vasodilator

activity of cromakalim and diazoxide (Cook & Quast, 1989). Other sulfonylureas or related K^+ channel blockers that lack the sulfonylurea group are also able to inhibit cromakalim stimulated $^{86}Rb^+$ efflux in vascular smooth muscle, (Weir & Weston, 1986) their relative binding potencies suggesting that the sulfonylurea binding site on vascular smooth muscle resembles that of the ATP sensitive potassium channel in the heart and pancreatic ß cells (Miller, 1990). There is considerable variation in the selectivities of the potassium channel opening drugs; cromakalim shows selectivity for the potassium channel in smooth muscle over the ATP sensitive potassium channels in ß cells, whereas diazoxide opens both channels with a similar potency (Quast & Cook, 1989).

A6. Therapeutic Potential

The involvement of ATP sensitive potassium channels in several disease states suggests that potassium channel openers would be therapeutically useful. It seems that the ATP sensitive channels are not open in resting conditions in normal (non-ischaemic) vessels but may constitute a cellular defence mechanism in responses to ischaemic insult. This function has been hypothesised for ATP-sensitive channels in cardiac muscle, skeletal muscle, and in brain (Ashcroft, 1988). In cardiac ischaemia or central anoxia, the changes in metabolic state might favour opening of the channels by potassium channel openers in the heart and brain respectively. But potassium channel opening in the heart is complicated by cardiac dysrythmia and in the brain by neurotoxicity following the release of excitatory amino acids (Miller 1990). It is possible that selective blockade of ATP sensitive potassium channels might be of use in cardiac dysrythmia and ischaemic damage of the nervous system after stroke for example. There is also a possible rôle for potassium channel openers selective for vascular, bronchial, intestinal or bladder smooth muscle for such diseases as hypertension, asthma, irritable bowel and bladder syndromes.

B THE EFFECT OF POTASSIUM CHANNEL OPENING DRUGS ON AIRWAY SMOOTH MUSCLE AND PULMONARY NERVES

B1. Nicorandil

The first potassium channel opening drug to be investigated in guinea-pig airways was nicorandil (Allen et al 1886). Nicorandil (1 -1000 μM)

caused concentration dependant relaxation and a hyperpolarisation of guinea-pig trachealis. Smooth muscle slow wave activity was abolished when hyperpolarisation was pronounced at higher doses. It was found that nicorandil had another mechanism in addition to potassium channel opening since it caused smooth muscle relaxation at doses that did not cause a hyperpolarisation and was 20 times more potent than non-nitrate containing derivatives. The nicorandil induced relaxation is partly due to the formation of nitric oxide from the nitrate moiety in its molecular structure (Ignarro et al, 1981; Allen et al, 1986). Low doses of nicorandil can evoke relaxation in the absence of membrane potential change but towards the upper end of its concentration range nicorandil increases membrane potassium conductance and thereby evokes hyperpolarisation of the trachealis muscle cells.

Pinacidil

Pinacidil has been classified as a potassium channel opening drug since it produces a hyperpolarisation close to the potassium equilibrium potential and stimulates ^{86}Rb efflux in smooth muscle. Pinacidil relaxed tracheal rings contracted by asthma mediators, histamine, $PGF_{2\alpha}$, LtC4 and carbachol (Nielsen-Kudsk, 1988).

Cromakalim

Cromakalim was first found to suppress tone of the guinea-pig trachealis using tracheal strips (Allen et al, 1886). Cromakalim caused only very minor rightward shifts of the concentration effect curves of acetylcholine and histamine.

Hall and Maclagan (1988) used the in vitro innervated tracheal tube preparation and found that cromakalim caused a slowly developing concentration-dependant reduction in response to preganglionic vagal stimulation. Since the inhibition of nerve-induced responses was much greater than the inhibition of responses produced by applied acetylcholine it was suggested that this effect of cromakalim could be due to inhibition of transmitter release from the cholinergic nerve terminals.

McCaig and Dejonkeere (1989) next found that in their preparation of the isolated innervated trachea, cromakalim attenuated responses to

preganglionic vagal stimulation at all frequencies tested. They reported that cromakalim did not affect the amplitude of responses to field stimulation and concluded that cromakalim does not interfere with acetylcholine release from postganglionic cholinergic nerves but is due to an action at the ganglia. These discrepancies have to be resolved and this issue is investigated and considered in more detail later on in this thesis

B2 Potassium channel opening drugs on the lung in vivo

In the anaesthetised guinea-pig Konsett Rossler preparation, both cromakalim and pinacidil i.v. produced a dose related inhibition of the bronchoconstrictor effect of 5HT (Arch et al, 1988; Buckle et al, 1988)

In conscious guinea-pigs oral cromakalim and pinacidil inhibited the bronchoconstriction induced by a 20 s exposure to histamine. Similarly cromakalim and pinacidil inhibited antigen-induced dyspnoea in sensitised conscious guinea-pigs exposed to an aerosol of ovalbumin (Arch et al, 1988).

B3. Potassium channel opening drugs on the lung in man

The effect of cromakalim was examined on histamine induced bronchoconstriction in healthy volunteers and it was found that cromakalim does inhibit bronchoconstriction to this spasmogen in humans. (Baird et al, 1987). When given to patients with nocturnal asthma, oral cromakalim seemed promising as a potential asthma therapy since it reduced the worsening of the asthmatic symptoms in the early morning known as morning dipping (Williams et al (1990).

C THE RESPIRATORY SYSTEM

On inspiration air enters the nose or mouth and then passes the upper airways, the pharynx and larynx. Next the inspired air enters the tracheo-bronchial tree. The central airways comprise the trachea which subdivides into the left and right main bronchi. After the bronchi, the airways further subdivide 23 times into bronchioles and terminal bronchioles finishing with the alveoli where gas exchange takes place. The conducting airways are anatomically incapable of gas exchange and gas exchange takes place in the alveoli only. The entire respiratory

tract from nose to terminal bronchioles is lined with ciliated cells interspersed with mucous secreting goblet cells and other secretory cells. In bronchioles the goblet cells become less frequent and are replaced by Clara cells. The ciliated epithelium, secreted mucus and secretory products of the goblet and Clara cells are important for the protection of the lung.

C1 Blood supply to the lung

The lungs receive blood via both the bronchial and pulmonary circulation. The bronchial arteries arise from the aorta and supply oxygenated blood to the tracheobronchial tree and the conducting tissue of the lung down to the level of the terminal bronchioles. The peripheral airways which include the repiratory bronchioles, alveolar ducts and sacs receive oxygen directly from alveolar air and nutrients from the pulmonary venous circulation.

The pulmonary artery carries non-oxygenated blood from the right ventricle of the heart into the lungs. Gas exchange occurs between the alveoli and the pulmonary arterial circulation. Oxygenated blood is then returned to the heart via the pulmonary vein. There are about 300 million alveoli and nearly 1000 pulmonary capillaries per alveolus resulting in up to 100 m^2 of surface area available for gas exchange by diffusion (Comroe, 1966).

C2 Airway smooth muscle

The fundamental plan of the musculature of the lungs was first described by Reisseisen (1822) who points out that its elastic structure permits ease of dimension changes which are an integral part of lung inflation and deflation. The structure of the tracheal musculature is that of a branched tubular network extending outwards as far as the alveoli, (Maklin, 1929; Vandam, 1952). The trachea and bronchi are supported by incomplete rings or plates of cartilage which are supported by a band of smooth muscle along the dorsal wall. The bronchioles contain a circular ring of smooth muscle and no cartilage which becomes thinner towards the alveoli. Smooth muscle can even be found in the wall of the alveolar duct. Throughout the airways, elastic fibres are associated with the muscle layer so it is called a myoelastic layer

(Maklin, 1929). Smooth muscle taken from different parts of the tracheal bronchial tree differs in its intrinsic tone and in its reaction to spasmogenic and relaxant drugs (Hahm & Nadel, 1981). Such variation may be due to differences in mediator release, in innervation and receptor distribution.

The airways are relatively sparcely innervated and so some smooth muscle cells are not directly innervated by the neurotransmitter but are activated indirectly via gap junctions (Daniel et al, 1986). These gap junctions form low resistance pathways which allow electrogenic coupling between cells. The proportion of gap junctions per membrane and the degree of cell to cell coupling varies down the airway muscle and is species dependant (Burnstock, 1988).

C3 PULMONARY INNERVATION

The airways contain both a motor (efferent) and sensory (afferent) innervation which controls the function of pulmonary smooth muscle and submucosal glands.

C3.1 Afferent innervation

Several types of afferent nerve fibres have been identified throughout the airways. They carry sensory impulses from the irritant, stretch and chemoreceptors to the central nervous system so that the appropriate changes in breathing patterns and bronchomotor tone may occur. The irritant and chemoreceptors arise in the airway epithelium, smooth muscle, submucosal layer and interalveolar spaces and terminate in the vagal nuclei (Richardson & Ferguson, 1979).

Slowly adapting stretch receptors are myelinated nerve terminals found mainly in the smooth muscle of the conducting airways. They are mechanoreceptors which respond to changes in the tension of the airway walls (Colerige and Colerige, 1986). The stretch receptors can modulate a reflex bronchodilation by inhibiting vagal tone and are also responsible for the Hering Breuer inflation reflex. (Widdicombe, 1963). Rapidly adapting irritant receptors adapt more quickly than the stretch receptors, fire regularly and are excited by a wide range of mechanical and chemical stimuli (Widdicombe, 1954 a & b). The fibres of the irritant receptors are myelinated but nonmyelinated terminals are

located below the epithelium and between epithelial cells. (Sant' Ambrogio, 1987). The rapidly adapting receptors in the larynx and trachea are very sensitive to mechanical stimuli such as cigarette smoke and dust. Experimentally the irritant receptors are stimulated by sulphur dioxide (SO_2) and ozone as well as inflammatory mediators, for example histamine and 5HT. (Colerige et al, 1976).

C-fibres are nonmyelinated nerve fibres found throughout the airway epithelium which respond to many pathological and chemical stimuli eg. embolism, histamine bradykinin, capsaicin and SO_2. Stimulation of the C-fibres results in apnoea and changes mediated via the autonomic nervous system: bronchoconstriction, mucus secretion and vasodilation. (Clozel et al, 1985).

Some nonmyelinated nerves contain sensory neuropeptides like substance P or neurokinin A which may be released by the axon reflex. Antidromic vagal stimulation or capsaicin also release the neuropeptides resulting in local changes in the tracheal vasculature or airway smooth muscle.

3.2 Efferent Innervation.

The efferent innervation of the lung comprises three types of nervous pathway; the parasympathetic cholinergic, the sympathetic noradrenergic and the nonadrenergic noncholinergic (NANC) nervous system. Each of the systems will be considered in turn

3.2a The cholinergic parasympathetic pulmonary innervation The dominant neuronal control of airway smooth muscle is exerted via excitatory cholinergic nerves (Widdicombe, 1963). Cholinergic efferent nerves arising in the vagal nuclei of the brainstem pass down the vagi and synapse in small ganglia located on an extensive nerve plexus within the airway walls. Short fibres from these ganglia supply the target cells, the airway smooth muscle and submucosal glands.

3.2a*(1)*. Airway parasympathetic ganglia

Preganglionic parasympathetic axons whose cell bodies are located in the medulla oblongata (nucleus ambiguus) descend in the vagus and synapse onto ganglion cell bodies in the airway wall (Mitchell et al, 1985). Ganglia have been observed in chains extending along the length of the trachea.

(Skoogh, 1988). Ganglia containing one to four ganglion cells have been identified in the superficial neuronal plexi of the airway smooth muscle and the mucosal glands.(Baker et al, 1986). Ganglia containing 10 to 40 larger cells were located along a longitudinal nerve trunk near the trachealis muscle and cartilage junction. In human airways ganglia consisting of up to 20 cells bodies have been observed. In the bronchioles, single ganglion cells may be found in the airway wall. Electron microscopic sections of the interganglionic nerve trunk show that most fibres are nonmyelinated although a portion are myelinated (Cameron and Coburn, 1984).

Intracellular recording techniques have been used to investigate the electrophysiological properties of the ganglion cells. Acetylcholine released from preganglionic nerve terminals induces a fast excitatory post synaptic potential (fEPSP) which is mediated by nicotinic cholinoceptors in the ganglion cells (Kuba & Kuketsu, 1978). In some ganglion cells the fEPSP is followed by a slow excitatory post synaptic potential (sEPSP) and a slow inhibitory postsynaptic potential (sIPSP) Coburn, 1987).

Two distinct types of ganglion cells were encountered which could be distinguished by their anatomical and electrophysiological features. Compact clusters of small ganglionic cells of diameter 36 ± 6 μM were located in the posterolateral tracheal adventitia near the intercartilagenous spaces. These cells fired with an expiratory rhythm or continously with increasing frequency during expiration.

A second population of cells of larger diameter were located in the adventitia in close apposition to the trachealis muscle near its attachment to tracheal cartilagenous rings. They fired with an inspiratory rhythm or with an increased frequency during inspiration, firing at a peak frequency of 10 to 15 Hz (Mitchel, 1989). This author concluded that modulation of the activity of parasympathetic ganglia is achieved by regulation in the central nervous system of a number of active excitatory preganglionic fibres innervating the ganglion cells not by IPSP acting at the level of the ganglia.

The existence of facilitatory M_1 muscarinic receptors on pulmonary parasympathetic ganglia has recently been proposed. However there are considerable species differences and conflicting results within species.

Lammas et al (1989) claim to have found evidence of a ganglionic M1 receptor. In healthy volunteers they found that a dose of pirenzepine, the M_1 antagonist, that did not antagonise muscarinic agonist induced bronchoconstriction was found to antagonise reflex bronchoconstriction. Since pirenzepine was not having a postjunctional action on the airway smooth muscle, the ganglia was proposed as the probable site of action. It has also been suggested that M_1 receptors are present in the ganglia of the rabbit bronchi as pirenzepine was more potent at inhibiting tracheal contractions to vagal stimulation than to field stimulation (Bloom et al, 1988). Recently Maclagan et al (1989) concluded that M_1 muscarinic receptors are not present in the pulmonary parasympathetic ganglia of the rabbit or the guinea-pig as pirenzepine was equipotent at antagonising bronchoconstrictor responses induced by intravenous acetylcholine and vagal stimulation.

Filtering may occur at parasympathetic ganglia, particularly at the higher frequencies (Skoogh, 1983). It was demonstrated that postganglionic vagal stimulation had a greater contractile effect on the ferret trachea than preganglionic vagal stimulation. Additionally, although preganglionic nerves may fire at frequencies as high as 50 Hz, and fire at 25 Hz during spontaneous inspiration, ganglion cells can only fire up to 15 Hz (Mitchel et al, 1985).

Evidence has been obtained to show that neural inputs other than cholinergic may be supplied to parasympathetic ganglia. VIPergic nerves have been demonstrated in airway parasympathetic ganglia (Yip, et al 1986). VIPergic immunoreactivity in dense cored vesicles has been detected in the ganglia providing some evidence of possible co-storage of VIP with acetylcholine (Uddman et al, 1978). Neuropeptides may potentiate parasympathetic ganglionic stimulation. It was found that removal of peptide containing fibres with capsaicin reduced the size of responses of the trachea to vagal stimulation in vivo (Martling et al, 1984). Inflammatory mediators may also influence ganglionic transmission and mast cells are often found in close association with airway ganglia (Barnes 1986).

C3.2a*(2)* Post ganglionic parasympathetic nerves

Post ganglionic nerve fibres innervate target cells such as airway smooth muscle and submucus glands (Richardson, 1979). The parasympathetic nervous system has been shown to provide the dominant neural control to the airway smooth and is responsible for airway tone. Resting tone may be abolished by muscarinic antagonists or vagal section. Direct recordings from vagal efferent fibres confirm an irregular firing at rest (Widdicombe, 1961). In human subjects muscarinic antagonists cause bronchodilation (Taylor et el, 1989) whereas inhalation of anticholinesterase causes bronchoconstriction in normal subjects (Quigley et al, 1985) These results confirm that there is a tonic release of acetylcholine in the airways. At night this tonic vagal activity increases (Morrison et al, 1988) and may be important in the pathophysiology of nocturnal asthma.

Transmitter recycling is a feature of the cholinergic parasympathetic nervous system. Acetylcholine synthesis by choline acetyl transferase occurs in the neurone body and it is stored in agranular vesicles of 30 to 60 nM diameter. Following release, acetylcholine is inactivated by the enzyme acetylcholine esterase. Choline produced from the hydrolysis of acetylcholine is taken up into the prejunctional nerve terminals and is reused to synthesise acetylcholine.

C3.2a*(3)* Modulation of transmitter release from parasympathetic nerve endings by muscarinic autoreceptors.

Postganglionic parasympathetic nerve endings are an important site for modulation of acetylcholine release via neuronal muscarinic receptors. Muscarinic receptors which modulate transmitter release have been identified in both the peripheral and central nervous systems. They may be located on fibres supplying the ganglia (preganglionic), on the ganglia cell body or on the prejunctional terminals innervating target organs such as the smooth muscle and gland cells. If the transmitter modulates its own release, the receptors are called autoreceptors.

Pre-synaptic muscarinic autoreceptors which inhibit release from cholinergic nerves were first discovered in the cat cerebral cortex (Mitchell, 1963). Muscarinic inhibitory autoreceptors have also been identified on prejunctional parasympathetic nerve endings, innervating

peripheral effector organs such as the ileum (Kilbinger & Wessler, 1980), heart (Wetzel & Brown, 1985) and airways (Fryer & Maclagan, 1984).

Muscarinic agonists such as acetylcholine and pilocarpine reduce acetylcholine release via activation of these prejunctional receptors. The inhibitory effect of muscarinic agonists can be blocked with selective M_2 muscarinic antagonists eg. gallamine, (Fryer & Maclagan, 1984) and methoctramine (Watson et al, 1989). The M_2 muscarinic antagonists also potentiate vagally induced bronchoconstriction. The results suggest that the inhibitory muscarinic autoreceptors are of the M_2 subtype.

C3.2a*(4)* Postjunctional pulmonary muscarinic receptors on airway smooth muscle.

Acetylcholine released from pulmonary postganglionic parasympathetic nerves activates muscarinic receptors present on airway smooth muscle. Direct receptor binding studies and autoradiographic mapping indicate a high density of muscarinic receptors on airway smooth muscle of the large airways, this density decreasing with airway size (Barnes et al, 1983). This distribution of pulmonary muscarinic receptors is consistent with the decreasing parasympathetic innervation with decrease in airway diameter down the lung.

The receptors on airway smooth muscle are usually classified as M_3 muscarinic receptors. Bronchoconstriction evoked by either muscarinic agonist or vagal stimulation is not affected by M_1 selective concentrations of pirenzepine. (Bloom et al, 1987) nor M_2 selective concentrations of gallamine or methoctramine (Fryer & Maclagan, 1984; Watson et al, 1989) but is antagonised by atropine and and the M_3 antagonists 4-DAMP and hexahydrasiladifenidol (Moore et al, 1989). Vagally induced, but not muscarinic agonist mediated bronchoconstriction, may be potentiated by M_2 antagonists due to an inhibition of the prejunctional muscarinic autoreceptors. Binding studies in guinea-pig lungs indicate a predominance of M_3 receptors while human lungs contain both M_1, M_2 and M_3 receptors in almost equal proportions.

C3.2b Overview of the sympathetic efferent innervation of the trachea. Two types of nerve-mediated inhibitory responses have been uncovered in the trachea: nonadrenergic noncholinergic and the sympathetic mediated responses. The sympathetic nervous system mediated responses are the more conspicuous and operate via noradrenergic nerve endings releasing noradrenaline onto beta adrenoceptors (Foster, 1964). This response is well documented in vitro (Fleisch et al, 1970; Suzuki et al, 1976) and can be elicited in vivo from the cervical trachea of the guinea-pig by transmural stimulation in the presence of atropine or more specifically by stimulation of the sympathetic nerve trunks (Yip et al, 1981). The sympathetic nerve stimulation induced response is abolished by hexamethonium indicating that the sympathetic nerve trunk contains preganglionic fibres that synapse within the stellate ganglion. The beta adrenoceptor antagonists, sotalol and propranolol, blocked responses of the trachea to sympathetic stimulation while phenoxybenzamine in doses that cause alpha adrenoceptor blockade had no effect (Yip et al, 1981). It was therefore concluded that sympathetic relaxation of the guinea-pig trachea is mediated by ß adrenoceptors.

Nerve fibres penetrate the muscle from the adventitial side and are gathered into small bundles. These nerve bundles are connected to meshes of the tracheal nerve plexus which contains the cholinergic ganglia and to perivascular nerves. Histochemistry and electron microscopy indicate that these bundles contain an admixture of nerve fibres of different type eg cholinergic and adrenergic (Gabella et al, 1987). The admixture of nerves of different type within the same bundle is common to all autonomic nerves and the closeness of the adrenergic and sympathetic axons offers the possibility of direct chemical interaction between the two pathways.

Histochemically, there are abundant adrenergic fibres in the bronchial muscles of the cat (Silva & Ross, 1974), goat, sheep, pig (Man, 1971) and man (Pack & Richardson, 1984). In the guinea-pig tracheal muscle adrenergic fibres are readily found by fluorescence microscopy in the cervical portion, whereas they are scanty in the thoracic portion (O'Donnell & Saar, 1973; O'Donnell et al, 1978) and are rare in the bronchiolar muscles. The uptake of tritiated noradrenaline by guinea-pig trachea has a similar gradient (Foster & O'Donnell, 1972)

and the pharmacological effect of ß blockers on relaxation elicited by transmural stimulation is markedly greater in the cervical than in the thoracic trachea of the guinea-pig. The findings of histochemical studies showed that adrenergic fibres are always less numerous than fibres interpreted as cholinergic. Adrenergic fibres originate from neurones in the sympathetic chain, whereas adrenergic neurones are not found in the ganglia near the trachea and bronchi (Jacobowitz et al, 1973). However in some animals such as the calf, some adrenergic fibres are seen closely associated with bronchial ganglion neurones (Jacobowitz et al, 1973), thus raising the possibility of a modulatory interaction between sympathetic and parasympathetic nerves.

C3.2(c) Nonadrenergic noncholinergic (NANC) innervation and function in the lung.

Evidence for NANC inhibitory nerves in the guinea-pig trachealis muscle in vitro was found in preparations treated with the muscarinic antagonist atropine, noradrenergic depletion by reserpine and ß adrenoceptor blockade with propranolol. Transmural stimulation of the trachealis muscle elicited a NANC relaxant response (Coburn and Tomita, 1973). These findings were confirmed by work in the tracheal tube preparation, (Coleman and Levy, 1974). Electrical stimulation of the guinea-pig isolated tracheal tube preparation causes a biphasic response, initially excitatory and then inhibitory. The excitatory response was abolished by atropine leaving the inhibitory response unaffected. The inhibitory response was reduced but not abolished by pretreatment with propranolol and noradrenergic depletion with 6-hydroxydopamine. The NANC inhibitory response was abolished by tetrodotoxin, suggesting that it is neuronal in origin.

The identity of the NANCergic transmitter has still not been verified. There is currently some speculation that this could be vasoactive intestinal polypeptide (VIP). Since the isolation of VIP from porcine small intestine (Said et al, 1970) interest in the physiologic and pharmacologic properties of this 28 amino acid peptide has increased progressively. It is now known that VIP occurs widely in the animal kingdom and is distributed throughout the gastrointestinal tract. VIP has also been demonstrated in adrenal medulla, pancreas, heart, brain,

peripheral nerve, urogenital tract and lung (Said et al 1980). In the brain VIP has been localised in synaptosomal and vesicular fractions. In peripheral tissues, the peptide has been found in neurones suggesting a possible neurotransmitter function (Said et al 1980). VIP relaxes a variety of smooth muscle tissues, influences salivary and gastrointestinal secretion, increases cardiac output, causes central nervous system arousal, and exerts multiple effects on metabolic and endocrine functions.

Evidence has been found to suggest that NANCergic relaxation of the guinea-pig trachea to electrical field stimulation could be mediated by VIP. Overnight incubation of the trachea with VIP antisera markedly reduced the inhibitory response of the guinea-pig trachea to field stimulation compared to time-matched controls (Ellis & Farmer, 1989). Peptide histidine isoleucine (PHI) antisera had a similar but smaller effect. It was also found that in a concentration of VIP that is maximal for its relaxant effect, inhibitory responses to electrical stimulation were greatly inhibited even after raising tone with histamine to counteract the fall in tone caused by the presence of VIP.

Evidence for the release of immunoreactive VIP from the guinea-pig trachea was found by Matsuzaki et al, (1980) The release was abolished by tetrodotoxin which blocks the relaxation suggesting a neural origin for the released VIP.

The localisation of VIP was investigated using an immunofluorescent technique (Dey et al, 1981). The occurrence of VIP immunoreactive, nerves has been demonstrated in walls of bronchi, bronchioles and pulmonary vessels, suggesting a physiological rôle for VIP in these locations. VIP positive nerve cell bodies were identified in ganglia located in the walls of the bronchi. VIP and acetylcholine have been found to occur in some of the same neurones including those supplying the salivary glands and sweat glands (Lundberg et al, 1979). In such locations the vasodilator action of VIP is believed to be coupled to the secretory actions of acetylcholine (Lundberg et al, 1980). Cholinergic nerve fibres have been identified in bronchial submucosal glands (El Bermani & Grant, 1975), but the possible coexistance of acetylcholine and VIP remains to be investigated.

VIP has been found to be an endogenous hyperpolarising vasodilator that acts directly on the smooth muscle (Lee et al, 1984). The hyperpolarisation of cerebral arterial smooth muscle cells induced by VIP was reversed by glibenclamide, a selective blocker of ATP sensitive potassium channels (Standen, 1989). Thus it is possible that opening of ATP sensitive potassium channels may contribute to the mechanism of relaxation to VIP.

α chymotrypsin, an enzyme that degrades peptides, was found to selectively abolish responses to VIP and PHI (Ellis and Farmer, 1989b). In a concentration that degrades VIP, α chymotrypsin reduces inhibitory responses to NANC stimulation by about 35 % in the trachea.

It is therefore probable that another substance apart from VIP contributes to the NANCergic relaxant response. Tucker et al (1990) have proposed an endogenous nitrate as a component of the NANC relaxations of the tracheal smooth muscle. They used L-nitroarginine (L-NOARG) an inhibitor of nitric oxide synthase to investigate the possibility that nitric oxide is the factor responsible for the α chymotrypsin resistant component of the electrically induced NANC relaxations of the guinea-pig trachea. L-nitroarginine inhibited the NANC relaxations after α chymotrypsin in a dose dependant manner. L-nitroarginine was without effect on acetylcholine-induced responses of the trachea or on relaxations to VIP. These results suggest that an endogenous nitrate or nitric oxide may contribute to the NANCergic relaxations of the tracheal smooth muscle. These observations are confirmed by the work of Li & Rand (1991).

In vivo demonstration of noradrenergic NANC inhibitory innervation of the guineapig trachea differs from the in vitro work since in vitro NANCergic relaxation of the trachea is elicited by transmural stimulation (Coburn and Tomita, 1973; Coleman and Levy, 1984). However in vivo relaxation is elicited by vagal stimulation (Chesrown et al, 1980; Diamond et al 1983). NANC relaxations of the trachea were investigated in vivo by Chesrown et al, (1980). Animals were pretreated with 6-hydroxydopamine, atropine and propranolol. Sympathetic nerve stimulation induced responses were abolished confirming the elimination of noradrenergic transmission. Vagal stimulation induced NANC relaxant responses which were unaffected by these pretreatments in cats in vivo.

It was found that NANCergic relaxations to vagal stimulation were diminished by sectioning of the recurrent laryngeal nerves which supply innervation to the tracheal muscle. These findings were supported by Yip et al (1981). Using the guinea-pig trachea in situ, it was shown that NANCergic relaxation to transmural stimulation was not inhibited by hexamethonium, while the response to sympathetic stimulation was completely abolished. Vagal stimulation induced- relaxations were completely resistant to propranolol at doses that had maximal effects on sympathetic stimulation. It was concluded that the vagal inhibitory motor fibres run in the recurrent laryngeal nerves since bilateral ligation reduced the relaxant responses to vagal stimulation. The vagal nerves appeared to contain preganglionic fibres which synapse in the paratracheal ganglion and continue on to form the recurrent laryngeal nerve. Transmural stimulation induced relaxations due to stimulation of postganglionic fibres with a NANCergic and adrenergic component.

It has been found that the putative NANC transmitter VIP is a potent bronchodilator in vivo (Diamond et al, 1983). Bolus intravenous injection of VIP in cats reversed the bronchoconstriction to infusion of 5-hydroxytryptamine (5HT). This relaxation to VIP was found to be independant of prostaglandin production or ß adrenoceptor activation since bronchodilatation mediated by VIP persisted after pretreatment with indomethacin and propranolol (Diamond et al, 1983). Although all levels of the tracheobronchial tree respond to VIP, relaxation appears to be most prominant in central airways. These findings are supported by the observation of VIP containing fibres are present in feline airways (Dey et al, 1981). In summary, it appears that VIP is a strong candidate for a neurotransmitter responsible for the NANCergic relaxation of the airways.

AIMS

1. The first and most important aim of the experiments in this thesis was to investigate the location and mechanism of the inhibitory action of the potassium channel opening drugs on contractions of the guinea-pig trachea elicited by stimulation of the pulmonary cholinergic nerves. Cromakalim, lemakalim and SDZPCO400 were applied cumulatively while

recording responses mediated by preganglionic vagal stimulation and transmural stimulation inorder to establish dose effect relationships.

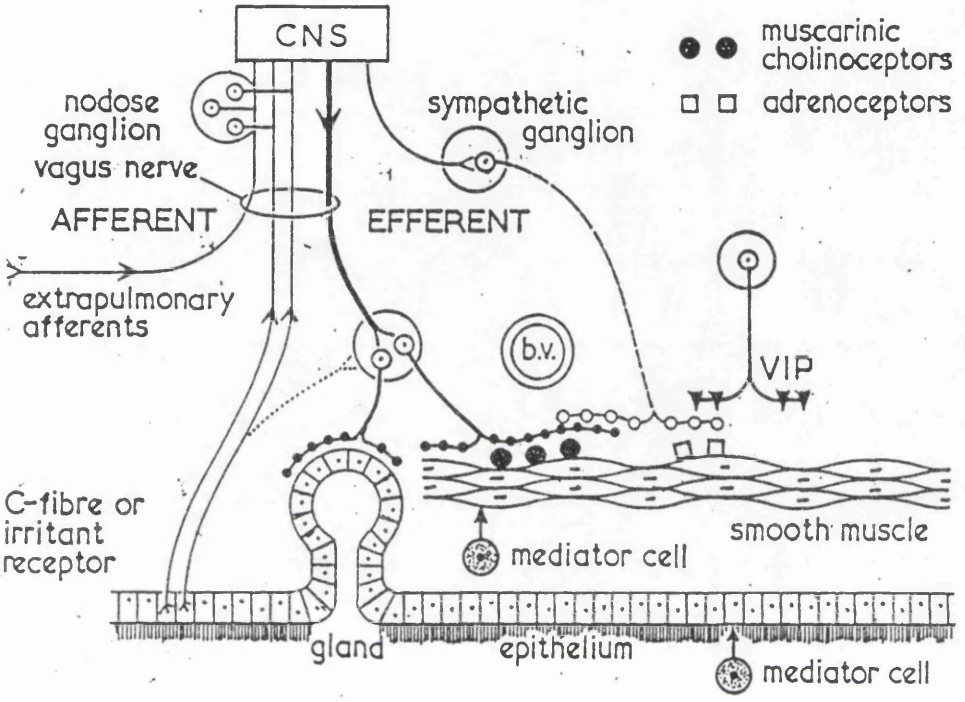
2. In order to investigate whether the potassium channel opening drugs had a postjunctional effect on smooth muscle muscarinic receptors, experiments were performed to investigate the effect of the presence of the potassium channel opening drugs on contractions of the trachea to exogenous acetylcholine. We also investigated the effect of cromakalim, lemakalim and SDZPC0400 on contractions of the trachea to histamine and the thromboxane mimetic, U46619.

3. We aimed to investigate the type of potassium channel opened by cromakalim and the other potassium channel opening drugs on pulmonary nerves. Glibenclamide, a selective blocker of ATP-sensitive potassium channels was used to investigate whether cromakalim, lemakalim and SDZPC0400 opened ATP-sensitive K^+ channels on pulmonary cholinergic nerves.

4. Also, we aimed to investigate and compare the actions of cromakalim, the racemate, and its constituent isomers, the (-) enantiomer lemakalim and the (+) enantiomer BRL38226 on the innervated guinea-pig trachea and to study the interaction of the (+) and (-) enantiomers.

5. It was also hoped to test the possibility that there could be an M_2 muscarinic autoreceptor linked to a potassium channel. We investigated the effect of cromakalim on responses to preganglionic vagal and transmural stimulation in the presence of methoctramine, an M_2 muscarinic antagonist. In addition, the effect of the presence of glibenclamide, a blocker of ATP operated K^+ channels, was studied on the inhibitory effect of pilocarpine, an M_2 muscarinic agonist on responses of the trachea to preganglionic vagal and transmural stimulation.

6. Finally, we aimed to investigate the action of cromakalim and lemakalim on NANCergic nervous system in the in vitro tracheal preparation. We also investigated the effects of cromakalim and lemakalim on relaxations of the trachea to exogenous VIP.



Pulmonary innervation

Diagram 2

METHODS.

1. Animals.

Male Guinea-pigs of the Dunkin Hartley strain were used in these experiments. They were housed in stock cages containing up to six animals with unlimited access to food and water. Animals weighed from 250g to 350g.

2. Preparation of the in vitro innervated trachea.

Dunkin Hartley guinea-pigs were killed by cervical dislocation. The trachea with both vagal and recurrent laryngeal nerves intact was removed according to the method of Blackman and McCaig, (1983). The preparation was mounted with one electrode inside the lumen of the trachea for transmural stimulation and the vagal nerves were looped through a ring electrode for preganglionic stimulation. An electrode specially designed for field stimulation was used in one group of experiments only. Two wires placed one either side of the trachea were used for field stimulation. The fluid-filled lumen of the trachea was cannulated and the intraluminal pressure (ILP) was measured via a Stratham pressure transducer. Contraction of the trachealis muscle was measured as a change in ILP. The tissue preparation and electrode were mounted in an organ bath of Krebs' solution and bubbled with 95 % O_2 and 5% CO_2. Both vagal nerves were stimulated at 30 V, 30 Hz, 0.2 ms for 5s with a 40s interval. The above parameters were also used for transmural stimulation in the presence of hexamethonium (50 μM) to ensure postganglionic stimulation of the cholinergic nerves. However when stimulating transmurally it was impossible to avoid stimulation of other types of nerves within the tissue eg sympathetic or peptidergic pathways.

A period of one hour was allowed for the tissue to equilibrate before any drugs were administered to the organ bath. Any preparation where the control response to electrical stimulation was less than 100 mmH_2O was discarded. The average range of control responses was between 150 to 250 mmH_2O.

In experiments where NANC relaxations to transmural stimulation were investigated, the tone was raised to 200 mmH_2O with histamine;

atropine (1 μM) and (-) propranolol (1 μM) were present to ensure inhibition of cholinergic and sympathetic pathways respectively.

3. Drugs.

BRL38226 SmithKline Beecham Pharmaceuticals
Cromakalim SmithKline Beecham Pharmaceuticals.
Lemakalim SmithKline Beecham Pharmaceuticals.
SDZPC0400 Sandoz Basle Switzerland.
Atropine BDH chemicals.
Acetylcholine BDH chemicals.
Histamine BDH chemicals
U46619 Semat Technical UK Ltd.
Glibenclamide Hoescht Pharmaceuticals
(-) Propranolol ICI Imperial Chemical Industries Ltd
Methoctramine Research Biochemicals
Pilocarpine BDH chemicals
Hexamethonium Koch Light.
VIP Sigma
(dp chloro phe leu 17) VIP Sigma
Indomethacin Sigma
Salts for Krebs BDH

Krebs' solution composition mM.

Nacl 118.4; Kcl 4.7; $NaHCO_3$ 25.0; Glucose 11.1; KH_2PO_4 1.16; $MgSO_47H_2O$ 1.19; CaCl2 2.6.

4. Solvents.

In preliminary experiments the stock solution of cromakalim (10^{-3} M) was prepared using 2 % ethanol to dissolve the powdered drug. Serial dilutions from the stock solution were used for the experiments. Time matched control experiments were performed using volumes of ethanol in saline corresponding to the dosing schedule but with no cromakalim present. It was found that ethanol had an inhibitory effect on responses to cholinergic nerve stimulation. Therefore in all further experiments

ethanol was ommited and drugs were dissolved in 0.9 % saline and sonicated with a Headland electrosonic sonicator where necessary.

5. **Statistics.**

In all experiments, unless stated otherwise, data points on graphs represent the mean ± standard error of the mean (sem) of group number (n) equal to or greater than 5. Post drug inhibition of contractions of the trachea were measured as % inhibitions of the predrug control value in the same tissue. There was no significant difference between the size of control contractions to cholinergic stimulation in different groups before the administration of potassium channel opening drugs. Unpaired t-tests were used to test for significance between groups from parallel experiments.

6. **Protocols**

6.1. The effect of potassium channel opening drugs on responses to preganglionic vagal stimulation and transmural stimulation.

The potassium channel opening drugs were administered in cumulative doses; cromakalim and SDZPC0400 (in the range of 0.1 to 26 μM), lemakalim (0.01 to 26 μM). An interdose interval of 10 minutes was chosen to allow the maximum inhibitory effect of the potassium channel opening drugs to develop on nerve stimulation induced contractions of the trachea. As the potassium channel opening drugs, cromakalim and lemakalim were very slow to wash out of the tissue, only one dose response curve was performed per tissue.

6.2. The effect of potassium channel opening drugs on dose related rise in intraluminal pressure to spasmogens.

ACETYLCHOLINE. In each preparation used, an acetylcholine dose response curve was performed twice in the presence of (-) propranolol (1 μM) and hexamethonium (50 μM) to establish a mean control dose response curve. A washout period of 20 minutes was allowed between each dose response curve to acetylcholine. Cromakalim (3.2 μM) was added to the organ bath and 10 minutes later a dose response curve to acetylcholine was

performed. This process was continued by adding cromakalim (12.8 μM) and then cromakalim (26 μM) between subsequent acetylcholine dose response curves.

HISTAMINE AND U46619

A similar protocol was used to investigate the effect of potassium channel opening drugs on the other spasmogens histamine and U46619.

6.3. The effect of the presence of glibenclamide on the inhibitory action of the potassium channel opening drugs on cholinergic nerve stimulation.

The effect of glibenclamide was studied using a single application to each tissue. In each preparation a period of 30 minutes was allowed for incubation of the tracheal preparation with glibenclamide (20 μM) followed by a dose response curve to one of the potassium channel opening drugs.

6.4. The effect of lemakalim on responses to cholinergic stimulation in the presence of BRL38226.

A dose of BRL38226 (26 μM) was applied and 10 minutes later a dose response curve to lemakalim was performed (0.01 to 26 μM).

6.5a. Investigation of a possible interaction of muscarinic autoreceptors and potassium channels using methoctramine.

The effect of cromakalim on responses to cholinergic nerve stimulation in the presence of an M2 muscarinic antagonist, methoctramine. Methoctramine (10^{-7}) was applied to the tissue and 10 minutes later a dose response curve to cromakalim was performed on either preganglionic vagal stimulation or transmural stimulation.

6.5b. **The effect of pilocarpine an M2 muscarinic agonist on responses to cholinergic nerve stimulation alone and in the presence of glibenclamide**

A dose response curve to pilocarpine (0.1 to 10 μM) was performed with a 6 minute interdose interval. After a washout period of about 1 hour, the tissue was incubated for 20 minutes with glibenclamide (20 μM) and then the dose response curve to pilocarpine was repeated. The same

protocol was used for both preganglionic vagal stimulation and transmural stimulation.

6.6 **The effect of potassium channel opening drugs on relaxant responses to NANC nerve stimulation**

The effects of cromakalim and lemakalim on NANC relaxations to transmural stimulation were investigated. Two consecutive control dose response curves to histamine (0.1 to 26 μM) were performed to raise tone to 200 mmH_2O. Transmural stimulation was continued throughout to evoke NANCergic relaxations. A 30 minute washout period was allowed between curves. Then a dose of the potassium channel opening drug was applied 10 min before the start of a new dose response curve to histamine while continuing transmural stimulation to elicit NANCergic relaxations..

6.7. **The effect of potassium channel opening drugs on responses of the trachea to exogenous VIP.**

The tone was raised to 200 mmH_2O by histamine and relaxation was measured after a 7 minute interval following application of VIP. The effects of the potassium channel opening drugs cromakalim or lemakalim were seen on responses to exogenous VIP. A dose of the potassium channel opening drug was applied 10 minutes before raising the tone with histamine and measuring relaxation to VIP at raised tone. A similar protocol was followed for the effect of the VIP antagonist on responses to exogenous VIP.

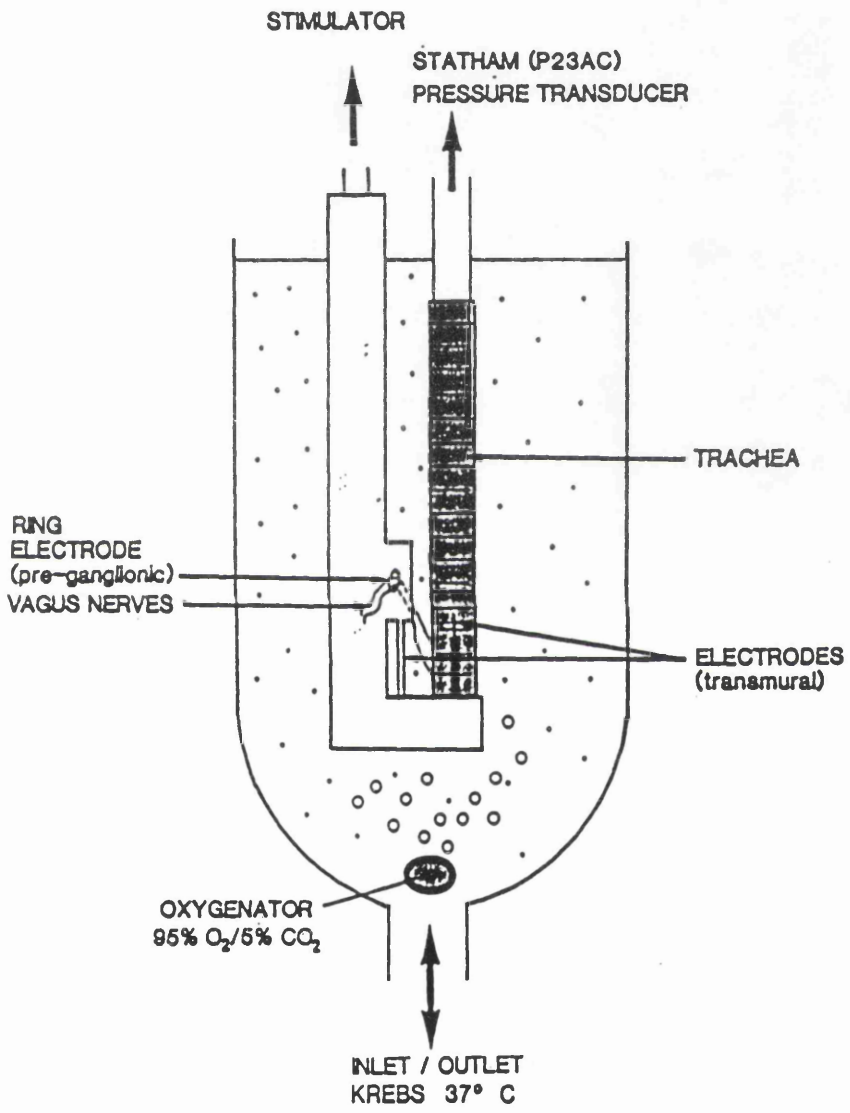


Diagram 3 Preparation of the in vitro innervated guinea pig trachea

RESULTS

PART A Section 1.1

The effect of the potassium channel opening drugs, cromakalim, lemakalim and SDZPC0400 on responses to preganglionic vagal stimulation and to transmural stimulation in the presence of hexamethonium (50 μM).

Contractions of the in vitro tracheal tube preparation were elicited by either preganglionic vagal stimulation or transmural stimulation (30 V, 30Hz, 0.2 ms pulse width, 5 s duration, 40 s interval). Indomethacin (5 μM) was present throughout to abolish prostaglandin induced tone. Hexamethonium (50 μM) was included when stimulating transmurally to ensure that stimulation was postganglionic. Contractions of the trachea to cholinergic nerve stimulation were measured as a rise in intraluminal pressure. Pre-drug control contractions were stabilised in all preparations to a value greater than 150 mmH_2O.

1.1a Cromakalim on responses to preganglionic vagal stimulation and transmural stimulation .

Graded cumulative doses of cromakalim (0.1 to 26 μM) given at 10 min intervals caused a dose related inhibition of nerve induced contractions. **Figure 1** is a typical trace showing the effect of cromakalim on responses to preganglionic vagal stimulation (upper trace) and transmural stimulation (lower trace). Cromakalim caused a dose-related inhibition of responses to transmural stimulation reaching a maximum 50.4 % inhibition at cromakalim 26 μM. Cromakalim had a greater effect on responses to preganglionic vagal stimulation reaching a maximum 63.3 % inhibition at 26 μM. **Figure 2** shows the mean ± sem % inhibition by cromakalim of responses to both types of nerve stimulation, (n=5) and thus allows a comparison to be made. In a group of experiments, the control contractions in preganglionically stimulated preparations were 158 ± 45.3 mmH_2O ILP and this value was reduced by cromakalim (26 μM) to 55.3 ± 2.9 mmH_2O (65 ± 1.1 % inhibition). The control intraluminal pressure to transmural stimulation was 155 ± 39.2 mmH_2O and this value was reduced by cromakalim (26 μM) to 82.2 ± 20.4 mmH_2O (53.3 ± 3.3 % inhibition). The maximum inhibitory effect of cromakalim on responses to preganglionic vagal stimulation (65 ± 1.1 %

inhibition) was greater than the maximum effect of cromakalim on responses to transmural stimulation (53.25 ± 3.28 % inhibition), but complete blockade of responses to cholinergic stimulation was never obtained with cromakalim.

Section 1.1b. The effect of cromakalim on responses to transmural stimulation and field stimulation under different conditions.

It was reported by McCaig and DeJonkeere (1989) that cromakalim did not affect responses of the guinea-pig trachea to field stimulation. This observation did not agree with our own findings that cromakalim and lemakalim both inhibit responses to transmural stimulation. Our experiments were carried out in the presence of indomethacin and hexamethonium while McCaig and Dejonkeere did not use any pretreatments. The effect of cromakalim on responses to transmural stimulation under different conditions was investigated in the presence and in the absence of indomethacin (5 μM) and hexamethonium (50 μM) using the usual stimulus parameters 30 V, 30 Hz, 0.2 ms, 5 s duration, 40 s interval **(Figure 3)**. In the absence of indomethacin and hexamethonium the size of responses to transmural stimulation was smaller and more variable due to the presence of spontaneous tone (71.478 ± 64.51 mmH_2O). After application of cromakalim (26 μM) contractions of the trachea were 19.93 ± 5.16 mmH_2O and mean % inhibition of responses was 73.57 ± 7.45 %. A different design of electrode for field stimulation was developed with wires placed either side of the tracheal tube. This new electrode was used because we hoped to reproduce the conditions used by McCaig & Dejonkeere and thus find the cause of our differing results. This field stimulation electrode required higher stimulus parameters (50 V, 40 Hz) to elicit contractions of comparable size to that obtained to transmural stimulation. The size of responses was 90.34 ± 25.4 mmH_2O and these responses were inhibited by cromakalim (88.43 ± 4.746 % inhibition) to a value of 10.46 ± 2.37 mmH_2O. Under the above stated conditions, cromakalim caused a dose related inhibition of responses to transmural and field stimulation. In all subsequent experiments parameters were 30 V, 30 Hz for both preganglionic vagal and transmural cholinergic nerve stimulation because this is closer to the physiological situation and gave larger very reproducible responses. Indomethacin (5 μM) and

hexamethonium (50 μM) were present when stimulating transmurally in all the following experiments.

Section 1.2. The effect of graded cumulative doses of lemakalim on preganglionic vagal stimulation and transmural stimulation in the presence of hexamethonium (50 μM).

A sample trace of the effect of graded cumulative doses of lemakalim (0.01 to 26 μM) on preganglionic vagal stimulation (upper trace) and transmural stimulation in the presence of hexamethonium (lower trace) is shown, **Figure 4a**. Complete abolition of responses to both preganglionic vagal stimulation and transmural stimulation occurred at lemakalim 12.8 μM. The mean values for a group of experiments is shown **Figure 4b**. Lemakalim (1.6 μM) caused an inhibition of responses to preganglionic vagal stimulation from 230 ± 30.33 mmH_2O to 26.09 ± 8.62 mmH_2O (90.46 ± 4.60 % inhibition). At lemakalim (26 μM) responses to preganglionic stimulation were virtually abolished at 6.7 ± 0.8 mmH_2O (98.46 ± 0.98 % inhibition). The effect of lemakalim on responses to transmural stimulation was greater than the effect on preganglionic vagal stimulation $p<0.05$. At lemakalim (1.6 μM) responses to transmural stimulation were reduced from 187.46 ± 18.919 mmH_2O to 13.53 ± 7.40 mmH_2O (93.625 ± 3.45 % inhibition). At the higher dose of lemakalim (26 μM) responses to transmural stimulation were reduced to 0.9 ± 0.89 mmH_2O (99.61 ± 0.89 % inhibition).

Section 1.3 The effect of graded cumulative doses of SDZPCO400 on responses to preganglionic vagal stimulation and transmural stimulation in the presence of hexamethonium (50 μM).

SDZPCO400 (0.1 to 26 μM) inhibited contractions of the isolated tracheal tube induced by both preganglionic vagal stimulation and transmural stimulation, **Figure 5**. There was no difference in the effect of SDZPCO400 on responses to either type of nerve stimulation in doses up to 12.8 μM. SDZPCO400 (26 μM) caused a fall in the size of contractions to preganglionic vagal stimulation from 143.716 ± 21.43 mmH_2O to 67.58 mmH_2O ± 39.14 mmH_2O (52.07 ± 7.6.83 % inhibition). Responses to transmural stimulation fell from control values 196.33 ±

5.8 to 58.688 ± 16.317 mmH_2O (71.40 ± 6.90 % inhibition) after SDZPCO400.

Section 1.4. Time matched control experiments on responses to preganglionic vagal stimulation and transmural stimulation.

The potassium channel opening drugs were dissolved in saline and well sonicated in all experiments. Saline control tests were performed where volumes of saline equivilent to those used as the solvent for cumulative dosing with potassium channel opening drugs were applied at 10 minute intervals to the organ bath during nerve stimulation. The time matched saline volume control tests did not show any change in the size of responses to either preganglionic vagal stimulation or transmural stimulation over the duration of the experiments which was 2 hours.

Section 2.1 The effect of potassium channel opening drugs on the dose related rise in intraluminal pressure elicited by exogenous acetylcholine

2.1a Cromakalim on acetylcholine induced contraction.

Control dose response curves to acetylcholine were established at the start of the experiments in the same tissue. Exogenous acetylcholine (0.1 to 12.8 μM, caused contraction of the guinea-pig trachea leading to a dose related rise in intraluminal pressure (0 to 200 mmH_2O). Preincubation of the tissue with doses of cromakalim (3.2, 12.8 & 26 μM) in between acetylcholine dose response curves did not inhibit the rise in tone to exogenous acetylcholine, **Figure 6a**.

2.1b Lemakalim on acetylcholine induced contraction

Preincubation with lemakalim (3.2, 12.8 & 26 μM) did not inhibit the rise in tone to exogenous acetylcholine, **Figure 6b**.

2.1c SDZPCO400 on acetylcholine induced contraction.

Preincubation with SDZPCO400 (12.8 μM) did not inhibit the rise in tone to exogenous acetylcholine, **Figure 6c**.

2.2 The effect of cromakalim on the dose related rise in intraluminal pressure to histamine.

Histamine (0.1 to 26 μM) caused a dose related rise in intraluminal pressure from 0 to 200 mmH_2O. Preincubation with cromakalim (3.2 or 12.8 μM) did not inhibit the rise in tone to exogenous histamine, **Figure 7**.

2.3 The effect of cromakalim on the dose related rise in ILP to U46619.

The thromboxane mimetic U46619 (1×10^{-8} M to 1.6×10^{-7} M) caused a dose related rise rise in intraluminal pressure from 0 to 200 mmH_2O. Preincubation with cromakalim 3.2 & 12.8 μM did not inhibit the rise in tone to U46619, **Figure 8**.

Section 3. The effect of cromakalim on responses to cholinergic stimulation in the presence of (-) propranolol.

Cromakalim alone caused a dose related inhibition of responses to preganglionic vagal stimulation and transmural stimulation (see section 1.1a for ILP values). In parallel experiments (-) propranolol (1 μM) was added 20 minutes before a dose response curve to cromakalim was performed. The control contraction to preganglionic vagal stimulation was 103.99 ± 24.38 mmH_2O which was reduced to 31.2 ± 15.02 mmH_2O (70.08 % inhibition) by cromakalim (26 μM). The control responses to transmural stimulation were 159.385 ± 29.45 mmH_2O which were reduced to 38.77 ± 10.01 mmH_2O (75.72 % inhibition) by cromakalim (26 μM). The presence of (-) propranolol potentiated the inhibitory effect of cromakalim on responses to both preganglionic vagal stimulation (**Figure 13a**) and transmural stimulation (**Figure 13b**).

Section 4. The effect of the presence glibenclamide on the inhibitory action of the potassium channel opening drugs on cholinergic stimulation.

The experiments in this section followed the same general protocol. A dose response curve to a potassium channel opening drug was performed using either preganglionic vagal stimulation or transmural stimulation. Then in separate experiments, glibenclamide (20 μM) was added 30 minutes before a cumulative dose response curve to the potassium channel opening

drug was performed. The mean values obtained in these parallel experiments were compared.

Section 4.1 The effect of glibenclamide on the inhibitory effect of cromakalim on cholinergic stimulation.

For absolute ILP values of the inhibitory effect of cromakalim alone on responses to preganglionic vagal stimulation and transmural stimulation see section 1.1a (Page 43). Preincubation of the tracheal preparation for 30 minutes with glibenclamide (20 μM) significantly reduced the inhibitory effect of cromakalim (0.1 to 26 μM) on responses to preganglionic vagal stimulation,(**Figure 9a**), and to transmural stimulation (**Figure 9b**). The values for the contraction of the trachea to preganglionic vagal stimulation in the presence of glibenclamide were 126.7 ± 27.9 mmH_2O and after cromakalim (26 μM) this was reduced to 83.6 ± 21.0 mmH_2O (34.5 ± 6.0 % inhibition). Values for contraction of the trachea to transmural stimulation in the presence of glibenclamide were 102.0 ± 68.1 mmH_2O. After cromakalim (26 μM) this value was only slightly reduced to 76.7 ± 54.9 mmH_2O (25.1 ± 4.5 % inhibition).

Section 4.2 The effect of glibenclamide on the inhibitory effect of lemakalim on cholinergic stimulation.

Preincubation of the tracheal preparation for 30 minutes with glibenclamide (20 μM) significantly reduced the inhibitory action of lemakalim (0.01 to 26 μM) on responses to both preganglionic vagal stimulation (**Figure 10a**) and on transmural stimulation (**Figure 10b**). For values of the effect of lemakalim alone on responses to preganglionic vagal stimulation and transmural stimulation see section 1.2 (Page 45). In the presence of glibenclamide, lemakalim (26 μM) reduced values for contraction to preganglionic vagal stimulation from 224.36 ± 32.5 to 0 mmH_2O (100 % inhibition). Contraction to transmural stimulation was reduced from 226.34 ± 35.6 mmH_2O to 55.18 ± 8.9 mmH_2O (77.36 % inhibition).

Section 4.3 The effect of glibenclamide on the inhibitory action of SDZPC0400 on cholinergic stimulation.

For values of the effect of SDZPC0400 (0.1 to 26 μM) on responses to preganglionic vagal stimulation and transmural stimulation see section 1.3 (Page 45). Preincubation of the tracheal preparation for 30 minutes with glibenclamide (20 μM) significantly reduced the inhibitory action of SDZPC0400 (0.1 to 26 μM) on responses to preganglionic vagal stimulation (**Figure 11a**) and on transmural stimulation,(**Figure 11b.**). In the presence of glibenclamide, the maximum dose of SDZPC0400 (26 μM) reduced responses to preganglionic vagal stimulation from 300.845 ± 37.085 mmH_2O to 207.54 ± 35.19 mmH_2O (38.2 % inhibition). Responses to transmural stimulation were reduced by SDZPC0400 (26 μM) in the presence of glibenclamide from 328.06 ± 34.65 mmH_2O to 205.89 ± 6.83 mmH_2O (33.698 % inhibition).

The presence of glibenclamide (20 μM) alone for 30 minutes had no effect on responses to either preganglionic vagal stimulation or transmural stimulation. The antagonism of potassium channel opening drugs by glibenclamide was significant but only partial; complete blockade was never obtained even at the relatively high dose of glibenclamide used.

Section 5.1 The effect of the (+) isomer of cromakalim BRL38226 on responses to preganglionic vagal stimulation and transmural stimulation.

BRL38226 in graded cumulative doses (0.1 to 26 μM) caused only a very slight inhibition of responses to nerve stimulation. At BRL38226 (26 μM) the maximum inhibition of responses to preganglionic vagal stimulation was from 330 to 249 mmH_2O (24.52 %) inhibition and for transmural stimulation from 182.92 mmH_2O to 145.26 mmH_2O (20 58 %, n=2). It appears that BRL28226 is the relatively inactive enantiomer of cromakalim.

Section 5.2 The effect of lemakalim on responses to preganglionic vagal stimulation and transmural stimulation alone and in the presence of BRL38226

Cumulative doses of the (-) isomer of cromakalim, lemakalim alone significantly inhibit contraction of the guinea-pig trachea to

preganglionic stimulation and to transmural stimulation, reaching 100 % inhibition at 12.8 μM (see section 1.2, page 45 for ILP values). In parallel experiments, preincubation with BRL38226 (26 μM) the (+) isomer, significantly reduced the inhibitory effect of lemakalim on responses to preganglionic vagal stimulation (**Figure 12a**) and transmural stimulation (**Figure 12b**). In the presence of BRL38226 lemakalim reduced responses to preganglionic vagal stimulation from 320.59 ± 28.95 mmH2O to 207.79 ± 30.06 mmH2O (33.70 % inhibition;). Responses to transmural stimulation in these conditions were reduced from 210.27 ± 62.65 mmH_2O to 126.87 ± 47.27 mmH_2O (40.23 % inhibition). The (+) isomer of cromakalim, BRL38226 opposes the inhibitory action of lemakalim on nerve stimulation.

Section 6. An investigation was made of a possible interaction of muscarinic autoreceptors and potassium channels.

Section 6.1 The effect of cromakalim on responses to preganglionic vagal stimulation and transmural stimulation in the presence of methoctramine, an M2 muscarinic receptor antagonist

Cromakalim alone caused a dose related inhibition of responses to nerve stimulation (see section 1.1a ,page 43 for ILP values). In parallel experiments, the trachea was preincubated with methoctramine (10^{-7} M) for 15 minutes then a dose response curve to cromakalim was performed. Responses to preganglionic vagal stimulation were reduced from 137.56 ± 18.97 mmH_2O to 54.732 ± 10.52 mmH_2O (60.224 ± 7.34 % inhibition) by cromakalim (26 μM) in the presence of methoctramine. Similarly responses to transmural stimulation were reduced from 137.08 ± 24.10 mmH_2O to 70.57 ± 6.25 mmH_2O (48.52 ± 5.51 % inhibition) by cromakalim in the presence of methoctramine. The presence of methoctramine did not affect the inhibitory action of cromakalim on responses to preganglionic vagal stimulation (**Figure 14a**) and transmural stimulation (**Figure 14b**) compared to values for cromakalim alone in parallel experiments.

Section 6.2. The effect of pilocarpine, an M_2 receptor agonist, on responses to preganglionic stimulation and transmural stimulation alone and in the presence of glibenclamide.

Pilocarpine an M_2 receptor agonist (0.1 to 10 μM) caused a dose related inhibition of responses to both types of nerve stimulation accompanied by a rise in tone. Pilocarpine (10 μM) reduced responses to preganglionic vagal stimulation from 225 ± 26.77 mmH_2O to 9.79 ± 4.10 mmH_2O (95.47 ± 0.68 % inhibition) and responses to transmural stimulation were reduced from 328 ± 38.12 mmH_2O to 47.25 ± 45.78 mmH_2O (87.40 ± 5.97 % inhibition).

After a washout period the same tissue was preincubated with glibenclamide (20 μM) a selective blocker of ATP-operated potassium channels. The presence of glibenclamide (20 μM) did not affect the dose related inhibition by pilocarpine. Pilocarpine (10 μM) in the presence of glibenclamide reduced contraction to preganglionic vagal stimulation from 196.35 ± 35.39 mmH_2O to 13.214 ± 3.97 mmH_2O (93.46 ± 1.79 % inhibition **Figure 15a**) and transmural stimulation from 273.49 ± 44.27 mmH_2O to to 9.58 ± 3.13 mmH_2O (93.27 ± 1.79 % inhibition **Figure 15b**).

PART B. Section 1a &b The effect of cromakalim and lemakalim on NANC relaxations of the trachea to transmural stimulation.

Preganglionic vagal stimulation (in the presence of atropine (1 μM), (-) propranolol, (1 μM) and histamine to raise tracheal tone) did not induce relaxant responses of the trachea (**Figure 16a** lower trace). Since NANC relaxant responses could be elicited by transmural stimulation but not preganglionic vagal stimulation, it is probable that the preganglionic nerves which activate the NANCergic fibres enter the trachea below the cervical vagal nerve trunk. NANC relaxant responses of the trachea were elicited by transmural stimulation 30 V, 30 Hz, 5 s duration, 40 s interval in the presence of hexamethonium (50 μM), atropine (1 μM), (-) propranolol (1 μM) and histamine to raise tone to 200 mmH_2O, (upper trace). In section A cromakalim and the other potassium channel openers inhibited contractions of the trachea to cholinergic nerve stimulation but in contrast the presence of cromakalim (12.8 μM) facilitated NANC relaxations of the trachea,(**Figure 16a** middle trace). The facilitatory effect of lemakalim on NANCergic responses was

more pronounced, (**Figure 16b**). Lemakalim (3.2 μM) slightly potentiated responses to NANC relaxation, while lemakalim (12.8 μM) approximately doubled NANC relaxations from 23.998 ± 5.73 mmH_2O to 41.256 ± 7.21 mmH2O mean ± sem. Time matched control experiments revealed that NANCergic relaxations did not change in size over the time course of these experiments which was about 5 hours.

Section 2.1 a & b The effect of cromakalim and lemakalim on responses of the trachea to exogenous VIP

After raising tone to 200 mmH_2O with histamine, exogenous VIP (10^{-9} M to 10^{-7} M) caused a slowly developing relaxation which was measured 10 minutes after its application. Mean relaxation to VIP (10^{-9} M) was 28.5 ± 7.33 mmH_2O which was increased by 51.01 % after preincubation with cromakalim (12.8 μM) to a mean value of 43.038 ± 12.47 mmH_2O. Similarly relaxation to VIP (10^{-8} M) was 27.71 ± 12.8 mm H_2O and this value was significantly increased by 100 % to 57.75 ± 18.86 mmH_2O by cromakalim 12.8 μM, (**Figure 17a**, $p<0.05$, n=5).

Lemakalim (12.8 μM) slightly facilitated relaxant responses to VIP 10^{-8} M causing an 18 % increase in the size of the response. Relaxation to VIP (10^{-7} M) was increased by 72.5 % from 83.39 ± 9.28 mmH_2O to 143.915 ± 33.57 mmH_2O by lemakalim ($p<0.05$ n=5 **Figure 17b**).

Figure 17c shows a typical trace showing relaxation of the trachea to exogenous VIP (10^{-8} M and 10^{-7} M) after raising tone with histamine. Lemakalim (12.8 μM) significantly potentiated relaxation of the trachea to the higher dose of VIP. Time matched control experiments showed that after raising tone with histamine, relaxation to exogenous VIP was greater than any decline in tone occurring over the same time period. Control experiments also established that there was no spontaneous variation in the size of relaxations to VIP over the duration of these experiments which was about 4 hours.

Section 3 The effect of the VIP antagonist ((dp chloro phe leu 17) VIP) on responses to exogenous VIP

The VIP antagonist ((d-p chloro phe leu 17) VIP) in the following concentrations (10^{-9} M, 10^{-8} M, 10^{-7} M) was not at all effective at antagonising responses to exogenous VIP (10^{-7}). The control relaxation

to VIP (10^{-7} M) was was not altered by the presence of the VIP antagonist (**Figure 18**). Due to the lack of effect of the VIP antagonist to antagonise exogenous VIP it was not used in any further experiments.

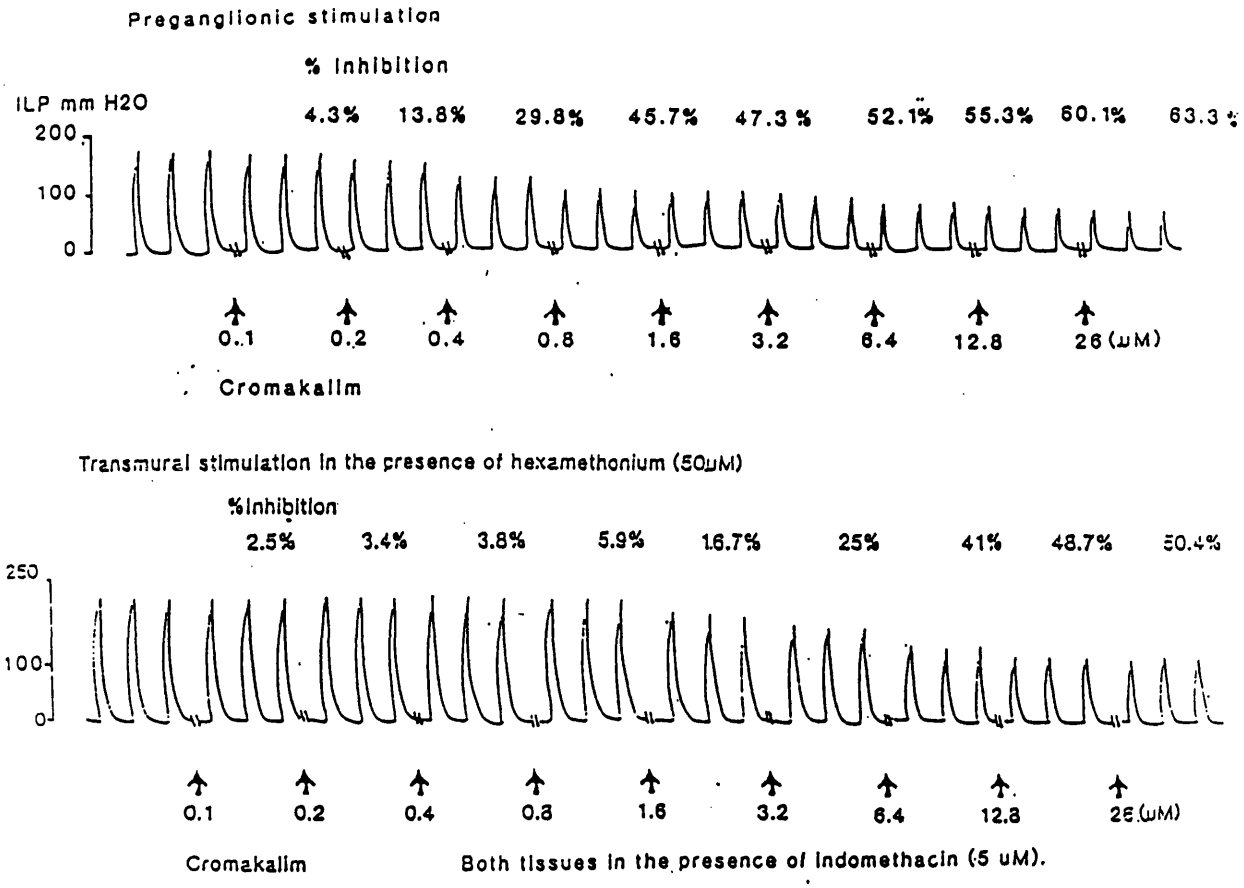


FIG 1. The effect of graded cumulative doses of cromakalim on changes in intraluminal pressure (ILP) of the guinea-pig isolated trachea evoked by preganglionic stimulation of the vagus nerve (upper section) and transmural stimulation (lower section). Hexamethonium (50 μM) was always present during transmural stimulation. Each concentration of cromakalim was applied for 10 minutes and the vertical bars indicate sections of the trace at the end of each application period. The % inhibition of the nerve induced contractions with respect to pre-drug control values are shown above each section. Stimulus parameters were 30 V, 30 Hz, 0.2 ms pulse width, 5s duration, 40 s interval and these were used in all subsequent figures unless otherwise stated.

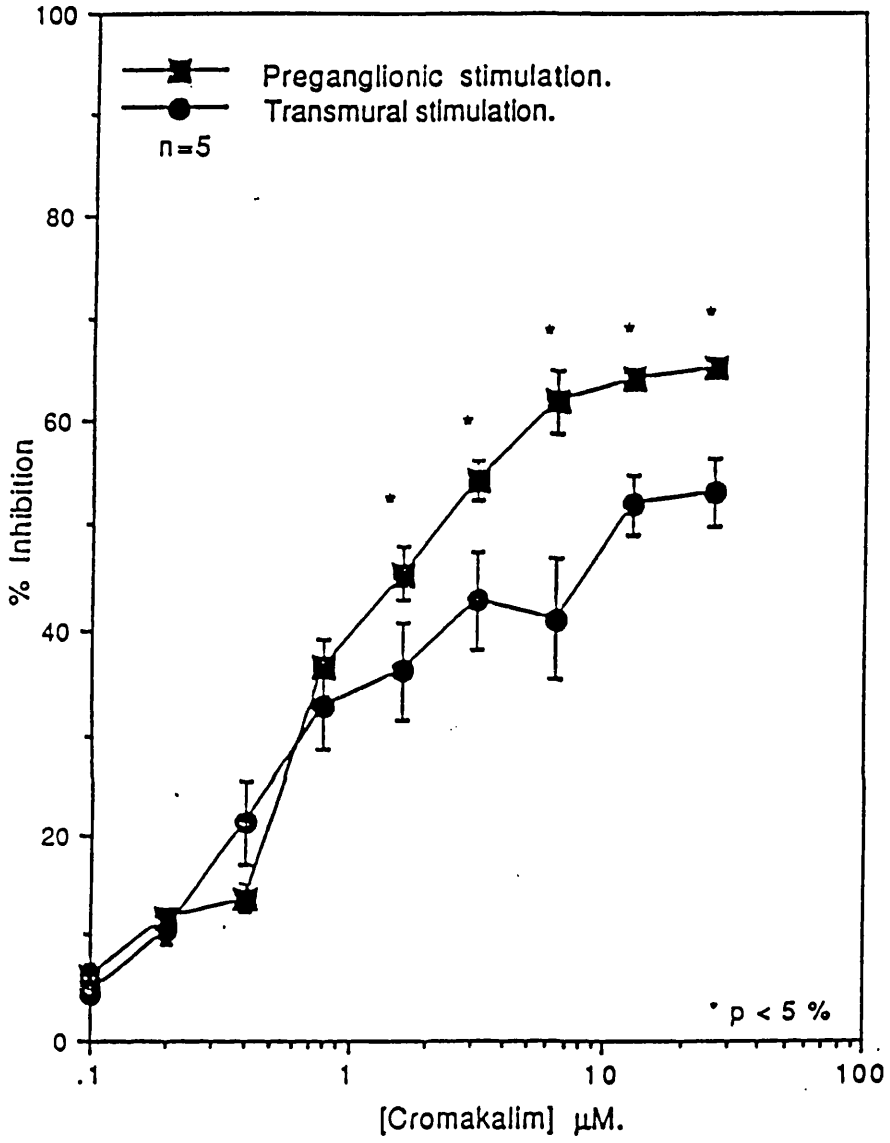


FIG 2. Cromakalim inhibited contractions of the isolated tracheal tube induced by both preganglionic(■) and transmural (●) stimulation. Values are mean ± sem % inhibition with respect to pre-drug control values (n=5). The effect of cromakalim on responses to preganglionic stimulation was greater than on transmural stimulation * $p<0.05$.

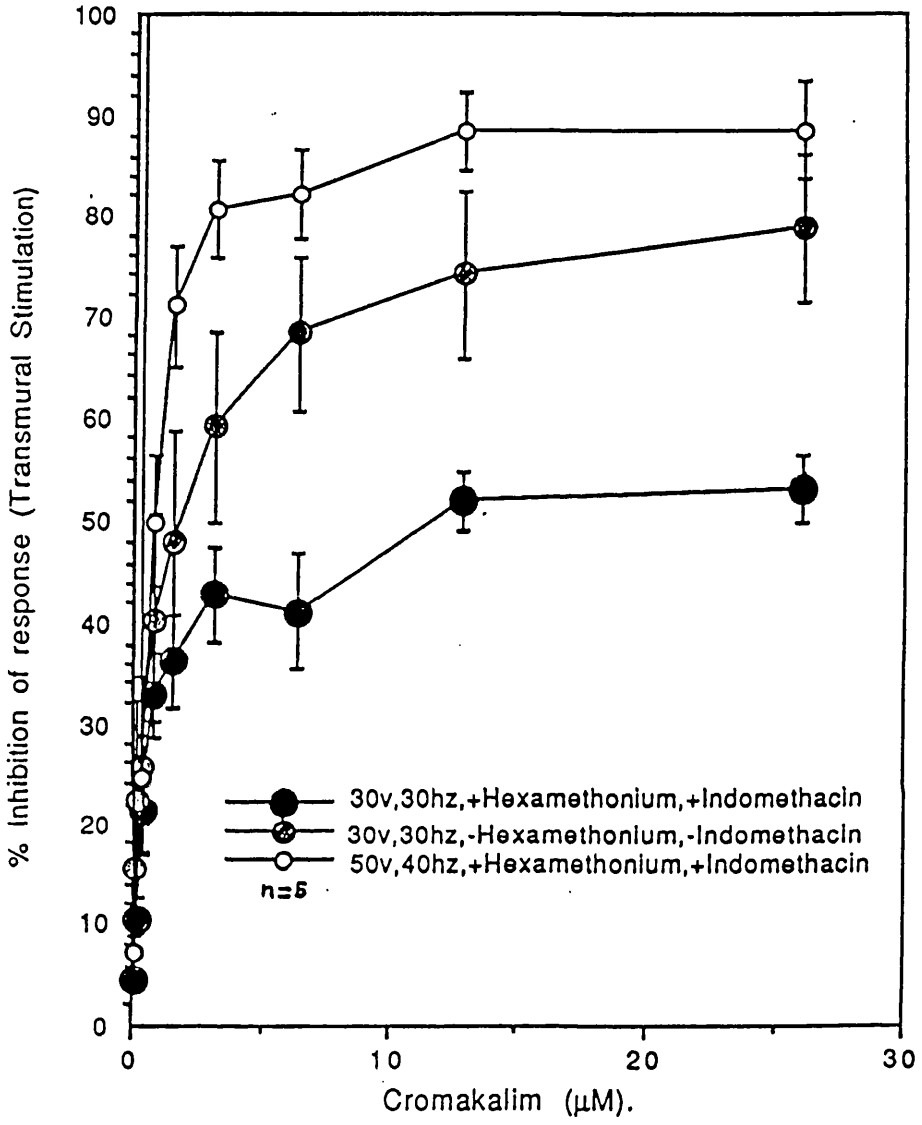


FIG 3. The effect of cromakalim on responses to transmural stimulation under different conditions; in the presence (●) and then in the absence (⊘) of hexamethonium (50 μM) and indomethacin (5 μM). Also a different design of electrode with wires placed either side of the closed tracheal tube was used for field stimulation at 50 V; 40 Hz in the presence of indomethacin and hexamethonium (○).

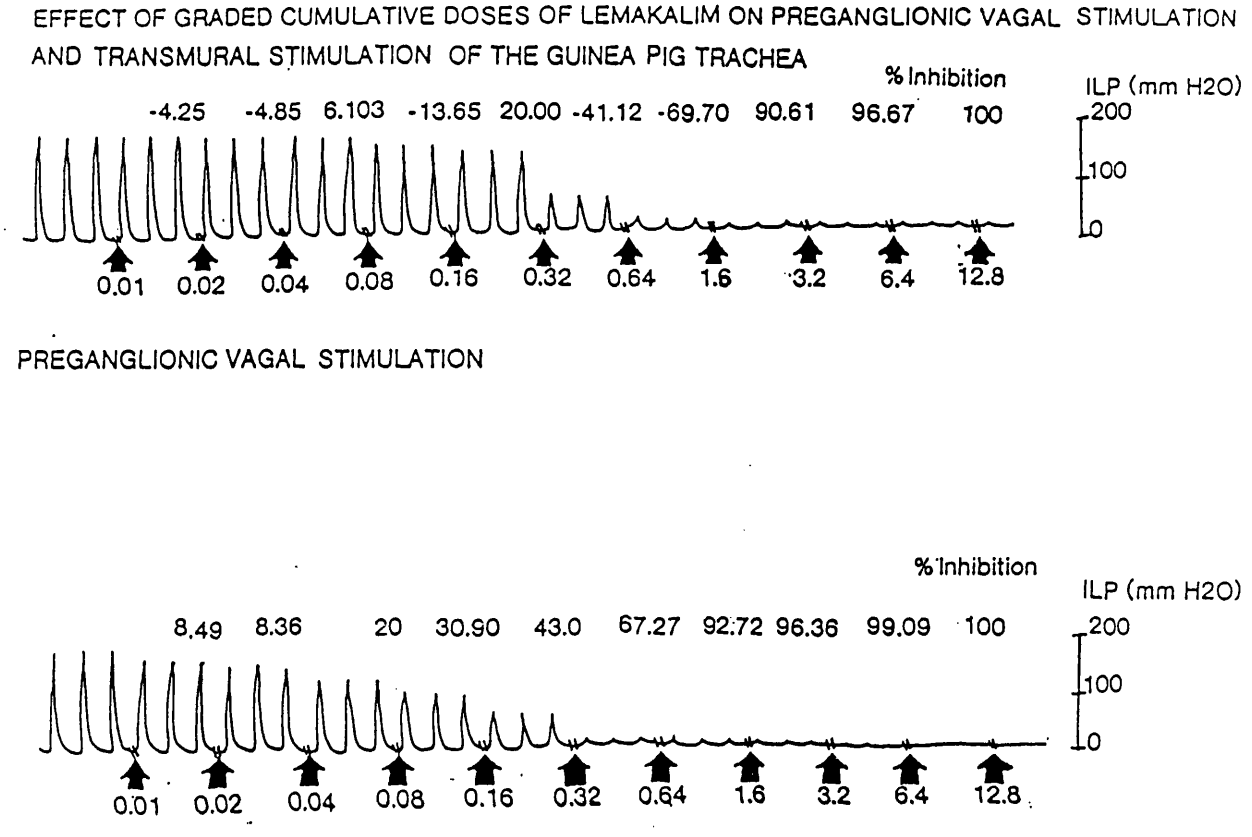


FIG 4a. The effect of graded cumulative doses of lemakalim on changes in ILP of the guinea-pig isolated trachea evoked by preganglionic stimulation of the vagus nerve (upper section) and transmural stimulation (lower section) Hexamethonium (50 μM) was present during transmural stimulation. Each concentration of lemakalim was applied for 10 minutes and the vertical bars indicate sections of the trace at the end of each application period. The % inhibition of nerve induced contractions with respect to the pre-drug control values are shown above each section. Stimulus parameters were 30 V, 30 Hz, 0.2 ms pulse width, 5s duration 40 s interval.

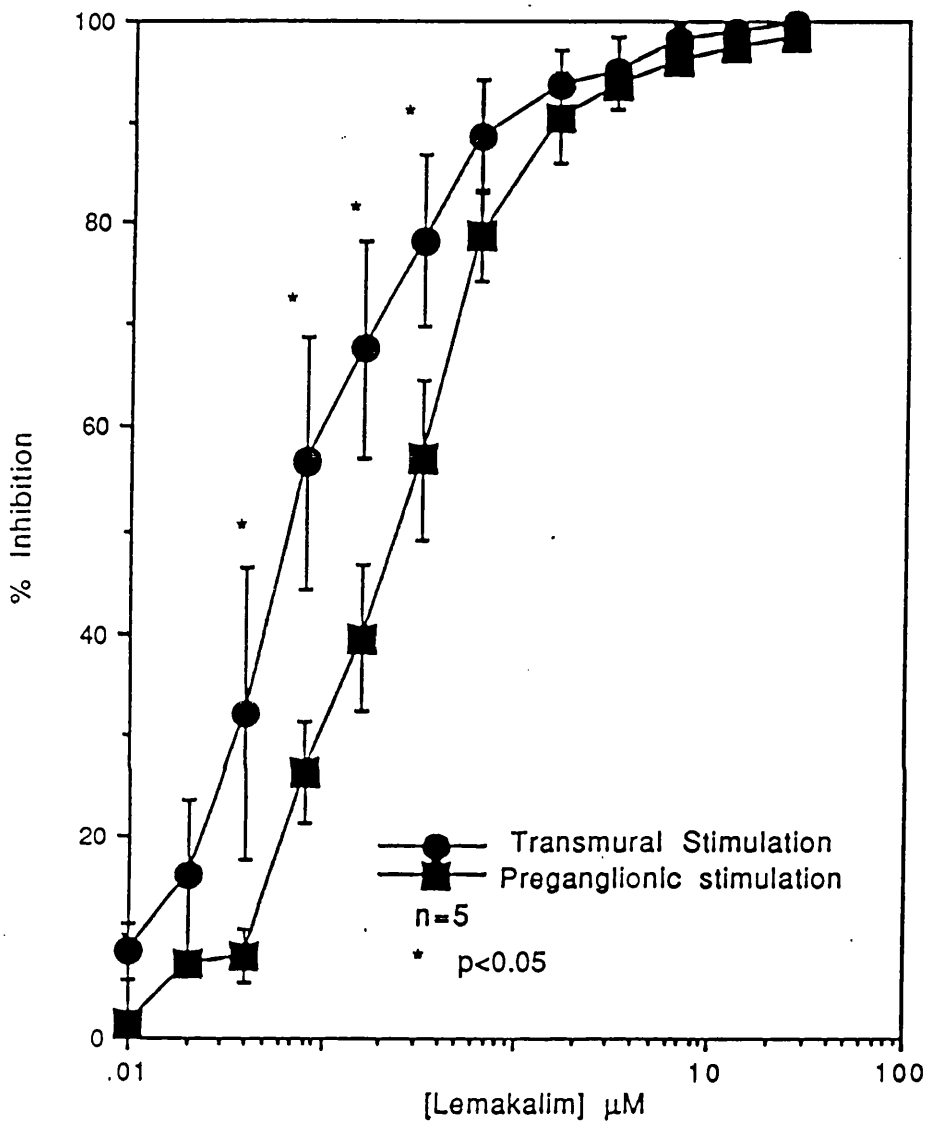


FIG 4b. Lemakalim inhibits contractions of the isolated tracheal tube induced by both preganglionic (■) and transmural (●) stimulation. Values are mean ± sem % inhibition with respect to pre-drug control values where n=5. The effect of lemakalim on transmural stimulation was greater than the effect on preganglionic vagal stimulation * $p<0.05$.

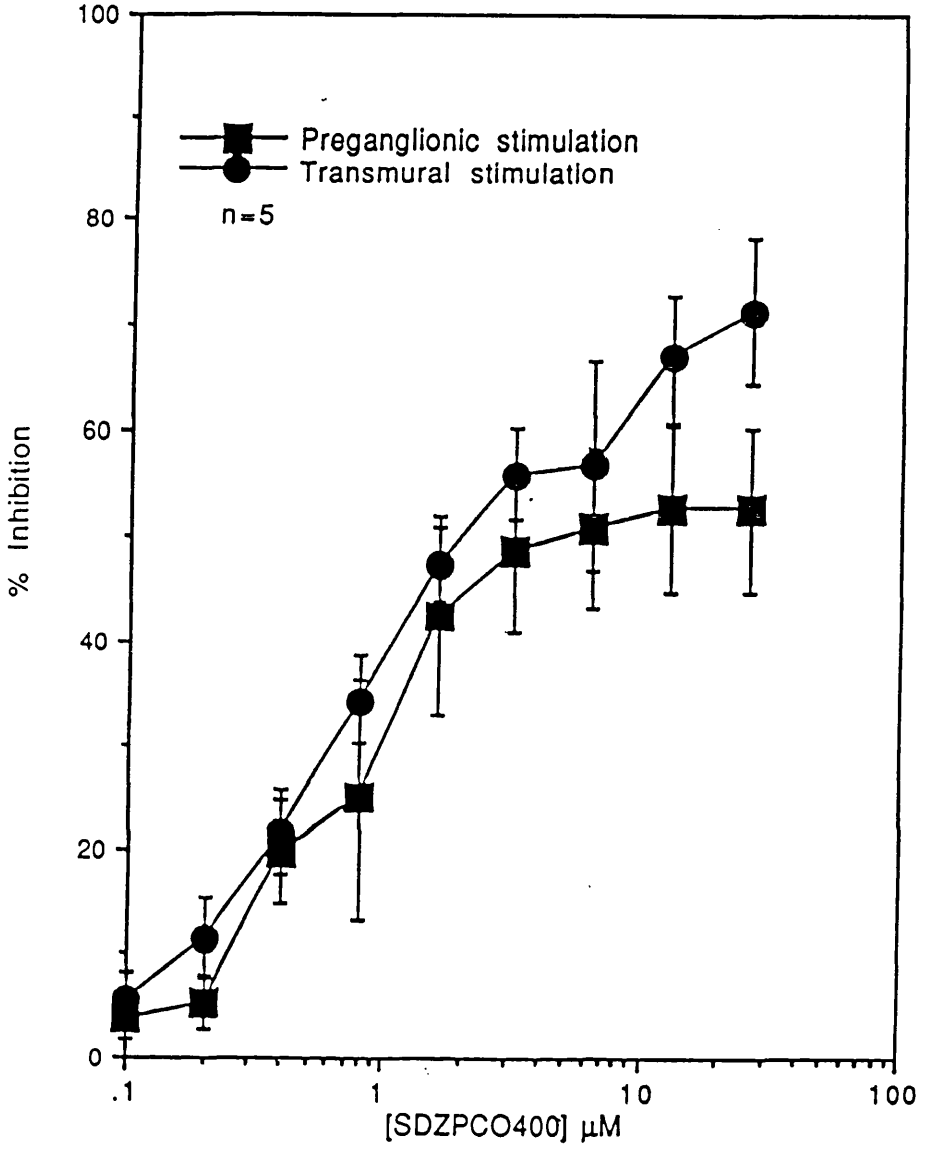


FIG 5. SDZPC0400 inhibited contractions of the isolated tracheal tube induced by both preganglionic vagal (■) and transmural stimulation (●). Values are mean ± sem % inhibition with respect to pre-drug control values (n=5). There was no significant difference between the effect of SDZPC0400 on either type of nerve-induced contraction .

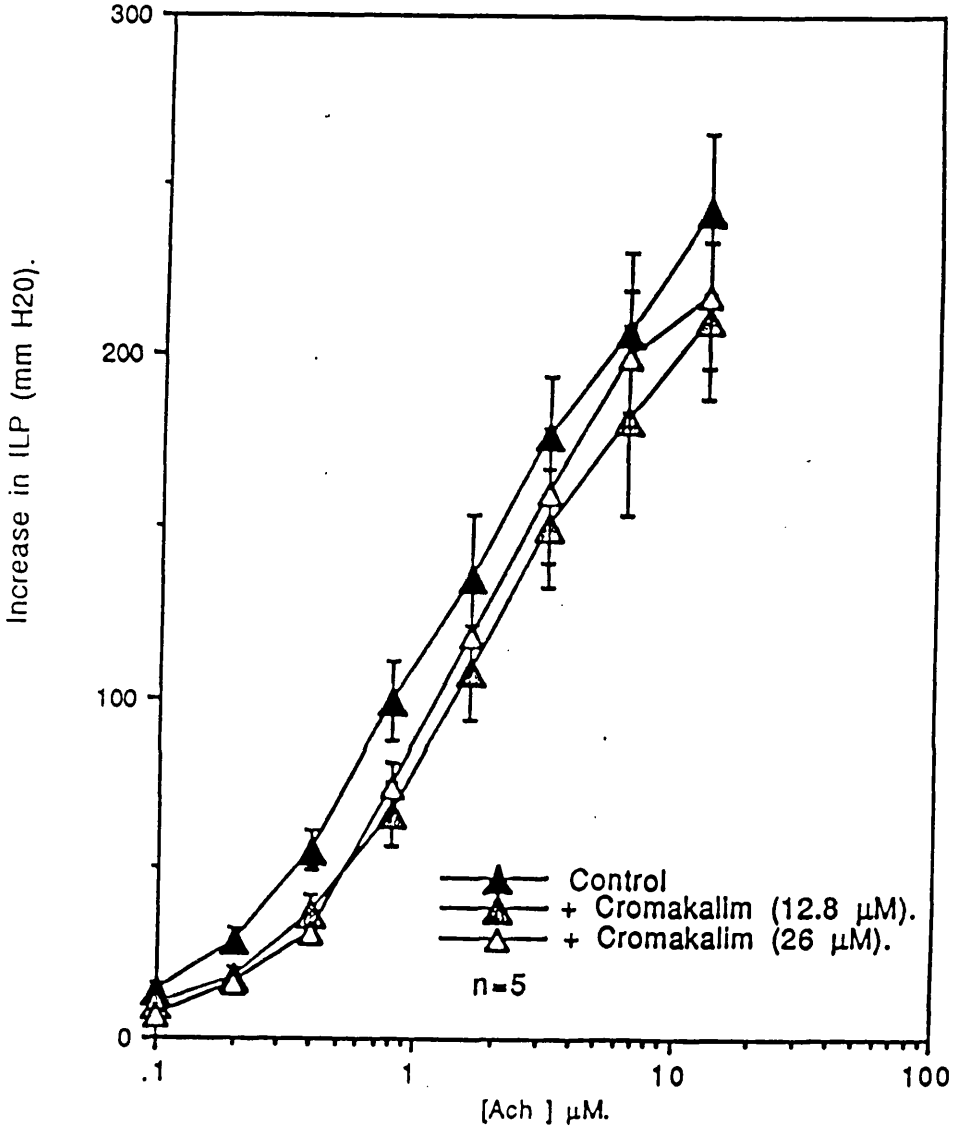


FIG 6a. Acetylcholine (0.1 to 26 μM) caused a dose related rise in ILP from 0 to 250 mmH20 (▲). Preincubation with cromakalim 12.8 μM (▲) and 26 μM (△) did not significantly affect the rise in tone to exogenous acetylcholine. The values represent mean ± sem of ILP (mmH20) (n=5).

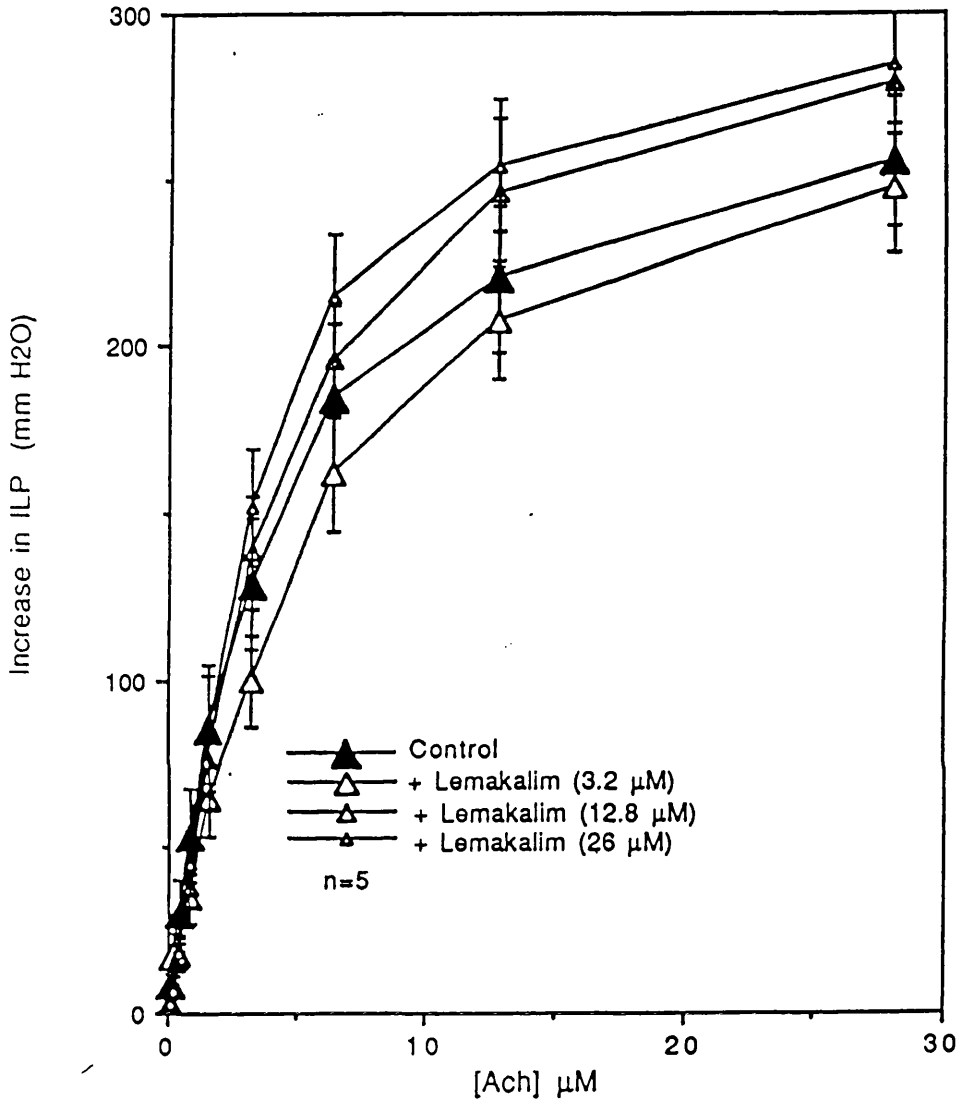


FIG 6b. Acetylcholine (0.1 to 26 μM) caused a dose related rise in ILP from 0 to 26 μM (▲). Preincubation with cromakalim 3.2 μM (Δ) ,12.8 μM (Δ) and 26 μM (Δ) did not significantly affect the rise in tone to exogenous acetylcholine. The values represent mean ± sem of ILP (mmH20), (n=5).

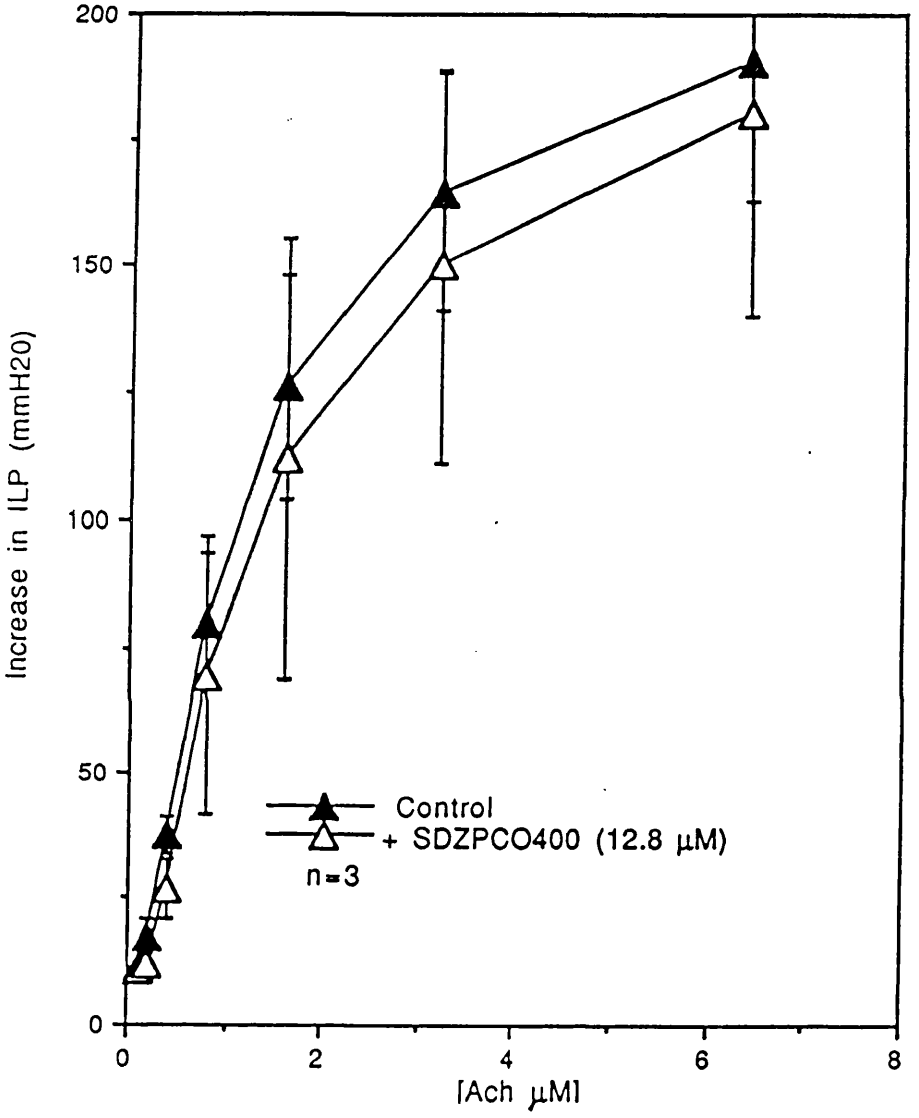


FIG 6c. Acetylcholine (0.1 to 12.8 µM) caused a dose related rise in ILP from 0 to 200 mmH20 (▲) . Preincubation with SDZPCO400 (12.8 µM △) did not affect the rise in tone to exogenous acetylcholine. The values represent mean increase ± sem (mmH20), (n=3).

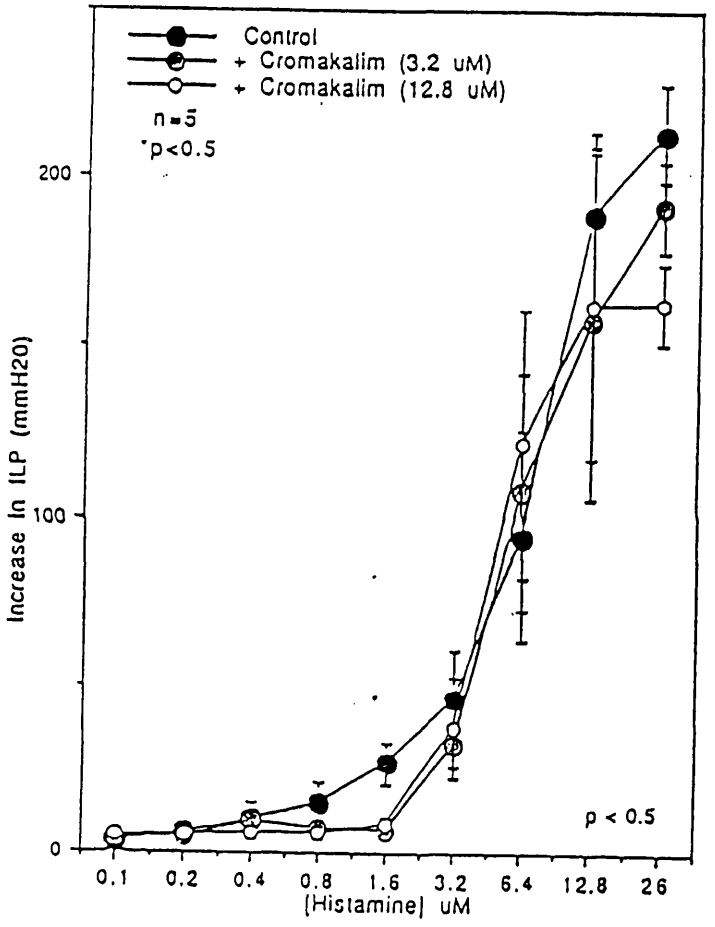


FIG 7. Histamine (0.1 to 26 μM) caused a dose related rise in ILP from 0 to 200 mmH20 (●) which was unaffected by preincubation with cromakalim 3.2 μM (◐) or cromakalim 12.8 μM (○). The values represent mean ± sem ILP (mmH20), (n=5).

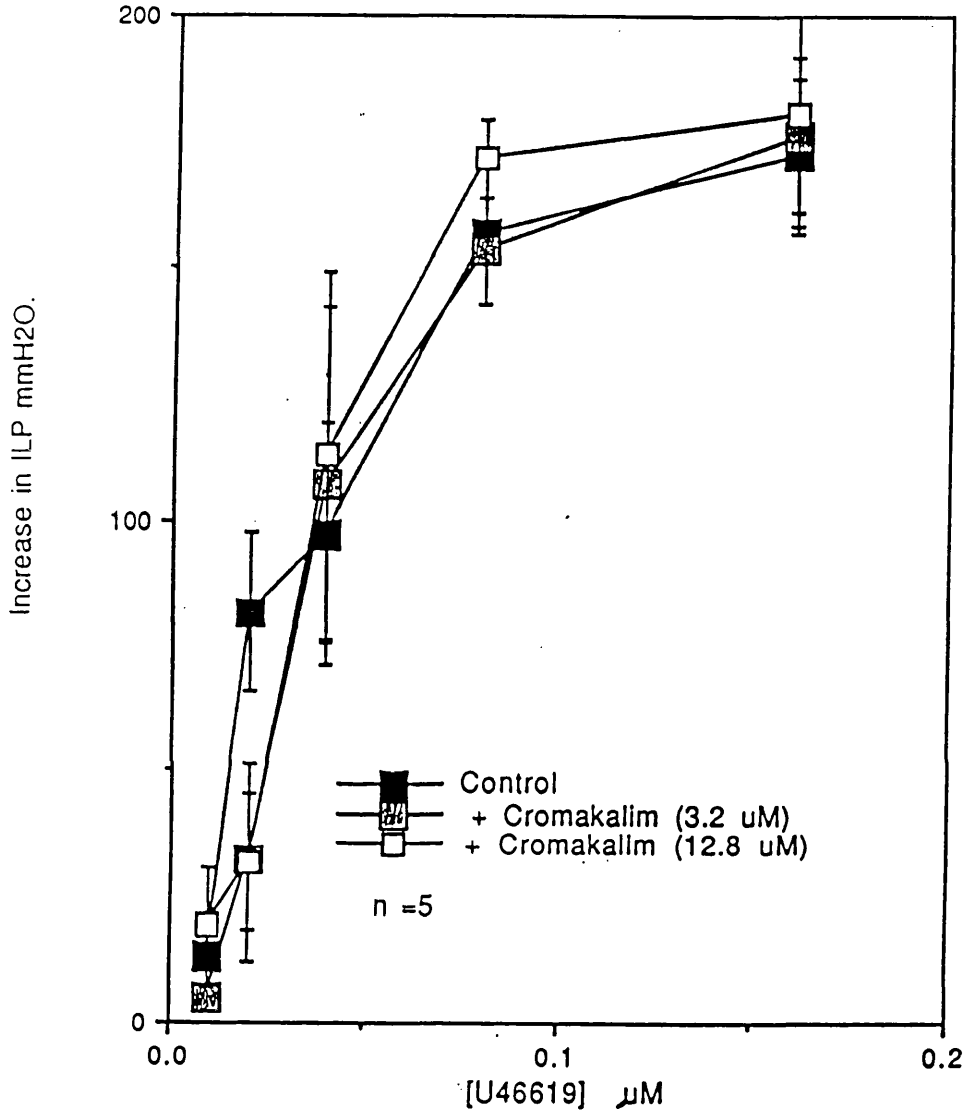


FIG 8. The thromboxane mimetic U46619 (1 x 10^{-8} M to 1.6 x 10^{-7} M) caused a dose related rise in ILP from 0 to 200 mmH20 (■). Incubation with cromakalim 3.2 µM (▨) or 12.8 µM (□) did not significantly affect the response to U46619. The values represent mean ± sem ILP mmH20, (n=5).

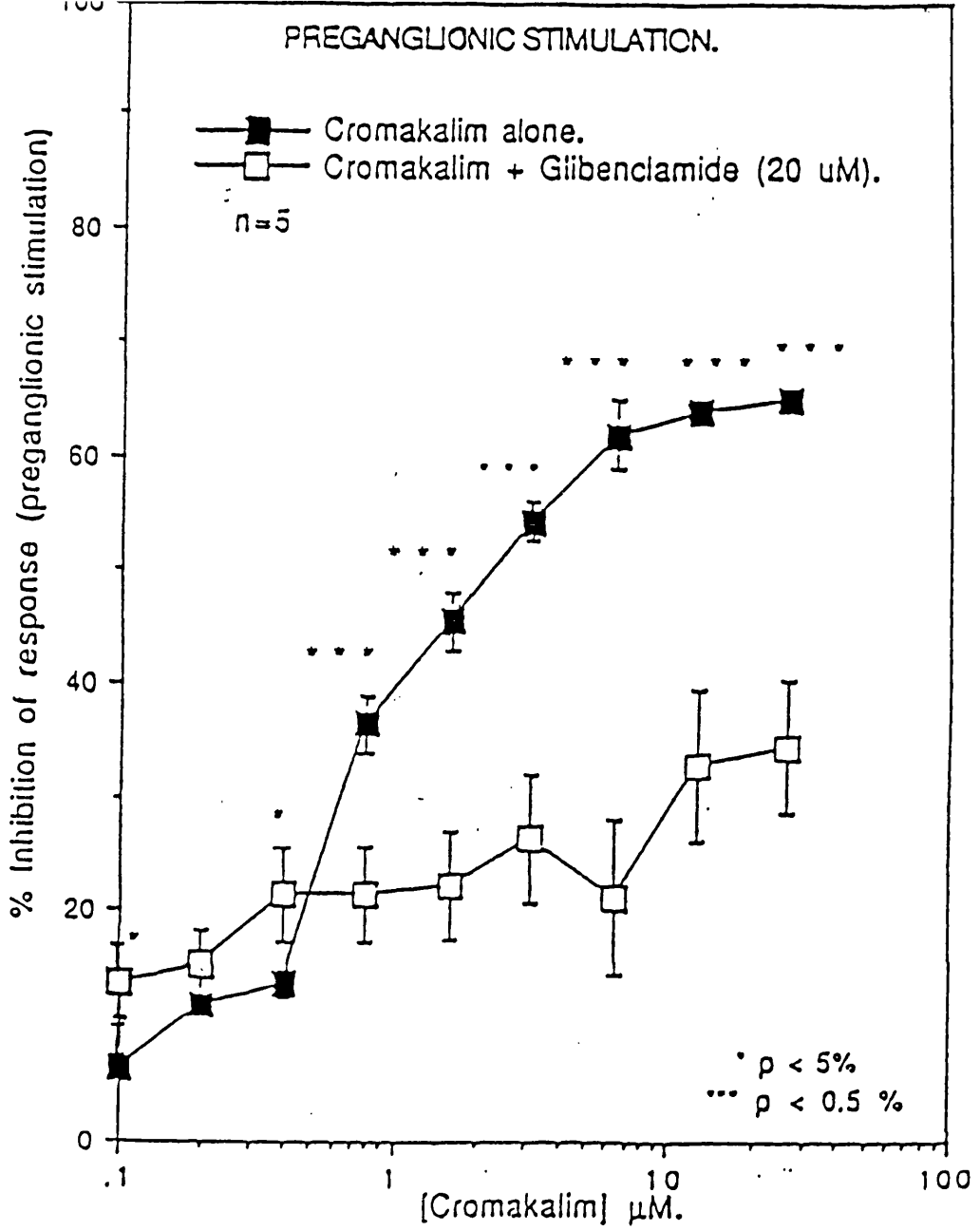


FIG 9a A dose response curve was performed to cromakalim alone on preganglionic vagal stimulation (■). In parallel experiments in different tissues, glibenclamide (20 µM) was added 30 min before a cumulative dose response curve to cromakalim was performed in the presence of glibenclamide (□). Glibenclamide reduced the effect of cromakalim on preganglionic vagal stimulation compared to the values for cromakalim alone. Values represent mean ± sem % inhibition with respect to control values before the application of cromakalim, (n=5) *** p< 0.005.

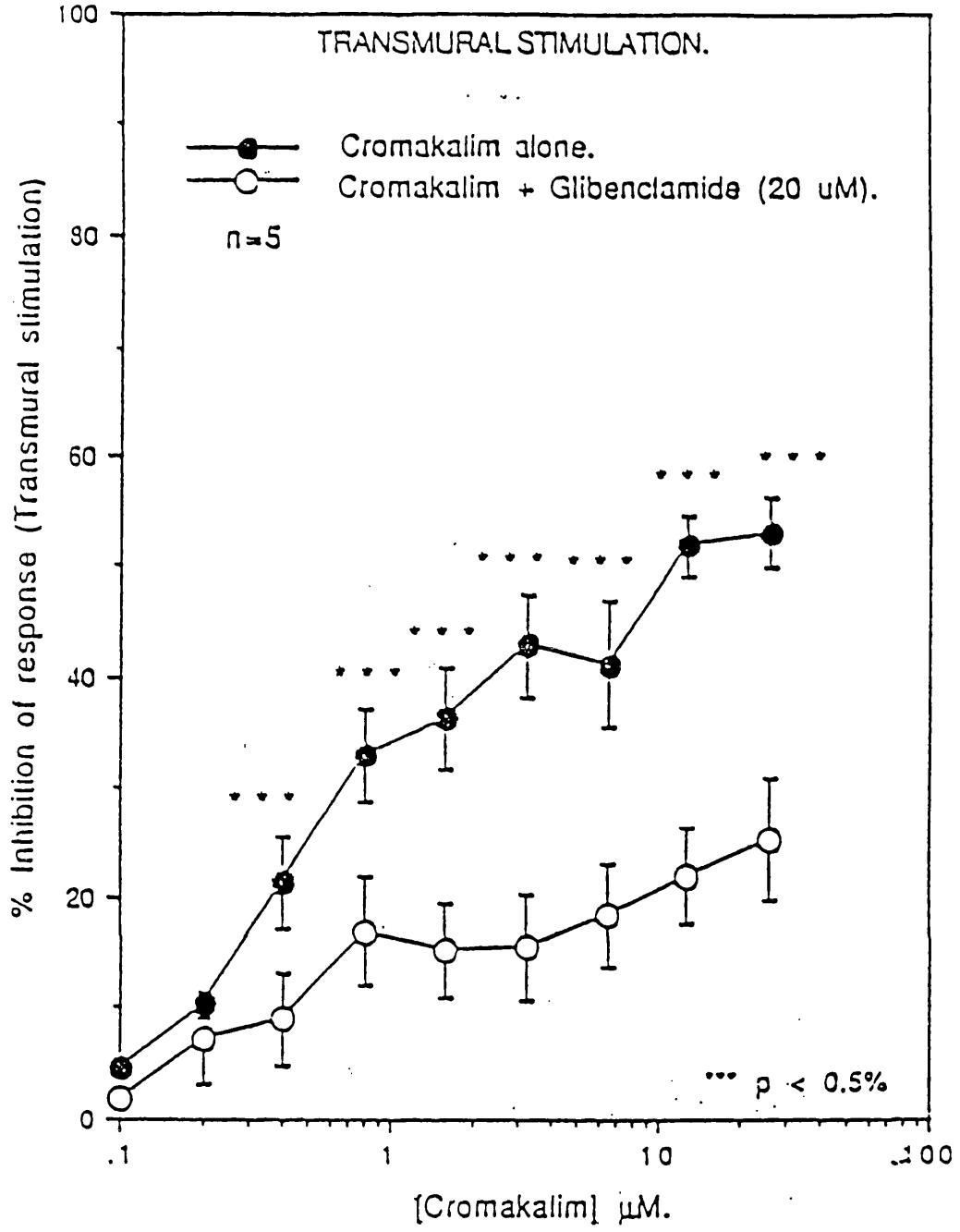


FIG 9b. A dose response curve was performed to cromakalim alone on transmural stimulation (●). In parallel experiments, a cumulative dose response curve was performed to cromakalim in the presence of glibenclamide (20 µM) (○). Glibenclamide reduced the effect of cromakalim on responses to transmural stimulation compared to values for cromakalim alone. Values are mean ± sem % inhibition with respect to control values before the application of cromakalim, (n=5) *** $p<0.005$, * $p<0.05$.

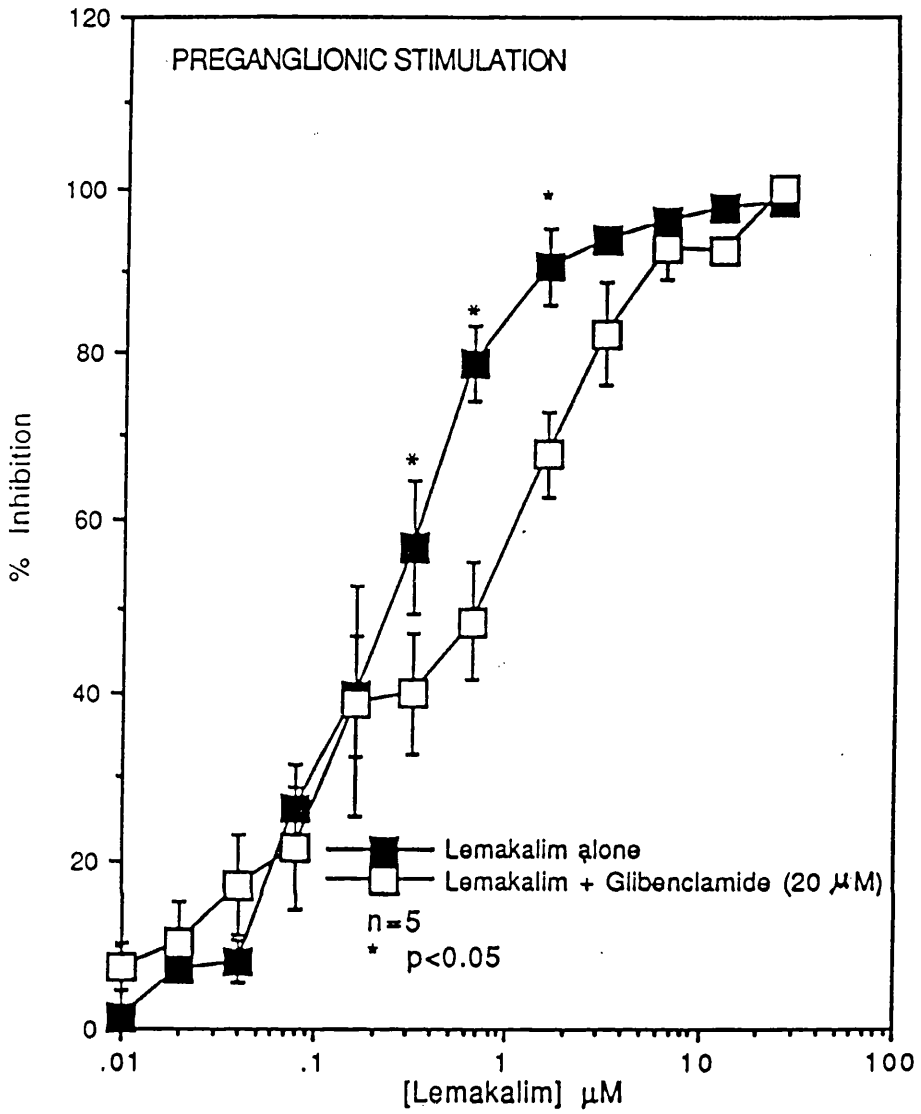


FIG 10a. A dose response curve was performed to lemakalim alone on preganglionic vagal stimulation (■) . In parallel experiments Glibenclamide (20 μM) was added 30 min before a cumulative dose response curve to lemakalim was performed (□). Glibenclamide reduced the effect of lemakalim on responses to preganglionic vagal stimulation compared to values for lemakalim alone. Values are mean ± sem % inhibition with respect control values before the application of lemakalim* $p<0.05$.

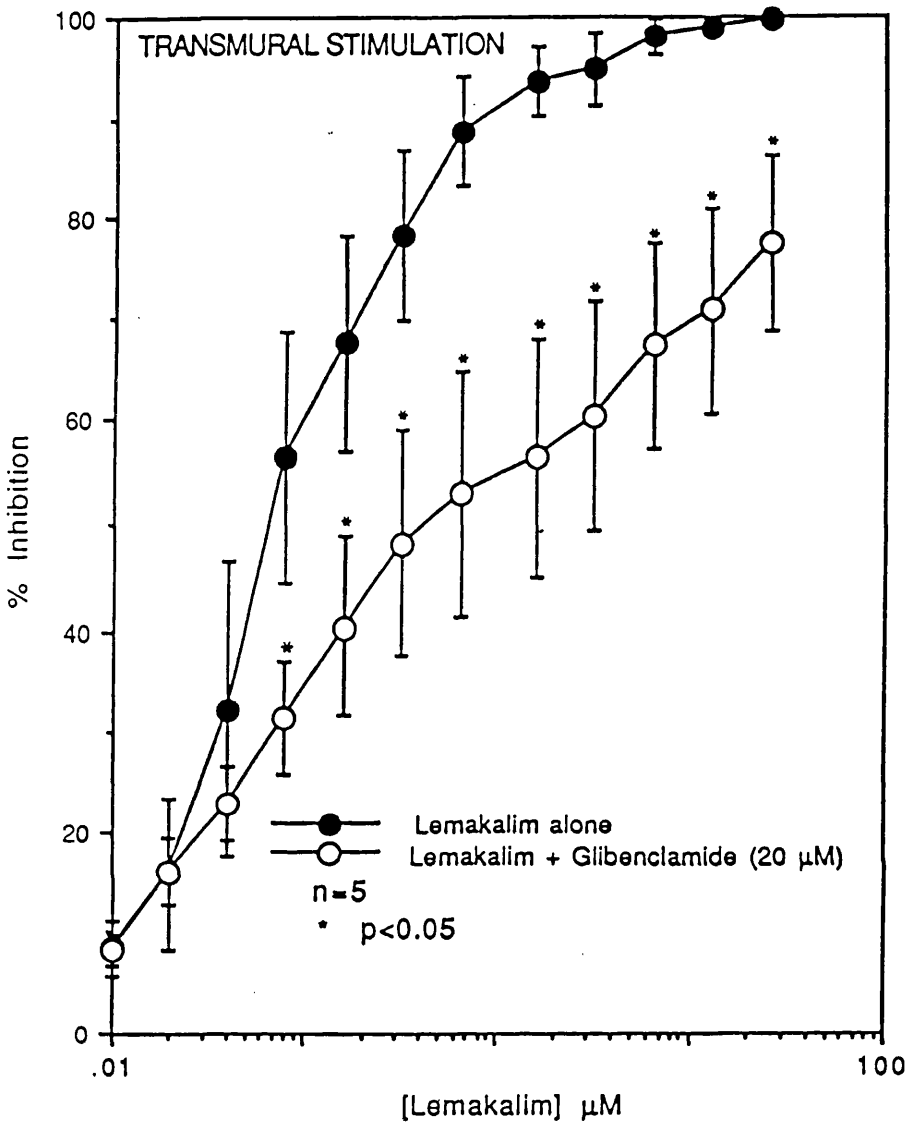


FIG 10b. A dose response curve was performed to lemakalim alone on transmural stimulation (●). In parallel experiments glibenclamide was added 30 min before a cumulative dose response curve to lemakalim was performed (○). Glibenclamide reduced the effect of lemakalim on responses induced by transmural stimulation compared to values for lemakalim alone. Values are mean ± sem % inhibition compared to control values before the application of lemakalim (n=5) *p< 0.055.

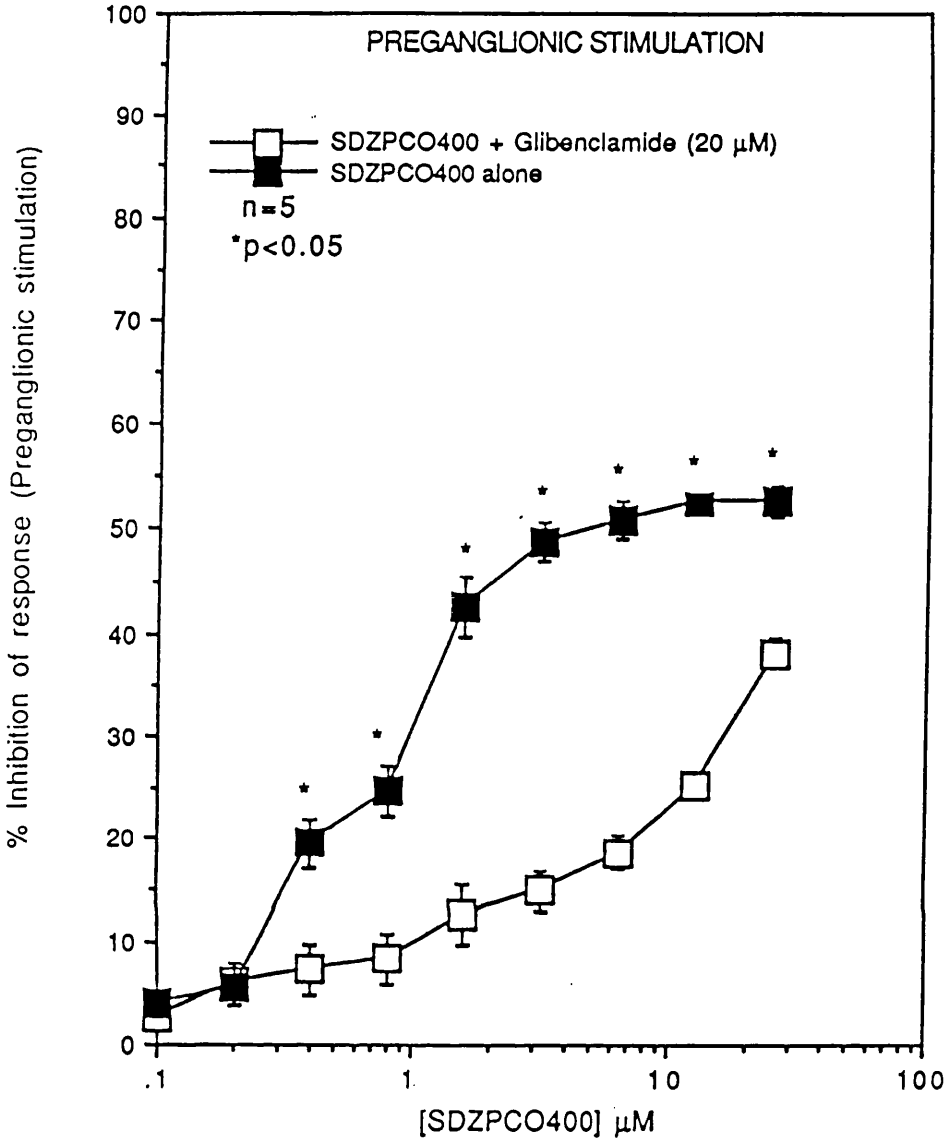


FIG 11a. A dose response curve was performed to SDZPCO400 alone (■) on preganglionic vagal stimulation. In parallel experiments, glibenclamide (20 μM) was added 30 min before a cumulative dose response curve to SDZPCO400 was performed (□). Glibenclamide reduced the effect of SDZPCO400 on responses to preganglionic vagal stimulation compared to values for SDZPCO400 alone. Values are mean ± sem % inhibition compared to control values before the application of SDZPCO400, (n=5), * $p<0.05$.

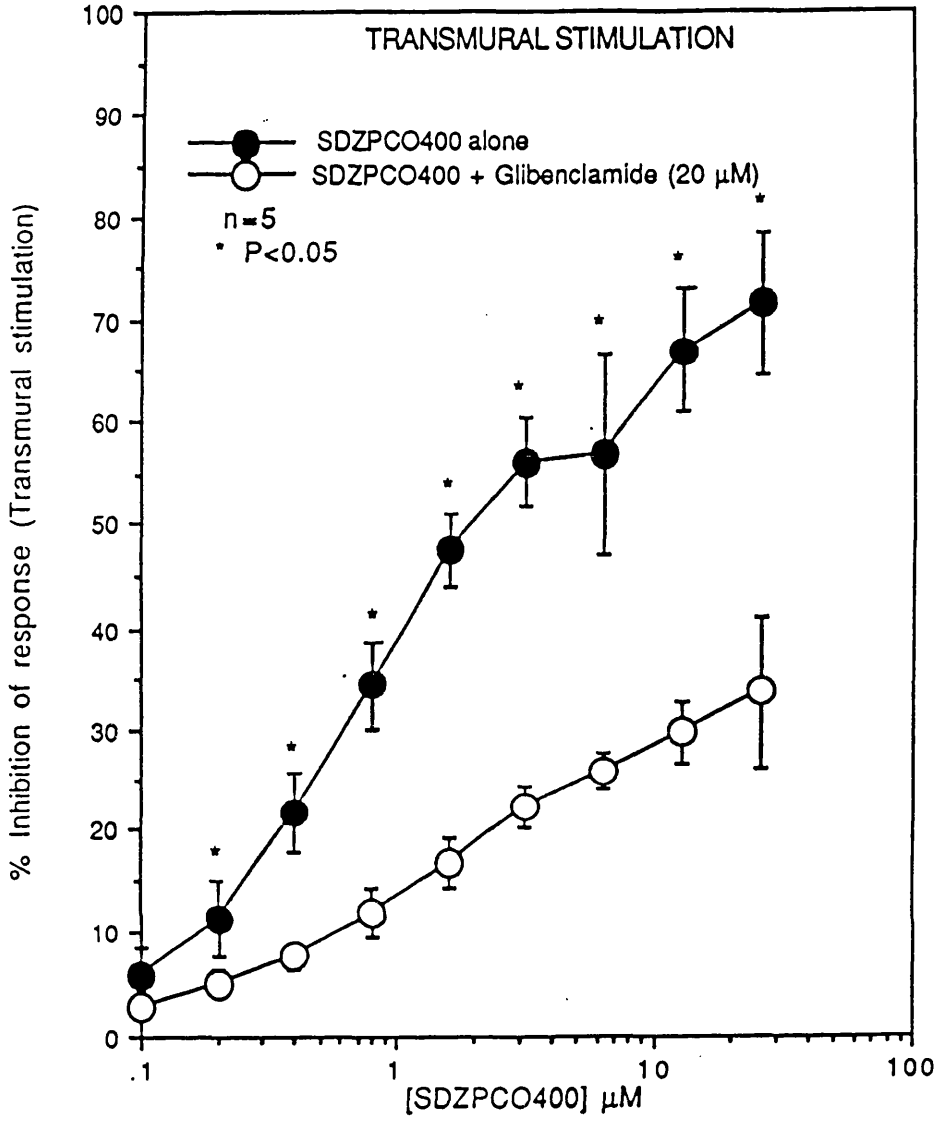


FIG 11b. A dose response curve was performed to SDZPCO400 alone on transmural stimulation (●). In parallel experiments glibenclamide (20 μM) was added 30 min before a cumulative dose response curve to SDZPCO400 was performed (○).Glibenclamide reduced the effect of SDZPCO400 on responses induced by transmural stimulation compared to values for SDZPCO400 alone. Values are mean ± sem % inhibition with respect to control values before the application of SDZPCO400, (n=5) *p<0.05.

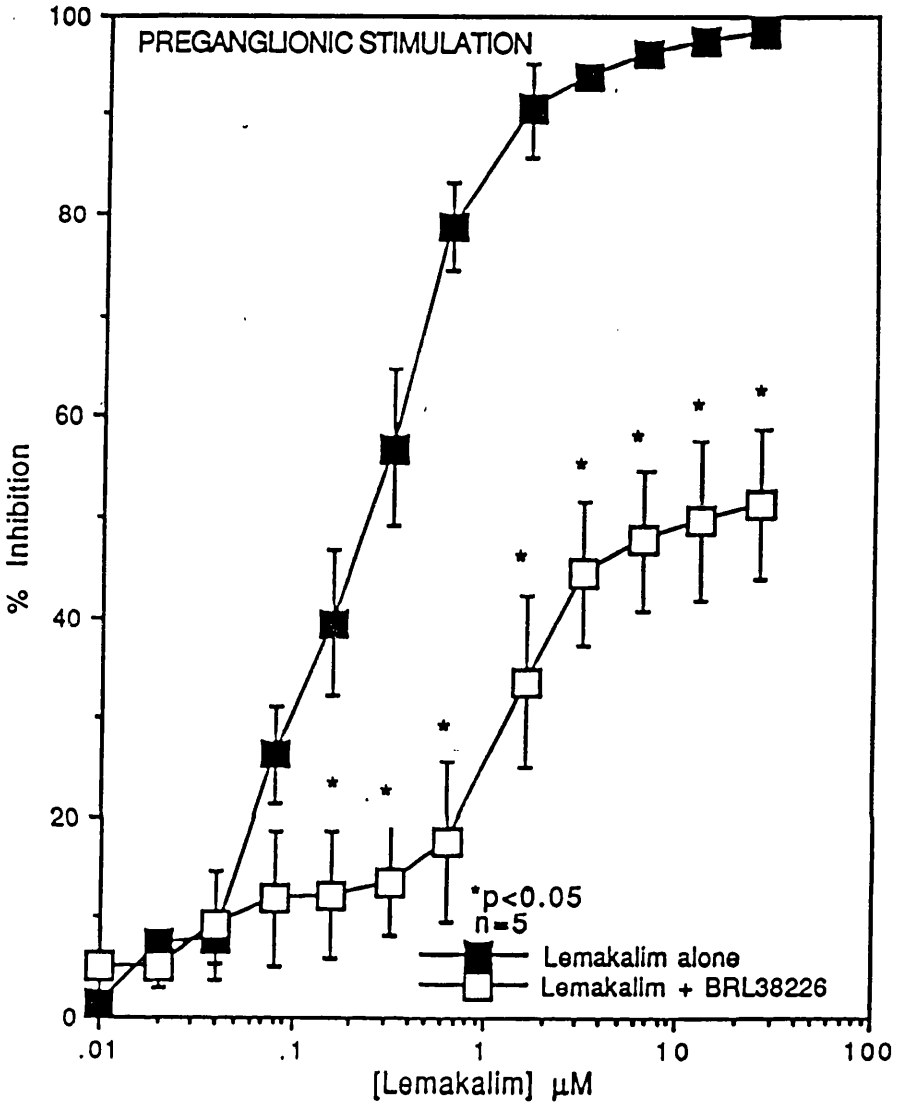


FIG 12.a. Cumulative doses of the (-) isomer of cromakalim known as lemakalim inhibited the contraction of the trachea to preganglionic stimulation (■). The presence of the + isomer of cromakalim BRL38226 (26 µM) (□) reduced the inhibitory effect of lemakalim on responses to nerve stimulation, * $p<0.05$. Values are mean ± sem % inhibition with respect to control values before the application of lemakalim, (n=5).

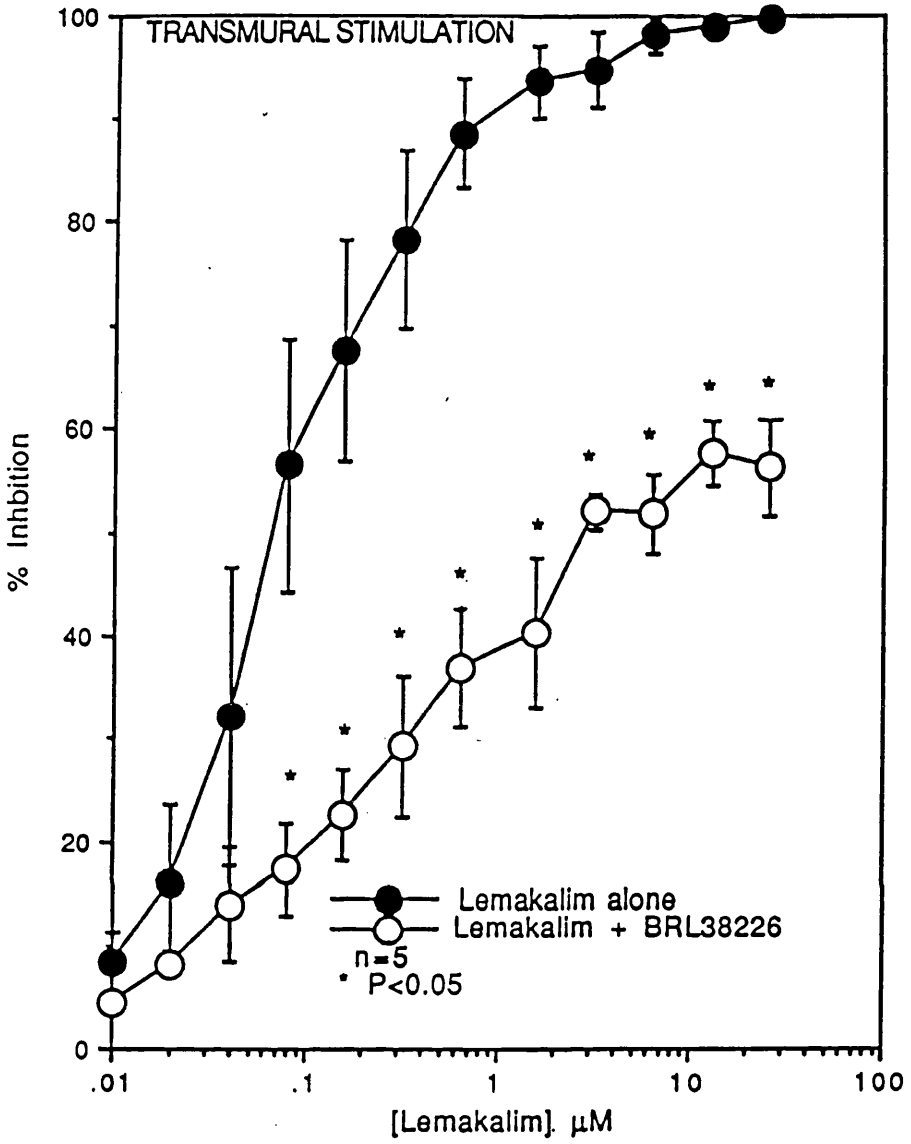


FIG 12.b. Cumulative doses of the (-) isomer lemakalim inhibits contraction of the trachea to transmural stimulation (●). The presence of the (+) isomer BRL38226 (○) reduces the inhibitory effect of lemakalim on nerve stimulation, * $p<0.05$. Values are mean ± sem % inhibition with respect to control values before the application of lemakalim.

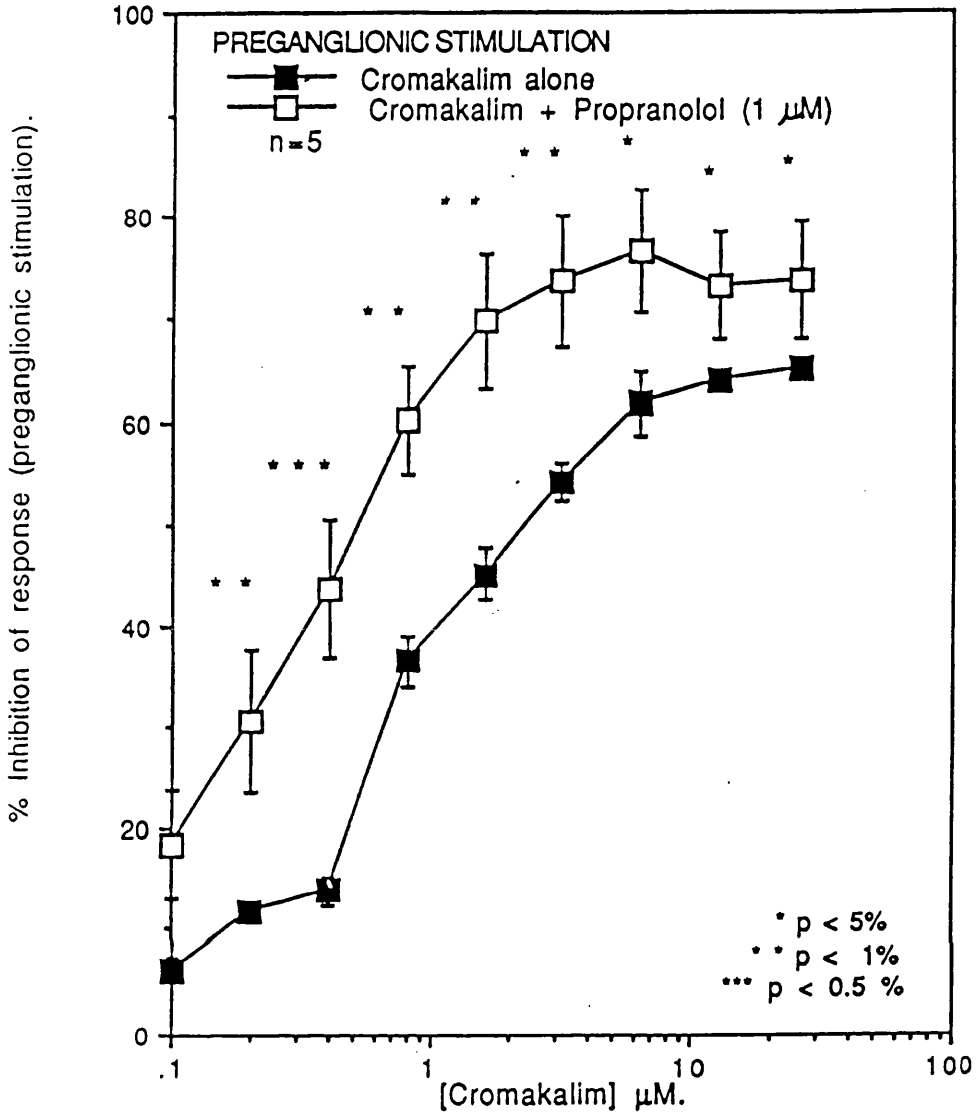


FIG 13a A dose response curve was performed to cromakalim alone on responses elicited by preganglionic vagal stimulation (■). In parallel experiments, (-) propranolol was added 20 min before a cumulative dose response curve to cromakalim was performed (□). (-) propranolol potentiated the effect of cromakalim on responses to preganglionic vagal stimulation compared to values for cromakalim alone(*** $p<0.005$, ** $p<0.01$, * $p<0.05$). Values are mean ± sem % inhibition with respect to control values before the application of cromakalim, (n=5).

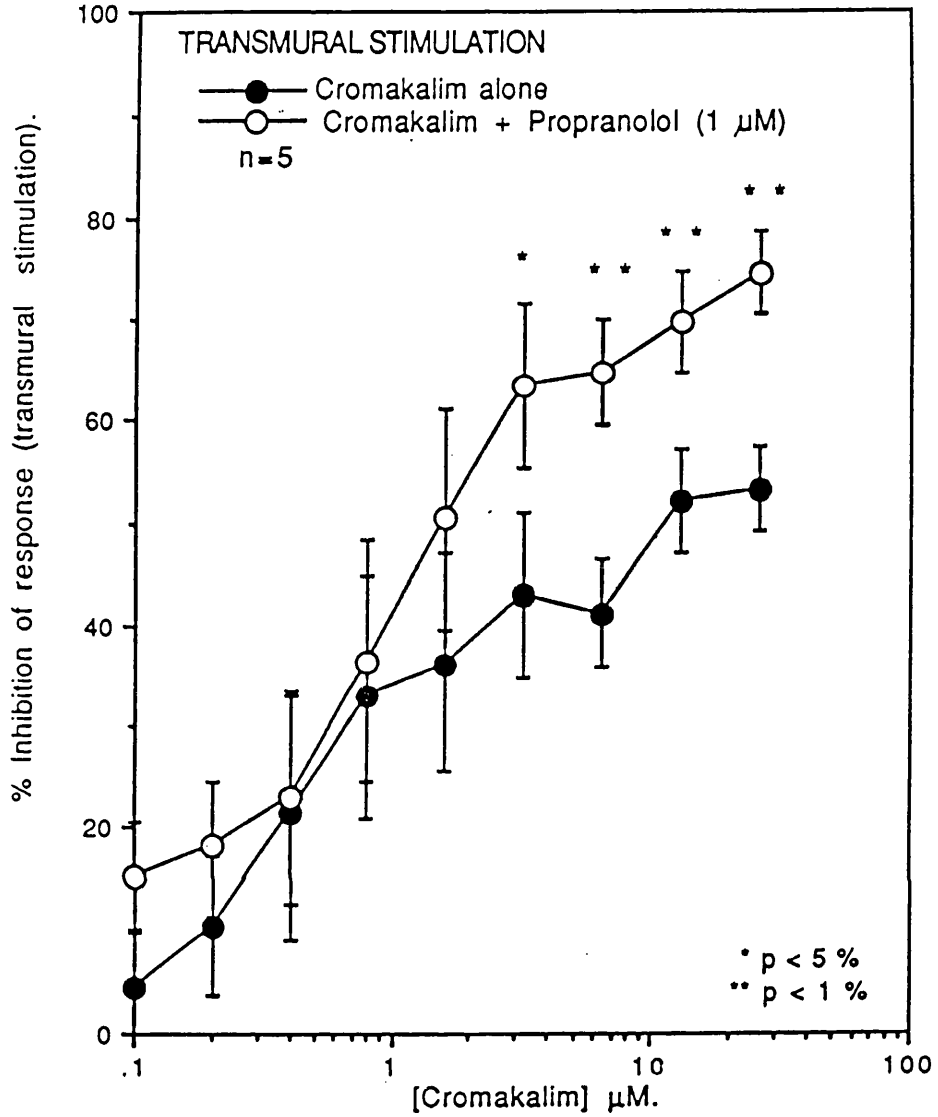


FIG 13b. A dose response curve was performed to cromakalim alone (●) . In parallel experiments, (-) propranolol was added 20 min before a cumulative dose response curve to cromakalim was performed (○) . (-) Propranolol potentiated the effect of cromakalim on responses to transmural stimulation compared to values for cromakalim alone (*** $p<0.005$, ** $p<0.01$). Values are mean ± sem % inhibition compared to control values before the application of cromakalim. n=5.

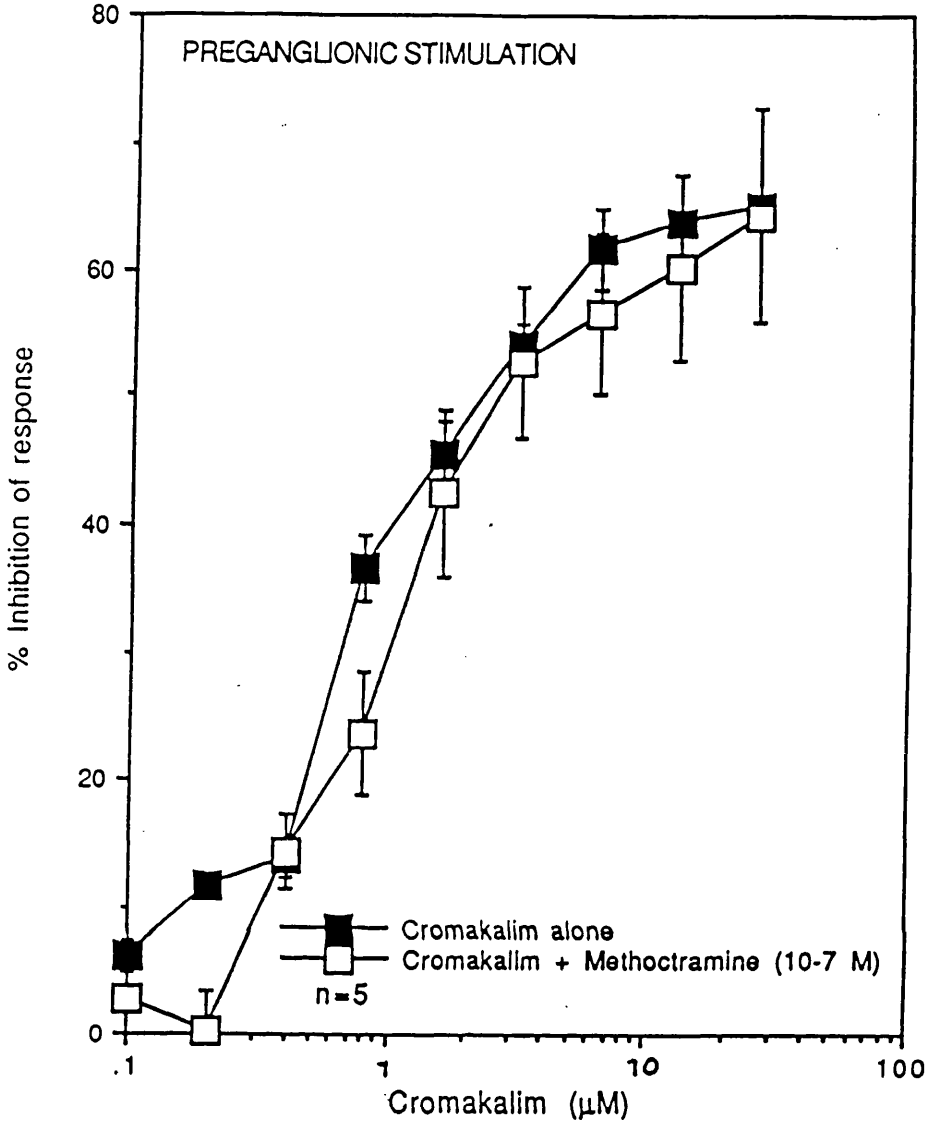


FIG 14a. A dose response curve to cromakalim alone was performed on preganglionic stimulation ■ . In parallel experiments Methoctramine (10^{-7} M) was added 15 min before a cumulative dose response curve to cromakalim was performed on preganglionic stimulation (□) . Values are mean ± sem % inhibition compared to control values before the application of cromakalim, (n=5). The presence of methoctramine did not affect the inhibition of responses to preganglionic vagal stimulation by cromakalim compared to values for cromakalim alone.

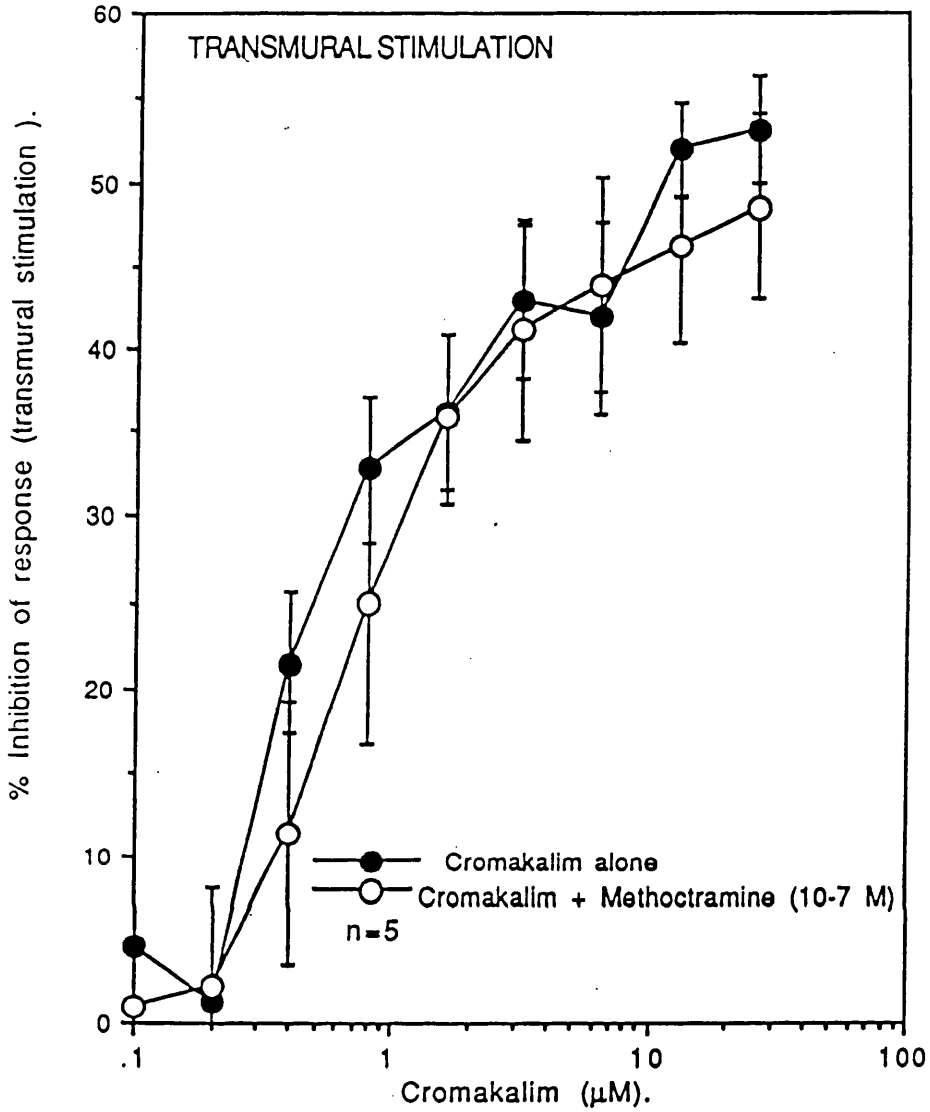


FIG 14b A dose response curve to cromakalim alone was performed on transmural stimulation (●). In parallel experiments methoctramine (10^{-7} M) was added 15 minutes before a cumulative dose response curve to cromakalim was performed on transmural stimulation (○) .Values are mean ± sem % inhibition with respect to control values before the application of cromakalim (n=5). The presence of methoctramine did not affect the inhibition of responses to transmural stimulation by cromakalim compared to values for cromakalim alone.

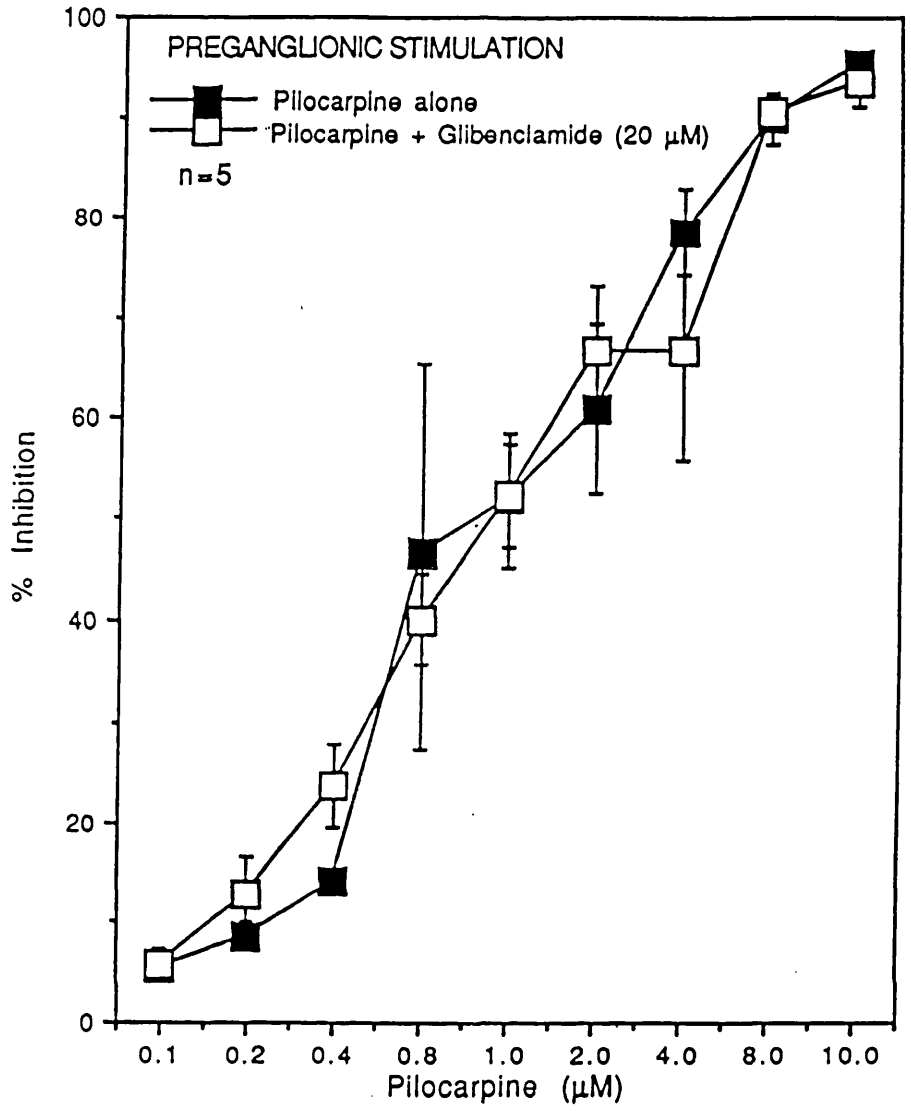


FIG 15a. A cumulative dose response curve was performed to pilocarpine alone on responses to preganglionic vagal stimulation (■). After a washout period the tissue was preincubated with glibenclamide (20 μM) and a cumulative dose response curve to pilocarpine in the presence of glibenclamide was obtained on responses to preganglionic vagal stimulation (□). The presence of glibenclamide did not affect the inhibition of responses to nerve stimulation by pilocarpine. Values represent mean ± sem % inhibition with repect to control values before the application of pilocarpine, (n=5).

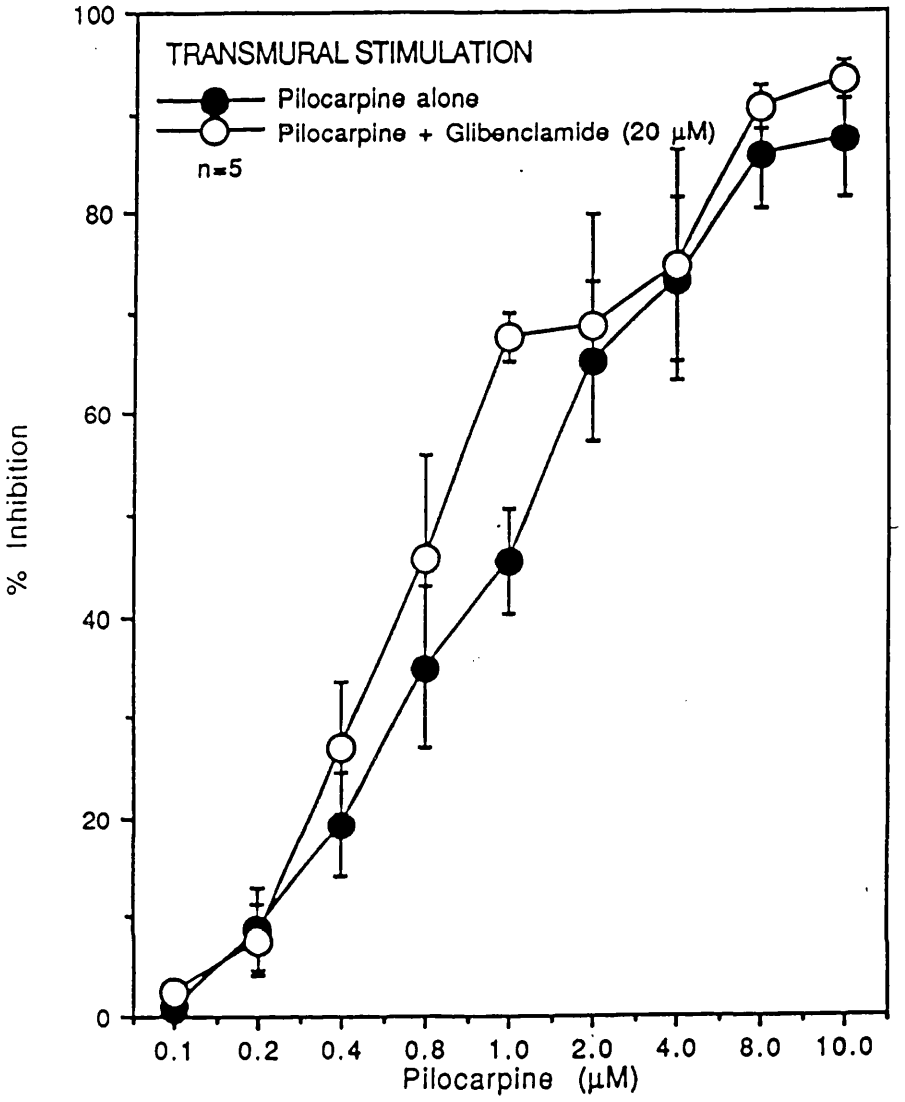


FIG 15b. A cumulative dose response curve was performed to pilocarpine alone on responses to transmural stimulation (●) . After a washout period, the tissue was preincubated with glibenclamide (20 μM) and a cumulative dose response curve to pilocarpine in the presence of glibenclamide was obtained on responses to transmural stimulation (○) . The presence of glibenclamide did not affect the inhibition of responses to nerve stimulation by pilocarpine. Values represent mean ± sem % inhibition with respect to control values before the application of pilocarpine.

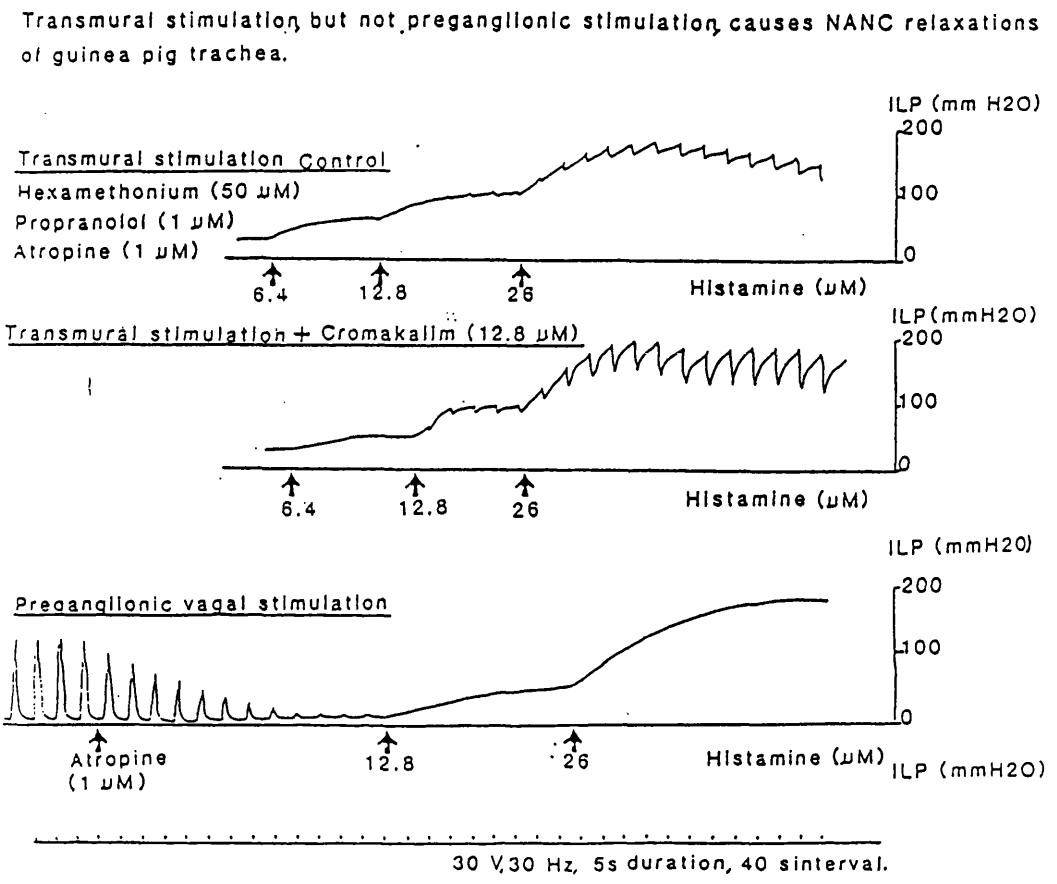


FIG 16a. NANCergic relaxations were elicited by transmural stimulation (30 V, 30 Hz, 0.2 ms, 5 s duration, 40 s interval in the presence of hexamethonium (50 μM), propranolol (1 μM) and atropine (1 μM) when tone was raised by histamine, upper trace. These NANCergic relaxations were potentiated by cromakalim (12.8 μM), middle trace. Preganglionic vagal stimulation in the presence of atropine and the spasmogen histamine did not elicit NANCergic relaxation (lower trace).

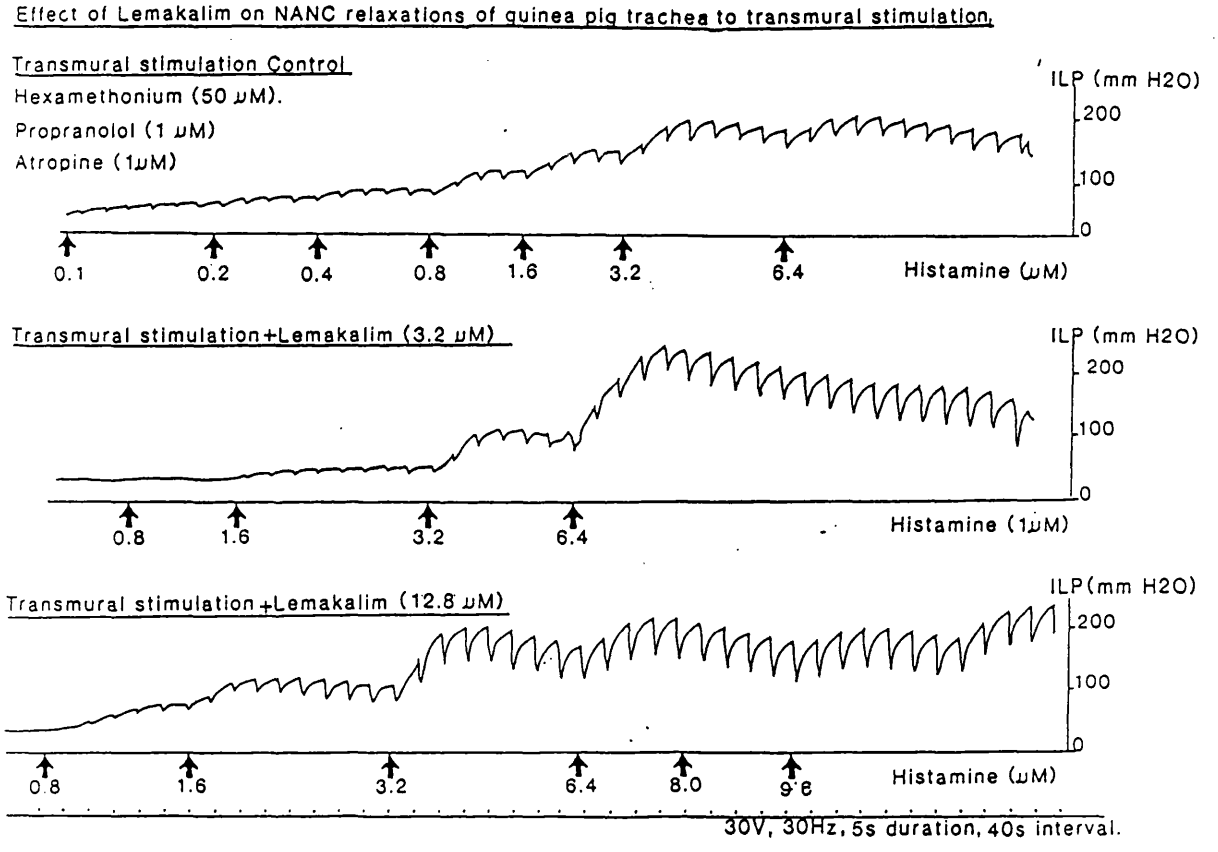


FIG 16b. Control NANCergic relaxations were established by transmural stimulation in the presence of, hexamethonium (50 μM), propranolol (1 μM) and atropine (1 μM) when tone was raised by histamine, upper trace. The NANCergic relaxations were potentiated by lemakalim (3.2 μM, middle trace) and (12.8 μM lower trace).

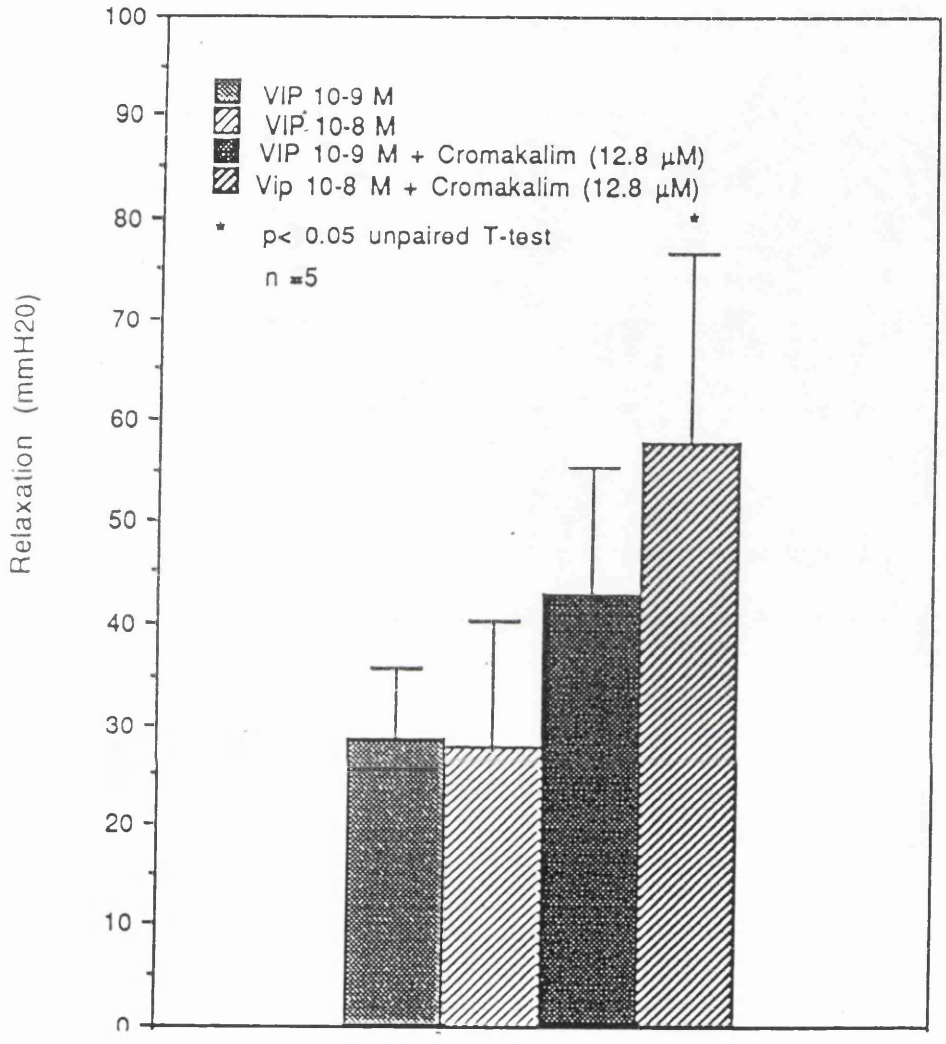


FIG 17a. Relaxations to exogenous VIP (10^{-9} M) ▩ and VIP (10^{-8} M) ▨ were established under control conditions after initially raising tone to 200 mmH_2O with histamine. After preincubation with cromakalim (12.8 μM) the responses to VIP (10^{-9} M) ▩ and VIP (10^{-8} M) ▨ were repeated. The response to the higher dose of VIP was potentiated by cromakalim * $p<0.05$. Responses are mean ± sem relaxation mmH_2O (n=5).

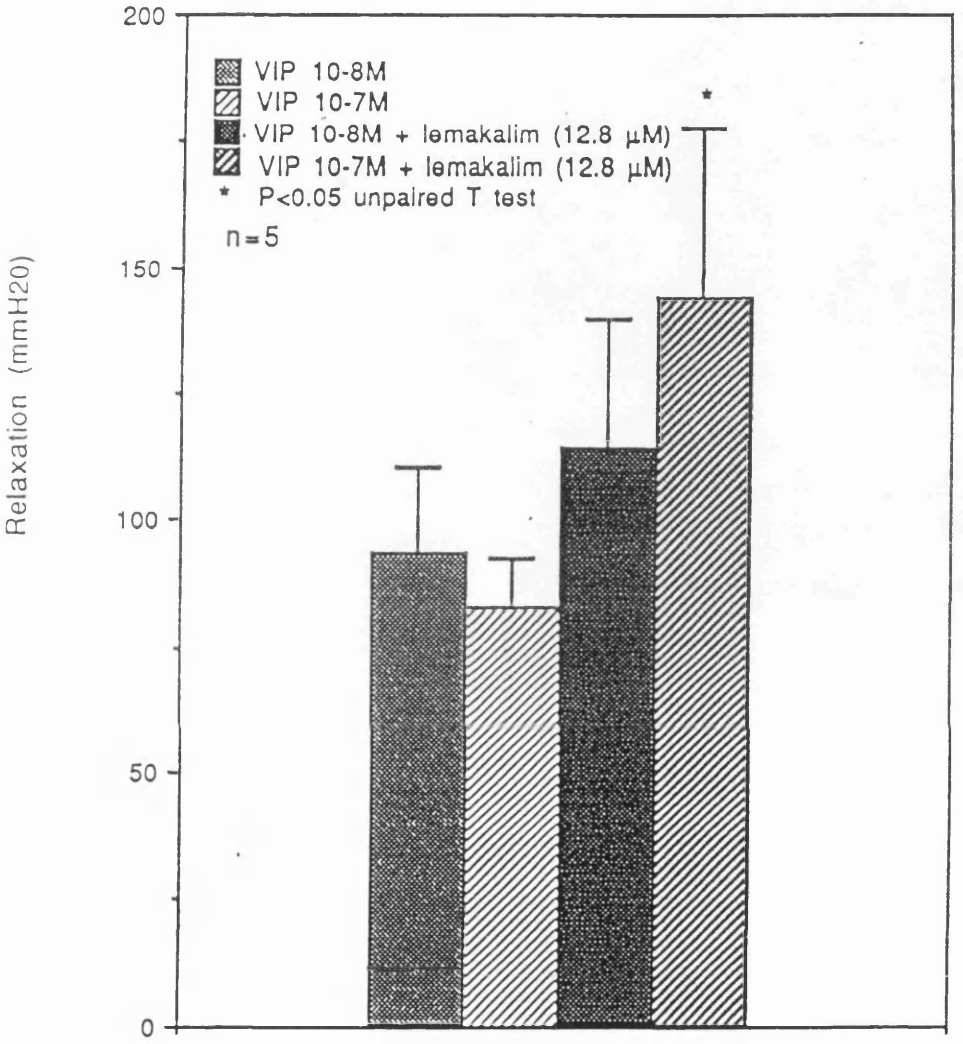


FIG 17b. Relaxations to exogenous VIP (10^{-8} M) and VIP (10^{-7} M) were established under control conditions after initially raising the tone to 200 mmH_2O with histamine. After preincubation with Lemakalim (12 μM) the responses to VIP (10^{-8} M) and VIP (10^{-7} M) were repeated. The response to the higher dose of VIP was potentiated by lemakalim * $p<0.05$. Responses are mean ± sem relaxation mmH_2O, (n=5).

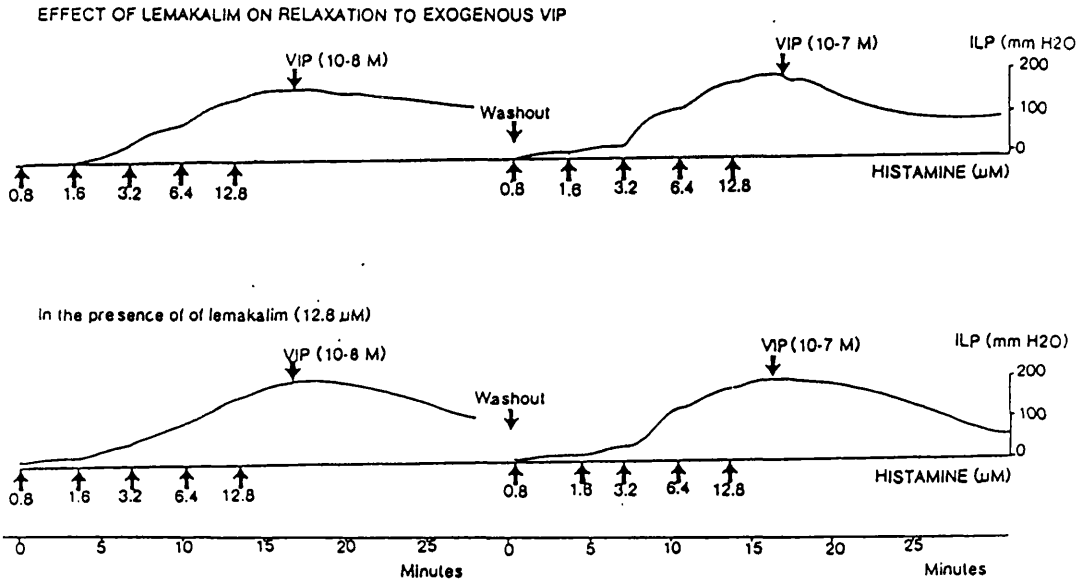


FIG 17c. A typical trace showing the relaxation of the trachea to exogenous VIP (10^{-8} M) and VIP (10^{-7} M). Responses to VIP (10^{-8} M) and then VIP (10^{-7} M) were repeated in the presence of Lemakalim (12.8 μM). The presence of lemakalim potentiated the relaxation to the higher dose of VIP. Hexamethonium (50 μM), propranolol (1 μM) and atropine (1 μM) were present throughout.

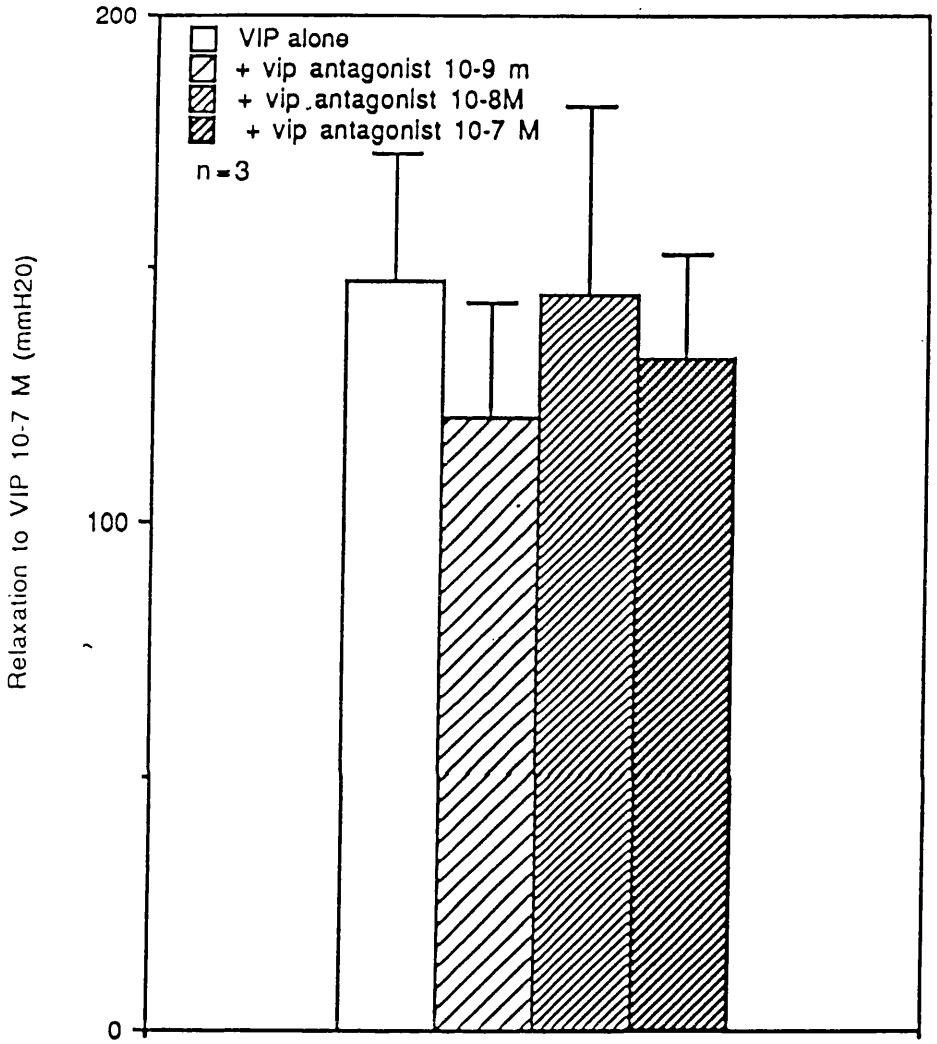


FIG 18. Relaxation to VIP (10^{-7} M) was obtained in the guinea-pig trachea □ . The presence of the VIP antagonist (dp chloro phe leu 17) VIP in the following concentrations (10^{-9} M ▨ ; 10^{-8} M ▨ and 10^{-7} M ▨) did not antagonise the relaxation of the trachea to VIP. Responses are mean relaxation ± sem (mm H_20) (n=3).

DISCUSSION

Part A. The effect of potassium channel opening drugs on pulmonary cholinergic nerves

The most important aim of the experiments in this thesis was to investigate the location and mechanism of the inhibitory action of the potassium channel opening drugs on contractions of the trachea elicited by stimulation of the cholinergic pulmonary nerves. It is important to note that in our preparation there was no endogenous tracheal tone since the vagus nerves were sectioned and indomethacin was present. Firstly we attempted to determine whether this inhibitory effect of cromakalim could be due to a postjunctional inhibition of the action of the transmitter, acetylcholine, which is released from pulmonary cholinergic nerves and contracts the trachealis muscle by acting on postjunctional muscarinic receptors on the tracheal smooth muscle. The potassium channel opening drug, cromakalim was shown to cause only a very slight inhibition of contractions of the guinea-pig trachea in vitro to exogenous acetylcholine (Allen et al, 1986; Hall & Maclagan, 1988). In vivo, cromakalim has been documented to inhibit bronchoconstriction to the spasmogens histamine and 5HT (Arch et al, 1988). The findings of the experiments described in this thesis show that in vitro each of the following potassium channel opening drugs, cromakalim, lemakalim and SDZPC0400 caused only a very slight nonsignificant rightward shift of the dose response curve to the spasmogens acetylcholine, histamine and U46619. It was decided that the potassium channel opening drugs do not inhibit the postjunctional actions of acetylcholine on tracheal smooth muscle.

Graded doses of cromakalim (0.1 to 26 μM) caused a dose related inhibition of contractions of the trachea to cholinergic nerve stimulation. Cromakalim caused a dose related inhibition of responses to transmural stimulation reaching a maximum of 53.3 ± 3.3 % inhibition at cromakalim (26 μM). Cromakalim had a greater effect on responses to preganglionic vagal stimulation reaching a maximim of 65.11 ± 11.1 % inhibition but complete blockade of responses to cholinergic stimulation was never obtained with cromakalim. This inhibitory action of cromakalim was due to an action located on the pulmonary parasympathetic cholinergic nerves since cromakalim had no effect on response of the

trachea to exogenous acetylcholine which contracted the trachea by acting on postjunctional muscarinic receptors. It seems probable that since responses to preganglionic vagal stimulation were reduced to a greater extent than responses to postganglionic transmural stimulation, cromakalim may act on postganglionic nerve terminals and may also have an additional action at the ganglia.

Other researchers have found that cromakalim does not inhibit acetylcholine postjunctionally but seems to have inhibitory actions on nerves. Cromakalim was found to cause more than 90 % inhibition of the excitatory nonadrenergic noncholinergic (eNANC) contractile response which was elicited by bilateral vagal stimulation in the presence of atropine and propranolol (Burka et al, 1991). The administration of glibenclamide reversed the inhibition of eNANC responses by cromakalim indicating a role for ATP dependant potassium channels. The invitro findings of Burka et al (1991) confirm the in vivo results of Ichinose and Barnes (1990) who found that cromakalim inhibited responses of the trachea to cholinergic vagal stimulation and also to eNANC stimulation The eNANC response to vagus nerve stimulation is due to the release of neuropeptides such as substance P (SP) and neurokinins from sensory nerve endings (Lundberg & Saria, 1987). The nomination of a peptidergic transmitter is derived from observations that the eNANC response is inhibited by an SP antagonist and is not present after depletion of tachykinins with capsacin (Lundberg et al, 1983). In the experiments of Ichinose and Barnes (1990), cromakalim had no effect on bronchoconstriction induced by exogenous SP suggesting that cromakalim inhibits release of the transmitter from sensory nerves rather than inhibiting its action on airway smooth muscle.

The converse situation is experienced with the potassium channel blocker 4 aminopyridine (4AP) which facilitates the release of the neurotransmiter acetylcholine. Since the relatively recent use of 4 aminopyridine as an anticurare agent its mechanism of action has been studied. 4 Aminopyridines have been shown to facilitate neurotransmission at synapses both excitatory and inhibitory in the brain, spinal cord, autonomic nerves and neuroeffector junctions. (Bowman, 1982).

Results obtained by us and others provide strong evidence that the inhibitory effect of cromakalim on cholinergic nerve stimulation is predominantly prejunctional and causes a reduction in neurotransmitter output. There are two ways in which potassium channel opening could modify electrical activity in the pulmonary cholinergic nerves. Opening of potassium channels would move the membrane potential closer to the equilibrium potential (E_k) causing hyperpolarisation and reduced firing frequency and hence reduced transmitter output (Cook, 1988). Additionally activation of potassium channels responsible for repolarisation may restrict the opening of voltage operated calcium channels, thus reducing the influx of calcium and the rise in intracellular calcium concentration necessary for neurotransmitter release (Allen et al, 1986).

It was reported by McCaig and DeJonkheere (1989) that cromakalim did not affect responses of the guinea-pig trachea to field stimulation. This observation does not agree with our results which show that cromakalim, and the other potassium channel openers investigated, inhibit responses of the trachea to transmural stimulation. We performed experiments to try to resolve this difference. We omitted our usual pretreatment with indomethacin (5 μM) and hexamethonium (50 μM), which McCaig & Dejonkheere did not use. In the absence of indomethacin and hexamethonium, the size of responses was smaller and more variable due to the development of prostaglandin-induced tone, but cromakalim still had a dose related inhibitory effect on responses to transmural stimulation. A new design of electrode was developed for field stimulation with electrodes placed either side of the tracheal tube since we hoped to reproduce the conditions of the experiments performed by McCaig & Dejonkheere. We found that cromakalim always produced a dose related inhibition of responses to postganglionic stimulation whether transmural or field stimulation was used. Unfortunately, it was not possible to offer an explanation for the difference in our results from those of McCaig and Dejonkheere. Recently, results that support our findings that cromakalim inhibits responses to transmural stimulation have been published by another laboratory (Burka, et al, 1991).

The (-) enantiomer of cromakalim, lemakalim is more than twice as potent as cromakalim in inhibiting contractions of the trachea to

preganglionic vagal stimulation and transmural stimulation at all doses. Almost complete blockade of responses to cholinergic stimulation was acheived at lemakalim (1.6 μM), whereas cromakalim never caused inhibition in excess of 65% on preganglionic vagal stimulation and complete blockade was unattainable. This is a surprising result as one would expect the active enantiomer to be exactly twice as potent as the racemic compound. SDZPC0400 was of comparable potency to cromakalim and inhibited responses to both preganglionic vagal and transmural stimulation.

In the presence of (-) propranolol (1 μM) the dose response curve for cromakalim on responses to transmural and preganglionic vagal stimulation was significantly shifted to the left. It is not clear why the presence of (-) propranolol potentiated the inhibitory effect of cromakalim on responses to cholinergic stimulation but this effect is not due to due to differences between the size of control responses between the control and the (-) propranolol treated group. It is possible that in addition to ß adrenoceptor blockade, (-) propranolol could have a blocking effect on potassium channels. This is supported by previous reports that propranolol competitively inhibited both potassium stimulation of cardiac Na^+/K^+ ATPase and $^{86}Rb^+$influx into trout erythrocytes (Nowell et al, 1987) and guinea-pig left ventricular slices (Nowell & Tiku, 1989).

Glibenclamide is an antidiabetic sulphonylurea drug which selectively blocks the ATP-operated potassium channels (Malaisse et al, 1983; Gylfe et al, 1984). Glibenclamide antagonised the inhibitory effect of cromakalim on contractions of the trachea to both preganglionic vagal and transmural stimulation (Cooper & Maclagan, 1990). Subsequently we found that glibenclamide significantly antagonised the inhibitory effect of both lemakalim and SDZPC0400 on responses to preganglionic vagal and transmural stimulation. It appears that ATP sensitive potassium channels represent a significant proportion of the channels opened by cromakalim, lemakalim and SDZPC0400 in the pulmonary cholinergic nerves of the guinea-pig.

Cromakalim is a racemic mixture of (+) and (-) isomers; the (+) isomer is known as BRL38226 and the (-) isomer is lemakalim (BRL38227). It has been suggested that these drugs have a stereospecific mechanism

of action. As described for their antihypertensive activity, the vasorelaxant properties of cromakalim reside primarily in the (-) enantiomer (Buckingham et al 1986; Bray et al, 1987). We found that the (+) isomer BRL38226 caused only a very slight inhibition of responses of the trachea to preganglionic vagal stimulation and transmural stimulation. From this small inhibition of BRL38226 on pulmonary cholinergic nerves it seems that BRL38226 is able to open neuronal potassium channels but is much less potent than cromakalim and may be acting as a partial agonist. Cumulative doses of lemakalim alone inhibit contraction of the trachea to cholinergic stimulation. However, in the presence of the (+) isomer BRL38226, the inhibitory effect of lemakalim on responses to both preganglionic vagal and transmural stimulation was significantly reduced. This was an unexpected finding. It is possible that the (+) isomer antagonises the actions of the potassium channel opener lemakalim by blocking the channels opened by lemakalim in the guinea-pig pulmonary nerves.

Postganglionic parasympathetic nerve endings are an important site for modulation of acetylcholine release via neuronal muscarinic receptors. Presynaptic muscarinic autoreceptors which inhibit release of acetylcholine from cholinergic nerves have been identified in the airways (Fryer & Maclagan, 1984). Muscarinic agonists such as acetylcholine and pilocarpine reduce acetylcholine release via activation of these prejunctional receptors. The use of M_2 muscarinic antagonists which potentiate vagal stimulation induced bronchoconstriction and inhibit the inhibitory effect of muscarinic agonists on cholinergic stimulation have led to the theory that these autoreceptors are of the M_2 subtype (Fryer & Maclagan, 1984; Watson et al, 1989). In the heart, the inhibitory action of acetylcholine on conductivity in the SA node is due to the opening of potassium channels linked to muscarinic receptors, (Sakman et al, 1983; Paffinger et al, 1985). However, experiments that we have performed to test for a possible link between prejunctional autoreceptors and the potassium channel opened by cromakalim and lemakalim gave only negative results. Inhibition by cromakalim of contractions of the trachea to cholinergic nerve stimulation was unaffected by the presence of the M_2 muscarinic antagonist, methoctramine.

The potassium channel blocker, glibenclamide was used as an investigative tool. We have found that glibenclamide antagonises the inhibitory action of the potassium channel opening drugs cromakalim, lemakalim and SDZPC0400 on pulmonary cholinergic nerves by blocking the ATP sensitive potassium channels. Thus glibenclamide can be used as a tool to investigate the role of this type of potassium channel in the muscarinic autoreceptor mechanism in pulmonary nerves. We found that the inhibitory effect of pilocarpine, an M_2 muscarinic agonist, on cholinergic nerve stimulation induced contractions was unaffected by the presence of glibenclamide. Thus it seems that the inhibitory action of pilocarpine on contractions of the trachea does not involve opening of ATP sensitive potassium channels. We concluded that the M_2 autoreceptors on pulmonary cholinergic nerves are not associated with the the type of potassium channels which are opened by cromakalim and blocked by glibenclamide.

To summarise, it seems that the potassium channel opening drugs inhibit contractions of the guinea-pig trachea to pulmonary cholinergic nerve stimulation via an action located at the ganglia and also at the final nerve terminals since inhibition of responses to cromakalim is greater on responses to preganglionic vagal stimulation than on responses to postganglionic transmural stimulation.

The use of glibenclamide indicates that a significant proportion of the potassium channels opened by cromakalim, lemakalim and SDZPC0400 are of the ATP sensitive class.

Part B. Nonadrenergic Noncholinergic (NANC) innervation and function in the lung.

When tracheal tone was raised by histamine, NANCergic relaxations of the trachea were elicited by transmural stimulation in the presence of the muscarinic antagonist atropine and during ß adrenoceptor blockade with propranolol. This is in agreement with the earlier in vitro work of Coburn & Tomita, (1973) and Coleman & Levy, (1974). The NANC inhibitory response was abolished by tetrodotoxin suggesting that it is neuronal in origin.

In vivo experiments have demonstrated NANCergic relaxations of the trachea to vagal stimulation (Chesrown et al, 1980; Diamond et al 1983).

In contrast, in vitro we found that preganglionic vagal stimulation in the presence of atropine and propranolol did not elicit a NANCergic relaxation of the trachea (Cooper & Maclagan, 1990). However NANCergic relaxations were elicited to transmural stimulation using the above pretreatments, indicating that NANC fibres enter the trachea below the point of preganglionic vagal stimulation.

Cromakalim and lemakalim facilitated NANC mediated relaxations of the guinea-pig trachea, approximately doubling the size of relaxant responses. This effect of the potassium channel opening drugs may be due to facilitation of transmitter release from the NANCergic nerves or may be due to a potentiation of the effect of the inhibitory transmitter on the trachealis muscle. However it is impossible to precisely predict the site of action of cromakalim and lemakalim on NANCergic responses since we do not yet know the identity of the the NANC transmitter. It appears that VIP is a strong candidate for the neurotransmitter responsible for the NANCergic relaxation of airways, (Dey et al, 1981; Diamomd et al, 1983). We found that relaxations of the guinea-pig trachea to exogenous VIP were similarly significantly potentiated by cromakalim and lemakalim leading to about 50 % increase in the size of relaxation to VIP. It is also interesting that VIP functions by opening ATP operated potassium channels in the smooth muscle. It has been reported that the smooth muscle hyperpolarisation to VIP in the rabbit middle cerebral artery is reversed by glibenclamide implying that VIP opens ATP sensitive potassium channels, (Standen et al, 1989). The mechanism underlying the stimulation of NANC inhibitory nerves by potassium channel opening drugs is however complicated by the important possibility that nitric oxide may contribute to NANCergic transmission since it was found that L-nitro arginine, the inhibitor of nitric oxide synthase, reduces the size of NANC relaxations of the trachea (Tucker et al, 1990; Li & Rand, 1991). In view of these uncertainties about the identity if the NANCergic transmitter, we can not be sure whether the potentiating effects of potassium channel opening drugs on NANC inhibitory responses are due to a pre or postjunctional mechanism.

To conclude, the potassium channel opening drugs cromakalim and lemakalim facilitate NANC mediated relaxation of the isolated guinea-pig trachea and also potentiate relaxation of the trachea to exogenous VIP

which is a putative NANC transmitter. In the isolated guinea-pig trachea, opening of potassium channels inhibits cholinergic neurotransmission but facilitates NANC mediated relaxations. The bronchodilator effect of cromakalim and lemakalim in vivo is probably due to a combination of inhibition of cholinergic bronchoconstriction, a faciliatation of NANC mediated relaxation and a direct relaxant effect on airway smooth muscle. These properties suggest that the potassium channel opening drugs may have potential for use in asthma therapy.

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