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ω-Conotoxin GVIA-resistant neurotransmitter release from postganglionic sympathetic nerves in the guinea-pig vas deferens and its modulation by presynaptic receptors

***Amanda B. Smith & [1]Tom C. Cunnane**

University Department of Pharmacology, Mansfield Road, Oxford, OX1 3QT, U.K. and *Department of Physiology and Pharmacology, University of Queensland, St. Lucia, Queensland 4072, Australia

1 Intracellular recording techniques were used to study neurotransmitter release mechanisms in postganglionic sympathetic nerve terminals in the guinea-pig isolated vas deferens.

2 Recently, a component of action potential-evoked release which is insensitive to high concentrations of the selective N-type calcium channel blocker ω-conotoxin GVIA termed 'residual release' has been described. Under these conditions, release of the neurotransmitter ATP evoked by trains of low frequency stimuli is abolished, but at higher frequencies a substantial component of release is revealed.

3 'Residual release' was studied with trains of 5 or 10 stimuli at stimulation frequencies of 10, 20 and 50 Hz. The α_2-adrenoceptor agonist clonidine (30–100 nM) inhibited 'residual release', the degree of inhibition being most marked at the beginning of a train.

4 The α_2-adrenoceptor antagonist yohimbine (1 μM) induced a marked increase in 'residual release' which was dependent on both the frequency of stimulation and the number of stimuli in a train.

5 Prostaglandin E_2 (30 nM) and neuropeptide Y (100 nM) caused a rapid inhibition of 'residual release' at all stimulation frequencies examined.

6 4-Aminopyridine (100 μM) induced a powerful potential of 'residual release' and could reverse the inhibition of ω-conotoxin GVIA.

7 'Residual release' was modulated through presynaptic α_2-adrenoceptors suggesting that (i) residual release of ATP is subject to α-autoinhibition through the co-release of noradrenaline, (ii) noradrenaline release can be triggered by calcium channels other than the N-type and (iii) when presynaptic receptors are activated, inhibition of transmitter release can occur by mechanisms other than modulation of calcium-entry through N-type calcium channels in postganglionic sympathetic nerves. Prostaglandin E_2 and neuropeptide Y also modulated neurotransmitter release.

Keywords: Neurotransmitter release; co-transmission; autoinhibition; N-type calcium channels; sympathetic nerves

Introduction

It is widely accepted that neurotransmitter release from nerve terminals can be modulated by a variety of drugs which act through presynaptic receptors located on nerve terminals, called autoreceptors. This phenomenon, whereby neurotransmitters or drugs act on presynaptic receptors; has been termed autoinhibition (for reviews see Langer, 1981; Starke, 1987; Starke *et al*., 1989).

The mechanisms whereby α-adrenoceptor agonists and antagonists and other agents which activate presynaptic receptors, such as prostaglandin E_1 (PGE_1), PGE_2 and neuropeptide Y (NPY) modify neurotransmitter release have received much attention but remain poorly understood (see Illes, 1986; Starke, 1987). At least three different mechanisms for presynaptic receptor-mediated inhibition of neurotransmitter release have been suggested, namely: (i) hyperpolarization of the nerve terminals by increasing K^+ conductances (see Morita and North, 1981; North & Surprenant, 1985; Williams *et al*., 1985), (ii) inhibition of voltage-sensitive calcium channels, thereby decreasing depolarization-induced calcium entry (see Hedqvist, 1976; Alberts *et al*., 1981; Starke, 1987; Lipscombe *et al*., 1989; Toth *et al*., 1993; McQuiston *et al*., 1996) and (iii) interference with depolarization-secretion coupling at some stage post calcium entry (see Güllner, 1983; Schoffelmeer & Mulder, 1983; Majewski *et al*., 1990; Xiang *et al*., 1990; Jackisch *et al*., 1992). In the guinea-pig vas deferens focal extracellular recording techniques have been used to show convincingly that these drugs do not produce their effects by interfering with impulse propagation in postganglionic sympathetic nerve terminals (Brock & Cunnane, 1990; 1996; Brock *et al*., 1990).

We have recently described a novel form of neurotransmitter release mechanism which occurs in the presence of high concentrations of the irreversible N-type calcium channel blocker ω-conotoxin GVIA (ω-CTX GVIA). This ω-CTX GVIA-insensitive 'residual release' has the following characteristics: (i) it is positively frequency-dependent, (ii) the magnitude is dependent on both the train length and interval between trains, (iii) α,β-methylene-ATP (1 μM) which desensitizes P2X-receptors abolished the residual excitatory junction potentials (e.j.ps) which therefore result from the actions of neuronally-released ATP acting through postjunctional P2X-purinoceptors, (iv) 'residual release' is insensitive to various combinations of selective calcium-entry blockers (L-, N-, P- and Q-type calcium channel blockers) but abolished by ω-Grammotoxin SIA (300 nM), cadmium ions (10 μM) and when the extracellular calcium concentration is reduced to 0.6 mM (Smith & Cunnane, 1996a) and (v) ryanodine (10 μM) inhibits 'residual release' in a time- and use-dependent manner (Smith & Cunnane, 1996b). ω-Grammotoxin SIA, a purified extract from the venom of the tarantula spider, *Grammastola spatulata*, inhibits calcium-entry through neuronal voltage-sensitive calcium channels but is not specific for any one type of channel (Keith *et al*., 1992; Lampe *et al*., 1993; Piser *et al*.,

[1] Author for correspondence.

1994). These results suggest that calcium-entry through a novel, pharmacologically uncharacterized, voltage-dependent calcium channel is responsible for 'residual release' in sympathetic nerve terminals. It has also been demonstrated in the rat anococcygeus that calcium channels other than N-type are involved in neurotransmitter release (Smith & Cunnane, 1997).

Since modulation of N-type calcium channel activity is widely believed to be a key mechanism by which activation of presynaptic receptors inhibits neurotransmitter release, it was interesting to investigate whether presynaptic receptor stimulation could inhibit neurotransmitter release when N-type calcium channels were irreversibly blocked by toxins. In the present study we have investigated whether 'residual release' can be modulated when presynaptic α_2-adrenoceptors, prostaglandin and neuropeptide Y receptors are stimulated with appropriate agonists. A preliminary account of these findings has been communicated to the Physiological Society (Smith & Cunnane, 1995).

Methods

Preparation

Male guinea-pigs (Dunkin Hartley strain, 300–600 g) were humanely killed and bled. The abdominal viscera were exposed with a midline incision and the vasa deferentia were removed. Preparations were pinned to the Sylgard (Dow-Corning) covered base of a 3 ml Perspex organ bath and gently stretched to approximately their *in situ* length. A small area of the connective tissue sheath was carefully removed to expose the outer smooth muscle layer and electrophysiological recordings were made from this region. Preparations were superfused with Krebs solution of the following composition (mM): NaCl 118.4, $NaHCO_3$ 25.0, NaH_2PO_4 1.13, $CaCl_2$ 1.8, KCl 4.7, $MgCl_2$ 1.3 and glucose 11.1, gassed with a mixture of 95% O_2 and 5% CO_2 to pH 7.4 and maintained at 36–37°C.

Intracellular recording

Changes in membrane potential of smooth muscle cells, on or near the serosal surface of the tissue, were measured with intracellular microelectrodes. Microelectrodes were filled with 5 M potassium acetate and had tip resistances of 60–120 MΩ. Electrical signals were fed into a high-impedance d.c. preamplifier ($\times$ 10, Neurolog NL102, Digitimer) and low-pass filtered (5 kHz, NL 125 Digitimer). The sympathetic nerves innervating the vas deferens were excited by field stimulation of the prostatic end of the vas deferens, with rectangular voltage pulses delivered from a digital stimulator coupled to an optically isolated stimulus unit (Applegarth Instruments, Oxford). The stimuli were applied through a pair of Ag-AgCl electrodes positioned across the prostatic end of the vas deferens. This stimulation protocol excites mainly postganglionic sympathetic nerve fibres (Ferry, 1967; Brock & Cunnane, 1988) and was usually subthreshold for stimulating muscle contraction. Some of the experiments were carried out in the presence of nifedipine (10 μM) to inhibit muscle contraction when high stimulation frequencies were employed.

Collection and analysis of electrophysiological data

The recorded signals were digitized at 5 kHz (Sony PCM - 701ES) and stored on a modified video tape recorder (Panasonic AG-6200). Evoked events were subsequently analysed by digitizing through an ITC-16 computer interface (0.1–1 kHz) connected to a Macintosh IIfx computer (Instrutech Corp., Elmont, NY, U.S.A.) by use of the programme Axodata (Axon Instruments Inc., Foster City, CA, U.S.A.). The amplitude, frequency, rise time and latency of excitatory junction potentials (e.j.ps) were analysed by means of the programme Axograph 2 (Axon Instruments Inc.). The mean baseline was determined by averaging the initial part of the digitized signal between the stimulus artefact and the onset of the response. The peak amplitude of individual e.j.ps evoked at frequencies >1 Hz was calculated as the difference between the mean of 3 points either side of the detected peak and the mean baseline. When trains of stimuli were applied, the peak e.j.p. amplitude was defined as the maximum amplitude obtained at any point in a train. The peak amplitudes of summating e.j.p. complexes evoked by higher frequency trains were measured manually. The e.j.p. or the e.j.p. complex with the largest amplitude in a train was defined as the fully facilitated e.j.p. Time-match control experiments showed that the responses did not vary with time. Data are expressed as the mean $\pm$ s.e.mean and the n value refers to the number of preparations. Data were analysed statistically by Student's paired t test with the level of significance being taken as $P<0.05$.

Drugs

The following drugs were used: α,β-methylene adenosine 5′-triphosphate (Sigma), 4-aminopyridine (Sigma), clonidine hydrochloride (Sigma), ω-conotoxin GVIA (Alamone labs), nifedipine (Sigma), neuropeptide Y (Peptide Institute), prostaglandin E_2 (Sigma), tetrodotoxin (Sigma) and yohimbine hydrochloride (Sigma).

Results

When the postganglionic nerves of the guinea-pig vas deferens are electrically stimulated excitatory junction potentials (e.j.ps) are evoked which increase in amplitude during the first six stimuli in a train, a phenomenon called facilitation (Figure 1a). Low concentrations of the irreversible and selective N-type calcium channel blocker ω-CTX GVIA (10 nM) abolished e.j.ps evoked at 1 Hz (Brock *et al*., 1989; Smith & Cunnane, 1996a) (Figure 1a). However, a component of action potential-evoked release, which was tetrodotoxin-sensitive but insensitive to high concentrations of ω-CTX GVIA (termed 'residual release'), was revealed at higher stimulation frequencies (Figure 1b). ATP is the neurotransmitter generating these residual depolarizations (Smith & Cunnane, 1996a) but it is not apparent whether the co-transmitter noradrenaline is also being released under these experimental conditions. One way to determine whether noradrenaline is being released together with ATP when N-type calcium channels are blocked is to apply the selective α_2-adrenoceptor antagonist yohimbine.

Is 'residual release' modulated by presynaptic receptor activation?

α_2-Adrenoceptor agonists reduce the amplitude of e.j.ps in the guinea-pig and mouse vas deferens and this effect can be prevented or reversed by selective α_2-adrenoceptor antagonists (Blakeley *et al*., 1981; Brock *et al*., 1990). When an α_2-adrenoceptor antagonist is applied alone, e.j.p. amplitude was

increased. This increase was not evident until the second to fifth e.j.p. in the train.

Prostaglandin E_2, a potent inhibitor of neurotransmitter release in the guinea-pig vas deferens, also acts through presynaptic receptors (Ito & Tajima, 1979; Brock & Cunnane, 1996), as does neuropeptide Y (Stjärne *et al*., 1986). The mechanism of action of these drugs is uncertain but it is clear that they act through G protein-coupled presynaptic receptors, which when activated inhibit calcium influx into the nerve terminal through N-type calcium channels (Hedqvist, 1976; Akasu *et al*., 1990; Xiang *et al*., 1990; Toth *et al*., 1993). It was therefore of interest to examine the effects of the α_2-adrenoceptor agonist clonidine and the competitive α_2-adrenoceptor antagonist yohimbine, prostaglandin E_2 and neuropeptide Y on 'residual release' evoked when N - type calcium channels are irreversibly blocked.

Effect of the α_2-adrenoceptor agonist clonidine

Bath application of the α_2-adrenoceptor agonist clonidine (30–100 nM) inhibited 'residual release' at all frequencies of stimulation employed. The effect of clonidine on e.j.ps evoked by trains of 5 stimuli at 10 Hz delivered every 2 s are shown in Figure 2. Fully facilitated e.j.ps, evoked by trains of 5 stimuli at 10 Hz delivered every 2 s, in the presence of ω-CTX GVIA (100 nM), had a mean amplitude of 14.1 ± 0.6 mV ($n=4$). After the addition of clonidine (30 nM and 100 nM) for 20 min, e.j.ps had mean amplitudes of 7.4 ± 0.3 mV and 3.0 ± 0.3 mV ($n=4$), respectively. Fully facilitated e.j.ps, evoked by trains of 5 stimuli at 20 Hz delivered every 2 s, in the presence of ω-CTX GVIA (100 nM), had a mean amplitude of 21.7 ± 0.7 mV ($n=4$). After the addition of clonidine (30 nM and 100 nM) for 20 min, e.j.ps had mean amplitudes of 11.7 ± 0.3 mV and 7.5 ± 0.2 mV ($n=4$), respectively. The degree of inhibition was most marked at the beginning of the train and became less pronounced during and between successive trains. For example, 30 and 100 nM clonidine inhibited e.j.ps evoked by the first train of 5 stimuli at 20 Hz delivered every 2 s by $64.5 \pm 1.8\%$ and $75.3 \pm 1.9\%$ ($n=4$), and the fully facilitated train by $47.8 \pm 0.6\%$ and $67.0 \pm 0.8\%$ ($n=4$), respectively, without affecting the amplitude distribution of spontaneous e.j.ps. (s.e.j.ps). Therefore, presynaptic α_2-adrenoceptors can modify neurotransmitter release when N-type calcium channels are blocked.

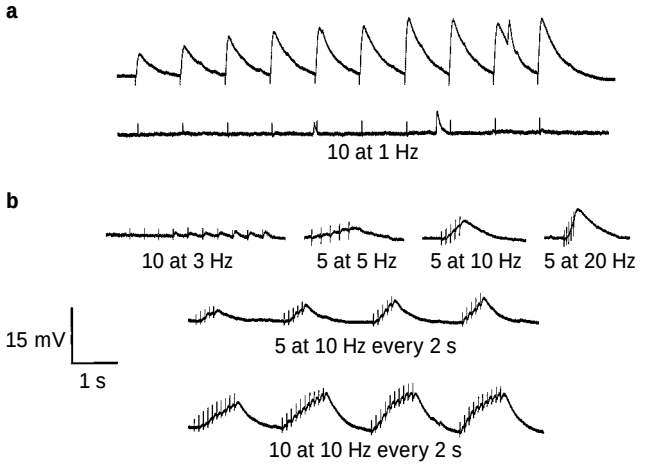

Figure 1 Effect of ω-conotoxin GVIA on e.j.ps in the guinea-pig vas deferens. (a) E.j.ps evoked by trains of 10 stimuli at 1 Hz before (upper panel) and 30 min after ω-conotoxin GVIA (10 nM) (lower panel). After 20–30 min superfusion with ω-conotoxin GVIA, all the e.j.ps in a train were abolished. (b) Frequency-dependent facilitation of e.j.ps evoked in the presence of a high concentration of the irreversible N-type calcium channel blocker ω-conotoxin GVIA (1 μM). The e.j.ps remaining after the application of ω-conotoxin GVIA presumably reflect neurotransmitter release evoked by calcium-entry through calcium channels other than the N-type and this phenomenon has been termed 'residual release'.

Effect of the α_2-adrenoceptor antagonist yohimbine

When the α_2-adrenoceptor antagonist yohimbine (1 μM) was applied there was a significant increase in 'residual release' at all stimulation frequencies employed (10, 20 and 50 Hz). The effect of yohimbine (1 μM) on e.j.ps evoked by trains of 10 stimuli at 10 Hz delivered every 2 s is shown in Figure 3. Control e.j.ps evoked by the third train of 10 stimuli at 10 Hz delivered every 2 s had a mean amplitude of 13.5 ± 4 mV ($n=4$), and in the presence of 1 μM yohimbine the amplitude was 18.5 ± 4 mV ($n=4$) ($P<0.01$), a 37% increase. Control e.j.ps evoked by the fifth train of 5 stimuli at 20 Hz delivered every 2 s had a mean amplitude of 9.3 ± 1.7 mV ($n=4$), and in the presence of yohimbine (1 μM) the amplitude was 12.6 ± 2.0 mV ($n=4$) ($P<0.01$), a 35% increase. However, there was no effect on the first train in the series. For example, control e.j.ps evoked by the first train of 5 stimuli at 20 Hz delivered every 2 s had a mean amplitude of 4.6 ± 0.6 mV and 20 min after the addition of yohimbine (1 μM) e.j.ps had mean amplitudes of 4.6 ± 0.6 mV ($n=4$). Control e.j.ps evoked by the first train of 10 stimuli at 10 Hz delivered every 2 s had a mean amplitude of 5.5 ± 0.6 mV and 20 min after the addition

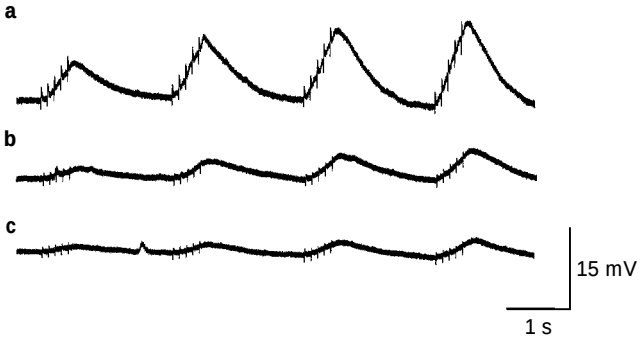

Figure 2 Effect of clonidine on ω-conotoxin GVIA-resistant e.j.ps in the guinea-pig vas deferens. E.j.ps were evoked by trains of 5 stimuli at 10 Hz delivered every 2 s>1 h after ω-conotoxin GVIA (100 nM). (a) Control; (b) 30 min after clonidine (30 nM) in the continued presence of ω-conotoxin GVIA; (c) 30 min after clonidine (100 nM) in the same cell.

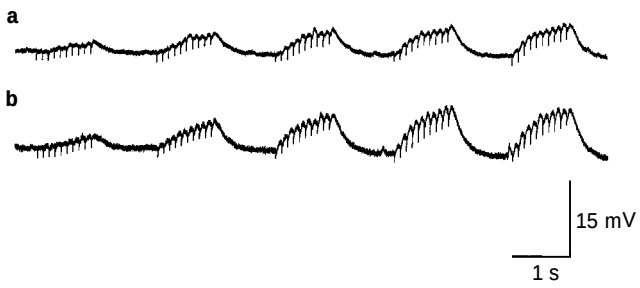

Figure 3 Effect of yohimbine on ω-conotoxin GVIA-resistant e.j.ps in the guinea-pig vas deferens. E.j.ps were evoked by trains of 10 stimuli at 10 Hz delivered every 2 s>1 h after ω-conotoxin GVIA (100 nM). (a) Control; (b) 30 min after yohimbine (1 μM) in the continued presence of ω-conotoxin GVIA.

of yohimbine (1 μM) e.j.ps had mean amplitudes of 5.5 ± 0.5 mV ($n=4$). At a stimulation frequency of 50 Hz, the vas deferens often contracted when yohimbine was present (even in the presence of nifedipine, results not shown). These results imply that both noradrenaline and ATP are released in the presence of high concentrations of ω-CTX GVIA and that 'residual release' is subject to α_2-autoinhibition.

Prostaglandin E_2

Bath application of prostaglandin E_2 (30 nM) caused a rapid inhibition of 'residual release' at all stimulation frequencies employed. The effect of prostaglandin E_2 on e.j.ps evoked by trains of 5 stimuli at 50 Hz delivered every 2 s is shown in Figure 4. Fully facilitated e.j.ps evoked by 10 trains of 5 stimuli at 50 Hz delivered every 2 s, in the presence of ω-CTX GVIA (100 nM), had a mean amplitude of 18.7 ± 0.5 mV, and 20 min after prostaglandin E_2 (30 nM) had a mean amplitude of 4.7 ± 0.4 mV ($n=4$), an inhibition of some 75%. Fully facilitated e.j.ps evoked by 10 trains of 5 stimuli at 20 Hz delivered every 2 s, in the presence of ω-CTX GVIA (100 nM), had a mean amplitude of 14.2 ± 1.5 mV, and 20 min after prostaglandin E_2 (30 nM) had a mean amplitude of 4.1 ± 0.6 mV ($n=4$), an inhibition of 71%. There was no detectable effect on s.e.j.ps. These results show that presynaptic prostaglandin receptors can inhibit 'residual release' when N-type calcium channels are irreversibly blocked.

Neuropeptide Y

Bath application of neuropeptide Y (100 nM) also caused inhibition of 'residual release' (Figure 5). Fully facilitated e.j.ps evoked by 10 trains of 5 stimuli at 10 Hz delivered every 2 s, in the presence of ω-CTX GVIA (100 nM), had a mean amplitude of 8.5 ± 0.8 mV, and 20 min after neuropeptide Y had a mean amplitude of 4.0 ± 0.6 mV ($n=4$), a 56% inhibition. Fully facilitated e.j.ps evoked by 10 trains of 5 stimuli at 20 Hz delivered every 2 s, in the presence of ω-CTX GVIA (100 nM), had a mean amplitude of 12.2 ± 4.5 mV, and 20 min after neuropeptide Y had a mean amplitude of 6.7 ± 3 mV ($n=4$), a 45% inhibition. Therefore presynaptic neuropeptide Y receptors are also functional when N-type calcium channels are blocked.

Effect of 4-aminopyridine on 'residual release'

The non-selective K^+ channel blocker, 4-aminopyridine (4-AP), potently enhances neurotransmitter release from many nerve terminals. 4-AP lengthens the duration of the nerve terminal action potential thereby increasing depolarization-induced Ca^{2+} entry. Bath application of 4-AP (100 μM) produced a powerful potentiation of 'residual release' at all frequencies of stimulation (Figure 6) and even partially reversed the ω-CTX GVIA block seen at 1 Hz (see Figure 6b). When trains of stimuli were delivered at 20 or 50 Hz, neurotransmitter release frequently evoked muscle contractions (even in the presence of 10 μM nifedipine). Fully facilitated e.j.ps evoked by trains of 10 stimuli at 1 Hz in the presence of ω-CTX GVIA (100 nM) had a mean amplitude of 0.3 ± 0.1 mV ($n=4$). Twenty minutes after 4-AP (100 μM) the mean amplitude of the fully facilitated e.j.ps was 3.3 ± 0.4 mV ($n=4$).

Discussion

Can 'residual release' be modulated through presynaptic receptors?

The molecular processes underlying presynaptic receptor-mediated inhibition in neurones are poorly understood but three different mechanisms have been suggested: (i) hyperpolarization of the nerve terminals caused by an increase in K^+ conductance, (ii) inhibition of voltage-sensitive calcium channels, thereby decreasing depolariza-

Figure 5 Effect of neuropeptide Y on ω-conotoxin GVIA-resistant e.j.ps in the guinea-pig vas deferens. E.j.ps were evoked by trains of 5 stimuli at 20 Hz delivered every 2 s > 1 h after ω-conotoxin GVIA (100 nM). (a) Control; (b) 30 min after neuropeptide Y (100 nM) in the continued presence of ω-conotoxin GVIA.

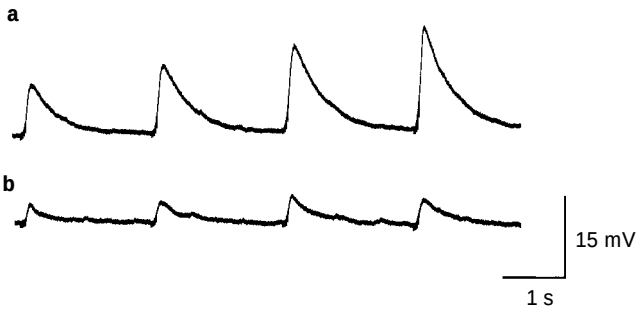

Figure 4 Effect of prostaglandin E_2 on ω-conotoxin GVIA-resistant e.j.ps in the guinea-pig vas deferens. E.j.ps were evoked by trains of 5 stimuli at 50 Hz delivered every 2 s > 1 h after ω-conotoxin GVIA (100 nM). (a) Control; (b) 30 min after prostaglandin E_2 (30 nM) in the continued presence of ω-conotoxin GVIA.

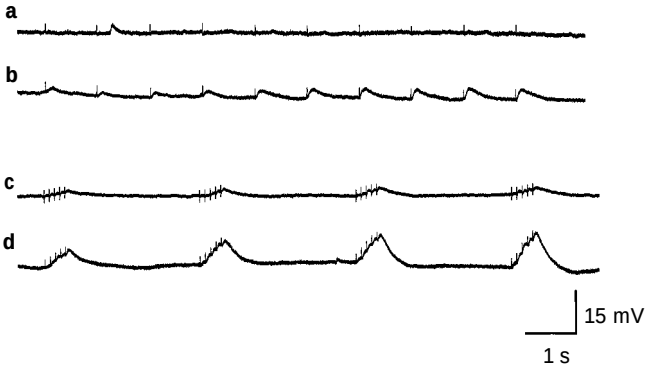

Figure 6 Effect of 4-aminopyridine (4-AP) on ω-conotoxin GVIA-resistant e.j.ps in the guinea-pig vas deferens. (a) Control e.j.ps were evoked by trains of 10 stimuli at 1 Hz; (b) 20 min after 4-AP (100 mM). (c) Control e.j.ps were evoked by trains of 5 stimuli at 10 Hz delivered every 3 s. (d) 20 min after 4-AP (100 μM).

tion-induced calcium entry and (iii) interference at some stage post calcium entry into the nerve terminal but before exocytosis occurs. The evidence presented so far has been mixed but a view held by many is that G protein-coupled autoreceptors inhibit calcium influx into the nerve terminal by modifying calcium entry through N - type calcium channels (Hedqvist, 1976; Akasu *et al*., 1990; Xiang *et al*., 1990; Toth *et al*., 1993). In the present study we examined the effects of three different presynaptic receptor agonists, namely the α_2-adrenoceptor agonist clonidine, prostaglandin E_2 and neuropeptide Y on 'residual release', i.e. neurotransmitter release occurring when N-type calcium channels were irreversibly blocked. In some experiments we also studied the effects of yohimbine to determine whether autoinhibition was operating under the conditions of these experiments.

Mechanisms underlying presynaptic receptor-mediated inhibition

The α_2-adrenoceptor agonist clonidine markedly inhibited 'residual release' at all stimulation frequencies employed (see Figure 2), the degree of inhibition being most marked at the beginning of the train and less pronounced during and between successive trains. Therefore α_2-adrenoceptor-induced inhibition occurs when N-type calcium channels are blocked, suggesting that the mechanism of inhibition, under the conditions of the experiments, is not a reduction of calcium influx through N-type calcium channels. Recent studies by Brock and Cunnane (1995) on the guinea-pig vas deferens suggested that hyperpolarization of the nerve terminal or inhibition of calcium entry through voltage-gated N-type calcium channels are not the mechanisms used by α-autoinhibition. The authors showed that a K^+ conductance, which was sensitive to a reduction in the extracellular calcium concentration and to ω-CTX GVIA (and therefore an indirect measure of calcium entry into nerve terminals), in sympathetic nerve terminals was unaffected by clonidine at concentrations which markedly inhibited or abolished neurotransmitter release.

The α_2-adrenoceptor antagonist yohimbine had no effect on the first train in a series but induced a significant potentiation of neurotransmitter release evoked by subsequent trains (Figure 3). This effect fits in well with the idea that preceding nerve activity is required before negative feedback mechanisms can be activated, i.e. release must first occur for subsequent release to be inhibited (Story *et al*., 1981). We have previously shown that the effects of clonidine can be antagonized by yohimbine over a wide range of concentrations and stimulation frequencies (Brock & Cunnane, 1990; Brock *et al*., 1990). The importance of the finding that yohimbine potentiates residual release is that it demonstrates unequivocally that noradrenaline is released when N-type channels are blocked. Therefore these results indicate that 'residual release' is subject to α_2-autoinhibition, and that both noradrenaline and ATP are released in the presence of high concentrations of ω-CTX GVIA. It should be remembered that, under the conditions of these experiments, N-type calcium channels were irreversibly blocked by ω-CTX GVIA. Therefore presynaptic modulation by neuronally-released noradrenaline, acting through presynaptic α_2-adrenoceptors, must occur by mechanisms other than regulation of calcium entry through N-type channels.

Little is known about the mechanism whereby prostaglandin E_1 (PGE_1) and PGE_2 inhibit neurotransmitter release, but it is thought that these agents inhibit neurotransmitter release by similar mechanisms to presynaptic α-autoreceptors. PGE_2 potently inhibited 'residual release' at all frequencies of stimulation (Figure 4). Therefore PGE_2 inhibits release by mechanisms other than inhibition of calcium entry through N-type calcium channels. In the guinea-pig vas deferens, focal extracellular recording has indicated that both PGE_1 and PGE_2 strongly inhibit ATP release without affecting the nerve terminal impulse (NTI) (Brock & Cunnane, 1987; 1996). Therefore, both sets of results provide strong evidence that PGE_2 inhibits neurotransmitter release by interfering with depolarization-secretion coupling.

It is generally accepted that neuropeptide Y (NPY) reduces calcium entry into the nerve terminal by selective inhibition of N-type calcium channels. Investigations of calcium currents in rat cultured superior cervical ganglia provided direct evidence that the presynaptic inhibition produced by NPY is associated with a reduction in calcium influx, due to selective inhibition of N-type calcium channels (Toth *et al*., 1993). More recents studies on calcium currents in rat cultured dentate granule cells have reached similar conclusions (McQuiston *et al*., 1996). NPY inhibited 'residual release' by 45% (Figure 5), demonstrating that in the sympathetic nerves innervating the guinea-pig vas deferens NPY-induced presynaptic inhibition is not mediated solely by N-type calcium channel inhibition.

Effects of the potassium channel blocker 4-aminopyridine

The potassium channel blocker 4-aminopyridine (4-AP) was used to establish whether procedures other than blockade of presynaptic receptors (e.g. with yohimbine) could enhance neurotrasmitter release when N-type calcium channels were blocked. 4-AP exhibited very potent effects on 'residual release'. Neurotransmitter release was even evoked by trains of stimuli at 1 Hz in the presence of 4-AP and ω-CTX GVIA (Figure 6). This can be explained by the broadening effect of 4-AP on the nerve action potential, induced by blocking various K^+ channels, allowing increased calcium influx into the nerve terminal. Augustine (1990) estimated that in the squid giant axon a 30% increase in presynaptic action potential duration would increase the total amount of calcium entry by about 230%. A similar increase in calcium entry into the nerve terminals through ω-grammotoxin SIA-sensitive calcium channels in the guinea-pig vas deferens would explain the marked potentiation of 'residual release' by 4-AP. These experiments demonstrate that even when N-type calcium channels are blocked, modification of K^+ channel activity can still powerfully increase neurotransmitter release.

In conclusion, in postganglionic sympathetic nerves innervating the guinea-pig vas deferens, (i) 'residual release' of ATP is subject to α_2-autoinhibition through the co-release of noradrenaline, (ii) noradrenaline release can be triggered by calcium channels other than the N-type and (iii) under these experimental conditions, clonidine, PGE_2 and NPY do not mediate their presynaptic inhibitory effects through modulation of N-type calcium channels. The intriguing possibility remains that presynaptic agonists may modulate neurotransmitter release at a step post calcium entry in nerve terminals.

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