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Sodium-dependent inhibition by PN200-110 enantiomers of nicotinic adrenal catecholamine release

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1 Dimethylphenylpiperazinium (DMPP) or high K concentrations evoke catecholamine release from perfused cat adrenal glands; in both cases the secretory response was significantly enhanced in the absence of Na. Tetrodotoxin did not modify the nicotinic secretory response.

2 The (+)- and (−)-enantiomers of the dihydropyridine Ca channel blocker PN200-110 show a high degree of stereoselectivity in the inhibition of catecholamine secretion evoked by high K or by DMPP in the presence of Na, the (+)-enantiomer being 57 and 80 times more potent, respectively, than the (−)-enantiomer. Both, noradrenaline and adrenaline release were equally depressed by PN200-110.

3 The IC_{50} values for (+)- and (−)-PN200-110 for blockade of the secretory response induced by K or DMPP in the presence of Na are in the same range. In the absence of Na, (−)-PN200-110 did not affect DMPP-evoked secretion; however, the (+)-enantiomer partially inhibited it.

4 The results suggest that the physiological catecholamine release from chromaffin cells is preceded by Na entry through the nicotinic receptor-associated ionophore; this causes cell depolarization, opening of voltage-dependent, dihydropyridine-sensitive Ca channels and Ca entry into the cell. In the absence of Na, additional Ca influx through an alternative pathway (the nicotinic cholinoceptor ionophore?) might also activate secretion.

Introduction

To trigger acetylcholine-evoked catecholamine release (Douglas & Rubin, 1961), Ca ions may gain access to the adrenal chromaffin cell secretory machinery through voltage-dependent and acetylcholine receptor-associated channels; the relative contribution of each channel type as well as the role of Na ions in secretion are matters of controversy in the literature (García *et al.*, 1984). While some authors conclude that the influx of Ca through voltage dependent channels is the common ionic event in triggering catecholamine release evoked either by K, veratridine or nicotinic receptor stimulation (Ceña *et al.*, 1983; Wada *et al.*, 1985), others suggest a major contribution of receptor-operated channels (Douglas & Rubin, 1963; Ishikawa & Kanno, 1978; Holz *et al.*, 1982; Kilpatrick *et al.*, 1982).

The fact that the dihydropyridine Ca channel blocker, PN200-110, potently inhibits K-evoked catecholamine secretion from, and Ca uptake into cat adrenomedullary tissues (Gandia *et al.*, 1987), and that its (+)- and (−)-enantiomers exhibit a high degree of stereoselectivity (Fonteriz *et al.*, 1987) prompted us to use them as tools to clarify further the role of external Na ions in triggering catecholamine release by nicotinic stimulation. The present paper describes experiments that demonstrate a strong Na-dependency of the blocking effects of PN200-110 on nicotinic catecholamine release from cat adrenal glands, suggesting a prominent role of Na ions in determining cholinoceptor-mediated secretion.

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Methods

Perfusion of cat isolated adrenal glands

Cats of either sex weighing 2.5–4 kg were anaesthetized with ether followed by chloralose (90 mg kg^{-1}, i.v.). Both adrenal glands were isolated and prepared for retrograde perfusion with Krebs-Tris solution at room temperature (22 ± 2°C) as previously described by García *et al.* (1980); the

perfusion rate was adjusted to 1 ml min^{-1}. Experiments with PN200-110 were performed under sodium light.

Perfusion media

The Krebs-Tris solution had the following composition (mM): NaCl 134, KCl 5.9, $CaCl_2$ 2.5, $MgCl_2$ 1.2, Tris 10 and glucose 11 and was bubbled with 100% O_2, the final pH being 7.4. Sodium-free solution was prepared by replacing NaCl by sucrose. Potassium-rich solution (35 mM) was made up by substituting appropriate amounts of NaCl by iso-osmotic concentrations of KCl. Both enantiomers of PN200-110 were dissolved in ethanol (at 10^{-2} M) and later diluted in Krebs solutions. Dimethylphenylpiperazinium iodide (DMPP) was directly dissolved in Krebs solution.

Collection of perfusate samples

After 1 h of initial perfusion with Krebs-Tris solution, collection of perfusate samples at 2 min intervals was started. In some experiments, glands were perfused with Na-free solution during the last 20 min. The last three samples were collected to determine the basal catecholamine output. Then, the appropriate modified solution was perfused through the gland and samples were collected and maintained in iced assay tubes containing enough perchloric acid to give a final concentration of 0.05 N.

Catecholamine assay

The total catecholamine content of perfusate samples (noradrenaline plus adrenaline) was determined according to Shellenberger & Gordon (1971) without further purification on alumina. In some experiments, adrenaline and noradrenaline were measured after separation with high performance liquid chromatography (h.p.l.c.) and electrochemical detection techniques (Borges *et al.*, 1986). Catecholamine release evoked by six 1-min pulses of high K or DMPP (30 min apart) plus that released during the subsequent 5 min was expressed as μg per stimulus and named S_1 to S_6 after subtraction of the basal release. The results obtained in individual experiments with identical protocol were expressed as means ± s.e. Analysis of significance of differences between means was performed by use of Student's *t* test. Differences were considered significant at the $P < 0.05$ level.

Sigmoid concentration-response curves were converted into straight lines to estimate the IC_{50}s of both enantiomers by plotting values in the ordinate as log (Y/100 − Y). The intercept with the abscissa (Y = 0) gives the IC_{50} values.

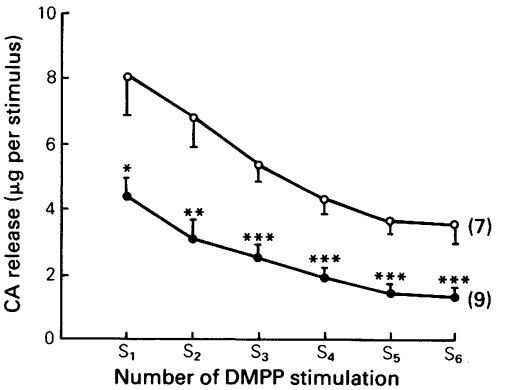

Figure 1 Catecholamine (CA) release evoked by dimethylphenylpiperazinium (DMPP, 5 μM for 1 min) applied at 30 min intervals (S_1 to S_6) to perfused cat adrenal glands, in the presence (●) or the absence (○) of Na. Results are means from the number of glands shown in parentheses; vertical bars show s.e.mean. * $P < 0.02$; ** $P < 0.005$; *** $P < 0.001$ compared with their respective stimulus number in Na-free solution.

Results

Catecholamine release evoked by DMPP in normal or sodium-free medium

Figure 1 shows the profiles of catecholamine release obtained in glands stimulated six times (S_1–S_6) with DMPP (5 μM) for 1 min, at 30 min intervals. The time course of secretion is quite similar with all stimuli: a quick rise that peaks at the first min of stimulation followed by a fall to basal levels 2–3 min after washing out the drug. In 9 glands, 4.3 ± 0.6 μg catecholamine was released during the first stimulus (S_1); of this, 48% was noradrenaline and 52% adrenaline. The secretory response decayed with subsequent stimuli to reach in S_6 a value around 30% of S_1. Tetrodotoxin (10^{-6} M) inhibited fully veratrine-evoked catecholamine release but did not modify DMPP-stimulated secretion (data not shown).

In the absence of Na, catecholamine release was significantly enhanced with all DMPP pulses. During S_1 8 ± 1.2 μg catecholamine was released, while during S_6, secretion was 3.5 ± 0.45 μg ($P < 0.01$ compared with the respective S_1 and S_6 pulses obtained in the presence of Na).

Catecholamine release evoked by high potassium concentrations in normal or sodium-free medium

The secretory response induced by six 1-min pulses of high K (35 mM) given at 30 min intervals is shown in Figure 2. In 10 glands, 5.7 ± 0.1 μg of cate-

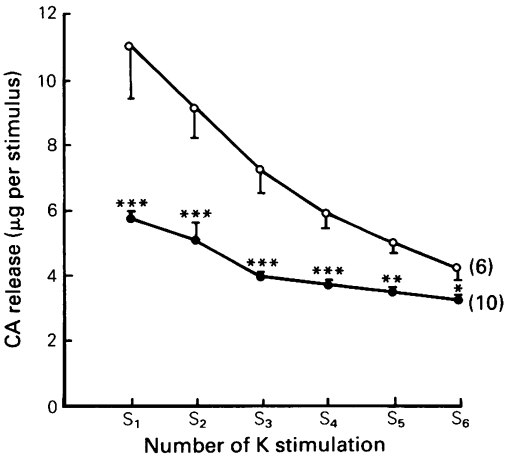

Figure 2 Catecholamine (CA) release evoked by high K concentrations (35 mM for 1 min) at 30 min intervals (S_1 to S_6) to perfused adrenals, in the presence (●) or the absence (○) of Na. Data are means of the number of glands shown in parentheses; vertical bars show s.e.mean. * $P < 0.025$; ** $P < 0.005$; *** $P < 0.001$ as compared with their respective stimulus number in Na-free solution.

cholamine was released during S_1; of this, 58% was noradrenaline and 42% adrenaline. The secretory response declined slightly during S_2 and S_3 and then stabilized in subsequent stimulations. Desensitization of secretion was only 20% in S_6 in contrast to the 70% observed with DMPP.

A significant increase of catecholamine release was also observed in the absence of Na. During S_1, $11 \pm 1.6\,\mu g$ was released which was almost double the release obtained in presence of Na ($P < 0.001$; Figure 2). Inactivation of secretion in subsequent stimuli was also more pronounced than in the presence of Na; in S_6, secretion was only 40% of S_1.

Effects of PN200-110 enantiomers on catecholamine release evoked by potassium or DMPP

Increasing concentrations of (+)- and (−)-PN200-110 inhibited catecholamine release evoked by K (35 mM) in a concentration-dependent manner (Figure 3a). The IC_{50} for the (+)-enantiomer was 20.3 nM and for the (−)-enantiomer 1150 nM; the eudismic ratio was 57, a figure similar to that previously reported by Gandia *et al.* (1987).

When DMPP (5 μM) was used as secretagogue in the presence of Na, the IC_{50}s of (+)- and (−)-PN200-110 were 58 and 4470 respectively, the eudismic ratio being 77. Even at 5 μM, the (−)-enantiomer inhibited secretion only by 60% (Figure 3b). Catecholamine release evoked by DMPP in the absence of Na was highly resistant to inhibition by both enantiomers. An IC_{50} of 740 nM was calculated for (+)-PN200-110; the (−)-enantiomer did not significantly inhibit secretion up to 5 μM (Figure 3c).

Effects of (+)-PN200-110 on the separate secretion of noradrenaline and adrenaline

Upon stimulation with high K (35 mM for 1 min), noradrenaline release rose from 34 to 1800 ng, an

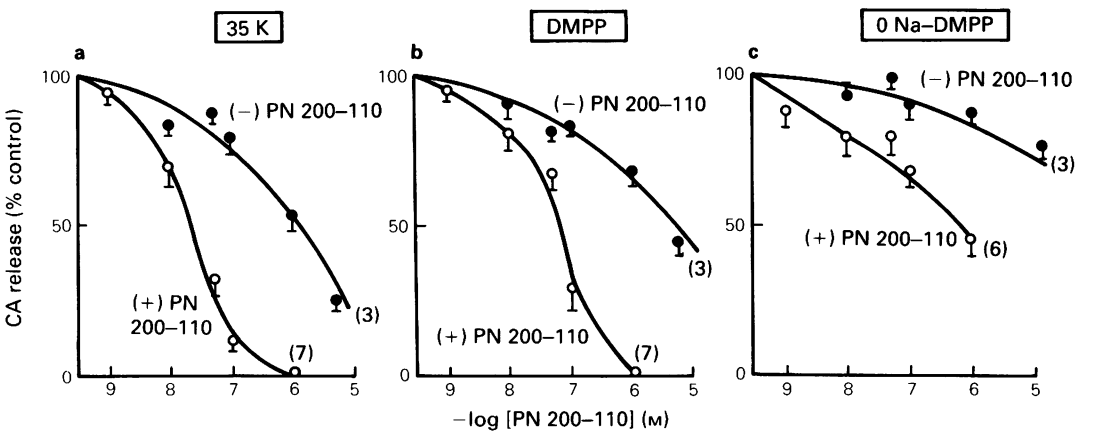

Figure 3 Effect of increased concentrations of (+)- (○) and (−)-PN200-110 (●) on catecholamine release evoked by K (35 mM for 1 min) or DMPP (5 μM for 1 min). Experiments were carried out in the presence (a and b) or the absence (c) of Na. Data are expressed as % of the release obtained in each stimulation in parallel experiments performed on control glands; therefore, the decay of the secretion not related to the drug action was corrected for. The results represent mean results from the number of glands shown in parentheses; vertical bars show s.e.mean.

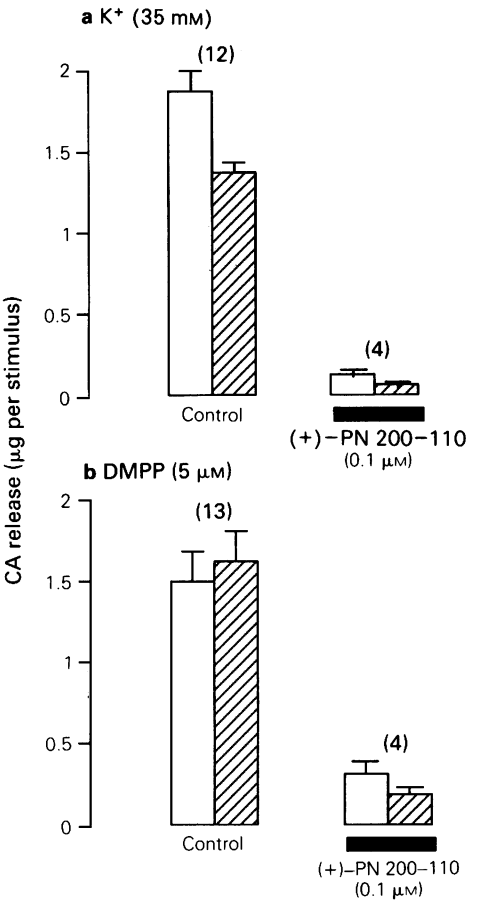

Figure 4 Effects of PN200-110 on the separate release of noradrenaline (open columns) and adrenaline (hatched columns) evoked by K (35 mM for 1 min; a) or DMPP (5 μM for 1 min; b). PN200-110 (0.1 μM) was present 10 min before and during the stimulation period. Data are means of the number of experiments shown in parentheses; vertical bars show s.e.mean.

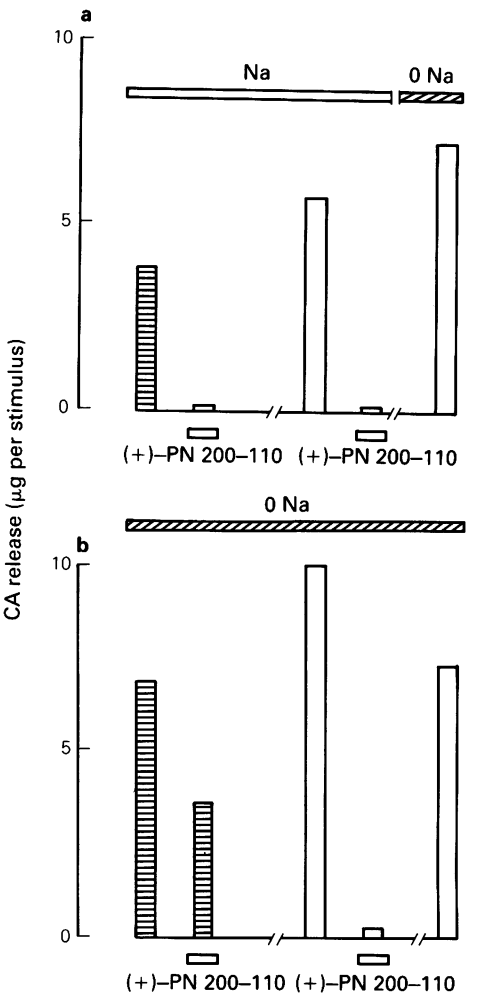

Figure 5 Differential effects of (+)-PN200-110 on catecholamine release evoked by DMPP (horizontally striped column) (5 μM for 1 min) or K (open column) (35 mM for 1 min), in the presence (a) or the absence (b) of Na ions. When added, (+)-PN200-110 (1 μM) was present from 10 min before and during the stimulation period. The experiment was performed in parallel in two glands of the same cat. Data are the results of one typical experiment of three.

amount equivalent to 58% of total catecholamine secreted; the remaining 42% (1.330 ng) accounted for adrenaline (Figure 4). PN200-110 (0.1 μM) inhibited by over 90% the secretion of both catecholamines.

Comparative effects of (+)-PN200-110 on catecholamine release evoked by potassium or DMPP from the same gland

Since (+)-PN200-110 seemed to distinguish clearly catecholamine release evoked by K from that induced by DMPP in a Na-free medium, it was reasoned that this drug could separate the two secretory responses and that the specificity of such a phenomenon would be enhanced if both stimuli (K and DMPP) were applied sequentially to the same gland. Figure 5 shows the results of such an experiment.

Upon stimulation of the gland with DMPP (5 μM for 1 min), 3.87 μg of catecholamine was released. The same stimulus, applied 30 min later in the presence of (+)-PN200-110 (1 μM, 10 min before), failed to increase the spontaneous catecholamine release. After a 2.5 h washout period with fresh Krebs solu-

tion, stimulation with K (35 mM for 1 min) produced a good secretory response (5.7 μg) which was also blocked 30 min later by the presence of (+)-PN200-110 (1 μM present for 10 min beforehand) in the perfusion fluid. Finally, after washing out the gland with Na-free solution for 1 h, K (35 mM) evoked a sharp secretory response (Figure 5a). These data clearly indicate that (+)-PN200-110 inhibited fully both DMPP and K-evoked catecholamine secretion in the presence of Na.

In the contralateral gland, a similar protocol was followed but Na was absent during the entire experiment. Under these conditions, DMPP produced a large secretory response (6.83 μg) which was reduced only by 45% in the presence of (+)-PN200-110 (1 μM). However, secretion evoked by K (9.9 μg) was completely inhibited by the drug. After a 60 min washing period, the secretory response evoked by a new K-pulse was similar to that obtained in the contralateral gland (Figure 5b).

Discussion

Data shown here demonstrate two main features of the catecholamine secretory response evoked by DMPP or high K stimulation of perfused cat adrenal glands: (1st) that Na deprivation improves it; and (2nd) that the dihydropyridine Ca channel blocker PN200-110, blocks the nicotinic response only in the presence of Na. The first observation might suggest that Na is playing no role in triggering the physiological acetylcholine-mediated response, as reported by Douglas & Rubin (1961), Lastowecka & Trifaró (1974), Kilpatrick *et al.* (1981) and Amy & Kirshner (1982) who observed that Na removal did not affect secretion; however, opposite results were found by Role *et al.* (1981), Lemaire *et al.* (1981), Ceña *et al.* (1983) and Wada *et al.* (1985). On the other hand, the results obtained with PN200-110 clarify this issue since this dihydropyridine is a potent and highly selective blocker of cat adrenal chromaffin cell Ca channels (Gandia *et al.*, 1987).

These results fit well with the following sequence of events taking place during the physiological exocytotic cycle at the splanchnic nerve-chromaffin cell synapse (Ceña *et al.*, 1983). On activation of the nicotinic cholinoceptor by endogenously released acetylcholine, external Na enters the cell through its ionophore, causing depolarization and opening of voltage-sensitive Ca channels, Ca entry and secretion. In these conditions, PN200-110 should inhibit DMPP- as well as K-evoked secretion; this was the case. However, in the absence of Na, depolarization is not taking place and most of the DMPP secretory response obtained in these conditions is probably due to Ca entry through the PN200-110-resistant acetylcholine receptor ionophore; this is strengthened by the strong stereoselective effects of its two enantiomers in the presence of Na (where Ca channels are recruited) and the weak blockade observed in the absence of the cation. Some blockade caused by PN200-110 in zero Na might be due to slight depolarization caused by Ca itself entering through the cholinoceptor ionophore. Although the enantiomers of PN200-110 were synthesized by stereoselective synthesis, some cross-contamination might exist; however, the less potent haemodynamic effects of the (−)-enantiomer cannot be explained only by contamination with the (+)-enantiomer (Hof *et al.*, 1985). In any case, the poor blocking effects on secretion of (−)-PN200-110 corroborates previous findings from our laboratory (Fonteriz *et al.*, 1987) and strengthens the view that the dihydropyridine receptor displays an exquisite stereoselectivity in chromaffin cells.

A last intriguing observation relates to the potentiation of secretion in the absence of Na. Since the classical observation of Luttgau & Niedergerke (1958) that external Na and Ca ions compete for common sites in the heart, it has been corroborated that Na removal facilitates Ca entry inside many cell types, including the chromaffin cell (Artalejo *et al.*, 1987). An alternative explanation relates to the blockade and/or reversal of the Na/Ca exchange mechanism that at the cat adrenal medulla is triggering a clear secretory response (Esquerro *et al.*, 1981; Abajo *et al.*, 1987); upon DMPP or high K stimulation in zero Na, Ca entering the cell is not pumped out by such a mechanism and its secretory effects are amplified in this manner.

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