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STIMULUS SECRETION COUPLING IN CULTURED CHROMAFFIN CELLS

DEPENDENCY ON EXTERNAL SODIUM AND ON DIHYDROPYRIDINE-SENSITIVE CALCIUM CHANNELS

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Abstract—Stimulus secretion in response to acetylcholine (or nicotine) in adrenal chromaffin cells involves calcium influx and is tetrodotoxin insensitive. The mechanism by which activation of the nicotinic acetylcholine receptor (AChR) leads to calcium influx is not clear, and the requirement for external sodium is controversial. We report that when bovine chromaffin cells are continuously perfused in the absence of sodium, secretion of catecholamines in response to 3×10^{-5} M nicotine is not impaired. Also, secretion stimulated by this concentration of nicotine is not potently and stereospecifically inhibited by the (+)- and (−)-isomers of nicardipine. At a concentration of (+)-nicardipine (10^{-7} M) which inhibits most of the release stimulated by 50 mM potassium, the response to 3×10^{-5} M nicotine remains; however, the dose–response curve to nicotine is shifted slightly to the left. The results are discussed with respect to models of stimulus secretion coupling in bovine adrenal chromaffin cells.

Studies on the stimulation of catecholamine release from the adrenal medulla by acetylcholine have played a central part in the understanding of the role of calcium in stimulus secretion coupling (e.g. [1, 2]). However, the mechanism by which acetylcholine acting at its receptor (the nicotinic AChR) leads to the influx of calcium in adrenal chromaffin cells is poorly understood. Release of catecholamines is not dependent upon depolarisation propagated by fast sodium channels leading to opening of voltage-sensitive calcium channels (VSCC), since release is not inhibited by the voltage-sensitive sodium channel blocker tetrodotoxin [3]. An alternative model, that sodium entering through the AChR channel causes local non-propagated depolarisation, which opens VSCC, predicts that external sodium is required and that release may be prevented by calcium channel blockers, such as the dihydropyridine calcium channel 'antagonists'. The role of external sodium is controversial [1, 3–9]; this is in part due to the difficulty of unequivocally washing cells free from sodium under the conditions used in most studies. The involvement of dihydropyridine-sensitive VSCC is clear for high potassium-stimulated release of catecholamines but not for the AChR-linked stimulus secretion coupling [6, 10].

The possibility remains from these studies that a significant proportion of calcium entering the cell may do so via the AChR channel, as suggested by Stallcup [11]. This route of entry would be expected to be independent of external sodium but not specifically blocked by dihydropyridines. With respect to dihydropyridine sensitivity of stimulus secretion coupling, we must also consider the possible involvement of dihydropyridine insensitive VSCC [12], and the observed voltage dependency of dihydropyridine binding [13].

In this communication we investigate the dependency of stimulus secretion coupling in cultured bovine adrenal chromaffin cells on external sodium under conditions of continuous perfusion of the cells, and the susceptibility of this coupling to inhibition by stereoisomers of the dihydropyridine calcium channel blocker nicardipine.

METHODS

In the experiments described here, we used primary cell cultures of bovine adrenal chromaffin cells at 3–7 days of culture. Cells were maintained as a flat monolayer in multiwells for the experiments on dihydropyridine sensitivity and as a monolayer on microcarrier beads, as recently described [13], for the experiments on sodium dependency.

Cells were isolated from fresh bovine adrenal medullae, essentially as described by Kilpatrick *et al.* [15], and were cultured in Eagle's minimal essential medium supplemented with 10% foetal calf serum, 27 mg/100 ml glutamine, 1 ml/100 ml of 100 × non-essential amino acids, 5 mg/100 ml gentamycin, 5000 i.u./100 ml penicillin, 5000 μg/100 ml streptamycin, 250 μg/ml fungizone, 5 μM fluorodeoxyuridine and 5 μM cytosine arabinoside. Cells were plated onto 24-well multiwell plates (Linbro, Flow Laboratories) at about 5×10^5 cells per well, and maintained at 37° in 95% air, 5% CO_2. Medium was changed every 2–3 days, and the cultures used at 3–7 days. For the experiments the medium was removed from the cell and the monolayer was washed with a modification of Earl's balanced salt solution (BSS) with HEPES

buffer (composition: NaCl 125 mM; KCl 5.4 mM; $NaHCO_3$ 16.2 mM; HEPES 15 mM, NaH_2PO_4 1 mM; $MgSO_4$ 0.8 mM; $CaCl_2$ 1.8 mM; glucose 5.5 mM). They were then pre-incubated with this balanced salt solution with nicardipine as necessary. After 8 min the preincubate was removed and the cells incubated with BSS with HEPES supplemented with nicardipine, 5×10^{-5} M nicotine and high potassium as necessary. The high potassium solution was 50 mM with concurrent reduction in sodium. After a 3-min incubation the supernatant was removed to tubes on ice, and the cells extracted into 0.1 M perchloric acid. Noradrenaline and adrenaline was estimated in each case by electrochemical detection (Bioanalytical Systems, Layfayette, IN, U.S.A.) after separation by reverse-phase ion-pair chromatography (Ultrasphere octadecylsilane column, Altex, Berkeley, CA, U.S.A.) [16]. Nicardipine isomers were supplied by Dr R. Whiting, Syntex, Edinburgh. Nicotine was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

The culture of bovine chromaffin cells on microcarrier beads and their subsequent perfusion in small chambers, has been described recently [14]. Briefly, the isolated percoll purified cells were mixed with either Cytodex 1 or Cytodex 3 beads (Pharmacia, Uppsala, Sweden), 50–100 mg dry wt of pre-swollen beads with $6–10 \times 10^6$ cells, and cultured at 37° in 5% CO_2, 95% air in the medium described above, in bacteriological petri dishes, for 3–7 days prior to use. Cells attached to beads, flattened and formed processes. Chambers holding 150 μl of settled beads with attached cells were perfused at 1 ml/min at 37° with a solution of NaCl 140 mM, KCl 4.7 mM, KH_2PO_4 1 mM, $MgSO_4$ 1.2 mM, $CaCl_2$ 2.2 mM, Tris–HCl 20 mM, glucose 10 mM, pH 7.4, gassed with 95% O_2, 5% CO_2. Following pre-perfusion for 10–15 min, 1 min fractions were collected and aliquots assayed for noradrenaline and adrenaline by HPLC with electrochemical detection. Nicotine was introduced into the perfusate at 5×10^{-5} M continuously from the point indicated, and sodium-free perfusion was effected by replacing the NaCl with choline chloride.

RESULTS

Perfusion in sodium-free medium

Removal of sodium has previously been shown to stimulate release [6] and so cells were perfused in the presence of sodium, and release estimated when sodium was removed. In one of several experiments illustrated in Fig. 1, the basal release over 17 min can be seen (Fig. 1A) followed by the usual rapid and transient secretion stimulated by nicotine. This is true for both noradrenaline and adrenaline release. When, in parallel experiments, sodium was replaced by choline, noradrenaline and adrenaline release was stimulated, but had returned to baseline before the challenge with nicotine (Fig. 1B). Here, in the absence of sodium, a response to nicotine was seen of the same order of magnitude as when sodium was present. Using this procedure whereby sodium outside the cell is being continuously removed during the course of the experiments, we have unequivocally demonstrated the capacity of these cells to respond fully to nicotine in the absence of external sodium. The same experimental procedure has been used to replace sodium containing salt solution with sucrose instead of choline. Again we were able to show that external sodium is not required for nicotine-stimulated release.

Effect of nicardipine stereoisomers

Results of experiments on release from multiwells, carried out in the presence of balanced salt solution containing sodium, are expressed as a percent of maximal stimulated release in order to facilitate comparison of inhibition by dihydropyridines. In a typical example, cell content per multiwell was 6872 ± 269 pmol of noradrenaline and 19092 ± 793 pmol of adrenaline. Basal release, during a 3-min period, as a percent of cell content was 1.75 ± 0.46 (noradrenaline) and 1.15 ± 0.30 (adrenaline); nicotine-stimulated release (percent of cell content during a 3-min incubation with nicotine at 5×10^{-5} M) was 16.5 ± 0.55 (noradrenaline) and 6.06 ± 0.10 (adrenaline); 50 mM potassium-stimulated release was 13.22 ± 0.31 (noradrenaline) and

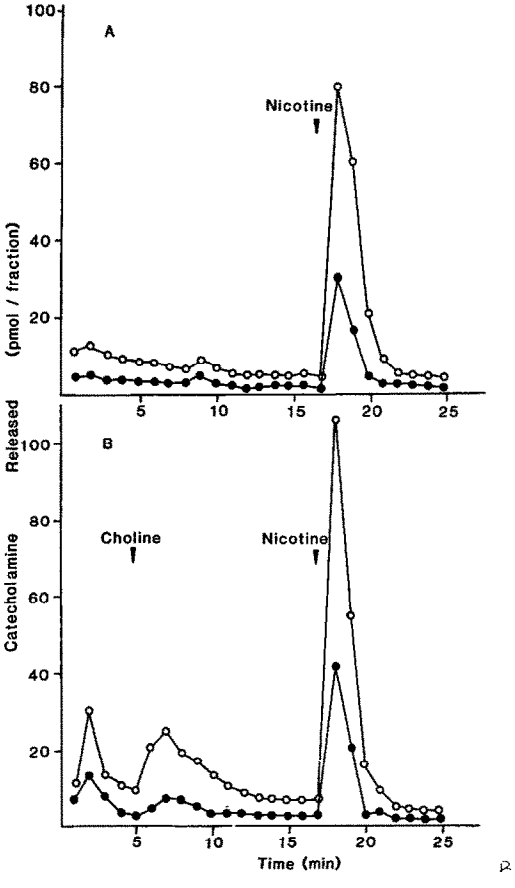

Fig. 1. Effect of replacement of sodium by choline on the nicotine-stimulated release of noradrenaline (○) and adrenaline (●) by perfused chromaffin cells maintained on microcarrier beads. The point of change of perfusing medium and of addition of nicotine are shown in each case. In (A) the medium contains sodium throughout, while in (B) the sodium is replaced by choline where indicated by the arrow.

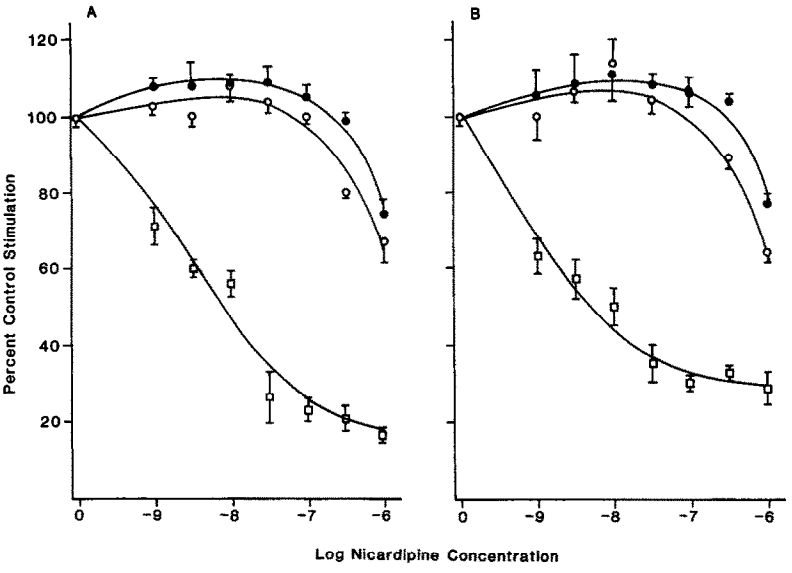

Fig. 2. Effect of (+)- and (−)-nicardipine (open and closed circles respectively) on nicotine-stimulated release of catecholamines and of (+)-nicardipine (open squares) on high potassium-stimulated release of catecholamines: (A) noradrenaline; (B) adrenaline.

6.58 ± 0.23 (adrenaline). Figures are means ± SEM, N = 4, in each case.

High potassium-stimulated catecholamine release was potently inhibited by the presence of the active (+)-isomer of nicardipine. This was true of both noradrenaline (Fig. 2A) and adrenaline (Fig. 2B) release. This was strikingly different from the absence of inhibition by low concentrations of (+)-nicardipine of nicotine-stimulated release for these two catecholamines. Furthermore, there was no difference in ability to inhibit nicotine-stimulated release between the active (+) and the inactive (−) stereoisomers of nicardipine. Inhibition by (−)-nicardipine of release stimulated by 50 mM potassium was not significantly different from inhibition of nicotine-stimulated release (data not shown). These results then show a high degree of stereospecific inhibition of potassium-stimulated release by nicardipine, but no stereospecific inhibition of nicotine-stimulated release. This is true for release of both noradrenaline and adrenaline.

Effect of nicardipine on nicotine dose–response curve

The above experiments were done at a concentration of nicotine which gave a maximal release of catecholamine, when influx of calcium may be supra-maximal with respect to release. To investigate the role of dihydropyridine-sensitive VSCC at lower concentrations when calcium entry is likely to be limiting, cells were stimulated with different doses of nicotine in the presence and absence of 10^{-7} M (+)-nicardipine (Fig. 3). This concentration effectively blocks the high potassium-stimulated release (Fig. 2). The experiment, repeated twice, shows that a small shift of the dose–response curve occurs, with an EC_{50} of $4.1 \pm 0.3 \times 10^{-6}$ M nicotine in the absence of nicardipine compared to $6.1 \pm 0.6 \times 10^{-6}$ M in the presence of nicardipine (data analysis by ALLFIT program). At lower nicotine concentrations as much as 40% of the release may be blocked by the dihydropyridine, indicating that these channels may play a significant role in augmenting the calcium signal when it is not sufficient for maximal release.

DISCUSSION

A classic picture of stimulus secretion coupling might involve a cell with nicotinic AChRs, in which contact with acetylcholine initiates depolarisation by sodium entry through the AChR channel, followed by propagation (dependent on fast sodium channels) to the site of release, where calcium entry occurs through the opening of voltage-sensitive calcium

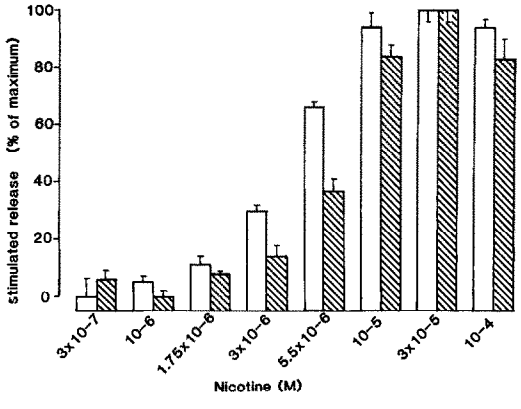

Fig. 3. Effect of (+)-nicardipine on the release of noradrenaline in response to different concentrations of nicotine. Stimulated release (after subtraction of basal) of noradrenaline, normalised as a percentage of the maximum release (3×10^{-5} M nicotine) in the presence (hatched bars) and absence (open bars) of 10^{-7} M (+)-nicardipine.

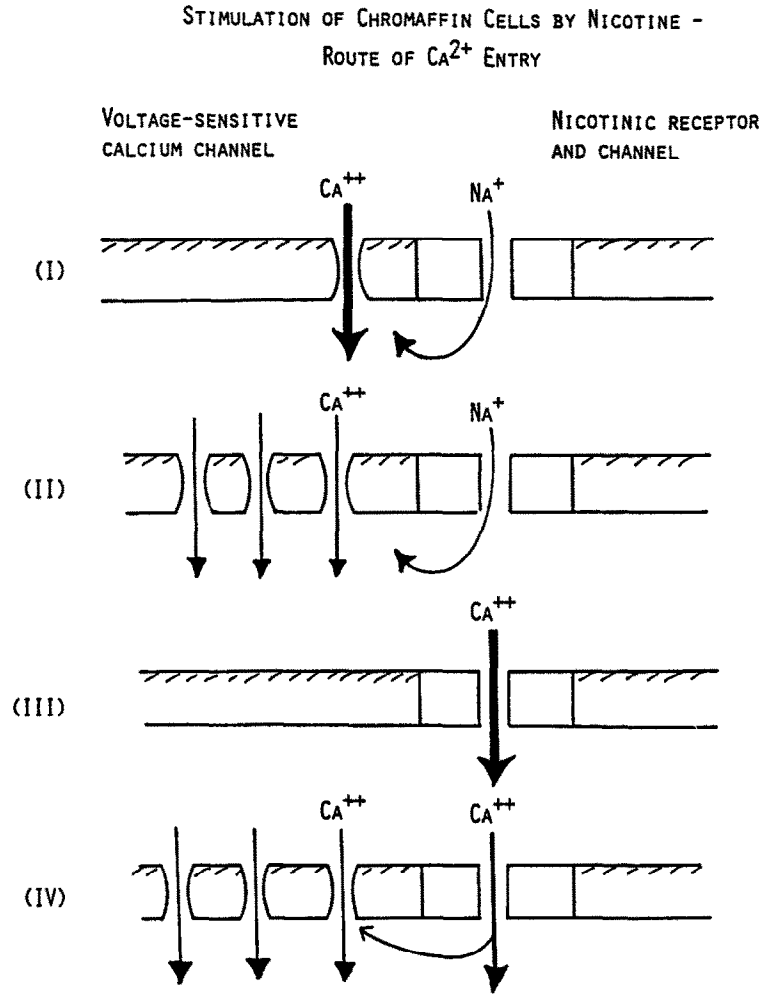

Fig. 4. Four models for the entry of [Ca^{2+}] following stimulation of cultured chromaffin cells by nicotine.

channels. Understanding of the initial steps in stimulus–secretion coupling which give rise to calcium entry has involved studies on the adrenal medulla [1, 2], and yet it is now known that this description cannot account for acetylcholine-mediated calcium influx in chromaffin cells since this is not blocked by tetrodotoxin, a drug which blocks the voltage-sensitive sodium channels [3]. We have considered four alternative models for calcium entry following stimulation of the AChR by acetylcholine or nicotine, indicated in Fig. 4. Model (I) supposes that sodium entry through the AChR channel causes sufficient depolarisation without propagation, to permit calcium entry through VSCC. Model (II) suggests sufficient VSCC density to permit propagation via a calcium action potential, permitting the involvement of calcium channels remote from the AChR. Model (III) suggests that sufficient calcium ions enter directly through the AChR channel to effect maximal release, and model (IV) suggests that calcium entry through this channel may initiate calcium entry through VSCC, possibly with propagation to remote sites via a calcium action potential.

The first two of these models require external sodium. This is an area of confusion in the literature since some studies show release independent of external sodium, while others show a varying degree of impairment when sodium is removed from the medium [1, 3–9]; it has been suggested that this is due to incomplete removal of sodium in those cases which show release in the absence of sodium [6]. Our results, however, are inconsistent with this observation. In conditions of continuous and highly efficient perfusion of cells in the absence of sodium added to the medium, we find no impairment of release. In our experiments, unlike some others reported [7], the medium in the absence of sodium is in other respects identical to the control medium. In the experiments reported here sodium chloride is replaced by choline chloride; in other experiments sucrose is used instead of choline chloride, with essentially the same results. Under these perfusion conditions the cells are continuously perfused during the experiments, so that even sodium coming from inside the cells will not accumulate in the medium.

These results indicate that stimulus secretion

mechanisms are not impaired under conditions in which models (I) and (II) of Fig. 4 cannot operate. Model (III) does not implicate external sodium, and should also be independent of the dihydropyridine VSCC blockers. This model then is quite consistent with our observation that with maximal nicotine stimulation, there is no stereospecific inhibition of catecholamine release by isomers of nicardipine. This observation is apparently in conflict with the conclusions of Cena *et al.* [6] but not with their published data, since these authors also show a much lower EC_{50} for nitrendipine against high potassium-stimulated release than against nicotine-stimulated release; they have no information on stereospecificity. Our results are fully consistent with those of Stallcup [11], who concluded that in the absence of external sodium 40% of $[^{45}Ca]^{2+}$ entering the cells in response to carbamylcholine passes directly through the acetylcholine receptor. If this is the case, then we may still see a dihydropyridine sensitivity to nicotine-stimulated release when submaximal concentrations of nicotine are used, a condition in which we can assume that rate of calcium influx is the limiting factor. That we see precisely this type of relationship in the studies reported here is consistent with the suggestion that at maximal nicotine levels sufficient calcium enters directly through the AChR channel to effect maximal stimulus secretion coupling. At submaximal nicotine levels dihydropyridine-sensitive VSCC apparently make a significant contribution [model (IV) in Fig. 4].

Patch-clamp experiments have recently indicated a multiplicity of VSCC in neurones in culture, both dihydropyridine-sensitive and insensitive [12]. We cannot exclude an involvement of these channels in the nicardipine insensitive release in response to nicotine; entry of cations directly through the AChR channel may cause sufficient depolarisation to open such channels. Furthermore, using whole cell patch-clamp techniques it has been shown that the apparent affinity of dihydropyridines for their site on the calcium channel is state dependent [13]. When cells were held at depolarised potentials, leading to channels being in an inactivated state, nitrendipine was much more potent than when the cells were maintained at a hyperpolarised potential. However, this seems unlikely to account for the difference between potency of (+)-nicardipine against high potassium and nicotine-stimulated release since opening of AChR channels in sufficient numbers would not be expected to produce a smaller depolarisation than 50 mM K^+. Furthermore, at concentrations of 10^{-6} M, and with a substantial pre-equilibration time with the drugs prior to the different stimuli, the extent of voltage dependency described by Bean [13] cannot account for the large difference in dihydropyridine sensitivity seen here.

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