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Effects of N- and L-type calcium channel antagonists and (±)-Bay K8644 on nerve-induced catecholamine secretion from bovine perfused adrenal glands

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1 The effects of N- and L-type calcium channel antagonists and (±)-Bay K8644 on catecholamine release from chromaffin cells and acetylcholine release from splanchnic nerve terminals was investigated in bovine perfused adrenal glands.

2 Adrenal glands were perfused retrogradely and preloaded with [^{3}H]-choline. Subsequent efflux of ^{3}H-labelled compounds was taken as an index of acetylcholine release from the splanchnic nerve terminals. Noradrenaline and adrenaline release from the glands was measured by h.p.l.c. with electrochemical detection.

3 A maximally effective frequency of field stimulation of the adrenal nerves, 10 Hz, induced release of catecholamines and ^{3}H-labelled compounds. Tetrodotoxin (1 μM) abolished release of both catecholamines and ^{3}H-labelled compounds. A combination of mecamylamine (5 μM) and atropine (1 μM) inhibited nerve-induced catecholamine release by about 75% but did not inhibit release of ^{3}H-labelled compounds. Reducing the concentration of extracellular calcium 5 fold to 0.5 mM inhibited nerve-induced catecholamine release by 80% and release of ^{3}H-labelled compounds by 50%.

4 (±)-Bay K8644 (1 μM), nitrendipine (1 μM), ω-conotoxin-GVIA (10 nM) and the combination of nitrendipine and ω-conotoxin-GVIA each had no effect on nerve-induced release of ^{3}H-labelled compounds.

5 (±)-Bay K8644 (1 μM) potentiated nerve-induced catecholamine release by 75%. Nitrendipine (1 μM) reduced release by 20% but this did not reach statistical significance. ω-Conotoxin-GVIA (10 nM) reduced nerve-induced catecholamine release by 75%, while the combination of ω-conotoxin-GVIA and nitrendipine reduced release to the same extent as ω-conotoxin-GVIA alone.

6 Exogenous acetylcholine perfusion through the glands produced a concentration-dependent increase in catecholamine release. The maximally effective concentration of acetylcholine for catecholamine release was $\geqslant$300 μM, while 30 μM acetylcholine gave comparable catecholamine release to that obtained with 10 Hz field stimulation.

7 (±)-Bay K8644 (1 μM), nitrendipine (1 μM) and ω-conotoxin-GVIA (10 nM) each had no significant effect on catecholamine release evoked by perfusion of the gland with either a near maximally effective concentration of acetylcholine, 100 μM, or with the lower concentration of 30 μM.

8 The results show that the ω-conotoxin-GVIA-sensitive N-type voltage-sensitive calcium channels located on the chromaffin cells are largely responsible for catecholamine release induced by nerve stimulation in bovine adrenal glands. In contrast, N-type calcium channels are not involved in catecholamine release induced by exogenous acetylcholine. L-type voltage sensitive calcium channels do not play a major role in nerve-induced or exogenously applied acetylcholine-induced catecholamine release. However, the L-type calcium channels do have the potential to augment powerfully nerve-induced catecholamine release. N- and L-type calcium channels do not play a major role in the presynaptic release of acetylcholine.

Keywords: ω-Conotoxin-GVIA; (±)-Bay K8644; nitrendipine; catecholamine release; calcium channels; splanchnic nerve; chromaffin cells; adrenal gland

Introduction

Extracellular calcium is critical for hormone release (Douglas & Rubin, 1961). Pharmacological, biophysical and molecular criteria have identified multiple subtypes of voltage-sensitive calcium channels which allow the influx of calcium into excitable cells (Olivera *et al*., 1994). Several types of calcium channels are present on adrenal chromaffin cells but the role each plays in the catecholamine release process remains controversial. The relative number and types of calcium channels and their contribution to catecholamine secretion show marked species variation. Cat chromaffin cells possess ω-conotoxin-GVIA-sensitive N-type calcium channels and dihydropyridine-sensitive L-type calcium channels which each carry 50% of the calcium current (Albillos *et al*., 1994). However, Lopez *et al*. (1994a) have shown that the L-type calcium channels dominate the exocytotic process in cat chromaffin cells. Rat chromaffin cells on the other hand possess not only N- and L-type calcium channels but also ω-agatoxin-IVA-sensitive P-type calcium channels (Gandia *et al*., 1995). Kim *et al*. (1995) showed that both L- and N-type calcium currents are recruited during exocytosis from rat chromaffin cells.

The situation in bovine adrenal chromaffin cells is more complex. Bovine chromaffin cells possess not only the L- (Artalejo *et al*., 1991), N-(Hans *et al*., 1990; Artalejo *et al*., 1992a) and P-type (Mintz *et al*., 1992; Gandia *et al*., 1993) calcium channels but also ω-conotoxin-MVIIC-sensitive Q-type voltage sensitive calcium channels (Lopez *et al*., 1994b). Artalejo and colleagues (1994) simultaneously measured changes in membrane capacitance, as an assay of catecholamine secretion, and calcium currents in bovine cultured chro-

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maffin cells. They found that L-, N-, and P-type calcium channels could each contribute to secretion induced by electrical depolarization, but that the L-type channels could increase secretion to a greater extent than either of the other channels for a given amount of calcium current. López *et al.* (1994b) showed that Q- and L-type calcium channels dominate the control of catecholamine secretion induced by potassium depolarization of bovine cultured chromaffin cells. In addition the study showed that L-, N-, P- and Q-type calcium channels all contribute to whole cell calcium currents.

Most investigations into the role of different calcium channels in adrenal catecholamine release have used bovine cultured adrenal chromaffin cells where catecholamine secretion is induced by the application of exogenous agonists or electrical depolarization. These methods have two significant limitations. Firstly, the application of exogenous agonist or electrical depolarization to induce catecholamine secretion does not closely mimic the normal release of neurotransmitters from presynaptic splanchnic nerve terminals onto the chromaffin cells. Secondly, the process of culturing chromaffin cells may alter the number and relative percentage of each calcium channel subtype contributing to cell function when compared with the cells *in situ*.

The aim of the present study was to use freshly prepared, intact, retrogradely perfused bovine adrenal glands to examine the role of L- and N-type calcium channels in catecholamine secretion. In particular, the role of the different calcium channels in catecholamine release induced by splanchnic nerve stimulation was compared to those involved when perfusing the glands with exogenous acetylcholine. In addition, the types of presynaptic calcium channels that control acetylcholine release from splanchnic nerve terminals was investigated. A preliminary account of this work has been published (O'Farrell & Marley, 1995a).

Methods

[^{3}H]-choline loading of bovine adrenal glands

Adult bovine adrenal glands (weight 11.5 ± 0.3 g, mean $\pm$ s.e.mean, range 5.1 – 20.5 g, $n = 167$) were collected from a local abattoir within 15 – 30 min of death from animals passed for human consumption. Adrenal glands were prepared for retrograde perfusion by the method described previously by Marley *et al.* (1993). The perfusion buffer comprised (mM): NaCl 118, $NaHCO_3$ 25, D-glucose 11.7, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, and $MgCl_2$ 1.18 and was continuously gassed with 5% CO_2-95% O_2 and maintained at 37°C. The technique of [^{3}H]-choline loading described by Wessler *et al.* (1991) was modified for the bovine perfused adrenal gland preparation. Glands were perfused at 15 ml min^{-1} and equilibrated for 15 min. After equilibration, maximal field stimulation of splanchnic nerves (5 min, 10 Hz, 1.5 ms square-wave pulses, 120 – 130 mA, Grass SS44 stimulator) was applied to facilitate the uptake of [^{3}H]-choline into the acetylcholine pool of the nerve terminals. The perfusion rate was then reduced to 3 ml min^{-1} and [^{3}H]-choline (0.1 μM, specific activity, 17.2 Ci.mmol^{-1}) added to 20 ml of perfusion buffer which was recycled through the glands for 20 min. Glands were then perfused at 15 ml min^{-1} with fresh choline-free buffer for 45 min and then with buffer containing 10 μM unlabelled choline for the remainder of the experiment to limit the reuptake of released [^{3}H]-choline. After 60 min of perfusion following the [^{3}H]-choline loading, glands were stimulated for 3 min (10 Hz, 1.5 ms square-wave pulses, 120 – 130 mA) to reduce [^{3}H]-choline adsorption to extracellular surfaces in the glands. After a further 27 min of perfusion to flush out non-neuronal [^{3}H]-choline, 3 min fractions were collected into tubes on ice. After each fraction was collected, a 1 ml aliquot was immediately removed, acidified with 2 M perchloric acid to give a final perchloric acid concentration of 0.4 M and stored at 4°C until assayed for the concentration of adrenaline and noradrenaline. At the completion of each experiment, a 4 ml aliquot of each fraction was removed and prepared for analysis of the total amount of ^{3}H-labelled compounds released.

Characterization of the release of ^{3}H-labelled compounds from perfused adrenal glands

In some experiments the nature of the released radioactivity was characterized. In these studies the cholinesterase inhibitor neostigmine (10 μM) was added to the perfusion buffer together with unlabelled choline after the first 45 min of washout and for the remainder of the experiment to inhibit the breakdown of [^{3}H]-acetylcholine to [^{3}H]-choline. Neostigmine was not present in any of the experiments in which catecholamine and the release of ^{3}H-labelled compounds were studied, since it would prolong the duration of action of acetylcholine on the chromaffin cells and therefore affect the release of catecholamines.

Aliquots (10 ml) of perfusate from fractions 1 and 3 were frozen in liquid nitrogen and then freeze dried. The powder was redissolved in 2 ml of high performance liquid chromatography (h.p.l.c.) mobile phase (see below), spun in a centrifuge (10 min, 2000 *g*) and the supernatant collected. Protein was removed from the solution by centrifuge filtration (60 min, 2000 *g*, 13 mm, 10 000 molecular weight acrylic housing centrifuge filter-ultra, AdeLab). A 1 ml aliquot of the filtrate was then applied to an h.p.l.c. column (5 μm-ODS2, 250 × 4.6 mm, Spherisorb). The mobile phase comprised (mM): $(CH_3)_4NCl$ 5.45, KH_2PO_4 50 and sodium octanesulphonate 1.24 and was delivered at 1 ml min^{-1} (See Bertrand *et al.*, 1990) and 1 min fractions collected for 60 min. A 0.5 ml aliquot was removed from each fraction, mixed with 5 ml of liquid scintillant (Emulsifier Safe, Packard) and radioactivity counted on a liquid scintillation analyser (2000 CA Tri-carb, United Technologies Packard).

To calibrate the elution positions of choline and acetylcholine in these chromatographic conditions, this procedure was repeated with buffer which was perfused through glands which had undergone the recycling procedure in the absence of [^{3}H]-choline. Before injection onto the h.p.l.c. column, the solution was spiked with either synthetic [^{3}H]-choline or [^{14}C]-acetylcholine. [^{3}H]-choline eluted as a sharp, symmetric peak separated from the void volume with a retention time of 10 min, while [^{14}C]-acetylcholine eluted much later, as a broad biphasic peak over a period of 22 – 36 min (Figure 1a). The latter elution profile was a chromatographic artefact, the result of the synthetic [^{14}C]-acetylcholine interacting with substances released from the stimulated adrenal glands into the perfusion buffer: fresh buffer that had been dried down, reconstituted and spiked with labelled acetylcholine produced a single, nearly symmetric peak with an elution time of 40 – 42 min (data not shown). Recovery of both labelled choline and acetylcholine from the chromatography was essentially quantitative (103% and 90%, respectively).

Measurement of catecholamines and ^{3}H-labelled compounds

Adrenaline and noradrenaline release were measured by h.p.l.c. with electrochemical detection as described by Livett *et al.* (1987). For determination of the release of ^{3}H-labelled compounds, a 4 ml aliquot from each 3 min fraction was mixed with liquid scintillant (Ultima Gold XR, Packard) and total radioactivity measured on a liquid scintillation analyser (2000 CA Tri-carb, United Technologies Packard).

Experimental design

Due to large variations in adrenal gland size and shape it was necessary for each gland to act as its own control (see Marley *et al.*, 1993). Hence, the experimental design involved two periods of stimulation during the 3rd (S1) and 23rd (S2)

fractions. Nerve-induced secretion involved maximal stimulation of the adrenal nerves (3 min, 10 Hz) during both the first and second periods of stimulation (S1 and S2). In experiments where exogenous acetylcholine was the stimulus, a near maximally effective concentration of acetylcholine (3 min, 100 μM) was perfused through the gland during S1 while the concentration of acetylcholine varied between 3 and 300 μM during S2. Evoked release of catecholamines from chromaffin cells and radioactivity from splanchnic nerves was calculated from the total release during the 3 min stimulation period and the subsequent 15 min, corrected for basal release during this time. The evoked secretion during S2 was then expressed as a fraction of the control response during S1 and will be referred to as the S2/S1 ratio. Some glands failed to yield good responses to stimulation (see Marley *et al*., 1993). Those in which the evoked release in S1 was less than twice the basal rate of secretion in the case of catecholamine release or with evoked release which did not exceed basal in the case of release of ^{3}H-labelled compounds were not included for analysis. Vehicle, the drug to be tested or low calcium buffer were perfused continuously during fractions 11–24 unless otherwise indicated. Thus, the second stimulation period was evoked after 36 min exposue to the vehicle, drug or low calcium buffer. In the case of tetrodotoxin, which was only present during fractions 21–24, the second stimulation period was evoked after a 6 min exposure of the gland to the toxin.

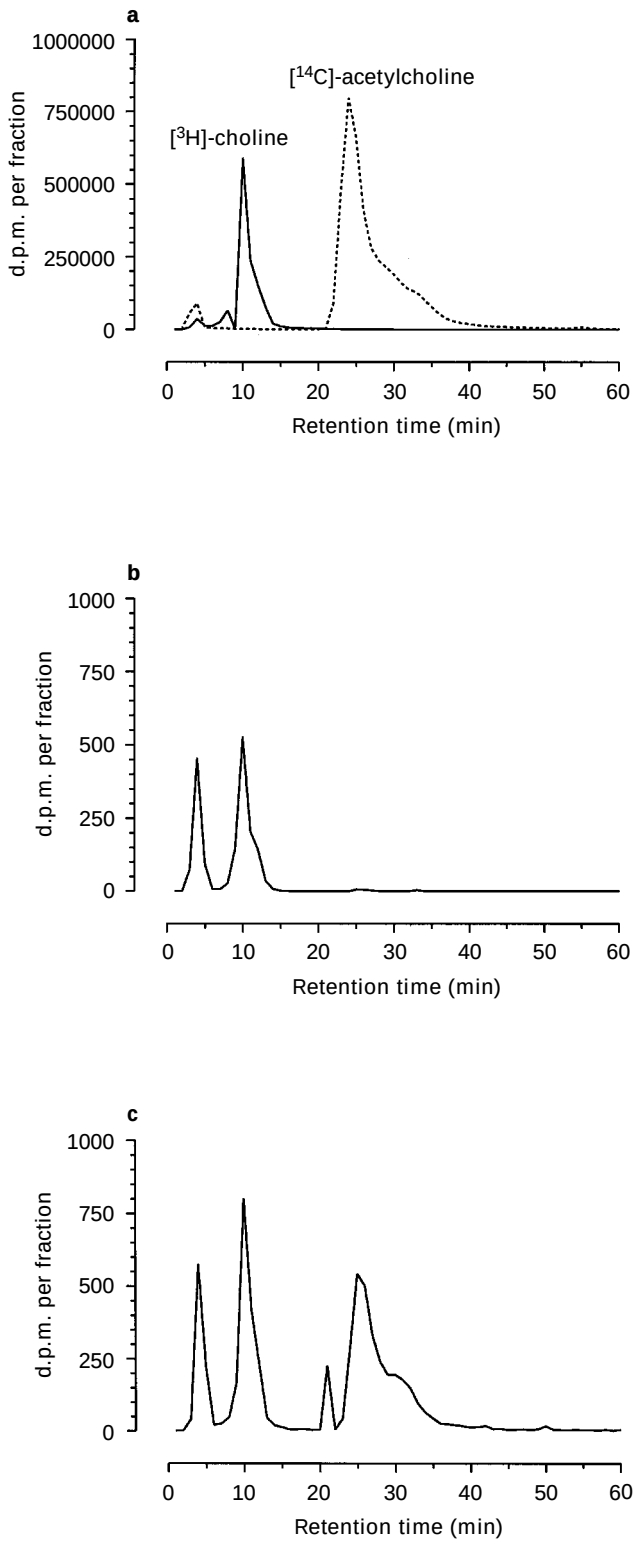

Figure 1 Characterization of [^{3}H]-choline and [^{14}C]-acetylcholine standards (a) and ^{3}H-labelled compounds in the perfusate of glands preloaded with [^{3}H]-choline (b, c) by h.p.l.c. followed by liquid scintillation spectrometry. (a) [^{3}H]-choline eluted as a sharp symmetrical peak with a retention time of 10 min while synthetic [^{14}C]-acetylcholine eluted as a broad biphasic peak over a period of 22–36 min. The radiochromatograms from basal (b) and 10 Hz nerve stimulation (c) fractions are from the same gland with the perfusion buffer containing 10 μM neostigmine and are representative of 4 experiments.

Data presentation and statistics

Data are presented as mean ± s.e.mean of the S2/S1 ratios for the indicated number of glands. Statistical significance was determined by one-way analysis of variance (ANOVA), followed by Dunnett's multiple comparisons test when $P<0.05$.

Drugs

Tetrodotoxin was from Sapphire Bioscience, Australia. Nitrendipine and (±)-Bay K8644 (1,4-dihydro-2,6-dimethyl-5-nitro-4-[2-(trifluoromethyl)-phenyl]-3-pyridine carboxylic acid methyl ester) were from Research Biochemicals International, U.S.A. Acetylcholine chloride, choline chloride, atropine sulphate and mecamylamine hydrochloride were from Sigma Chemical Co., U.S.A. ω-Conotoxin-GVIA was from Auspep Pty Ltd, Australia. [Methyl-^{3}H]-choline chloride (specific activity 86 Ci mmol^{-1}) and [1-^{14}C]-acetylcholine (specific activity 60 mCi mmol^{-1}) were from Amersham Life Science. The buffer used for the low calcium experiments had the same composition as normal perfusion buffer with the $CaCl_2$ concentration reduced to 0.5 mM from 2.5 mM. Nitrendipine and (±)-Bay K8644 were dissolved in dimethylsulphoxide before dilution in buffer. Appropriate solvent (vehicle) controls were performed in all experiments.

Results

Release of ^{3}H-labelled compounds from [^{3}H]-choline loaded adrenal glands in the presence of neostigmine

To characterize the nature of released ^{3}H-labelled compounds, some glands were perfused in the presence of neostigmine to protect any released acetylcholine from degradation (see Methods). In these glands, 80 ± 7% ($n=4$) of the ^{3}H-labelled compounds released during basal fractions eluted from the h.p.l.c. column in the position of [^{3}H]-choline with no detectable level of [^{3}H]-acetylcholine (Figure 1b). The remaining ^{3}H eluted in the void volume as an unidentified metabolite. Fractions collected during nerve stimulation contained ^{3}H-labelled compounds which eluted in the void volume, in the position of choline and in the position of acetylcholine (Figure 1c). In the stimulated fractions, 53 ± 7% ($n=4$) of the ^{3}H-labelled compounds eluted as [^{3}H]-choline and 30 ± 12% of the ^{3}H-labelled compounds eluted as [^{3}H]-acetylcholine. The majority of the increase in the release of ^{3}H-labelled compounds which occurred during nerve stimulation was due to an increase in acetylcholine release (compare Figure 1b and c).

Consequently, measurements of the evoked release of ^{3}H-labelled compounds, in the form of S2/S1 ratios, corrected for basal release (see Methods) were taken as an indication of the amount of acetylcholine release evoked by stimulation of the adrenal nerves.

Release of ^{3}H-labelled compounds following nerve stimulation in the absence of neostigmine

Experiments were performed to measure the release of ^{3}H-labelled compounds and catecholamines from the same glands. Consequently, in these experiments neostigmine was not present, since it would have perturbed the catecholamine release. In the absence of neostigmine, all the ^{3}H-labelled compounds released under both basal and nerve stimulation conditions eluted in the void volume and in the position of [^{3}H]-choline (data not shown). The failure to detect an increase in [^{3}H]-acetylcholine upon nerve stimulation in the absence of neostigmine may be due to the rapid hydrolysis of acetylcholine by cholinesterase in the preparation.

Basal secretion of ^{3}H-labelled compounds amounted to $24\,700 \pm 850$ d.p.m./3 min fraction ($n = 85$). Maximal nerve stimulation (10 Hz) for 3 min during S1 increased the efflux of ^{3}H-labelled compounds 1.96 ± 0.2 fold above basal ($n = 85$). The secretion of ^{3}H-labelled compounds induced by field stimulation was abolished when tetrodotoxin (1 μM) was present for 6 min before and during stimulation (Figure 2a). Reducing extracellular calcium levels to 0.5 mM from 2.5 mM for 36 min before and during S2 inhibited nerve-induced release of ^{3}H-labelled compounds by 50%. The combination of mecamylamine (5 μM) and atropine (1 μM) which together block nicotinic and muscarinic receptors present on chromaffin cells did not modify the release of ^{3}H-labelled compounds (Figure 2a).

Effects of (±)-Bay K8644, nitrendipine and ω-conotoxin-GVIA The activator of L-type calcium channels, (±)-Bay K8644 (1 μM) had no significant effect on the nerve-induced release of ^{3}H-labelled compounds (Figure 3). Nitrendipine (1 μM, an antagonist of L-type calcium channels), ω-conotoxin-GVIA (10 nM, an antagonist of N-type channels), and the combination of both antagonists had no effect on the S2/S1 ratio for the released ^{3}H-labelled compounds (Figure 3).

Nerve-induced catecholamine release

Control experiments Basal secretion of adrenaline and noradrenaline was 153 ± 7 and 50 ± 2 nmol/3 min, respectively, with an adrenaline : noradrenaline ratio of 3.3 ± 0.1 ($n = 85$). Maximal nerve stimulation (10 Hz, see Marley *et al.*, 1993) for 3 min during S1 increased secretion of adrenaline and noradrenaline 8.8 ± 0.7 fold and 15.7 ± 0.8 fold, respectively ($n = 85$). The increase in catecholamine release induced by maximal nerve stimulation was significantly lower than the 11.9 ± 0.9 and 28 ± 2 fold increases obtained by Marley and coworkers (1993) ($P < 0.001$, Welch's t test). In the two studies identical procedures were used to perfuse the adrenal glands except that in the present study the glands were loaded with [^{3}H]-choline which involved a period of reduced perfusion rate and a long washout period. Presumably this longer protocol resulted in slight deterioration of the glands and reduced secretion in response to nerve stimulation.

The secretion of noradrenaline and adrenaline induced by field stimulation (10 Hz, 3 min) was abolished when 1 μM tetrodotoxin was present for 6 min before the S2 stimulation period (Figure 2b and c). Reducing extracellular calcium levels from 2.5 mM to 0.5 mM inhibited nerve-evoked catecholamine release by about 80% (Figure 2b and c). The combination of the nicotinic and muscarinic receptor antagonists mecamylamine (5 μM) and atropine (1 μM) reduced the nerve-evoked catecholamine S2/S1 ratio by 75% (Figure 2), similar to that obtained by Marley *et al.* (1993).

Effects of (±)-Bay K8644, nitrendipine and ω-conotoxin-GVIA Figure 4 shows the effect of modifying calcium entry through L- and N-type calcium channels on nerve-induced

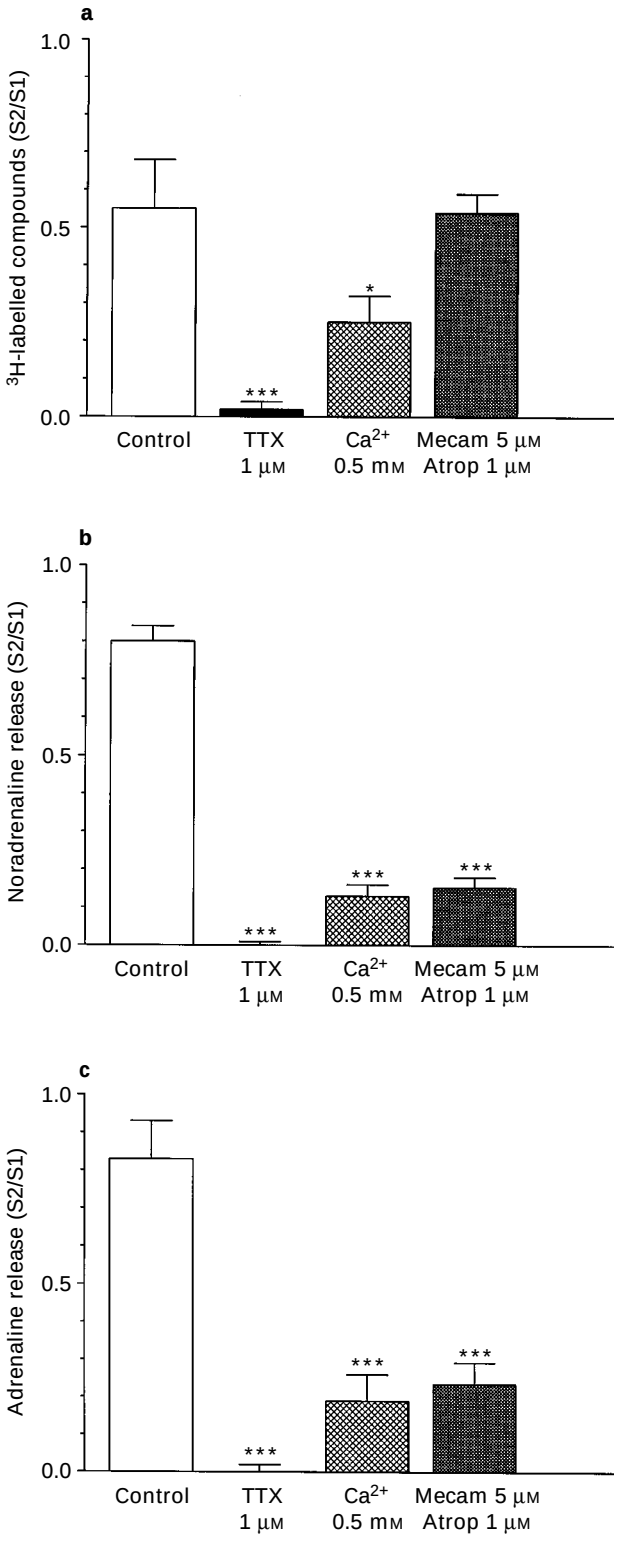

Figure 2 Effects of tetrodotoxin (TTX, 1 μM, solid columns), 0.5 mM extracellular calcium (Ca^{2+} 0.5 mM, hatched columns) or the combination of mecamylamine (Mecam, 5 μM) and atropine (Atrop, 1 μM, stippled columns), on release of ^{3}H-labelled compounds (a), noradrenaline (b) and adrenaline (c) induced by nerve stimulation at a frequency of 10 Hz. All drugs were present for 36 min before S2 except tetrodotoxin which was present for 6 min before S2. Results are means ± s.e.mean, from $n = 6-17$ bovine perfused adrenal glands. $^*P < 0.05$, $^{***}P < 0.001$ compared to control (open columns) Dunnett's test.

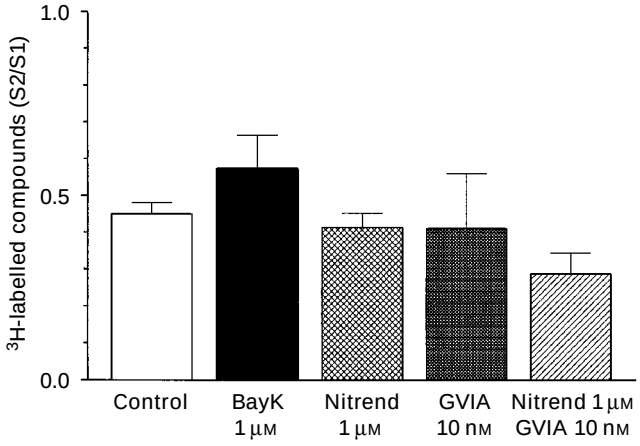

Figure 3 Effects of (±)-Bay K8644 (Bay K, 1 μM, solid columns), nitrendipine (Nitrend, 1 μM, hatched columns), ω-conotoxin-GVIA (GVIA, 10 nM, stippled columns) or the combination of nitrendipine (Nitrend, 1 μM) and ω-conotoxin-GVIA (GVIA, 10 nM, diagonally striped columns) on the S2/S1 ratio for the release of ^{3}H-labelled compounds from bovine retrogradely perfused adrenal glands induced by 10 Hz nerve stimulation. All drugs were present for 36 min before S2. Results are means±s.e.mean, from $n=6-15$ glands ($P>0.05$, ANOVA).

catecholamine release. Secretion of catecholamines was enhanced by approximately 75% by (±)-Bay K8644 (Figure 4, $P<0.001$ for noradrenaline and $P<0.05$ for adrenaline, Dunnett's test). The dihydropyridine nitrendipine at 1 μM decreased nerve-induced noradrenaline and adrenaline secretion by 21% and 33%, respectively, but this failed to reach statistical significance (Figure 4, $P>0.05$, Dunnett's test). ω-Conotoxin-GVIA (10 nM), reduced nerve-induced catecholamine release by approximately 75% (Figure 4). The combination of nitrendipine and ω-conotoxin-GVIA did not reduce the catecholamine S2/S1 ratio further than ω-conotoxin-GVIA alone (Figure 4).

Acetylcholine-induced catecholamine release

Control experiments Basal secretion of adrenaline and noradrenaline was 187.9±10.7 and 60.2±3.6 nmol/3 min, respectively, with an adrenaline : noradrenaline ratio of 3.5±0.2 ($n=87$). Perfusing glands with 100 μM acetylcholine in S1 increased adrenaline and noradrenaline release by 26.4±1.5 fold and 53.5±3.6 fold, respectively ($n=87$). When the concentration of acetylcholine in S2 was raised over the range 3–300 μM, there was a concentration-dependent increase in both adrenaline and noradrenaline release (Figure 5). The secretion of adrenaline and noradrenaline in S2, induced by 30 μM acetylcholine was 741±165 and 345±53 nmol/fraction ($n=6$), and this was not significantly different from that produced by 10 Hz nerve stimulation in S2 (556±66 and 338±53 nmol/fraction, $n=6$, $P>0.05$ Welch's t test). With 100 μM acetylcholine in S2, a concentration that is maximal for catecholamine secretion from bovine cultured chromaffin cells (e.g., see Schneider *et al*., 1977; Kilpatrick, 1984; O'Farrell & Marley, unpublished observations), the secretion of adrenaline and noradrenaline from perfused glands was not maximal, but during S2 produced secretion of 2111±323 and 1074±167 nmol/fraction ($n=8$). These responses were significantly greater than the maximal response to nerve stimulation (10 Hz in S2, see above; $P<0.05$, Welch's t test). The effects of calcium channel blockers on acetylcholine-induced catecholamine secretion during S2 was therefore assessed with 30 μM acetylcholine (a concentration that gave similar levels of secretion to the maximal nerve stimulation, 10 Hz) and 100 μM acetylcholine (a concentration that gives maximal catecholamine release from bovine cultured chromaffin cells).

Effects of (±)-Bay K8644, nitrendipine and ω-conotoxin-GVIA The dihydropyridines (±)-Bay K8644 (1 μM) and

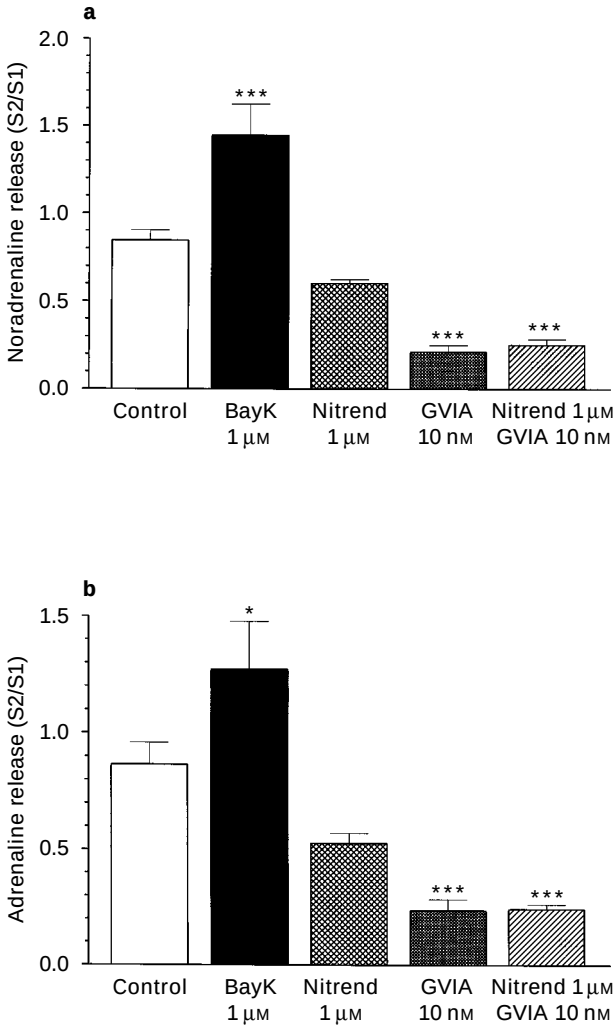

Figure 4 Effects of (±)-Bay K8644 (BayK, 1 μM, solid columns), nitrendipine (Nitrend, 1 μM, hatched columns) ω-conotoxin-GVIA (GVIA, 10 nM, stippled columns) or the combination of nitrendipine (Nitrend, 1 μM) and ω-conotoxin-GVIA (GVIA, 10 nM, diagonally striped columns) on noradrenaline (a) and adrenaline (b) secretion induced by 10 Hz nerve stimulation. All drugs were present for 36 min before S2. Results are means±s.e.mean, from $n=7-17$ glands. *$P<0.05$, ***$P<0.001$ compared to control, Dunnett's test.

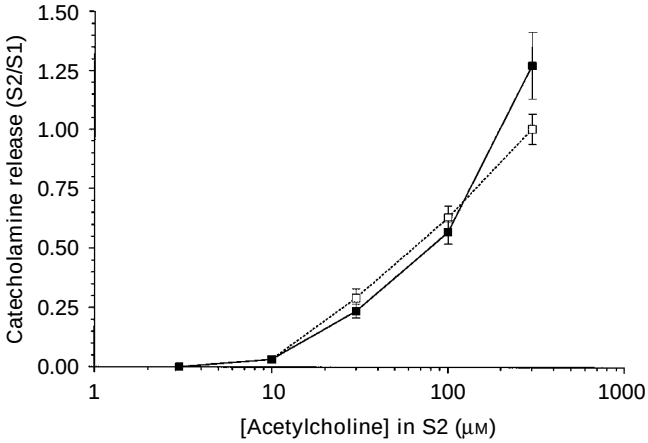

Figure 5 Acetylcholine concentration-effect curve for the release of noradrenaline (■) and adrenaline (□) from bovine retrogradely perfused adrenal glands. Acetylcholine at a concentration of 100 μM was perfused during S1, and the concentration of acetylcholine ranged from 3–300 μM in S2. Results are mean from $n=6-16$ glands; vertical lines show s.e.mean.

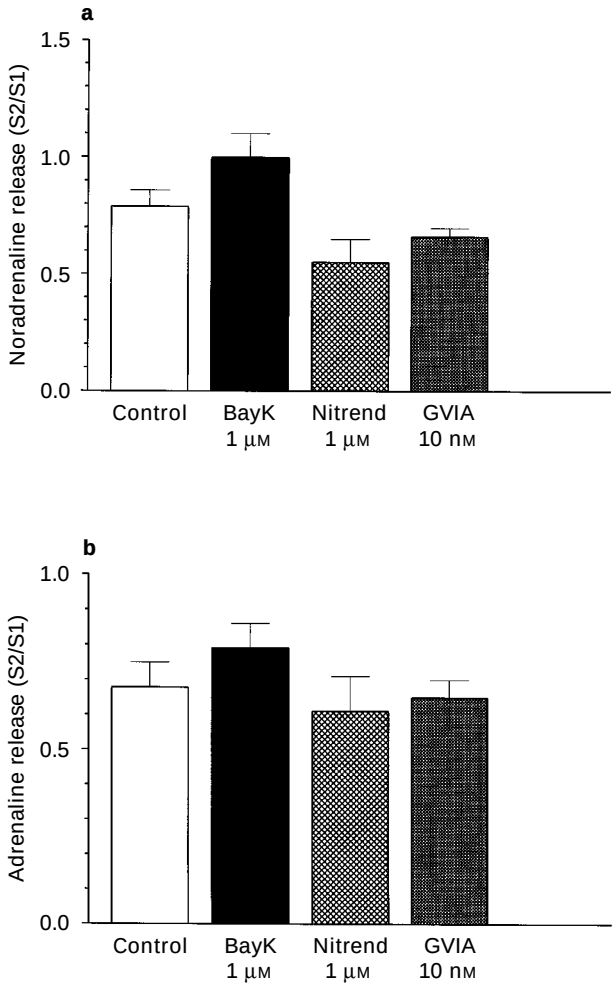

Figure 6 Effects of (±)-Bay K8644 (Bay K, 1 μM, solid columns), nitrendipine (Nitrend, 1 μM, hatched columns) and ω-conotoxin-GVIA (GVIA, 10 nM, stippled columns) on noradrenaline (a) and adrenaline (b) secretion evoked by retrograde perfusion of bovine adrenal glands with 100 μM acetylcholine in both S1 and S2. All drugs were present for 36 min before S2. Results are means ± s.e.mean, from $n=6-8$ glands, and are relative to the response to 100 μM acetylcholine in S1 (see Methods) ($P>0.05$, ANOVA).

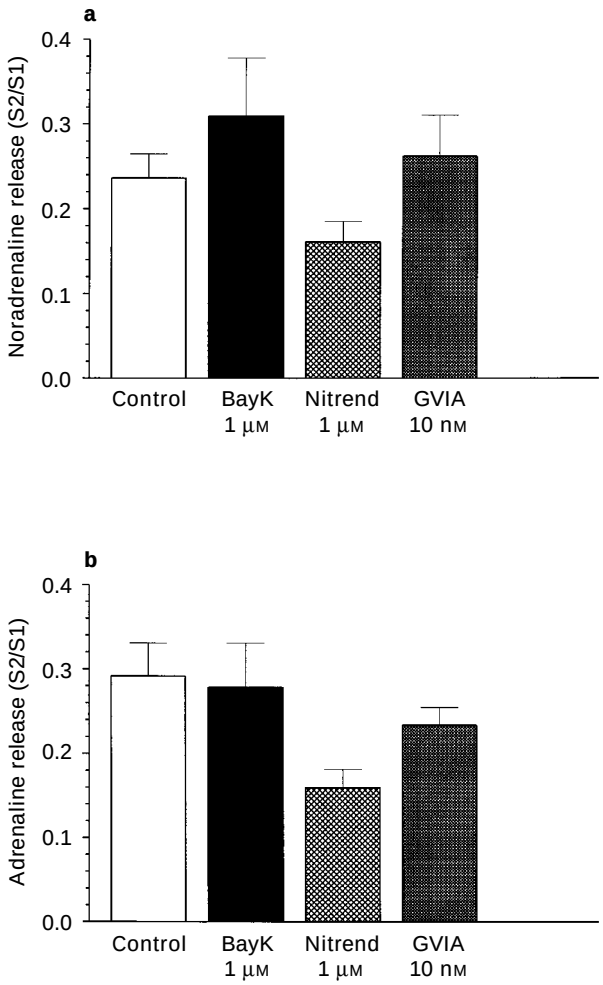

Figure 7 Effects of (±)-Bay 8644 (BayK, 1 μM, solid columns), nitrendipine (Nitrend, 1 μM, hatched columns) and ω-conotoxin-GVIA (GVIA, 10 nM, stippled columns) on noradrenaline (a) and adrenaline (b) secretion evoked by retrograde perfusion of bovine adrenal glands with 30 μM acetylcholine during S2. All drugs were present for 36 min before S2. Results are means ± s.e.mean, from $n=6-8$ glands and are relative to the response to 100 μM acetylcholine in S1 (see Methods) ($P>0.05$, ANOVA).

nitrendipine (1 μM) did not significantly affect the S2/S1 ratio for acetylcholine-induced catecholamine release when release in S2 was induced by 100 μM or 30 μM acetylcholine (Figures 6 and 7). In contrast to its effect on nerve-induced catecholamine release, ω-conotoxin-GVIA (10 nM) did not alter the S2/S1 ratio for catecholamine release induced by perfusing the glands with exogenous acetylcholine at either 100 μM or 30 μM (Figures 6 and 7).

Discussion

The present study shows that ω-conotoxin-GVIA sensitive N-type calcium channels are largely responsible for the influx of calcium required for nerve-induced catecholamine secretion from the bovine adrenal gland. ω-Conotoxin-GVIA acts postsynaptically on the chromaffin cells since the toxin did not affect the release of acetylcholine from the presynaptic nerve terminals (see below). In contrast, dihydropyridine-sensitive L-type calcium on their own play a minor role in nerve-induced catecholamine secretion. However, these channels have the potential to augment release induced by nerve-stimulation powerfully. N- and L-type calcium channels are not involved in catecholamine release induced by perfusing the glands with acetylcholine.

Effect of calcium channel antagonists on nerve-induced catecholamine release

Previous studies on primary cultures of bovine chromaffin cells have shown that dihydropyridines can partially inhibit catecholamine secretion induced by depolarization with acetylcholine, nicotine or K^+. The degree of inhibition varied between studies depending on the dihydropyridine used, its concentration, and the concentration of agonist (Cena *et al*., 1983; Boarder *et al*., 1987; Owen *et al*., 1989). However, high concentrations ($\geqslant 1$ μM), dihydropyridines block the nicotinic receptor ion channel and at these concentrations they inhibit calcium uptake and catecholamine secretion induced by nicotinic agonists without comparable effects on K^+-evoked responses (Lopez *et al*., 1993). At concentrations below 1 μM, the majority of secretion evoked by nicotinic agonists was resistant to dihydropyridines (Cena *et al*., 1983; Boarder *et al*., 1987). In agreement with these cultured chromaffin cell studies, the present study found that nerve-induced catecholamine secretion from bovine intact perfused adrenal glands was largely resistant to 1 μM nitrendipine. Nitrendipine at a concentration of 1 μM was sufficient to reduce 30 mM potassium-induced contraction of pig coronary artery rings mounted in organ baths (data not shown). In addition, 1 μM nitrendipine significantly reduced PACAP-27-induced

catecholamine release from bovine cultured chromaffin cells (O'Farrell & Marley 1995b), and it also prevented the increase of PACAP-27-induced catecholamine secretion induced by 1 μM ($\pm$)-Bay K8644 (O'Farrell & Marley, unpublished observations). Consequently, the failure of nitrendipine to inhibit significantly nerve-induced catecholamine release was not due to the lack of activity of this batch of drug or due to an insensitivity of bovine chromaffin cell L-channels to nitrendipine.

Studies on bovine cultured chromaffin cells found that depolarization-induced catecholamine secretion was unaffected by high (1 μM) concentrations of ω-conotoxin-GVIA, suggesting N-type calcium channels were not involved (Owen *et al*., 1989). However, using the bovine intact perfused adrenal gland, we found the majority of nerve-induced catecholamine secretion was blocked by a very low concentration of this toxin (10 nM; Figure 4). The effect of ω-conotoxin-GVIA was postsynaptic, on the chromaffin cells, since it had no effect on the release of acetylcholine from the splanchnic nerve terminals (see below). This result suggests catecholamine secretion induced by nerve stimulation in the intact adrenal gland recruits different calcium channels in the chromaffin cell membrane than those recruited during depolarization-induced secretion from cultured chromaffin cells.

The lack of direct involvement of L-type channels and the significant role of N-type channels in nerve-induced catecholamine secretion from the bovine adrenal gland is similar to that of the dog adrenal. Takeuchi *et al*. (1993) found that administration of nifedipine or Bay K8644 (10 μg kg^{-1} or 30 μg kg^{-1}) did not modify catecholamine release induced by splanchnic nerve stimulation of the dog adrenal gland at either 1 or 3 Hz. In addition, Kimura *et al*. (1994) found that catecholamine secretion induced by splanchnic nerve stimulation in the anaesthetized dog was reduced by ω-conotoxin-GVIA. However, in the rat isolated adrenal gland sub-micromolar concentrations of isradipine reduced secretion in response to 10 Hz field stimulation by up to 50% (Lopez *et al*., 1992), suggesting a significant involvement of L-type channels as well as dihydropyridine-resistant channels in this species. Species differences in the types of calcium channel involved in controlling calcium influx and secretion have been shown with rat, cat and bovine cultured chromaffin cells (Albillos *et al*., 1993; 1994; Artalejo *et al*., 1994; Gandia *et al*., 1995).

The majority of 10 Hz nerve stimulation-induced catecholamine secretion in the perfused bovine adrenal gland is mediated by acetylcholine (Figure 2b, c, see also Marley *et al*., 1993). However, in the present study, similar levels of catecholamine secretion induced by exogenous acetylcholine were unaffected by ω-conotoxin-GVIA while that due to nerve stimulation was reduced by over 70%. This suggests that acetylcholine release from the splanchnic nerve terminals activates different chromaffin cell calcium channels than exogenous acetylcholine delivered in the perfusion buffer. A similar finding was obtained in rat perfused adrenal glands, where nicotine-evoked catecholamine secretion was much more sensitive to isradipine than was the response to nerve-stimulation (Lopez *et al*., 1992), which at high frequency is mostly cholinergic (Malhotra & Wakade, 1983). Although nitrendipine had no significant effect on acetylcholine-evoked catecholamine secretion for the bovine perfused adrenal gland, secretion from bovine cultured chromaffin cells evoked by nicotinic agonists was reduced by 40–50% by nicardipine or nitrendipine (Cena *et al*., 1983; Boarder *et al*., 1987; Koyama *et al*., 1988). This suggests that culturing chromaffin cells may affect the types of calcium channel that contribute to secretion. The resistance of acetylcholine-induced catecholamine secretion from the bovine perfused adrenal gland to nitrendipine is in contrast to the effect of 1 μM nisoldipine obtained by Jimenez *et al*. (1993) in this preparation, which inhibited secretion by about 50%. The reason for this difference is not clear, but the same concentration of nisoldipine failed to affect secretion induced by K^+ (Jimenez *et al*., 1993). Given the effect of dihydropyridines on the nicotinic receptor (Lopez *et al*., 1993), nisoldipine may have been acting at the level of the nicotinic receptor rather than the L-type channels.

Although L-type calcium channels did not appear to be involved in nerve-induced catecholamine secretion from the bovine perfused adrenal gland, they have the potential to contribute to secretion as shown by the effects of ($\pm$)-Bay K8644 (Figure 4). This is consistent with previous electrophysiological studies on bovine chromaffin cells that have shown L-type channels are not activated by a short depolarization, but can be recruited by prolonged or repetitive depolarizations or by increasing cellular adenosine 3′:5′-cyclic monophosphate (cyclic AMP) levels and are then very efficient at evoking secretion (Artalejo *et al*., 1990; 1991; 1992b; 1994). Also, it is possible that the residual catecholamine secretion seen after blocking N-type calcium channels was supported by calcium entering the chromaffin cell through both L- and P- or Q-channels, and that sufficient calcium enters by each pathway to sustain this residual secretion. With such redundancy, blocking L-type calcium channels would not significantly inhibit secretion even though they are contributing to the calcium entry which supports secretion.

Presynaptic calcium channels and acetylcholine release from splanchnic nerve terminals

During nerve stimulation, the majority of the increase in release of ^{3}H-labelled compounds was due to a broad peak which eluted in the same position and with the same biphasic profile of synthetic [^{14}C]-acetylcholine (see Figure 1). This material was sensitive to cholinesterases, since it eluted in the position of choline in the absence of neostigmine. All the stimulation-evoked release of ^{3}H was sensitive to tetrodotoxin (Figure 2a). Taken together these results show the stimulation-induced release of ^{3}H-labelled compounds above basal release of ^{3}H-labelled compounds was mostly acetylcholine of neuronal origin. Therefore, the ^{3}H-choline loading technique provides a valid method of measuring evoked acetylcholine release from the splanchnic nerve terminals.

The release of acetylcholine evoked by nerve stimulation was unaffected by nitrendipine, ($\pm$)-Bay K8644 or ω-conotoxin-GVIA, suggesting neither L- nor N-type calcium channels contribute to acetylcholine release from splanchnic nerve terminals. Many previous studies have shown a variety of calcium channels can contribute to neurotransmitter release from different nerve terminals. Noradrenaline release from sympathetic nerve terminals is blocked by ω-conotoxin-GVIA, indicating N-type calcium channels mediate transmitter release from these nerves (Hirning *et al*., 1988). In neurohypophyseal nerve terminals, both L- and N-type channels are involved (Lemos & Nowycky, 1989), while in brain synaptosomes glutamate release is unaffected by blockade of L- and N-channels but is inhibited by ω-agatoxin-IVA, an inhibitor of P-type channels (Turner *et al*., 1992). Neurotransmission in rat cerebellum, spinal cord and hippocampus was largely dependent on N- and P-type channels with little contribution from L-type (Takahashi & Momiyama, 1993), while at the neuromuscular junction P- and Q-type channels appear to be involved (Olivera *et al*., 1994). Due to the large cost involved in purchasing sufficient amounts of ω-agatoxin-IVA and the lack of selectivity of ω-conotoxin-MVIIC for Q-type calcium channels, the role of P- and Q-type calcium channels in catecholamine secretion and splanchnic nerve transmission in the bovine perfused adrenal gland is yet to be determined.

In summary, chromaffin cell N-type calcium channels play a crucial role in nerve-induced catecholamine release in the bovine perfused adrenal gland. In addition, L-type calcium channels can powerfully enhance catecholamine release induced by nerve stimulation. The physiological conditions leading to the activation of the chromaffin cell L-type calcium channels and the presynaptic calcium channels controlling splanchnic nerve transmitter release remain to be elucidated.

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