Inhibition of neurally-evoked transmitter release by calcium channel antagonists in rat parasympathetic ganglia

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1 Excitatory postsynaptic potentials (e.p.s.ps) were recorded from the submandibular parasympathetic ganglia of newborn rats (10–20 days old), by intracellular microelectrode recording and a suction electrode to deliver stimulus trains to the lingual nerve (15 stimuli at 0.1, 0.3, 1, 3, and 10 Hz, 22°C). Only evoked responses without voltage-dependent action potentials were analyzed (observed at membrane potentials negative to -70 mV), and e.p.s.p. amplitudes were determined for the plateau responses during each train (5–15th response).

2 Cadmium, an inorganic calcium channel antagonist, reduced e.p.s.p. amplitudes in a dose-dependent manner (K_d $74 \mu\text{M}$, $P < 0.01$). Nickel (1 – $300 \mu\text{M}$) did not attenuate the amplitude of evoked responses.

3 Verapamil (0.1 – $30 \mu\text{M}$), a phenylamine, had no significant effects upon e.p.s.p. amplitudes at any frequency examined. Higher concentrations of verapamil ($100 \mu\text{M}$) blocked neurally evoked responses in a manner consistent with the antagonism of voltage-sensitive sodium currents.

4 Diltiazem, a benzothiazepine, reduced e.p.s.p. amplitudes in a dose-dependent manner, the depression being accentuated at high stimulation frequencies (80% block at $30 \mu\text{M}$ and 10 Hz). The pure (–)-*cis* enantiomer of diltiazem (10 – $30 \mu\text{M}$) was without effect.

5 Amlodipine, a 1,4-dihydropyridine, did not antagonize synaptic transmission at any stimulus frequency examined (10 – $30 \mu\text{M}$, 0.1 – 10 Hz, $n = 3$).

6 Amiloride, a potassium-sparing diuretic, depressed the amplitudes of evoked responses in a dose-dependent manner (one-site K_d $31 \mu\text{M}$, $P < 0.005$), although the extent of the block was alleviated with high stimulus frequencies. The effects of $30 \mu\text{M}$ amiloride were unlikely to be of postsynaptic origin as both the amplitudes of miniature e.p.s.ps, and the iontophoretic potentials induced by exogenous acetylcholine, were not attenuated by treatment with this compound. The amiloride derivative, 3',4'-dichlorobenzamil was ineffective in reducing the amplitude of e.p.s.ps (30 – $100 \mu\text{M}$).

7 ω -Conotoxin GVIA, a marine neurotoxin, which depressed whole cell calcium currents recorded from cultured rat parasympathetic cardiac neurones (up to 90% block at 10 nM), was ineffective at blocking synaptic transmission in submandibular ganglia (0.1 – $1 \mu\text{M}$).

8 The differential effects of these calcium channel antagonists upon synaptic transmission in rat parasympathetic ganglia, suggest that either more than one type of calcium channel may be involved in transmitter release, or that the presynaptic calcium channels possess pharmacological sensitivities different from those of channel types described in neuronal cell bodies.

Introduction

Calcium channel antagonists continue to be an essential tool for investigating calcium channel function (Janis *et al.*, 1987; Vanhóutte *et al.*, 1988). It transpires that at least three types of calcium antagonist binding sites are associated with certain voltage-

sensitive calcium channels, these include sites for benzothiazepines, phenylamines, and dihydropyridines (Wagner *et al.*, 1988). Other distinct sites may exist for the inorganic calcium channel blockers, as well as for neurotoxins such as ω -conotoxin (CGTX)

GVIA, VIIA, and maitotoxin. Calcium channel antagonists also exhibit modulatory actions upon other ligand binding sites under certain conditions, for example, the phenylamine and benzothiazepine sites interact both in their binding and efficacy (Wagner *et al.*, 1988). The influence of physiological concentrations of cations, such as H^+ and Ca^{2+} , on calcium antagonist binding, and the use-dependence of many of these drugs, also complicate the interpretation of data from different cell types and species. Although several types of calcium channels may be distinguished on the basis of biophysical and pharmacological criteria in some cell types, including chick dorsal root ganglion (DRG) neurones where three calcium channels types (designated L, N, and T) have been described (McCleskey *et al.*, 1986; Fox *et al.*, 1987), the relevance of such a classification for other cell types and species is unclear. Differences may also exist between the types of calcium channels which reside in the cell soma, and in neuronal processes which participate in neurotransmitter release.

Investigation of the role of calcium channel types in neurotransmitter release processes has been prompted by the availability of specific antagonists of presynaptic calcium channels such as the shellfish peptide toxin, ω -CGTX GVIA (Kerr & Yoshikama, 1984; Olivera *et al.*, 1985; McCleskey *et al.*, 1987). The high potency of ω -CGTX in blocking neurotransmitter release at the frog neuromuscular junction (Kerr & Yoshikama, 1984), and noradrenaline release from sympathetic neurones (Hirning *et al.*, 1988), compared with the ineffectiveness of some dihydropyridines, have led to the suggestion that the N-type calcium channels are responsible for mediating transmitter release from nerve terminals. However, one cannot exclude a role for L-type calcium channels in neurotransmission on the basis of dihydropyridines not inhibiting the electrically evoked release of substance P, given the voltage-dependence of dihydropyridine block of calcium channels (Holz *et al.*, 1988). At the lizard neuromuscular junction, presynaptic calcium currents, and neurotransmitter release induced by prolonged presynaptic depolarizations, are attenuated by dihydropyridines, indicative of the modulation of L-type currents (Lindgren *et al.*, 1988). However, like the latter study, many recent investigations of the calcium channel types involved in neurotransmitter release have been conducted under conditions whereby the presynaptic depolarization is prolonged by the application of 4-aminopyridine, tetraethylammonium ions, or by high potassium containing saline (Penner & Dreyer, 1986; Holz *et al.*, 1988; Pun *et al.*, 1988). At the mouse neuromuscular junction more than one type of calcium channel is present (Penner & Dreyer, 1986). The temporal characteristics of activation and inactivation of different

types of calcium channels differ, and consequently with these varied methodologies a confusing pharmacological profile of the calcium current(s) involved in transmitter release has emerged.

In this study, the actions of calcium channel antagonists on synaptic transmission were investigated to determine if a pharmacologically distinct calcium channel type may be involved in neurally evoked transmitter release in parasympathetic ganglia. The parasympathetic neurones used in this study belong to that part of the mammalian autonomic nervous system in which preganglionic axons emerge from the cranial region of the CNS. Synaptic transmission was examined between submandibular ganglia, and whole-cell calcium currents were recorded from cultured cardiac parasympathetic neurones. The differential sensitivity of synaptic transmission to a variety of calcium channel antagonists suggest that either more than one type of calcium channel is involved in transmitter release in submandibular ganglia, or that these presynaptic calcium channels possess pharmacological sensitivities different from those channel types described in neuronal cell bodies. A preliminary presentation of some of these results was made at the September meeting of the British Pharmacological Society (Seabrook & Adams, 1988).

Methods

Preparation and solutions

Submandibular ganglia were excised from 10–20 day old Sprague Dawley rats according to the methods of Ascher *et al.* (1979). The preparation was bathed in physiological salt solution which contained (mM): NaCl 119, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 11, $CaCl_2$ 7.5, KCl 4.7, $MgSO_4$ 5, 346 mOsm. A high extracellular divalent cation concentration was essential to obtain successful (>1 h) recordings from these neurones. The saline was equilibrated with 95% O_2 + 5% CO_2 , and the pH was adjusted to 7.2. Drug solutions were freshly made before each experiment, and were perfused onto the preparation at a rate of ca. 5 ml min^{-1} (bath volume 2 ml) for ca. 10 min, at 22°C. In the case of ω -CGTX GVIA, 1.65 ml of 1 μM stock solution was applied directly to the preparation without any background perfusion. Dichlorobenzamil was applied in 0.1% dimethylsulphoxide, at which concentration no significant effects of the solvent were observed on e.p.s.p. amplitudes.

Parasympathetic cardiac neurones obtained from newborn rat hearts were grown in tissue culture (Adams *et al.*, 1987). A dissociated neuronal cell culture was prepared from explants of the cardiac

ganglion plexus by dissecting out individual ganglia following enzyme treatment (collagenase). Neuronal cell cultures were used 24–72 h after plating. Cells were perfused in external saline of the following ionic composition (mM): NaCl 82, tetraethylammonium. Cl 50, HEPES 10, glucose 20, CaCl₂ 10, KCl 2.5, MgCl₂ 0.8 and 300 nM tetrodotoxin, adjusted to pH 7.4 with NaOH. The internal (pipette) solution contained (mM): CsCl₂ 150, Cs.BAPTA 5, HEPES 10, Mg.ATP 2, adjusted to pH 7.2 with KOH. High resistance seals (>5 GΩ) were formed against the soma membrane and the membrane was ruptured with increased suction to obtain the whole-cell recording configuration.

Electrophysiological recordings and analysis

Intracellular recordings were made from neurones visualized with Hoffman modulation contrast optics ($\times 400$ magnification), using an Axoclamp-2A amplifier and 90–120 MΩ 4 M K-acetate electrodes. Few successful recordings were obtained with alternative intracellular electrode solutions (3 M KCl, 2 M K-citrate pH 7.5). Stimuli were applied to the lingual nerve via a suction electrode at a basal frequency of 0.1 Hz. Stimulus protocols of 15 stimuli at 0.3, 1, 3, and 10 Hz were conducted at 5–7 min intervals (without any significant decline in response amplitudes over 5 protocols) to examine the use-dependence of drug action. Only evoked responses without voltage-dependent action potentials were analyzed (observed at membrane potentials negative to -70 mV), and e.p.s.p. amplitudes were determined for the plateau responses during each train (5th to 15th response). Means are presented $\pm$ s.d. of either the sample size, or the averaged responses as indicated.

Responses were recorded on video tape after A-D conversion via a PCM-2 recorder adaptor (Medical Systems, N.Y., U.S.A.). For analysis, waveforms were digitized at 10 kHz on a Tetrax 5223 oscilloscope. After acquisition through a GPIB interface the waveforms were averaged (10 consecutive e.p.s.ps at each frequency), and parameters (including amplitude, rise and decay times) were determined with customized software on an IBM 80286 computer. Waveforms were acquired, their offset zeroed at the rise of the e.p.s.p. and then averaged. The rise times and rate of decay were determined from this averaged waveform. Graphical interpretation of the data was executed using GOS (OLIS Inc.).

The dose-response relationships obtained for the effects of calcium channel antagonists on e.p.s.p. amplitudes were fitted by one- or two-site adsorption isotherms:

$$F(x) = (Y_{\max} \times K_d^{-1} \times [X]) / (1 + K_d^{-1} \times [X]) + C$$

Where K_d is the equilibrium dissociation constant, $[X]$ is the concentration of the test compound, and $F(x)$ represents the amplitude of the response normalized with respect to the control. The significance of the dose-response curves were tested on ASYSTANT-plus (MacMillan Software Co.) by a Gauss-Newton algorithm.

Membrane potential transients evoked by ionophoretically-applied acetylcholine (ACh) were recorded after pretreatment of ganglia with 1 μ M tetrodotoxin (TTX), and 3 μ M neostigmine, to block voltage-dependent action potentials and acetylcholinesterase activity, respectively. A microelectrode filled with 2 M ACh (-3 nA retaining current) was positioned proximal to the neurone from which intracellular potentials were being recorded, and $+400$ nA pulses of 5 ms duration were applied every 30 s. Resultant membrane potential transients were averaged (5 consecutive responses) and digitized at 10 kHz prior to analysis by computer.

Calcium currents were recorded from rat parasympathetic cardiac neurones by use of the whole cell configuration of the patch clamp technique. Membrane current and voltage were recorded with a List EPC-7 patch clamp amplifier (List Electronics), digitized at 20 kHz (Tecmar Labmaster A/D interface) and stored on an IBM/PC AT 80286 computer. Cells were voltage clamped at -70 mV, and whole cell currents were evoked by depolarizing voltage steps applied in 10 mV increments from -80 mV to $+80$ mV following preconditioning pulses to -100 mV (0.5–1 s). Pulse protocols were provided by pCLAMP 4.1 software (Axon Instruments) at 5 s intervals. Membrane capacitative transients and leak currents were uncompensated.

Drugs

Drugs were obtained from the following companies: amiloride hydrochloride dihydrate (Merck); cadmium chloride 99.999% (Aldrich); diltiazem hydrochloride (Calbiochem); (–)-*cis* diltiazem (gift from Dr J. Stern); 3',4'-dichlorobenzamil (gift from Dr P. Siegl, Merck Sharp & Dohme, West Point, PA, U.S.A.); neostigmine (Sigma); nickel chloride (Sigma); tetrodotoxin (Sigma); verapamil hydrochloride (Sigma); ω -CGTX GVIA (Calbiochem).

Results

Inorganic calcium channel antagonists

The inorganic calcium channel antagonist cadmium (Cd^{2+}), which blocks neurotransmitter release at the

squid giant synapse (Llinas *et al.*, 1981) and is a potent antagonist of L- and N-type calcium channels (Fox *et al.*, 1987), blocked synaptic transmission in rat parasympathetic ganglia in a dose-dependent manner (Figure 1a, K_d 74 μM , $P < 0.01$). The peak amplitude of control e.p.s.ps was significantly reduced ($> 50\%$) by stimulation at 10 Hz compared to 0.1 Hz, this attenuation of e.p.s.p. amplitudes with increasing stimulus frequency was more accentuated when more than five stimulation protocols were performed on any one preparation (see methods). Even so, the block by 300 μM Cd^{2+} was alleviated with increasing stimulus frequency, in that stimulation at 10 Hz did not significantly reduce the amplitude of the evoked response compared to the control (Figure 1b, means $\pm$ s.d.; average of 10 e.p.s.ps from the same cell). Bath application of nickel (1–300 μM), a T-type calcium channel antagonist (Fox *et al.*, 1987), did not depress the peak amplitude of neurally evoked e.p.s.ps in parasympathetic neurones (Figure 1c), although the rate of e.p.s.p. decay in 300 μM Ni^{2+} was prolonged.

Organic calcium channel antagonists

Verapamil, a phenylamine calcium channel antagonist which attenuates the high threshold calcium currents (L-type) in cardiac muscle (Lee & Tsien, 1983), had little effect on synaptic transmission in the present study, even at 30 μM and 10 Hz (Figure 2a,b). At 0.1 Hz the effect of 30 μM verapamil on the peak e.p.s.p. amplitude was insignificant ($n = 3$). Higher concentrations of verapamil (100 μM) were observed to block axonal conduction in this preparation, as well as at the rat hemidiaphragm neuromuscular junction. This was apparent from the intermittent failure of normal sized e.p.s.ps, whose high probability of failure in verapamil deviated from the low probability expected according to the binomial theorem for large (≥ 10) multiquantal discharges (Boyd & Martin, 1956). Unlike the rat diaphragm NMJ (personal observations), and the mouse and frog NMJ (Publicover & Duncan, 1979; Chang *et al.*, 1988), no significant increase in miniature e.p.s.p. (m.e.p.s.p.) frequency was detected after treatment with verapamil.

In contrast to verapamil, the benzothiazepine diltiazem was a relatively effective antagonist of synaptic transmission. Diltiazem depressed e.p.s.p. amplitudes in both a dose-dependent and frequency-dependent manner (Figure 2c,d), without any change in the resting membrane potential. Recovery from treatment with 30 μM diltiazem was observed after prolonged washing, > 20 min (Figure 2d). Increasing the stimulus frequency increased the degree of block of evoked release by diltiazem. Almost complete block ($> 80\%$) was observed at 30 μM diltiazem with

Figure 1 Effects of Cd^{2+} and Ni^{2+} on neurally evoked e.p.s.ps. (a) The attenuation of e.p.s.p. amplitudes by Cd^{2+} was dose-dependent (mean control amplitude 17.4 mV), the relationship was fitted according to a one-site adsorption isotherm (smooth curve) with a K_d of 74 μM ($P < 0.01$, $n = 3$ cells). No change in the resting membrane potential was evident after treatment with cadmium. (b) The depression of e.p.s.p. amplitudes by 300 μM Cd^{2+} was stimulus-dependent, in that by increasing the stimulus frequency to 10 Hz no significant block by cadmium was observed (means $\pm$ s.d., 10 consecutive e.p.s.ps from the same cell); ($\square$) = control, ($\blacksquare$) = 300 μM Cd^{2+} . (c) Ni^{2+} (300 μM) did not depress the amplitude of neurally evoked e.p.s.ps ($n = 3$), although the rate of decay of the potential was slower than that of the control. Waveforms represent the average of ten consecutive responses at 0.1 Hz, membrane potential -74 mV.

Figure 2 Effects of verapamil and diltiazem on the amplitude of neurally evoked e.p.s.ps. (a) Application of 30 μ M verapamil did not block neurally evoked e.p.s.ps at either 0.1, 1.0 or 10 Hz (average 10 consecutive waveforms), resting membrane potential -75 mV. An increase in e.p.s.p. delay was noted in 30 μ M verapamil, a phenomenon which may relate to verapamil's non-specific effects on voltage-sensitive sodium channels (see Table 2). (b) At the highest stimulus frequency examined, 10 Hz, no significant reduction in e.p.s.p. amplitude was observed in verapamil (V) compared to the control (C). The slight depression with prolonged stimulation (>15 th response) reflects the decrease in quantal efficacy with time (R = recovery). (c) Diltiazem (30 μ M) depressed e.p.s.p. amplitudes in a stimulus-dependent manner (■); no significant block by diltiazem was noted at a stimulus frequency of 0.1 Hz. Recovery of e.p.s.p. amplitudes following treatment with diltiazem (○) approached that of control levels (□). Responses were normalised with respect to the amplitude of e.p.s.ps elicited at 0.1 Hz in each test solution. (d) The block by diltiazem at 10 Hz was more pronounced after the first few responses (C = control, D = 30 μ M diltiazem, R = recovery), this is consistent with the use-dependence of block seen when different stimulus intensities are compared in (c).

stimulus frequency of 10 Hz. The (–)-*cis* isomer of diltiazem, which blocks a cyclic GMP-activated conductance in salamander retina (Stern *et al.*, 1986), was ineffective in depressing the amplitude of neurally evoked e.p.s.ps in parasympathetic neurones.

Dihydropyridine calcium channel antagonists are considered specific for L-type calcium currents (Fox *et al.*, 1987), but the voltage- and time-dependent antagonist actions of such drugs have been implicated in their inability to inhibit electrically evoked release of substance P from dorsal root ganglia (Holz *et al.*, 1988). Nevertheless, at high stimulus frequencies and with a long lasting dihydropyridine, one may expect to observe antagonist effects of these drugs on synaptic transmission if an L-type calcium current were involved in neurotransmitter release processes. The 1,4-dihydropyridine amlodipine maleate, which is a relatively light-stable and water-soluble derivative of nifedipine, did not depress e.p.s.p. amplitudes at concentrations of up to 30 μ M even with prolonged application (Figure 3).

Amiloride, a potassium sparing diuretic which has recently been shown to be an antagonist of T-type calcium channels in DRG neurones and neuroblas-

toma cells (Tang *et al.*, 1988), blocked synaptic transmission between rat parasympathetic ganglia (one-site K_d 31 μ M, $P < 0.005$, $R^2 = 0.974$). However, the dose-response relation between 10 and 100 μ M amiloride was not simply due to an inflexion in the dose-response relation (Figure 4a), suggesting that more than one effect of amiloride may contribute to the depression of e.p.s.ps at high concentrations (2-site K_d s of 10 and 560 μ M respectively with $R^2 = 0.979$). The block of e.p.s.ps by amiloride (30 μ M) was rapid (<1 min) and readily reversible, without any significant depression or prolongation of e.p.s.p. rise or decay times (Figure 4b,c). The depression of the e.p.s.p. amplitudes by 30 μ M amiloride was partly relieved by raising the stimulus frequency to 10 Hz (Figure 4d), in that only a 40% depression was observed at 10 Hz compared to the 47% depression at 0.1 Hz. To ensure that the site of action of amiloride was not a consequence of the postsynaptic block of synaptic transmission, the effect of this diuretic upon spontaneous transmitter release and upon ionophoretic-ACh responses were examined. The amplitude and frequency of m.e.p.s.ps (Figure 5a) were unaffected by treatment with 30 μ M amiloride

Figure 3 Inability of amlodipine, a 1,4-dihydropyridine, in blocking neurally evoked e.p.s.ps. (a) Averaged e.p.s.ps at 0.1 Hz recorded before and 10 min after application of 30 μ M amlodipine. (b) The reduction in e.p.s.p. amplitudes with increasing stimulus frequency was not significantly affected by 30 μ M amlodipine (■) compared to the control (□).

Figure 4 (a) Amiloride reduced e.p.s.p. amplitudes in a dose-dependent manner with complete block at 1 mM, smooth curve represents fit of the data to a single-site adsorption isotherm with a K_d of 31 μ M ($P < 0.001$, number of cells in parentheses). (b) Stimulus protocols of 15 stimuli conducted at 0.1–10 Hz in control saline, 1 mM amiloride and after washing. The block by amiloride was rapid and readily reversible. Vertical deflections represent e.p.s.ps superimposed upon stimulus artifacts (which also have a negative component to the transient). In amiloride the responses were completely blocked and the residual transient reflects just the stimulus artifact. In this cell, e.p.s.ps elicited postsynaptic action potentials, hence the large amplitude of the evoked responses. After washing, e.p.s.ps occasionally failed to elicit an action potential at 1.0 Hz due to incomplete washout of amiloride and the normal stimulus-dependent depression of e.p.s.p. amplitudes. (c) The depression of e.p.s.p. amplitudes by amiloride (30 μ M) did not involve a distortion of the e.p.s.p. time course, or a change in the resting membrane potential (–76 mV). (d) Increasing the stimulus frequency alleviated the reduction in e.p.s.p. amplitudes by amiloride compared to the amplitude of control e.p.s.ps evoked at an identical stimulus frequency: control = (□); treated = (■); recovery = (○).

Figure 5 Depression of e.p.s.p. amplitudes by amiloride did not involve a postsynaptic antagonism of synaptic transmission. (a) Treatment of neurones with amiloride (30 μ M) did not affect the frequency or amplitude of m.e.s.ps (0.053 Hz, 2.6 ± 0.7 mV) compared to the control (0.045 Hz, 2.9 ± 0.8 mV). (b) The amplitude of depolarizations induced by ionophoretic pulses of acetylcholine (ACh, indicated by lower trace) were not significantly reduced by 30 μ M amiloride. Waveforms represent the average of 5 consecutive depolarizations in response to ACh pulses (0.033 Hz) in the presence and absence of amiloride, recorded from the same cell in the presence of 3 μ M neostigmine and 1 μ M tetrodotoxin.

(2.6 ± 0.7 mV, 0.053 Hz compared to the control 2.9 ± 0.8 mV, 0.045 Hz). Similarly, membrane potential transients induced by the ionophoretic application of ACh to the soma (Figure 5b) were not significantly affected by 30 μ M amiloride (for example control 21.1 ± 4.8 mV, treated 18.9 ± 10.2 mV; means $\pm$ s.d. of 5 pulses from the same cell). The amiloride derivative 3',4'-dichlorobenzamil (30–100 μ M), which like amiloride blocks Na^+ - H^+ exchange, did not suppress e.p.s.p. amplitudes (Figure 6).

Figure 6 The amiloride derivative dichlorobenzamil (Dcb) did not significantly depress the peak amplitude or alter the waveform of e.p.s.ps. (a) Averaged e.p.s.p. recorded from the same cell before, during and after treatment with 100 μ M Dcb. (b) In a cell sensitive to the application of 30 μ M amiloride (Am), 30–100 μ M Dcb applied in 0.1% DMSO failed to depress the amplitude of neurally evoked responses.

The marine neurotoxin, ω -conotoxin GVIA (0.1–1 μ M), which has been shown to block neurotransmitter release at the frog neuromuscular junction (Kerr & Yoshikama, 1984) but not at the mouse neuromuscular junction (Anderson & Harvey, 1987), did not block synaptic transmission in rat parasympathetic ganglia ($n = 4$, Figure 7a). Furthermore, increasing the stimulus frequency (0.1–10 Hz) did not affect this result.

To examine the effect of this toxin on the calcium currents in the neuronal soma, whole cell calcium currents were recorded from cultured parasympathetic neurones of the newborn rat heart. Voltage-sensitive calcium currents were recorded in the presence of TTX, as well as internal Cs_2 and external TEA to block voltage-sensitive sodium, and potassium conductances respectively. Inward current amplitude was dependent on the external $[\text{Ca}^{2+}]$ and in the following experiments calcium currents were elicited in the presence of elevated, 10 mM,

Figure 7 (a) Application of $1\ \mu\text{M}$ ω -conotoxin GVIA (CGTX) did not block neurally evoked e.p.s.ps in parasympathetic ganglia ($n = 4$). Average of ten consecutive e.p.s.ps evoked at 0.1 Hz, membrane potential $-72\ \text{mV}$. (b) and (c) Whole cell calcium currents elicited with depolarizing steps to $+10\ \text{mV}$ were strongly attenuated after application of $10\ \text{nM}$ ω -CGTX, indicative of the high potency of this conotoxin fraction toward somal calcium currents (scale bar $2\ \text{nA}$, $10\ \text{ms}$). The block by this peptide was irreversible but was distinguishable from calcium current rundown, because over a similar time course the block of somal calcium currents by $100\ \mu\text{M}$ Cd^{2+} (recorded from a different neurone) was readily alleviated upon washing (scale bar $2\ \text{nA}$, $10\ \text{ms}$), compared to the control (scale bar $1.32\ \text{nA}$, $10\ \text{ms}$). A voltage-dependent increase in the latency of onset of the somal calcium current was observed in washout following treatment with cadmium.

external calcium. The whole-cell calcium currents recorded from cardiac neurones were predominantly high-threshold and exhibited only 20% inactivation during a 500 ms depolarising pulse to $+20\ \text{mV}$. Application of prior conditioning hyperpolarizations ($1\text{--}60\ \text{s}$) to $-100\ \text{mV}$ failed to reveal any transient calcium current. A marked reduction of the whole cell calcium currents (up to 90%) by $10\ \text{nM}$ ω -CGTX GVIA was observed (Figure 7b). The irreversibility of calcium current blockade by ω -CGTX was distinct from calcium current 'run-down', evident in many whole-cell patch clamp experiments (Yaari *et al.*, 1987), because the inhibition of soma calcium currents with $100\ \mu\text{M}$ Cd^{2+} , obtained in a different neurone, was readily reversible over a time course similar to the duration of the ω -CGTX experiments (Figure 7c). Calcium currents recorded from the soma of parasympathetic cardiac neurones were also inhibited by the dihydropyridine, amlodipine ($10\ \mu\text{M}$) but not by $30\ \mu\text{M}$ amiloride (data not shown).

Discussion

This study presents pharmacological evidence that the calcium channels involved in synaptic transmission between parasympathetic ganglia are distinct from the calcium channels characterized in dorsal root ganglia and N1E-115 neurones (Table 1). Apart from the insensitivity of neurally evoked transmitter release in parasympathetic ganglia to Ni^{2+} , it appears that the calcium current involved has pharmacological characteristics in common with the T-type currents, although it cannot be unambiguously classified with present criteria (Fox *et al.*, 1987).

Following vagal stimulation, the cardiac interval of spontaneously beating bullfrog heart is prolonged, an effect which is accompanied by an increase in the release of ACh. Application of $5\ \mu\text{M}$ Cd^{2+} attenuates these effects of vagal stimulation leading to the suggestion that Cd^{2+} suppresses the release of neurotransmitter from parasympathetic ganglia (Hayashi & Takayama, 1978). However, Cd^{2+} was much less potent in reducing the amplitude of neurally evoked e.p.s.ps in rat parasympathetic ganglia ($K_d\ 74\ \mu\text{M}$; $P < 0.01$), than in reducing the somatic L-type currents in cardiac parasympathetic neurones (Figure 7c), neuroblastoma (Narahashi *et al.*, 1987), and in sympathetic neurones (Fox *et al.*, 1987). Increasing the stimulus frequency (from 0.1 to 10 Hz) suppressed the degree of antagonism of e.p.s.p. amplitudes by Cd^{2+} . This finding is consistent with the occluded calcium channels being cleared of this blocker upon repetitive depolarizations. However, one cannot exclude the possibility that additional calcium channels (or types) are being recruited at

Table 1 Potency ranking of calcium channel block

Calcium channel antagonist	Calcium channel type*			Synaptic transmission in rat parasympathetic ganglia (this study)
	L	N	T	
Cadmium (inorganic)	+++	++	+	++(1/S)
Nickel (inorganic)	—	—	++	—
Verapamil (phenylamine)	+++ (S)	—	+(S)	—
Diltiazem (benzothiazepine)	+++ (S)	?	?	++ (S)
(-)-cis-Diltiazem (benzothiazepine)	—	—	—	—
Amlodipine (dihydropyridine)	+++	—	—	—
ω -CGTX GVIA (peptide)	+++	+++	—	—
Amiloride	+	?	+++	+++ (1/S)

*Criteria used in the characterization of calcium channel types in this table were based upon observations made from the dorsal root ganglia of the frog, rat and chick, as well as from cultured neuroblastoma cell lines (McCleskey *et al.*, 1986; Fox *et al.*, 1987; McCleskey *et al.*, 1987; Narahashi *et al.*, 1987; Hirning *et al.*, 1988; Tang *et al.*, 1988).

Key

—	Negligible	> 1 mM, or ineffective at concentrations which block other calcium currents.
+	Low	(IC ₅₀ 100 μ M to 1 mM)
++	Average	(IC ₅₀ 10 μ M to 100 μ M)
+++	High	(IC ₅₀ < 10 μ M)

(S) denotes increase in block with increasing stimulus frequency.

(1/S) alleviation of block with increasing stimulus frequency.

high stimulus frequencies, or that the reduced calcium influx in Cd²⁺ may unmask an underlying calcium-dependent mechanism involved in facilitation.

Additional evidence against the involvement of L-type channels in synaptic transmission in rat submandibular ganglia, was the lack of effect of verapamil, even at 30 μ M and a stimulus frequency of 10 Hz. Although the binding of calcium channel antagonists like verapamil are sensitive to millimolar concentrations of Ca²⁺ (Kostyuk *et al.*, 1975; Triggle & Swamy, 1983; Triggle & Janis, 1987), the calcium concentration used in this study (7.5 mM) is considered insufficient to account for the failure of verapamil to suppress the e.p.s.p. amplitude. The depression of synaptic transmission by 30 μ M diltiazem at high frequencies (10 Hz), would appear indicative of a role for L-type calcium channels in synaptic transmission, due to diltiazem's potent antagonism of slowly-inactivating high-threshold (presumably L-type) calcium currents (Uehara & Hume, 1985; Penner & Dreyer, 1986). However, diltiazem's potency was low compared to these other studies, and at 30 μ M the specificity of diltiazem for L-type calcium currents remains questionable (Tables 1 and 2). The absence of a role for L-type calcium currents is further supported by the inability of 1,4-dihydropyridine amlodipine maleate, to block synaptic transmission.

The kinetics of L-type calcium channels would favour the predominance of this conductance at high

stimulus intensities, and thus this may support the differential release of neurotransmitters discharged from the same nerve terminal (Hirning *et al.*, 1988). However, as benzothiazepines and phenylamine calcium channel antagonists are markedly voltage-dependent in their efficacy (Uehara & Hume, 1985), facilitation of block by these antagonists at high stimulus frequencies cannot be used alone as an argument for the role of more than one calcium channel type in neurotransmitter release.

The block of neurally-evoked transmitter release in rat parasympathetic ganglia by amiloride may relate to the recent finding that this K⁺-sparing diuretic acts as a potent and reversible blocker of low threshold (T-type) calcium channels in neuroblastoma and chick DRG neurones (Tang *et al.*, 1988). However, it is uncertain as to whether or not the depression of synaptic transmission is a consequence of either the block of a population of calcium channels, or an increase in intraterminal pH due to a suppression of Na⁺-H⁺ exchange. Several lines of evidence indicate that the effect of amiloride may be specific for calcium currents. Varying intraterminal pH (7.8 to 6.7) does not block transmitter release at the squid giant synapse (Adams & Thomas, 1989). In rat brain synaptosomes acidification from pH 7.2 to 5.8 increases the efflux of dopamine in a [Ca²⁺]_o-independent manner (Drapeau & Nashchen, 1988). In addition, the potent Na⁺-H⁺ exchange inhibitor dichlorobenzamil did not suppress e.p.s.p. amplitudes. The findings that

Table 2 Non-specific effects of various calcium channel antagonists

Calcium antagonist	'Non-specific' effects (IC_{50} s/ K_d s)	Tissue	Reference
Cadmium	(1) fast Na current antagonist ($180\text{ }\mu\text{M}$) (2) H^+ activated Na-current ($0.5\text{ }\mu\text{M}$)	Calf purkinje fibres Chick DRG neurones	DiFrancesco <i>et al.</i> (1985) Konnerth <i>et al.</i> (1987)
Nickel	(1) nAChR antagonist (10 mM)	Frog NMJ	Magleby & Weinstock (1980)
Verapamil	(1) α -adrenergic ligand ($<1\text{ }\mu\text{M}$) (2) mAChR antagonist ($>10\text{ }\mu\text{M}$) (3) Na + H-ATPase inhibitor ($>15\text{ }\mu\text{M}$) (4) nAChR ($>20\text{ }\mu\text{M}$) antagonist ($>20\text{ }\mu\text{M}$) (5) delayed K-current antagonist ($\geq 50\text{ }\mu\text{M}$) (6) Na channel antagonist ($\geq 100\text{ }\mu\text{M}$)	Platelets/heart Rabbit Purkinje fibres Hog gastric membranes Frog NMJ Mouse NMJ/BC3H1 Helix neurones Frog/crayfish NMJ Mouse NMJ Rat brain/frog NMJ	Motulsky <i>et al.</i> (1983) Mubagwa & Carmeliet (1987) Blakeman <i>et al.</i> (1984) Edeson <i>et al.</i> (1985, 1988) Wachtel (1987) Nishi <i>et al.</i> (1983) Van der Kloot & Kita (1975) Chang <i>et al.</i> (1988) Nashchen & Blaustein (1979)
Diltiazem	(7) Na-dependent transmitter uptake (1) nAChR antagonist ($\geq 20\text{ }\mu\text{M}$) (2) K channel antagonist ($\geq 30\text{ }\mu\text{M}$) (3) Na channel antagonist ($\geq 100\text{ }\mu\text{M}$) (4) H^+ activated Na-current (1 mM)	PC12 cells Mouse NMJ/BC3H1 cells Frog atria Mouse NMJ Chick DRG neurones	McGee & Schneider (1979) Wachtel (1987) Nishi <i>et al.</i> (1983) Chang <i>et al.</i> (1988) Konnerth <i>et al.</i> (1987)
(-)-cis-Diltiazem	Cyclic GMP conductance antagonist ($10\text{ }\mu\text{M}$)	Salamander retina	Stern <i>et al.</i> (1986)
ω -Conotoxin GVIA	(1) inhibits Na-fluxes ($1\text{ }\mu\text{M}$) (2) Na-H exchange inhibitor ($1\text{ }\mu\text{M}$) (3) mAChR antagonist ($\geq 40\text{ }\mu\text{M}$) (4) Na-Ca exchange inhibitor ($\geq 1\text{ mM}$)	None-identified Epithelia Epithelia/frog skin Rabbit pancreas Rat brain	Dale (1982) Dale (1982) Kuijpers <i>et al.</i> (1984) Dale (1982) Drapeau & Nashchen (1988)

NMJ = neuromuscular junction; mAChR = muscarinic acetylcholine receptor; nAChR = nicotinic acetylcholine receptor.

m.e.s.p. amplitudes were unchanged by $30\text{ }\mu\text{M}$ amiloride, and that the amplitude of potentials induced by the iontophoretic application of ACh were unaffected, suggest that the depression of e.p.s.p. amplitudes by amiloride is unlikely to be a postsynaptic effect.

To test the hypothesis that T-type calcium channels may be involved in synaptic transmission the activity of ω -CGTX GVIA, a specific blocker of N- and L-type currents in dorsal root ganglion neu-

rones (McCleskey *et al.*, 1987), was examined. In parasympathetic ganglia, $1\text{ }\mu\text{M}$ ω -CGTX was ineffective in blocking synaptic transmission. It has been demonstrated that the block of calcium currents by a synthetic omega-conotoxin is alleviated by raising the extracellular calcium concentration (Oyama *et al.*, 1987), but even with 10 mM $[Ca^{2+}]_o$, significant block of the calcium conductance still may be obtained with $1\text{ }\mu\text{M}$ ω -CGTX, as seen with 10 nM ω -CGTX in cardiac parasympathetic neurones

(Figure 7). However, in certain tissues such as frog and guinea-pig ventricular muscle, and in rabbit ear artery smooth muscle, high threshold calcium currents (presumably L-type) are reported to be insensitive to this toxin (see McCleskey *et al.*, 1987), and a verapamil-sensitive calcium current in the mouse neuromuscular junction is also unaffected by ω -CGTX (Anderson & Harvey, 1987). Neurotransmitter release at the squid giant synapse, which is sensitive to the inorganic blocker Cd^{2+} (K_d 70 μM), is also insensitive to ω -CGTX (M.P. Charlton personal communication; Llinas *et al.*, 1989). These data are indicative of an additional, but as yet unidentified, calcium conductance involved in neurotransmitter release processes.

Nickel blocks T-type calcium currents approximately ten times more potently than other calcium currents (Fox *et al.*, 1987; Narahashi *et al.*, 1987). Thus, one would predict that if T-type channels were responsible for eliciting e.p.s.ps, a potent antagonism of the e.p.s.p. by low doses of nickel (<100 μM) would be evident. However, no such effect was observed, which may suggest the absence of T-type calcium currents in neurotransmitter release processes. But, this observation contrasts with the block observed with amiloride, and the actions of other calcium channel antagonists used in this study (Table 1). It has also been noted at the

lizard neuromuscular junction that 100 μM Ni^{2+} is ineffective in blocking the normal presynaptic calcium currents (Lindgren *et al.*, 1988). This discrepancy may relate to the pharmacological sensitivities of the underlying calcium current involved in transmitter release, or alternatively to differing accessibilities/binding characteristics of this cation relative to Cd^{2+} .

In conclusion, the pharmacological characteristics of synaptic transmission in rat submandibular ganglia are inconsistent with the involvement of only one of the three identified neuronal calcium channel types. The calcium current(s) underlying neurally evoked transmitter release in parasympathetic ganglia share some characteristics with the T-type channels described in chick DRG neurones and neuroblastoma cell lines. However, the possibility of a different calcium current type in parasympathetic nerve terminals exists, as the current cannot be unambiguously defined within the existing pharmacological definitions of calcium channel types.

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