Glycine receptors in cultured chick sympathetic neurons are excitatory and trigger neurotransmitter release

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1. Total RNA isolated from embryonic chick paravertebral sympathetic ganglia was used in a reverse transcription-polymerase chain reaction (RT-PCR) assay with a pair of degenerate oligonucleotide primers deduced from conserved regions of mammalian glycine receptor α-subunits. Three classes of cDNA were identified which encode portions of the chicken homologues of the mammalian glycine receptor α1, α2 and α3 subunits.

2. The presence of functional glycine receptors was investigated in the whole-cell configuration of the patch-clamp technique in neurons dissociated from the ganglia and kept in culture for 7–8 days. In cells voltage clamped to −70 mV, glycine consistently induced inward currents in a concentration-dependent manner and elicited half-maximal peak current amplitudes at 43 μM.

3. The steady-state current–voltage relation for glycine-induced currents was linear between +80 and −60 mV, but showed outward rectification at more hyperpolarized potentials. Reversal potentials of these currents shifted with changes in intracellular chloride concentrations and matched the calculated Nernst potentials for chloride.

4. β-Alanine and taurine were significantly less potent than glycine in triggering inward currents, with half-maximal responses at 79 and 86 μM, respectively. At maximally active concentrations, β-alanine-evoked currents were identical in amplitude to those induced by glycine. Taurine-evoked currents, in contrast, never reached the same amplitude as glycine-induced currents.

5. The classical glycine receptor antagonist strychnine reversibly reduced glycine-induced currents, with half-maximal inhibition occurring at 62 nM. Two more recently characterized glycine receptor antagonists, isonipecotic acid (half-maximal inhibition at 2 mM) and 7-trifluoromethyl-4-hydroxyquinoline-3-carboxylic acid (half-maximal inhibition at 67 μM), also blocked glycine-evoked currents in a reversible manner. The chloride channel blocker picrotoxin reduced glycine-evoked currents, with half-maximal effects at 348 μM. Inhibition by the glycine receptor channel blocker cyanotriphenylborate was half-maximal at 4 μM.

6. Apart from evoking inward currents, glycine occasionally triggered short ($<$ 100 ms) spike-like currents which were abolished by hexamethonium and thus reflected synaptic release of endogenous acetylcholine. In addition, glycine caused Ca^{2+}-dependent and tetrodotoxin-sensitive tritium overflow from neurons previously labelled with [^{3}H]noradrenaline. This stimulatory action of glycine was reduced in the presence of strychnine and after treatment with the chloride uptake inhibitor furosemide (frusemide).

7. In 65% of neurons loaded with the Ca^{2+} indicator fura-2 acetoxymethyl ester, glycine increased the ratio of the fluorescence signal obtained with excitation wavelengths of 340 and 380 nm, respectively, which indicates a rise in intracellular Ca^{2+} concentration.

8. The results show that sympathetic neurons contain transcripts for different glycine receptor α-subunits and carry functional heteromeric glycine receptors which depolarize the majority of neurons to trigger transmitter release.

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Glycine and γ-aminobutyric acid (GABA) represent the predominating inhibitory neurotransmitters in the central nervous system, with glycine being of major importance in the spinal cord, whereas GABA prevails in the brain (Aprison, 1990). In general, these two amino acids exert their inhibitory actions by binding to ligand-gated chloride channels with ensuing anion influx and hyperpolarization of postsynaptic neurons. More recently, however, glycine and GABA have also been reported to cause neuronal depolarization (Reichling, Kyrozis, Wang & MacDermott, 1994; Owens, Boyce, Davis & Kriegstein, 1996), particularly in developing central neurons (Cherubini, Gaiarsa & Ben-Ari, 1991; Wang, Reichling, Kyrozis & MacDermott, 1994). This effect is most commonly related to high intraneuronal chloride concentrations (Reichling *et al.* 1994; Owens *et al.* 1996). In the peripheral nervous system, roles for glycine or GABA as neurotransmitters are less well defined.

Receptors for glycine are characterized by nanomolar affinities for strychnine (Young & Snyder, 1973) and are widely distributed in the central nervous system (for review see Betz, 1991). There, these receptors are composed of two types of integral, membrane-spanning subunits with molecular weights of 48 kDa (α) and 58 kDa (β). The α-subunits contain the ligand binding sites of glycine receptors. Currently, at least four different mammalian α-subunits (α1 to α4) have been characterized by molecular cloning (Matzenbach *et al.* 1994), and alternative splicing of α-subunits may result in further heterogeneity (for review see Kuhse, Betz & Kirsch, 1995). Glycine receptor α-subunit transcripts are differentially expressed in various areas of the central nervous system, whereas the β-subunit mRNA is abundant throughout the brain and spinal cord (Betz, 1991). Upon heterologous expression, α- and β-subunits form hetero-oligomers with a stoichiometry of $3\alpha:2\beta$ (Kuhse, Laube, Magalei & Betz, 1993), but α-subunits can also form homomeric receptors (Schmieden, Grenningloh, Schofield & Betz, 1989). Despite detailed knowledge about glycine receptors in heterologous expression systems, the composition of native glycine receptors in the central nervous system still remains unknown.

Very little is known about glycine receptors in the peripheral nervous system. There is only one recent report which demonstrated glycine-induced currents in cultured neurons of embryonic chick ciliary ganglia (Zhang & Berg, 1995). To unravel whether glycine receptors are restricted to these neurons or whether they are more widespread in the peripheral nervous system, we searched for glycine receptors in sympathetic ganglia of the same species. Embryonic chick sympathetic neurons *in vitro* constitute a frequently used model system to investigate neuronal differentiation (e.g. Ernsberger & Rohrer, 1996) as well as the function of neurotransmitter receptors (Boehm & Huck, 1997). Our results show that these neurons contain transcripts for at least three different α-subunits as well as functional strychnine-sensitive glycine receptors. Furthermore, these receptors are revealed to be excitatory rather than inhibitory.

METHODS

Reverse transcription-polymerase chain reaction (RT-PCR) amplification

Total RNA was isolated from paravertebral sympathetic ganglia, dissected from 14-day-old chick embryos killed by decapitation, using RNAzol B (AGS, Heidelberg, Germany), treated with RQ1 RNAse-free DNAse (Promega, Mannheim, Germany), and first-strand cDNA was synthesized using random nonamer primers (Stratagene, Heidelberg, Germany) and moloney murine leukaemia virus reverse transcriptase (Promega). Partial cDNAs encoding chicken glycine receptor α-subunits were amplified using two degenerate oligonucleotide primers: DGA1, 5′-TAC**GTCGAC**GCXAT(ATC)GA(TC)AT(ATC)TGGATG-3′ (where X = G, A, T and C), which is based on the DNA sequences that encode a region spanning the start of the third membrane-spanning domain [YVKAIDIWM] and DGA2, 5′-GTA**GAATTC**CCA(GA)TA(GA)AAXAT(GA)TT(GA)AA-3′, which is based on the DNA sequences that encode part of the fourth membrane-spanning domain [FN(I/M)FYW(V/I)(T/I)Y] of mammalian glycine receptor α-subunits (Matzenbach *et al.* 1994). Amplification was for 40 cycles of 94 °C for 1 min, 50 °C for 1 min and 72 °C for 1 min. Products were cloned into pBluescript SK- (Stratagene), taking advantage of restriction endonuclease recognition sites (in bold) incorporated into the PCR primers, and sequenced.

Cell culture

Chick embryos (14 days old) were killed by decapitation, and lumbosacral paravertebral sympathetic ganglia were dissected as previously described in more detail (Boehm *et al.* 1991; von Holst *et al.* 1995). Cells were resuspended in either Dulbecco's modified Eagle's medium (Gibco BRL) or Ham's F-14 Medium (Gibco BRL) containing 25000 IU l^{-1} penicillin and 25 mg l^{-1} streptomycin (Gibco BRL), 10 μg l^{-1} nerve growth factor (prepared according to Suda *et al.* 1978, or purchased from Gibco BRL), 5% (v/v) fetal calf serum and 10% (v/v) horse serum and were plated on polystyrol discs (diameter 5 mm) coated with rat tail collagen (Biomedical Technologies, Stoughton, MA, USA) for superfusion experiments, on glass coverslips coated with polyornithine (Sigma) and laminin (Gibco BRL) for fura-2 imaging, and on 35 mm culture dishes coated again with polyornithine and laminin for electrophysiological experiments. Cultures were kept at 37 °C in a humidified 5% CO_2 atmosphere, and two thirds of the medium were exchanged every 3 days.

Electrophysiological experiments

Experiments were performed at room temperature (20–24 °C) on the somata of neurons after 7–8 days *in vitro*, using the whole-cell mode of the patch-clamp technique (Hamill, Marty, Neher, Sakmann & Sigworth, 1981) as described previously (Boehm & Betz, 1997). The internal (pipette) solution contained (mM): CsCl, 140; $CaCl_2$, 1·59; EGTA, 10; Hepes, 10; adjusted to pH 7·3 with NaOH. In order to change intracellular chloride concentrations, 120 mM CsCl was replaced by iso-osmotic concentrations of sodium isethionate. The bathing (extracellular) solution contained (mM): NaCl, 140; KCl, 6·0; $CaCl_2$, 2·0; $MgCl_2$, 2·0; glucose, 20; Hepes, 10; adjusted to pH 7·4 with NaOH.

Glycine and all other drugs were applied via a DAD-12 drug application device (Adams and List, Westbury, NY, USA). This superfusion system delivers buffers from twelve reservoirs under pressure (200–400 mm H_2O) via a capillary with an inner diameter of about 100 μm and permits a complete exchange of solutions surrounding the cells under investigation within less than 100 ms. Currents were induced every 20 s by the application of glycine and were quantified by measuring peak current amplitudes. Glycine-

induced currents in the presence of various antagonists were compared with control currents recorded before and after the application of antagonists. Unless stated otherwise, antagonists were always applied before glycine.

[^{3}H]Noradrenaline uptake and superfusion experiments

The methods for superfusion experiments with cultured chick sympathetic neurons have previously been described in detail (Boehm, Huck, Drobny & Singer, 1991). After 7–8 days *in vitro*, the cultures were incubated in 0·03 μM [^{3}H]noradrenaline in culture medium containing 1 mM ascorbic acid for 60 min at a temperature of 36 °C. Thereafter, culture discs were transferred to small chambers and superfused with a buffer containing (mM): NaCl, 120; KCl, 6·0; $CaCl_2$, 2·0; $MgCl_2$, 2·0; glucose, 20; Hepes, 10; fumaric acid, 0·5; sodium pyruvate, 5·0; ascorbic acid, 0·57; adjusted to pH 7·4 with NaOH. Superfusion was performed at a temperature of 25 °C and at a rate of 1·0 ml min^{-1}. After a 60 min washout period, 4 min fractions of superfusate were collected. Glycine was included in the superfusion medium from 72 to 76 min, and electrical stimuli (24 monophasic rectangular pulses (0·5 ms) at 0·1 Hz, 50 V cm^{-1}, 50 mA) were applied from 92 to 96 min of superfusion. Modulatory agents (tetrodotoxin, $CdCl_2$, strychnine and furosemide) were present in the buffer from 50 min of superfusion (i.e. 10 min before the beginning of sample collection) and were kept at constant concentrations until the end of experiments. Then, the residual radioactivity was extracted from the cultures by immersion of the discs in 2% (v/v) perchloric acid, followed by sonication. Radioactivity in extracts and collected fractions was determined by liquid scintillation counting.

Spontaneous tritium outflow per 4 min fraction represents the amount of radioactivity in a 4 min superfusate fraction given as a percentage of the radioactivity in the cells at the beginning of the respective collection period. Stimulation-evoked overflow was calculated as the difference between total outflow during and after stimulation and the estimated basal outflow, which was assumed to decline linearly from the sample preceding stimulation to that 8–12 min after commencement of stimulation. Glycine-evoked overflow was expressed as a percentage of the fractional basal outflow preceding the application of the amino acid. Effects of modulatory agents on glycine- and electrically induced overflow were calculated as a percentage of the respective overflow in their absence (% of control).

Fura-2 imaging

Neuronal cell cultures on glass coverslips were incubated in culture medium containing 2% (w/v) bovine serum albumin (instead of serum) and 5 μM fura-2 acetoxymethyl ester (fura-2 AM) for 30 min at 36 °C in 5% CO_2. Thereafter, coverslips were transferred to a coverslip chamber (Adams and List), which was placed on an inverted microscope (Nikon Diaphot 300), and the cultures were washed with and incubated in the same buffer as used for superfusion experiments (see above). Drugs were applied via a gravity-driven six-barrel needle device capped by a glass capillary with a tip diameter of about 200 μm. This tip was placed in close proximity ($<$ 300 μm) to the cells under investigation in order to permit a complete exchange of the solutions surrounding these cells within about 1 s.

Changes in intracellular Ca^{2+} concentration were determined in single neurons by the two-wavelength method described by Grynkiewicz, Poenie & Tsien (1985) with excitation at 340 and 380 nm, and emission at 500 nm, where increases in the ratio of the fluorescence signal obtained with excitation at 340 and 380 nm (F_{340}/F_{380}), respectively, reflect rises in the Ca^{2+} concentration. Excitation was performed with light from a 100 W xenon lamp (Nikon), which was directed via appropriate excitation filters, a dichromatic mirror and a Nikon Fluor ×100/1·3 oil-immersion objective to the sample. Images of fluorescence signals were registered via an intensified CCD camera (Photonic Sciences, East Sussex, UK). Positioning of the excitation filters in a filterwheel with a stepping motor and registration of images once in 5 s was controlled by the QuantiCell 700 software (version 1.7; Applied Imaging, Sunderland, UK). The ratio F_{340}/F_{380} was registered on-line and was subsequently averaged (off-line) over the entire area of single neuronal somata.

Statistics

All data are given as arithmetic means ± S.E.M. and n is the number of cell culture discs in superfusion experiments and the number of single cells in electrophysiological and fura-2 imaging experiments. Concentration–response curves were fitted to experimentally obtained data points by using the ALLFIT program (DeLean, Munson & Rodbard, 1978). This program determines qualities of fitted results and significances of differences between single concentration–response curves by simultaneous fitting with shared parameters and subsequent calculation of the F statistic on the resulting 'extra sum of squares' (DeLean *et al.* 1978). Significance of differences between single data points was evaluated by Student's unpaired t test.

Materials

(−)-[^{3}H]Noradrenaline (59·7 Ci mmol^{-1}) was obtained from NEN (Dreieich, Germany); glycine, β-alanine, taurine, strychnine, furosemide (frusemide) from Sigma; 7-trifluoromethyl-4-hydroxy-quinoline-3-carboxylic acid (7TFQA) and isonipecotic acid from Aldrich; tetrodotoxin (TTX) from Latoxan (Rosans, France); cyanotriphenylborate (CTB) from Johnson Matthey Alfa Products (Karlsruhe, Germany); and fura-2 AM from Molecular Probes.

RESULTS

Amplification of chicken glycine receptor subunit partial cDNAs

To investigate whether glycine receptor subunit genes are expressed in embryonic sympathetic ganglia we performed a PCR-based survey, using a pair of degenerate oligonucleotide primers that are predicted to amplify cDNA sequences encoding the large presumed intracellular loop of glycine receptor α-subunits. Using these primers in the PCR, a cDNA product of ~400 bp could be readily amplified from 14-day-old chick embryo paravertebral sympathetic ganglia first-strand cDNA (Fig. 1*A*). This product did not derive from contaminating genomic DNA, since when reverse transcriptase was omitted from the cDNA synthesis reaction, no product was observed (Fig. 1*A*). Cloning of the sympathetic ganglion PCR product and subsequent DNA sequencing resulted in the identification of multiple clones for three different cDNAs that encode parts of polypeptides (named chick α1, α2 and α3; Fig. 1*B*) which show high sequence similarity (94, 91 and 85% identity, respectively) to the corresponding portions of the rat glycine receptor α1, α2 and α3 subunits (Fig. 1*C*).

Glycine-induced currents in chick sympathetic neurons

To investigate whether the presence of α-subunit transcripts was accompanied by the expression of functional glycine

receptors, whole-cell patch-clamp recordings were performed on chick sympathetic neurons after 7–8 days *in vitro*. The intracellular solution routinely contained 143 mM chloride, whereas extracellular chloride amounted to 154 mM. Under these ionic conditions, at a holding potential of −70 mV, the application of glycine at concentrations from 10 μM to 1 mM elicited inward currents of increasing amplitudes: in an initial set of nine neurons, peak amplitudes of glycine-evoked inward currents were half-maximal at 46·4 ± 12·3 μM and reached a maximum of 469 ± 44 pA. The Hill coefficient for glycine derived from this concentration–response curve was 1·8 + 0·7 (not shown).

Steady-state current–voltage (*I–V*) curves were obtained by measuring peak currents induced by 300 μM glycine at membrane potentials between −100 and +80 mV (Fig. 2). With 143 mM intracellular chloride (mainly CsCl), the *I–V* curve was linear between −60 and +80 mV, but showed

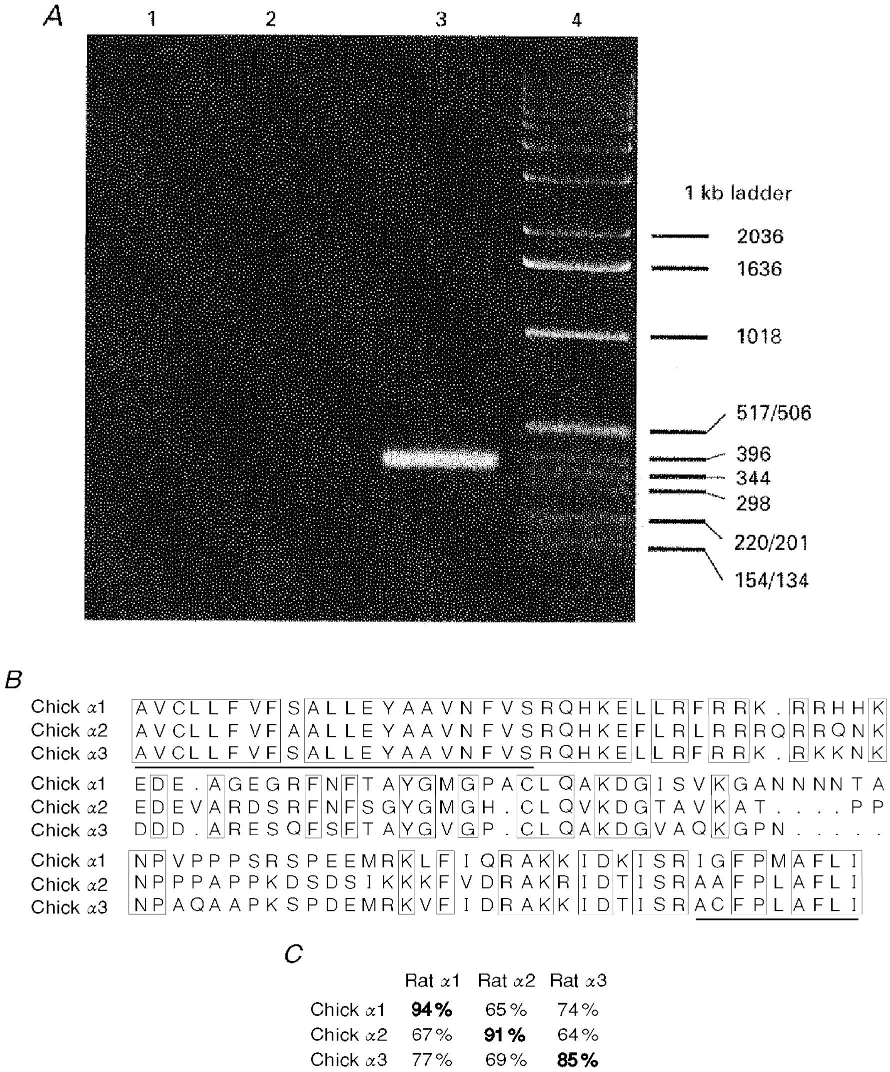

Figure 1. Isolation of chicken glycine receptor α-subunit partial cDNAs from embryonic chick sympathetic ganglia

A, agarose gel electrophoretic analysis of PCR products amplified using the degenerate primers DGA1 and DGA2. Lane 1, no DNA control; lane 2, a control in which reverse transcriptase was omitted from the first-strand cDNA synthesis reaction; lane 3, chick embryonic day 14 sympathetic ganglion first-strand cDNA; lane 4, molecular weight marker (1 kb ladder, Gibco BRL). *B*, alignment of the deduced partial sequences of the chicken glycine receptor α1, α2 and α3 subunits generated with the aid of the PILEUP programme. Bars below the sequences represent parts of the third and fourth membrane-spanning segments; positions at which all three of the sequences are identical are boxed. *C*, similarity of avian and rat glycine receptor α-subunit sequences. To determine percentage identities, the partial amino-acid sequences of the chicken glycine receptor subunits were aligned with the corresponding portions of the rat glycine receptor α1, α2 and α3 subunits (see Matzenbach *et al.* 1994, and references cited therein) using the programme GAP of the Wisconsin Software Package (Genetics Computer Group, Wisconsin, USA).

outward rectification at more hyperpolarized potentials (Fig. 2*B*). Similar outward rectification has previously been reported for glycine-evoked currents in central neurons (e.g. Akaike & Kaneda, 1989). The reversal potential was $-4{\cdot}8 \pm 4{\cdot}6$ mV ($n = 4$), which was close to the calculated Nernst equilibrium potential for chloride (−1·8 mV). Replacement of 120 mM CsCl by sodium isethionate (i.e. 23 mM intracellular Cl^-) shifted the reversal potential to $-48{\cdot}3 \pm 2{\cdot}7$ mV ($n = 4$), which again matched the calculated equilibrium potential for chloride (−47·9 mV).

Pharmacology of glycine receptors in chick sympathetic neurons

Peak amplitudes and activation kinetics of glycine-induced currents were concentration dependent with maximal amplitudes and shortest time-to-peak intervals at 1 mM glycine (Fig. 3*A*); currents induced by glycine reached half-maximal peak amplitudes at $43{\cdot}4 \pm 4{\cdot}0$ μM (Fig. 3*D*). Apart from glycine itself, the most potent agonists at native glycine receptors in central neurons are the amino acids β-alanine and taurine (Betz, 1991; Tokutomi, Kaneda & Akaike, 1989). In chick sympathetic neurons clamped at a membrane potential of −70 mV, these two amino acids also elicited inward currents with kinetics similar to those of glycine-evoked currents (Fig. 3*B* and *C*). However, β-alanine and taurine were significantly less potent than glycine ($P < 0{\cdot}01$), with half-maximal effects at $79{\cdot}1 \pm 13{\cdot}0$ and $86{\cdot}1 \pm 16{\cdot}1$ μM, respectively (Fig. 3*D*). β-Alanine, at 1 mM, induced currents of similar amplitude as glycine and thus behaved as a full agonist (Fig. 3*C*). By contrast, current amplitudes evoked by 1 mM taurine were always smaller than those induced by the same concentration of glycine (Fig. 3*B*). Hence, taurine is only a partial agonist at glycine receptors of chick sympathetic neurons.

Glycine receptors in central neurons are characterized by their high affinity for strychnine, which acts as an antagonist at nanomolar concentrations (Young & Snyder, 1973). In chick sympathetic neurons, strychnine potently reduced glycine-evoked currents, but only when applied before glycine, due to the slow on-rate of this alkaloid (Fig. 4*A* and *B*). In this case, strychnine reduced the currents elicited by 100 μM glycine with half-maximal inhibition at 62 ± 11 nM. If strychnine was, however, co-applied together with 100 μM glycine, half-maximal inhibition was seen at only 753 ± 168 nM (Fig. 4*B*). The inhibitory effect of strychnine was entirely reversible within 40 s of washout (not shown). Apart from reducing peak amplitudes of glycine-evoked currents, strychnine also slowed activation kinetics in a concentration-dependent manner (Fig. 4*A*).

A number of glycine antagonists, apart from strychnine, have previously been tested at heterologously expressed glycine receptors. The amphiphilic anion CTB causes an open channel block of α1 homomeric and heteromeric glycine receptors expressed in HEK-293 cells with half-maximal inhibition between 2 and 8 μM (Rundström, Schmieden, Betz, Bormann & Langosch, 1994). In the present study, CTB reduced currents elicited by 100 μM glycine and yielded half-maximal inhibition at $3{\cdot}8 \pm 4{\cdot}0$ μM (Fig. 4*B*). The inhibition by CTB was entirely reversible, but it took between 1 and 2 min to achieve complete recovery (not shown), as previously described for heterologously expressed glycine receptors (Rundström *et al.* 1994).

Picrotoxinin, the active isomer of picrotoxin, blocks currents through α-homomeric glycine receptors in HEK-293 cells with half-maximal inhibition at 5–9 μM. Heteromeric glycine receptors, however, are less sensitive to an inhibition by picrotoxinin, the effects being half-maximal near 1 mM (Pribilla, Takagi, Langosch, Bormann & Betz, 1992). In chick sympathetic neurons, picrotoxin reduced glycine-induced currents at high micromolar concentrations, and half-maximal inhibition occurred at $347{\cdot}9 \pm 22{\cdot}8$ μM (Fig. 4*B*). After inhibition by picrotoxin, glycine-evoked current amplitudes returned to control values within 20 s of washout.

Recently, isonipecotic acid (Schmieden & Betz, 1995) and 7TFQA (Schmieden, Jezequel & Betz, 1996) were found to be competitive antagonists at α1 homomeric glycine receptors expressed in *Xenopus laevis* oocytes. There, these

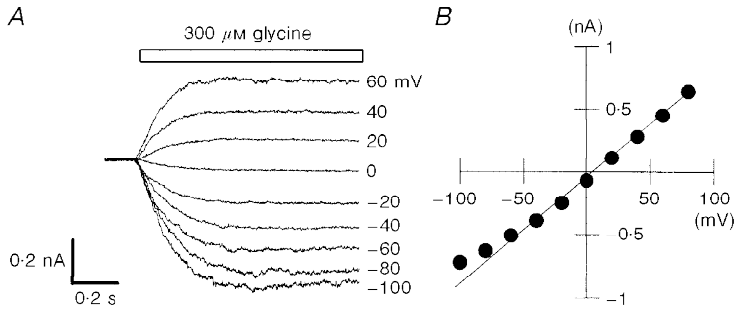

Figure 2. Current–voltage relation of glycine-induced currents in cultured chick sympathetic neurons

A, currents were induced by 300 μM glycine in a neuron clamped at the potentials indicated. The recordings were obtained with 143 mM intracellular and 154 mM extracellular Cl^-. *B*, *I–V* plot for peak amplitudes of currents shown in *A*. Note that the currents show outward rectification at membrane potentials negative to −60 mV.

carboxylic acids reduced glycine-evoked currents with half-maximal effects at 230 and 36 μM, respectively. In cultured chick sympathetic neurons, isonipecotic acid inhibited currents induced by 100 μM glycine at low millimolar concentrations, the effect being half-maximal at $1{\cdot}8 \pm 0{\cdot}3$ mM. 7TFQA reduced these currents with half-maximal inhibition at $67{\cdot}4 \pm 11{\cdot}9$ μM (Fig. 4*B*). The effects of both antagonists were reversed entirely after 20 s of washout.

Characterization of glycine-induced spike-like currents

In a few recordings (7 out of 66 cells), the application of 0·1 to 1 mM glycine to sympathetic neurons clamped at −70 mV elicited not only inward currents, but also spike-like currents, which were superimposed onto the inward currents (see Figs 3*A* and 5). The occurrence of these spike-like currents (as well as of glycine-induced inward currents) could be prevented by strychnine, but the underlying mechanisms were not clear.

Sympathetic neurons in cell culture form functional cholinergic synapses (e.g. O'Lague, Obata, Claude, Furshpan & Potter, 1974). We therefore speculated that glycine-induced spike-like currents reflected synaptic release of endogenous acteylcholine. To test for this hypothesis, glycine was applied in the absence and presence of the nicotinic blocking agent hexamethonium (100 μM). Unlike d-tubocurarine (e.g. Zhang & Berg, 1995), hexamethonium did not alter the amplitudes of glycine-evoked currents, and peak amplitudes (disregarding spike-like currents) in the presence of hexamethonium were $92{\cdot}4 \pm 5{\cdot}3$% of control ($n = 7$). However, the spike-like currents superimposed on the inward currents caused by glycine were completely abolished in the presence of hexamethonium (Fig. 5), but reappeared after 20 s of washout of the nicotinic antagonist (not shown).

Glycine-induced [^{3}H]noradrenaline release from chick sympathetic neurons

From the results presented above we concluded that glycine could depolarize chick sympathetic neurons in cell culture to an extent sufficient to trigger transmitter release. Since synaptic events triggered by glycine were rare, the secretagogue action of glycine was investigated in more detail by determination of the outflow of radioactivity from cultures loaded with tritiated noradrenaline. This procedure measures transmitter release independently of the formation of functional synapses and determines the activity of a large number of neurons at the same time (for review see Boehm & Huck, 1997).

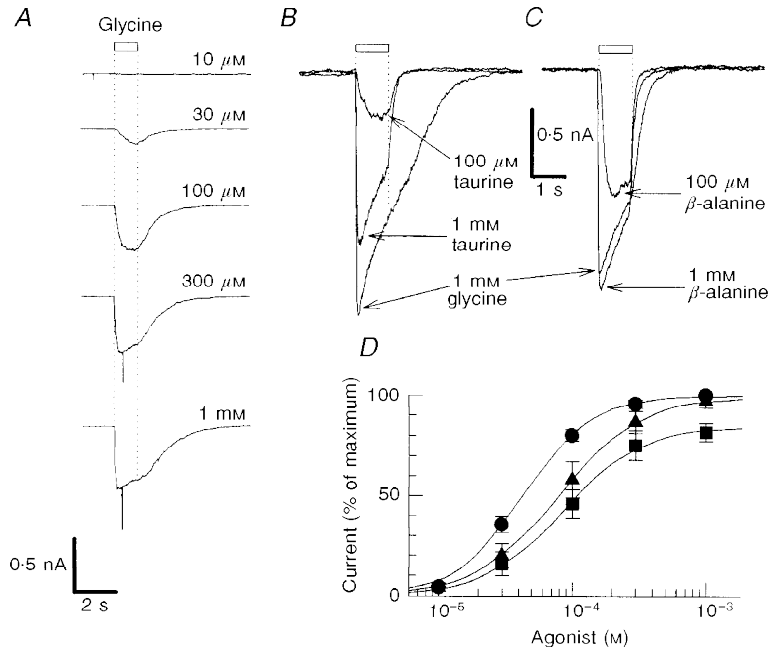

Figure 3. Agonist pharmacology of glycine receptors in cultured chick sympathetic neurons

A, currents induced by the indicated concentrations of glycine in a neuron clamped at −70 mV. At high glycine concentrations, neurons occasionally displayed spike-like events, as shown here for 300 μM and 1 mM. *B*, currents were induced by the indicated concentrations of glycine and taurine in another neuron clamped at −70 mV. *C*, currents were induced by the indicated concentrations of glycine and β-alanine in yet another neuron also clamped at −70 mV. The calibration (0·5 nA and 1 s) applies to *B* and *C*. *D* shows concentration–response curves for the peak amplitudes of currents induced by glycine (●), β-alanine (▲) and taurine (■); $n = 6$–13. The amplitudes were expressed as a percentage of the current induced by 1 mM glycine. Half-maximal concentrations were $43{\cdot}4 \pm 4{\cdot}0$ μM for glycine, $79{\cdot}1 \pm 13{\cdot}0$ μM for β-alanine ($P < 0{\cdot}01$ *vs.* glycine) and $86{\cdot}1 \pm 16{\cdot}1$ μM for taurine ($P < 0{\cdot}01$ *vs.* glycine). The deduced Hill coefficients were $1{\cdot}7 \pm 0{\cdot}2$ for glycine, $1{\cdot}4 \pm 0{\cdot}3$ for β-alanine and $1{\cdot}5 \pm 0{\cdot}4$ for taurine.

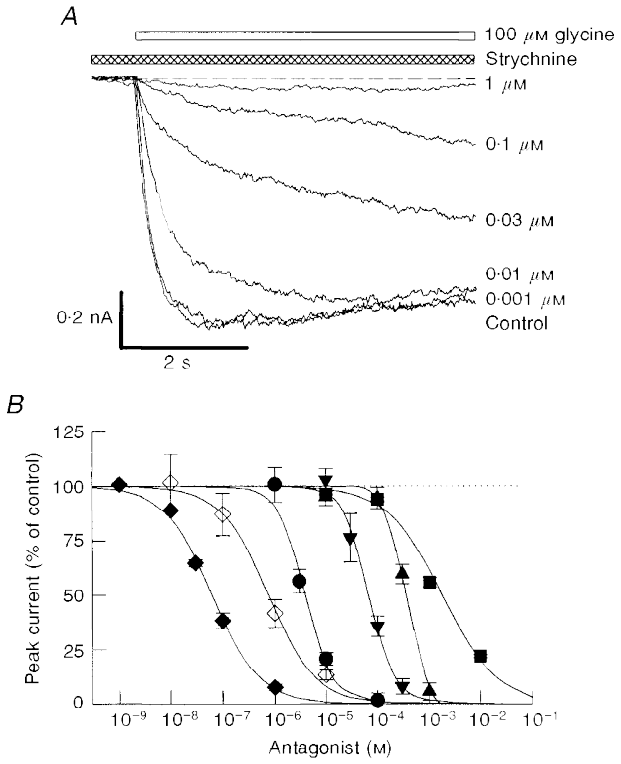

Figure 4. Antagonist pharmacology of glycine receptors in cultured chick sympathetic neurons

A depicts the reduction of currents induced by 100 μM glycine in the presence of the indicated concentrations of strychnine in a neuron clamped at −70 mV. Note that strychnine delays the activation of glycine-evoked currents. *B* shows concentration–response curves for the reduction of peak amplitudes of currents induced by 100 μM glycine by either co-applied (◇) or pre-applied (as shown in *A*; ◆) strychnine, and by the pre-application of CTB (●), 7TFQA (▼), picrotoxin (▲) and isonipecotic acid (■). Results are shown as a percentage of control currents recorded in the absence of antagonists; $n = 4$–8. Inhibition was half-maximal at 62 ± 11 nM (pre-applied strychnine), 753 ± 168 nM (co-applied strychnine), 3·8 ± 4·0 μM (CTB), 67·4 ± 11·9 μM (7TFQA), 347·9 ± 22·8 μM (picrotoxin) and 1·8 ± 0·3 mM (isonipecotic acid), respectively.

After labelling with [^{3}H]noradrenaline, chick sympathetic neurons steadily released radioactivity into the superfusion buffer when excess tritium had been removed during a 60 min washout period (see Fig. 6*A* for the time course of tritium outflow). Exposure of the neurons to 30 μM to 1 mM glycine for 4 min caused a concentration-dependent increase in [^{3}H] outflow, which was half-maximal at around 100 μM and reached a maximum at about 300 μM (Fig. 6*B*). Subsequent stimulation of the neurons by 0·5 ms electrical pulses (50 V cm^{-1}, 50 mA), delivered at 0·1 Hz for 4 min, also caused [^{3}H] overflow. When Ca^{2+} was omitted from the superfusion buffer, neither glycine nor electrical stimulation caused any alteration in [^{3}H] outflow (Fig. 6*A*).

Blockade of voltage-gated Na^+ channels by 1 μM TTX, and of voltage-dependent Ca^{2+} channels by 100 μM Cd^{2+}, both abolished overflow whether induced by 300 μM glycine or by electrical field stimulation (Fig. 6*C* and *D*). Strychnine (0·3 μM) reduced tritium overflow caused by 300 μM glycine, but left electrically induced overflow unchanged (Fig. 6*C* and *D*). These results indicated that glycine triggered transmitter release via strychnine-sensitive receptors and through mechanisms similar to those underlying electrically evoked noradrenaline release (see Boehm *et al.* 1991). Depolarization of neurons by activation of ligand-gated chloride channels is most commonly related to high intracellular chloride concentrations ($[Cl^-]_i$; Staley, Smith, Schaack, Wilcox &

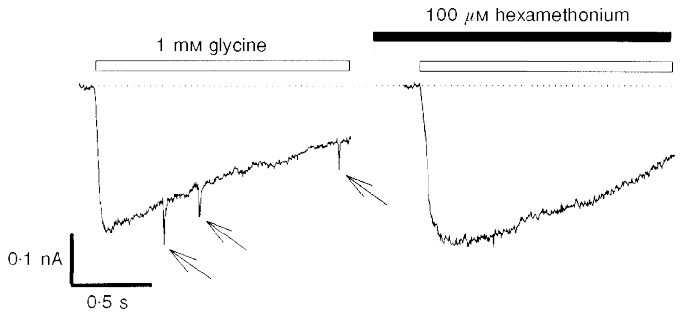

Figure 5. Hexamethonium abolishes glycine-induced spike-like currents in cultured chick sympathetic neurons

An inward current in a neuron clamped at −70 mV was induced by 1 mM glycine and carried three short spike-like currents (marked by arrows in the left trace). When glycine was applied in the continuous presence of 100 μM hexamethonium, inward currents still occurred, but spike-like currents were completely abolished (right trace). This effect of hexamethonium was entirely reversible.

Jentsch, 1996). Accumulation of high $[Cl^-]_i$ in neurons relies on a chloride uptake system, which can be blocked by furosemide (e.g. Ballanyi & Grafe, 1985; Owens *et al.* 1996). Inclusion of 2 mM furosemide in the superfusion buffer reduced glycine-evoked overflow by 75%, but increased electrically induced overflow (Fig. 6*C* and *D*). This result is consistent with high $[Cl^-]_i$ being essential for the stimulatory action of glycine.

Glycine-induced changes in intracellular Ca^{2+} in chick sympathetic neurons

The above results indicated that glycine was, in principle, able to depolarize sympathetic neurons. However, it remained unknown, whether all or just some of the neurons responded to glycine by depolarization. To resolve this issue, neurons were loaded with fura-2 AM and changes in the ratio of the fluorescence signal evoked at excitation wavelengths of 340 and 380 nm (F_{340}/F_{380}), respectively, were determined in single neurons. This ratio directly reflects the concentration of free Ca^{2+} (Grynkiewicz *et al.* 1985). Of the twenty-three neurons investigated, fifteen displayed significant ($P < 0{\cdot}05$) increases in the ratio F_{340}/F_{380} in the presence of 300 μM glycine (Fig. 7). This effect of glycine was always antagonized by 0·3 μM strychnine (Fig. 7). For a comparison, the neurons were also exposed to 100 μM nicotine (Fig. 7), which raised the ratio F_{340}/F_{380} in all of the neurons tested. Hence, all neurons were depolarized by the opening of ligand-gated cation channels, but only 65% were depolarized by the activation of glycine receptors.

DISCUSSION

α- and β-subunits of the inhibitory glycine receptor are widely distributed throughout the central nervous system (Betz, 1991), and glycine-evoked currents have been demonstrated, for instance, in neurons from spinal cord (Bormann, Hamill & Sakmann, 1987), hippocampus (Shirasaki, Klee, Nayake & Akaike, 1991) and hypothalamus (Akaike & Kaneda, 1989). In the present study, we show that neurons of sympathetic ganglia of chick embryos

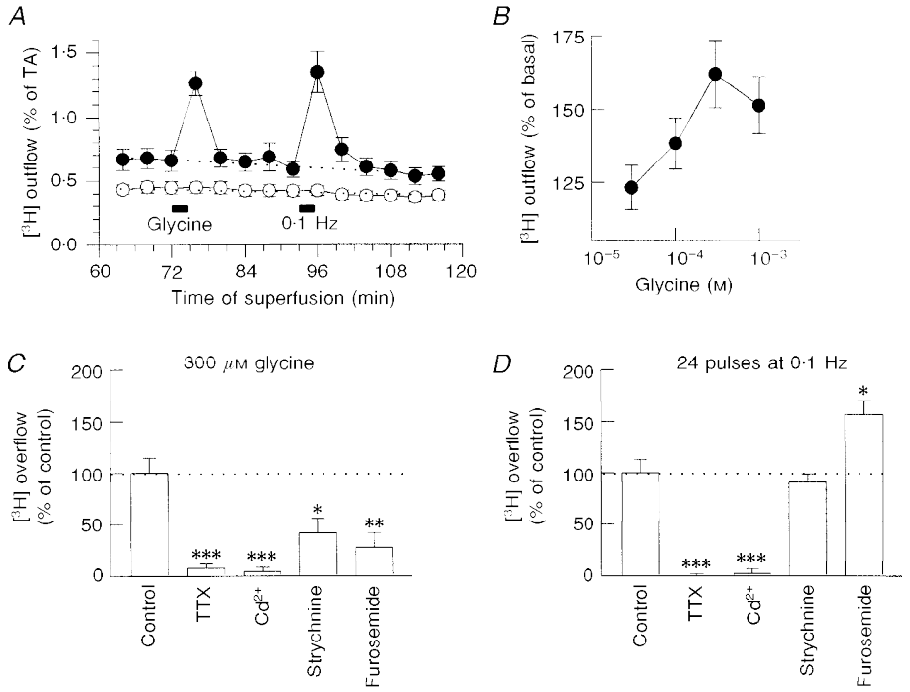

Figure 6. Glycine-induced [^{3}H] overflow from cultured chick sympathetic neurons previously labelled with [^{3}H]noradrenaline and comparison with the overflow triggered by electrical field stimulation

A, cultures were superfused after labelling with [^{3}H]noradrenaline, and subsequent to a 60 min washout period, 4 min fractions of superfusate were collected. From 72 to 76 min, the superfusion medium contained 300 μM glycine, from 92 to 96 min electrical pulses were applied at a frequency of 0·1 Hz. Results were obtained in either the presence (●) or the absence (○) of 2 mM Ca^{2+} and are shown as a percentage of total radioactivity (TA) in the cultures; $n = 5$–6. *B* shows the concentration–response relation for the secretory effect of glycine, determined as shown in *A*. The [^{3}H] overflow induced by glycine was calculated as a percentage of basal tritium outflow; $n = 6$–9. For the results in *C* and *D*, the superfusion medium contained either no additives (control), 1 μM TTX, 100 μM Cd^{2+}, 0·3 μM strychnine, or 2 mM furosemide, and the experiments were performed as shown in *A*. Results are depicted as a percentage of the overflow triggered by 300 μM glycine (*C*) and electrical field stimulation (*D*), respectively, in the absence of these drugs (control). Significant differences from control are indicated by * $P < 0{\cdot}05$, ** $P < 0{\cdot}01$ and *** $P < 0{\cdot}001$; $n = 6$–9.

contain transcripts for three different α-subunits of glycine receptors and present evidence that these neurons carry heteromeric glycine receptors, which are in most instances excitatory. Previously, glycine-induced currents have been described in chick ciliary neurons (Zhang & Berg, 1995), but information on the composition of glycine receptors in the peripheral nervous system and on pharmacological characteristics of these receptors has been lacking.

Chick sympathetic neurons contain transcripts for three glycine receptor α-subunits

We performed PCR with a set of degenerate oligonucleotide primers as an assay for glycine receptor subunit gene expression in chick sympathetic neurons. The primers used were designed to specifically amplify cDNA sequences encoding the large presumed intracellular loop of glycine receptor α-subunits. Since this portion of glycine receptor polypeptides shows the most sequence variation between the known mammalian α-subunits (Matzenbach *et al.* 1994), amplification, cloning and sequencing of PCR products allowed us to unequivocally identify cDNAs for the chicken homologues of the glycine receptor α1, α2 and α3 subunits (Fig. 1). Our data clearly demonstrate that at least three glycine receptor α-subunit genes are transcribed in the sympathetic ganglia. In addition, we recently also detected transcripts of the avian α4 subunit gene (R. J. Harvey, unpublished observations), a glycine receptor locus of unknown function (Matzenbach *et al.* 1994). To elucidate possible physiological roles of glycine receptors in chick sympathetic neurons, we performed whole-cell patch-clamp, radiotracer release, and fura-2 imaging experiments.

Chick sympathetic neurons carry functional heteromeric glycine receptors

Glycine reproducibly induced rapidly activating inward currents in chick sympathetic neurons at negative membrane potentials. Reversal potentials of glycine-evoked currents depended on the intracellular Cl^- concentrations and were close to the Nernst equilibrium potential calculated for Cl^-. This is consistent with glycine acting at ligand-gated anion channels. Comparison of the present pharmacological data with results previously obtained with either native glycine receptors in central neurons or with heterologously expressed glycine receptor subunits indicate that sympathetic neurons carry functional glycine receptors.

At native receptors in central neurons, glycine-evoked currents were half-maximal at 74 μM (in hippocampal neurons; Shirasaki *et al.* 1991) to 104 μM (in olfactory bulb neurons; Trombley & Shepherd, 1994). Receptors produced by the expression of α- and β-subunits in either *Xenopus* oocytes or HEK-293 cells show affinities for glycine between 40 and 400 μM (Schmieden *et al.* 1989; Bormann, Rundström, Betz & Langosch, 1993; Kuhse *et al.* 1993). In the present study, glycine-evoked currents occurred in the same range of concentration and were half-maximal at about 45 μM.

The rank order of agonist potency (glycine > β-alanine > taurine) observed here has also been reported for native glycine receptors (e.g. Tokutomi *et al.* 1989) and for homomeric α1 or α2 glycine receptors in *Xenopus* oocytes (Schmieden, Kuhse & Betz, 1992).

Glycine receptors in central neurons are characterized by nanomolar affinities for strychnine (e.g. Young & Snyder, 1973), which also blocks currrents through native (e.g. Tokutomi *et al.* 1989; Shirasaki *et al.* 1991) as well as heterologously expressed (e.g. Schmieden *et al.* 1989) receptors in the same range of concentration. In our experiments, glycine-induced currents were blocked by strychnine, with inhibition being half-maximal at 62 nM.

Recently, isonipecotic acid (Schmieden & Betz, 1995) and 7TFQA (Schmieden *et al.* 1996) have both been introduced as competitive antagonists of α1 homomeric glycine receptors. There, these compounds caused half-maximal inhibition at 0·23 mM and 36 μM, respectively. Although higher concentrations (half-maximal inhibition at 1·8 mM and 67 μM, respectively) were required in the present study, both antagonists fully blocked glycine-evoked currents.

Taken together, the above results clearly show that chick sympathetic neurons are equipped with *bona fide* glycine receptors. These receptors display pharmacological characteristics comparable to those of glycine receptors in heterologous expression systems and in central neurons and most closely resemble the glycine receptors previously described for chick ciliary neurons (Zhang & Berg, 1995). Glycine receptors may contain different α-subunits and one type of β-subunit at a stoichiometry of 3α : 2β, but α-homo-oligomers are sufficient to form functional receptors (Kuhse

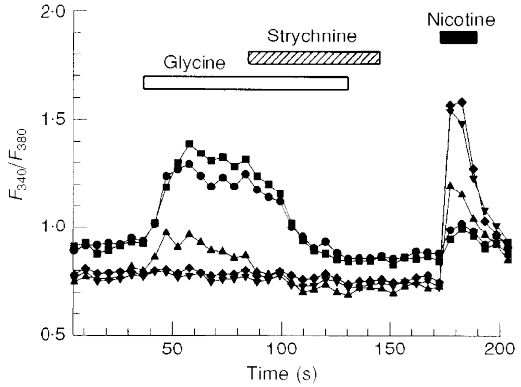

Figure 7. Changes in the ratio of the fluorescence signal evoked by excitation at 340 and 380 nm wavelength in chick sympathetic neurons loaded with fura-2 AM

In neurons loaded with fura-2 AM, fluorescence was excited once every 5 s by exposure to 340 and 380 nm light. Images of fluorescence signals were registered under 100-fold magnification with an intensified CCD camera and the ratio of the two fluorescence signals (F_{340}/F_{380}) was averaged over the entire region of each soma of 5 neighbouring neurons within the microscopic field. Glycine (300 μM), strychnine (0·3 μM) and nicotine (100 μM) were applied as indicated by the bars.

et al. 1993). The following results indicate that glycine receptors in sympathetic neurons are likely to be α–β-hetero-oligomers.

The receptors investigated here had Hill coefficients for glycine of 1·7–1·8, which are identical to the Hill coefficients for glycine at receptors in central neurons (e.g. Tokutomi *et al.* 1989; Trombley & Shepherd, 1994) and at heteromeric receptors generated by the co-expression of α- and β-subunits in *Xenopus* oocytes or HEK-293 cells (Bormann *et al.* 1993; Kuhse *et al.* 1993). By contrast, homomeric α1 or α2 receptors have Hill coefficients for glycine of $> 2{\cdot}4$ (Schmieden *et al.* 1992; Kuhse *et al.* 1993; Bormann *et al.* 1993).

The chloride channel blocker picrotoxinin blocks homomeric α-receptors at low ($< 10\ \mu$M) concentrations, but glycine receptors containing α- and β-subunits are only affected at concentrations $> 300\ \mu$M (Pribilla *et al.* 1992). In the present study, half-maximal inhibition by picrotoxin occurred at 348 μM.

CTB blocks the channels of hetero-oligomeric and of α1 homo-oligomeric glycine receptors at low micromolar concentrations, whereas α2 homo-oligomers are affected at concentrations well above 20 μM (Rundström *et al.* 1994). Here, CTB-induced inhibition of glycine-evoked currents was half-maximal at 4 μM.

All these data are consistent with glycine receptors of chick sympathetic neurons being predominantly α–β-hetero-oligomers. Since transcripts for α1, α2 and α3 subunits are present in chick sympathetic neurons (see above), all three subunits might contribute to the formation of heteromeric receptors. Unfortunately, pharmacological tools to precisely differentiate between various hetero-oligomeric glycine receptors are currently not available. Glycine receptors in HEK-293 cells produced by the expression of α1 subunits display higher affinities for glycine (around 40 μM) than those generated by α2 subunits (around 90 μM; Rundström *et al.* 1994). The glycine receptors of sympathetic neurons had affinities for this amino acid of about 45 μM and thus appear more closely related to α1 subunit-containing receptors. Furthermore, in our experiments, glycine and β-alanine displayed equal agonistic efficacies while taurine turned out to be only a partial agonist. Similar results have been obtained with homomeric α1 receptors (Schmieden *et al.* 1992), whereas at homomeric α2 receptors both β-alanine and taurine were only partial agonists (Schmieden *et al.* 1992). This may again indicate a major role of glycine receptor α1 subunits in chick sympathetic neurons. However, as different α-subunits can co-assemble within a single glycine receptor channel (Kuhse *et al.* 1993), oligomers containing two types of α-subunit may also be present in sympathetic neurons.

Functional consequences of glycine receptor activation in chick sympathetic neurons

In central neurons, glycine most commonly exerts inhibitory actions (Aprison, 1990). Nevertheless, recent reports have indicated that glycine may also cause neuronal depolarization (e.g. Reichling *et al.* 1994), particularly in developing neurons (Wang *et al.* 1994). In line with this idea, glycine has been found to elicit noradrenaline release from rat hippocampus *in vitro* (e.g. Schmidt & Taylor, 1990). In our cultures of chick sympathetic neurons, glycine also caused depolarization and transmitter release, at least in a subset (~65%) of neurons. This was evidenced threefold: (i) glycine occasionally evoked spike-like currents that were abolished by the nicotinic blocking agent hexamethonium, suggesting synaptic release of endogenous acetylcholine (see O'Lague *et al.* 1974); (ii) glycine triggered Ca^{2+}-dependent and TTX-sensitive [^{3}H]noradrenaline release, which shows that glycine may depolarize the neurons to an extent sufficient to trigger Na^+-carried action potentials (see Boehm & Huck, 1997); (iii) glycine raised intracellular Ca^{2+} concentrations as evidenced by increases in the ratio F_{340}/F_{380} of the fura-2 fluorescence signal (Grynkiewicz *et al.* 1985), but only in 65% of the neurons. The observation that intracellular Ca^{2+} concentrations changed in only a proportion of the cultured sympathetic neurons indicates that the neuronal population is heterogeneous; this might relate to differences in either glycine receptor expression or Cl^- equilibrium potentials. It remains to be shown whether this heterogeneity reflects neuronal subpopulations that can be distinguished in sympathetic ganglia (Heller, Ernsberger & Rohrer, 1996).

Considering the stimulatory actions of glycine the question arises as to what the underlying mechanisms might be. Depolarization of neurons due to activation of ligand-gated anion channels is generally believed to depend on high $[Cl^-]_i$ (Owens *et al.* 1996; Staley *et al.* 1996). Intracellular accumulation of Cl^- in sympathetic neurons depends on a Na^+–K^+–Cl^- cotransport, which can be blocked by furosemide (Ballanyi & Grafe, 1985). When the neurons were stimulated with glycine after exposure to furosemide, the stimulatory action of the amino acid was lost. Hence, triggering of transmitter release by glycine apparently required intraneuronal accumulation of high $[Cl^-]_i$, which then permitted Cl^- efflux and concomitant depolarization upon glycine receptor activation.

When considering the physiological role of glycine receptors in sympathetic ganglia, it should be noted that glycine reaches submillimolar concentrations in blood and extracellular fluids (e.g. McGale, Pye, Stonier, Hutchinson & Aber, 1977). In the central nervous system, neurons are shielded from such high concentrations by glycine uptake through two types of specific transporters present in neurons and glia, respectively (e.g. Adams, Sato, Shimada, Tohyama, Püschel & Betz, 1995; Jursky & Nelson, 1996). A cellular uptake mechanism for glycine has also been described for rat sympathetic ganglia (Bowery *et al.* 1979). Sequestration of glycine from the extracellular space in ganglia may prevent the neurons from being continuously exposed to active glycine concentrations. The preferential uptake of glycine into neuronal compartments of the ganglia reported by Bowery, Brown, White & Yamini (1979)

may indicate that glycine can function as a ganglionic neuromodulator or even transmitter. In addition, the depolarizing effect of glycine may have a trophic influence on developing sympathetic neurons, a role of glycine previously suggested for spinal cord neurons (Wang *et al.* 1994).

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