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Evidence for a dihydropyridine-sensitive and conotoxin-insensitive release of noradrenaline and uptake of calcium in adrenal chromaffin cells

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1 It has been suggested that neuronal voltage-sensitive calcium channels (VSCC) may be divided into dihydropyridine (DHP)-sensitive (L) and DHP-insensitive (N and T), and that both the L and the N type channels are attenuated by the peptide blocker ω-conotoxin. Here the effects of ω-conotoxin on release of noradrenaline and uptake of calcium in bovine adrenal chromaffin cells were investigated.

2 Release of noradrenaline in response to 25 mM K^+, 65 mM K^+, 10 nM bradykinin or 10 μM prostaglandin E_1 was not affected by ω-conotoxin in the range 10 nM–1 μM.

3 $^{45}Ca^{2+}$ uptake stimulated by high K^+ and prostaglandin was attenuated by 1 μM nitrendipine and enhanced by 1 μM Bay K 8644; these calcium fluxes were not modified by 20 nM ω-conotoxin.

4 With superfused rat brain striatal slices in the same medium as the above cell studies, release of dopamine in response to 25 mM K^+ was attenuated by 20 nM ω-conotoxin.

5 These results show that in these neurone-like cells, release may be effected by calcium influx through DHP-sensitive but ω-conotoxin-insensitive VSCC, a result inconsistent with the suggestion that ω-conotoxin blocks both L-type and N-type neuronal calcium channels.

Introduction

The neuronal voltage-sensitive calcium channels (VSCC) act at the nerve terminals as transducers of electrical information into chemical signals and so play a central role in synaptic function. Neuronal VSCC have been characterized by Nowycky *et al.* (1985), using electrophysiological criteria, into three types (L, N and T); only L-type are responsive to the dihydropyridine (DHP) calcium channel drugs. A number of reports show that depolarization-evoked calcium flux and neurotransmitter release at the nerve terminal is largely or completely insensitive to DHP antagonists (e.g. Nachsen & Blaustein, 1979; Rampe *et al.*, 1984; Perney *et al.*, 1986) suggesting that L channels are not responsible for most of the calcium influx which causes neurotransmitter release upon depolarization. However, in some studies depolarization-evoked calcium influx and neurotransmitter release is partly sensitive to DHP blockers in the brain (Turner & Goldin, 1985; Herdon & Nahorski, 1988), and most substance P release from cultured dorsal root ganglion cells elicited by depolarizing concentrations of potassium can be blocked by the drugs (Perney *et al.*, 1986; Rane *et al.*, 1987; Holz *et al.*, 1988).

The peptide ω-conotoxin has been shown in certain preparations to attenuate depolarization-evoked release of neurotransmitter (Olivera *et al.*, 1984; Kerr & Yoshikama, 1984; Reynolds *et al.*, 1986; Rivier *et al.*, 1987; Sano *et al.*, 1987; Dooley *et al.*, 1987; Mohy El-Din & Malik, 1988; Maggi *et al.*, 1988; Herdon & Nahorski, 1988). On the basis of electrophysiological evidence it has been suggested that this toxin blocks both L and N type neuronal VSCC (McCleskey *et al.*, 1987; Cruz *et al.*, 1987), consistent with the suggestion that release is mediated principally by these two channel types (Miller, 1987). However neurotransmitter release has not always been found to be sensitive to ω-conotoxin, perhaps reflecting heterogeneity of VSCC between different types of neurone (Anderson & Harvey, 1987; Sano *et al.*, 1987; Maggi *et al.*, 1988). In these earlier studies ω-conotoxin insensitivity was apparent with depolarization-evoked transmitter release

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from preparations expected to be resistant to DHP antagonists. In the work described in this paper we used the cultured bovine adrenal chromaffin cell, a neurone-like cell in culture, and show that depolarization-evoked release and calcium influx which are known to be blocked by DHP antagonists are not blocked by ω-conotoxin. This suggests that some neuronal L-type VSCC are ω-conotoxin-insensitive.

Methods

Cells were prepared from fresh bovine adrenal glands by digestion with collagenase/protease (Marriott *et al.*, 1988) and purified by an adaptation of the differential plating procedure of Waymire *et al.* (1983) as described in Owen *et al.* (1988). Purified chromaffin cells were cultured at 0.5×10^6 cells per well in 24 well 'Primaria' plates in a complete medium with cell growth inhibitors (Owen *et al.*, 1988). Used after 3–7 days in culture, the monolayers were first washed twice with 1 ml of HEPES buffered balanced salt solutions (BSS) as follows (mM): NaCl 125, KCl 5.4, $NaHCO_3$ 16.2, N-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid 30, NaH_2PO_4 1, $MgSO_4$ 0.8, $CaCl_2$ 1.8 and glucose 5.5; gassed with 95% O_2:5% CO_2 and buffered to pH 7.4. A 12 min preincubation period preceded a 3 min stimulation period; calcium channel drugs, where appropriate, were present through both preincubation and stimulation period.

For estimation of noradrenaline release supernatants were removed, centrifuged and acidified to a final concentration of 0.1 M HCl. The remaining monolayer was extracted into 0.1 M HCl, and noradrenaline content of both supernatant and cell extract was estimated by high pressure liquid chromatography followed by electrochemical detection, essentially as described by Mefford (1981). Results are expressed as release of noradrenaline as a percentage of cell content, each figure being the mean of quadruplicates.

For estimations of calcium uptake incubations were in 0.5 ml BSS in the presence of 0.037 MBq $^{45}Ca^{2+}$; after 3 min the medium was aspirated and the monolayer was rapidly washed with 2 ml of cold BSS three times. Finally the monolayer was extracted into 10% trichloroacetic acid and the radioactivity determined by liquid scintillation counting. Results, expressed as c.p.m. per well, are the mean of quadruplicates.

To investigate release of dopamine from rat brain, 300 μm cross chopped slices of striatum were preincubated for 1 h in the BSS described above with three changes of medium. The slices were then packed into a four chamber perfusion apparatus and perfused at 1 ml min^{-1} for 20 min before starting the collection of 1 min fractions of the perfusate. Conotoxin, where appropriate, was introduced into the perfusing medium as the fraction collection was started and was present throughout the rest of the procedure. Stimulation of the slices was effected by raising the concentration of the potassium in the perfusing medium to 25 mM. Dopamine content of the fractions was measured by high performance liquid chromatography followed by electrochemical detection. Assessment of significant differences was by two tailed Student's *t* test.

Materials

Tissue culture medium, plastics and supplements were from GIBCO (Paisley, Scotland) except for Primaria plates (Falcon), which were from Becton-Dickinson, Oxford. $^{45}Ca^{2+}$ was from Amersham Corp. Bay K 8644 (methyl 1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate) was a gift from Bayer (Leverkusen, F.R.G.). Synthetic ω-conotoxin GVIA was from Peptide and Protein Research Institute, Osaka, Japan, DHPs were kept as a stock solution in dimethylsulphoxide, the final concentration of which was 0.01%, at which concentration it was present in appropriate controls.

Results

Figure 1 shows the effect of 10 nM and 100 nM ω-conotoxin on basal release of noradrenaline and on release stimulated by 65 mM K^+ or 10 nM brady-

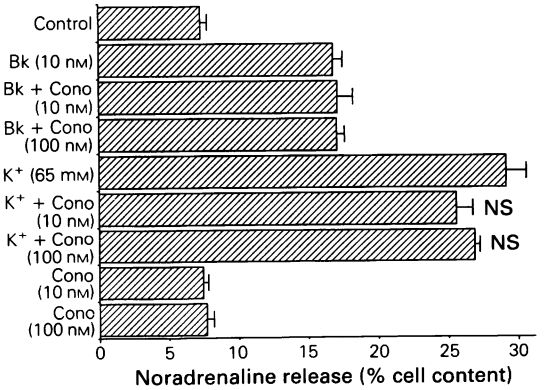

Figure 1 Effect of ω-conotoxin (Cono, 10 and 100 nM) on bradykinin (Bk) and high potassium stimulated release of noradrenaline from chromaffin cells. Bradykinin 10 nM or 65 mM potassium were present for the 3 min stimulation period. Conotoxin was present through this and the preceding 12 min preincubation period. Error bars are s.e.mean ($n = 4$). NS indicates no significant effect of the presence of ω-conotoxin.

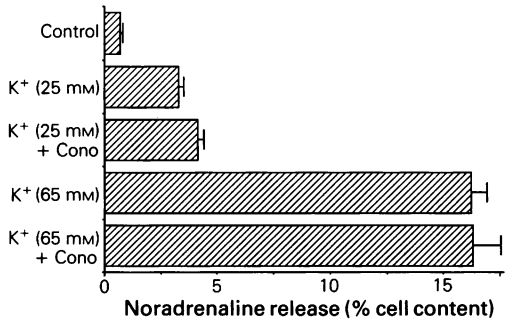

Figure 2 Effect of 1 μM ω-conotoxin (Cono) on release from chromaffin cells stimulated by 25 mM or 65 mM potassium. Error bars are s.e.mean ($n = 4$).

kinin. It can be seen that ω-conotoxin had no effect on either basal or stimulated release (the small apparent decrement in K^+-stimulated release in the presence of ω-conotoxin was not significant nor reproducible over a number of similar experiments). In order to see whether a smaller degree of depolarization, or a higher concentration of ω-conotoxin, would reveal an effect we tested 1 μM ω-conotoxin with either 25 mM or 65 mM K^+. As can be seen in Figure 2 the toxin still had no effect on noradrenaline release.

Figure 3 shows the effect of nitrendipine and ω-conotoxin on both noradrenaline release and calcium uptake stimulated by 65 mM K^+: nitrendipine reduced both stimulated noradrenaline release (down to 34% of control values, Figure 3a) and stimulated calcium uptake (to 52% of controls, Figure 3c). Neither release nor calcium uptake were affected by ω-conotoxin (Figure 3b and d).

Stimulation of calcium uptake by submaximal concentrations of extracellular K^+ (25 mM) was itself insensitive to 20 nM ω-conotoxin, but it could be enhanced by the DHP calcium channel agonist, Bay K 8644. This agonist-enhanced calcium uptake was itself insensitive to 20 nM conotoxin (data not shown).

Prostaglandin E_1 stimulated the release of noradrenaline from chromaffin cells, an effect powerfully potentiated by the calcium channel activator Bay K 8644 at 1 μM. Both the release stimulated by prostaglandin E_1 alone and in the presence of Bay K 8644 were insensitive to the presence of ω-conotoxin (Figure 4).

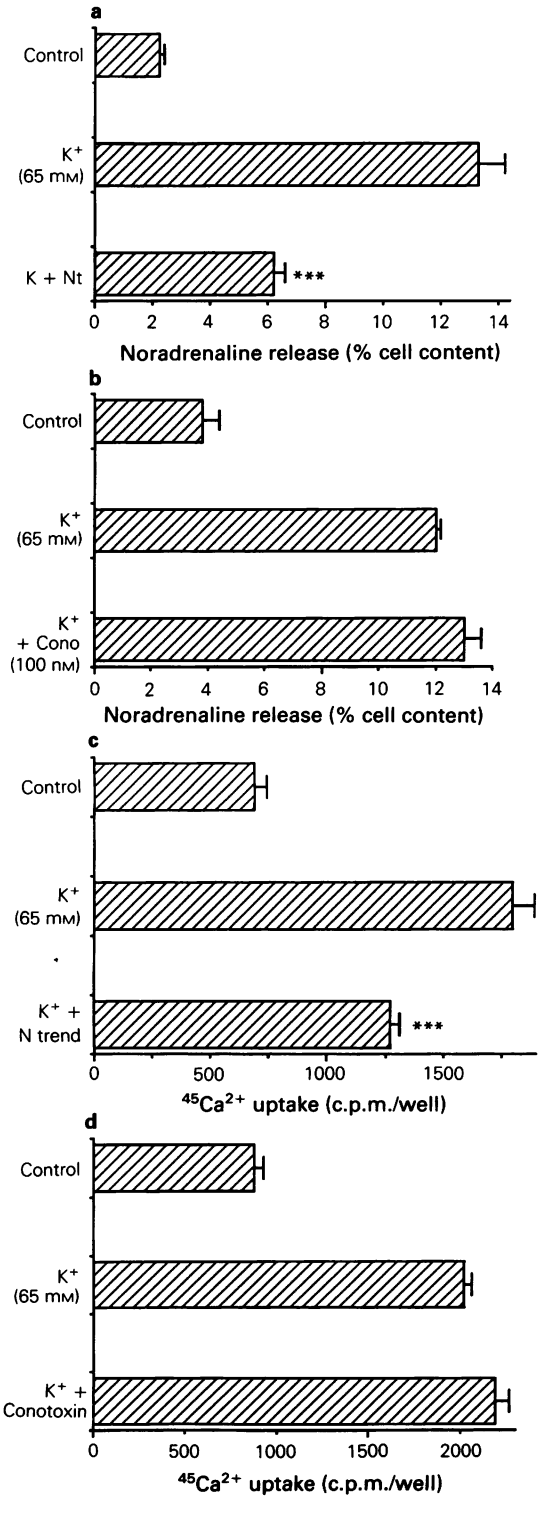

Figure 3 Effect of nitrendipine or conotoxin on noradrenaline release (a and b) and calcium uptake (c and d) stimulated by high potassium, with chromaffin cells. Nitrendipine (Nt) was 1 μM in (a) and (c), ω-conotoxin (Cono) 100 nM in (b) and 20 nM in (d). Error bars are ±s.e.mean ($n = 4$), ****$P < 0.005$ compared to potassium stimulation with no nitrendipine.

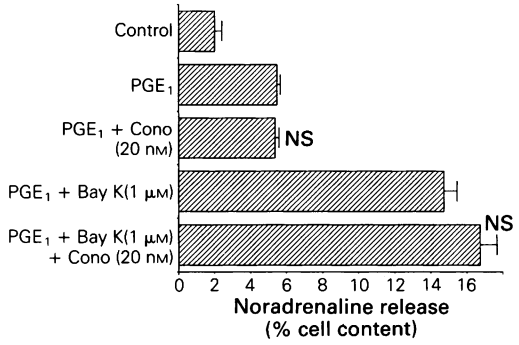

Figure 4 Effect of 1 μM Bay 8644 (Bay K) and 20 nM ω-conotoxin (Cono) on release of noradrenaline from chromaffin cells stimulated by 10 μM prostaglandin E_1 (PGE_1). Error bars are s.e.mean ($n = 4$). NS indicates no significant effect of the presence of ω-conotoxin.

To demonstrate that the toxin is active under conditions used here, we perfused rat striatal slices with the same BSS, stimulated with high K^+ in the presence and absence of ω-conotoxin and measured dopamine content of the perfusate. One of three similar experiments, each with a four channel perfusion system, is shown in Figure 5 where it can be seen that the presence of 20 nM conotoxin caused a substantial attenuation of dopamine release.

Discussion

In the studies described here, much of the depolarization-induced release of noradrenaline from chromaffin cells was found to be sensitive to 1 μM nitrendipine, consistent with earlier observations showing inhibition of release by DHPs in a stereospecific manner (Boarder *et al.*, 1987; Fonteriz *et al.*, 1987). Voltage and DHP sensitivity of calcium channels is characteristic of L type channels (Nowycky *et al.*, 1985). We have recently shown that bradykinin-stimulated release from chromaffin cells is largely dependent on extracellular calcium but is insensitive to DHP blockers (Owen *et al.*, 1988). Since ω-conotoxin has been reported to block both L and N types of neuronal channel (McCleskey *et al.*, 1987), we initiated experiments such as that shown here in Figure 1 with the expectation that K^+ depolarization-stimulated release would be sensitive to ω-conotoxin. We wished to see if bradykinin-stimulated release was attenuated by this peptide toxin. The evidence presented in this paper clearly shows however that high K^+-stimulated noradrenaline release and calcium uptake is not conotoxin-sensitive. This demonstrates that DHP-sensitive VSCC may be insensitive to conotoxin. This is clearly different from some recent reports of insensi-

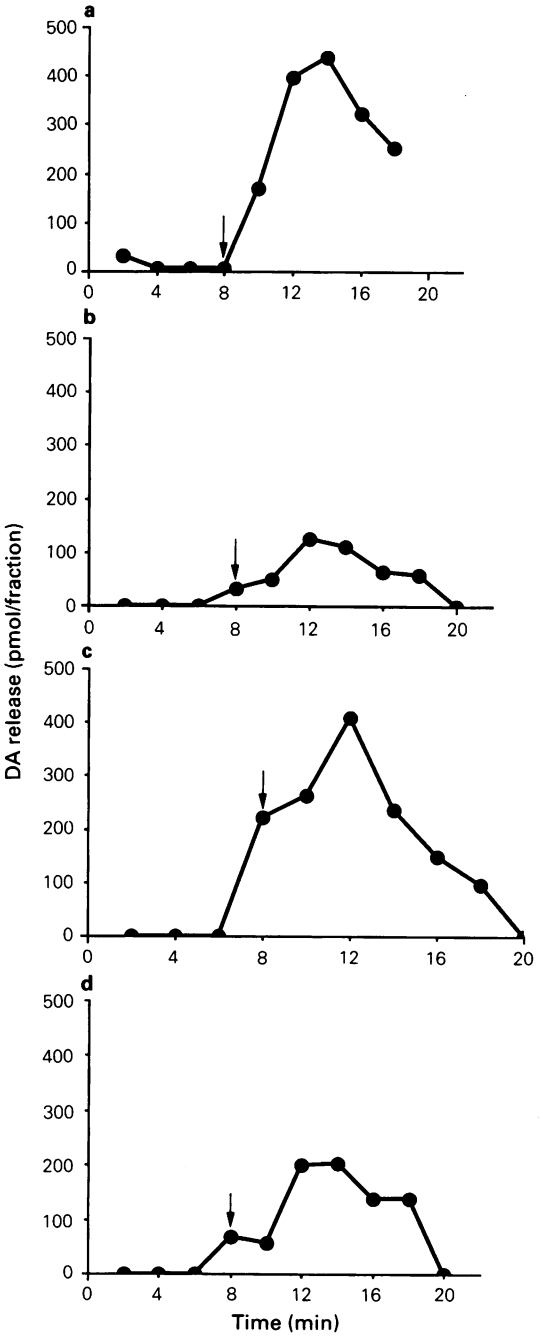

Figure 5 Release of dopamine (DA) from perfused slices of rat brain striatum. Slices were perfused in the presence (b and d) and absence (a and c) of 20 nM ω-conotoxin for the time shown, and the slices stimulated with 25 mM potassium introduced when indicated by the arrow, and present for the rest of the procedure.

tivity of neurotransmitter release to ω-conotoxin in preparations which would not be expected to be sensitive to DHP antagonists (Anderson & Harvey, 1987; Sano *et al.*, 1987; Maggi *et al.*, 1988).

A possible explanation for the failure to block the DHP-sensitive channels in the chromaffin cells is that ω-conotoxin is effective at N channels but not at neuronal L channels, contrary to the conclusion reached by others. The most substantial evidence for the effectiveness of conotoxin at neuronal L type channels is the electrophysiological data of McCleskey *et al.* (1987) obtained with a variety of patch clamp configurations. While this study provides evidence for action at the two channel types, other studies are not so unequivocal. For example Reynolds *et al.* (1986) using microspectrofluorimetry of fura-2 loaded cells showed that depolarization-induced calcium fluxes in cell bodies are sensitive to conotoxin as well as nitrendipine. However, rather than because these two drugs are acting on the same channels, this may be a reflection of channel heterogeneity in the cell bodies, with both N and L types being present. Militating against action at both channel types for ω-conotoxin is a study published as the work reported in this paper was being concluded, showing in two neuronal like cell lines (a human neuroblastoma cell line and the phaeochromocytoma-derived PC12 line) that ω-conotoxin was capable of modifying only those depolarization-induced calcium fluxes that are DHP-insensitive (Sher *et al.*, 1988). In agreement with the results presented here, this leads to the conclusion that ω-conotoxin is not active against neuronal L type channels.

This conclusion may be modified by considering L type channel heterogeneity. Perhaps different neurones and neurone-related cells will contain different channels of this type, all DHP-sensitive but only some ω-conotoxin-sensitive. Heterogeneity of L type channels with respect to ω-conotoxin-sensitivity has always been recognised in the comparison between neurones and muscle cells. No conotoxin binding or calcium channel activity is apparent on smooth, cardiac or skeletal muscle (Yeager *et al.*, 1987; McCleskey *et al.*, 1987; Cruz *et al.*, 1987; Barhanin *et al.*, 1988; Mohy El-Din & Malik, 1988) despite the presence of DHP-sensitive L type channels. Indeed Cruz *et al.* (1987) have proposed two types of L channel, L_n (neuronal and ω-conotoxin-sensitive) and L_m (ω-conotoxin-insensitive). The present results and those of Sher *et al.* (1988) are clearly in conflict with such a scheme, but may be reconciled with the work of McCleskey *et al.* (1987) by proposing that some neurones have mainly ω-conotoxin-sensitive L type channels while some have predominantly ω-conotoxin-insensitive L type channels. In considering this hypothesis it should be noted that McCleskey *et al.* (1987) investigated a number of neuronal cell types, including sympathetic postganglionic cells closely related to the chromaffin cells investigated here and the PC12 cells used by Sher *et al.* (1988) and reported no evidence for such heterogeneity. It is possible that neurone-like cells of adrenal origin differ from true neurones with respect to their calcium channel complement. It is also possible that ω-conotoxin sensitivity is more apparent under the conditions of patch clamp studies than with the potassium depolarization of populations of whole cells reported here. However, the prostaglandins also stimulate release from these cells in a DHP-dependent (Koyama *et al.*, 1988; and our unpublished observations) but ω-conotoxin-insensitive manner. Regardless of the mechanism of prostaglandin enhancement of calcium influx and catecholamine release, it is important in the present context in that it shows ω-conotoxin insensitivity of DHP-sensitive channels is not limited to the conditions of channel opening by high extracellular K^+.

Bradykinin-stimulated release is clearly by a different mechanism from prostaglandin-stimulated release: release in response to bradykinin is largely dependent on external calcium, but the route of calcium entry is different as shown by the lack of DHP-sensitivity (Owen *et al.*, 1988). The data presented here show that the route of calcium entry stimulated by bradykinin is also not sensitive to conotoxin, and therefore is presumably not via N-type VSCC.

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