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M_2 MUSCARINOCEPTOR-ASSOCIATED IONOPHORE AT THE CAT ADRENAL MEDULLA

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SUMMARY: Atropine and pirenzepine displaced ^{3}H-quinuclydinyl-benzylate binding and inhibited methacholine-evoked catecholamine release with a similar order of potencies, atropine being 200 fold more potent than pirenzepine. In contrast to high-K, methacholine-evoked ^{45}Ca uptake or catecholamine release were not blocked by (+)PN200-110. Bay-K-8644 did not modify the secretory response to methacholine either in the presence of Ca or Sr but potentiated K-evoked secretion. In depolarized glands, methacholine still evoked its usual secretory response. The results suggest that muscarinic stimulation of cat adrenal chromaffin cells stimulates Ca entry though an ionophore other than voltage-dependent Ca channels; such ionophore seems to be chemically operated through a M_2 muscarinoceptor.

Muscarinoceptor stimulation causes different biochemical and physiological responses in chromaffin cells from various animal species; the subtype of muscarinoceptor mediating those responses is unknown. In the cat (1,2), gerbil (3), guinea-pig (4) and rat (5-7) a muscarinoceptor mediates an increase of the rates of catecholamine release. In contrast, muscarinic stimulation does not enhance catecholamine secretion from freshly isolated (8,9) or cultured bovine adrenal chromaffin cells (10-14), yet it increases cGMP levels (10,15), phospholipid turnover (12,13) and intracellular free Ca concentrations (16). How these changes relate to the physiological control of the stimulus-secretion coupling process following stimulation of chromaffin cells by endogenously released acetylcholine, and why in several species (feline, rodents), but not in others (bovine), muscarinoceptors trigger catecholamine release, are fundamental questions that might be explained by assuming a coupling of this receptor to a specific membrane ionophore or channel in the feline, but not in the bovine adrenal gland. In this paper, we provide evidence suggesting that the cat adrenal chromaffin cell preferentially secretes adrenaline in response to

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methacholine by activating Ca entry into the cell through an ionophoric channel associated to or controlled by a M_2-type muscarinic cholinoceptor; such channel is chemically operated and seems to be unrelated to voltage sensitive Ca channels.

METHODS

^{3}H-quinuclydinyl benzylate binding

Aliquots (100 µg protein/ml) of an 800xg supernatant homogenate from cat adrenomedullary or atrial tissues (20 volumenes of 50 mM Tris-HCl buffer, pH 7.4) were incubated at 37ºC for 1 h in 50 mM phosphate buffer pH 7.4 containing 0.02-1 µM (-)^{3}H-QNB (Amersham, specific activity 33 Ci/mmol). The procedure was based in that of Kayaalp and Neff (17). Non-specific binding was defined as the radioactivity bound in the presence of 1 µM atropine.

Catecholamine release

Both cat adrenal glands were isolated and perfused at room temperature (25 + 2ºC) with Krebs-bicarbonate solution bubled with 95% O_2-5% CO_2 at pH 7.4. Solutions containing high K were prepared by adding KCl_2 and reducing isoosmotically NaCl. Catecholamine release were continuously monitored by on-line connection of the perfusion fluid emanating from the glands to a Metrohm electrochemical detector; in some experiments, noradrenaline and adrenaline were separated by high performance liquid chromatography (Series 10 Perkin-Elmer) (18).

^{45}Ca uptake

After equilibration with Krebs-bicarbonate solution, glands were perfused at 1 ml/min during 90 min with solutions containg 16 µCi/ml of ^{45}Ca (Amersham, sp., act. 40 mCi/mg) and then washed for 5 min with radioactive-free fresh solution. The stimulating and washing solutions contained 25 mM Na^+ (as $NaHCO_3$), 236 mM sucrose and 250 µM $CaCl_2$ as well as the rest of the components of the Krebs solution. At the end of the washing period, glands were frozen in liquid nitrogen, their medullae carefully dissected out, digested overnight in 1 ml of 2% sodium dodecylsulfate at 37ºC and their radioactivity contents counted in a Beckman 2800 model scintillation counter.

RESULTS AND DISCUSSION

Both, radioligand binding studies and secretion suggest the M_2 nature of the cat adrenal medulla muscarinoceptor (Fig. 1). ^{3}H-quinuclydinyl benzylate (^{3}H-QNB) binding to cat adrenomedullary and atrial membranes was highly specific (more than 90%), saturable and the Scatchard analysis revealed a single population of receptors with a n_H Hill coefficient of 1.07 for the adrenal medulla. ^{3}H-QNB bound was displaced by atropine and pirenzepine with a similar order of potency, atropine (a non-specific antagonist) being 200 fold more potent than pirenzepine (a M_1 specific antagonist) (9). Various muscarinic agonists given at 3-30 µM for 30 s enhanced catecholamine release from perfused cat

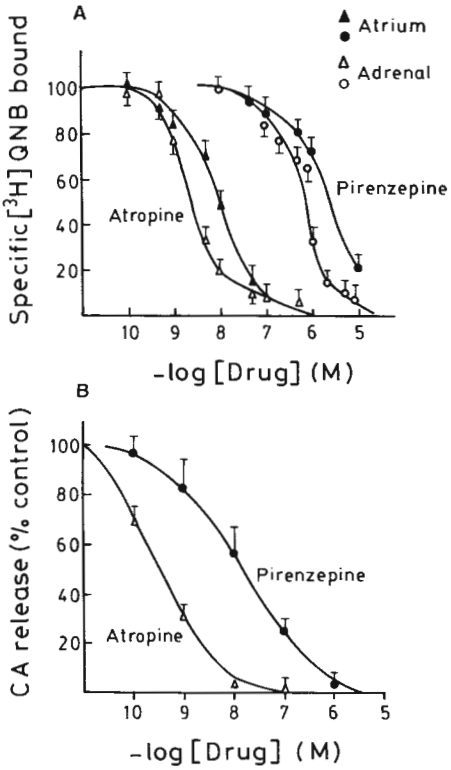

Fig. 1. A. Displacement by muscarinoceptor antagonists of ^{3}H-Quinuclydinyl benzylate (^{3}H-QNB) binding to cat adrenomedullary and atrial membranes. Atropine or pirenzepine were preincubated for 10 min with the homogenate at the concentrations shown in the abscissa; a concentration of 0.5 nM of ^{3}H-QNB was used. Displacement data are the means ± s.e. of 3 experiments made in triplicate.

B. Inhibition by muscarinoceptor antagonists of catecholamine release evoked by methacholine. Methacholine pulses (3 μM for 30 s) were given at 30 min intervals. Once the secretory response stabilized, cumulative concentrations (abscissa) of atropine or pirenzepine were added and the methacholine pulses repeated in their presence 10 min later. Data are means ± s.e. of 4 experiments.

adrenal glands with the following relative order of potencies: methacholine > pilocarpine > oxotromerine > McN-A-343 > betachenchol > muscarine. Being the most potent, methacholine was selected to perform the following experiments. Atropine was 100-fold more potent than pirenzepine in inhibiting methacholine-evoked release, suggesting again that the muscarinoceptor involved in this response is of the M_2 subtype.

Methacholine- and K^+-evoked catecholamine release with 30 s pulses have in common (Fig. 2): (1st) that secretion reaches a quick rise to a peak and a decline to basal levels; (2nd) that repetitive identical stimuli (applied at 15-min intervals) give similar responses showing little

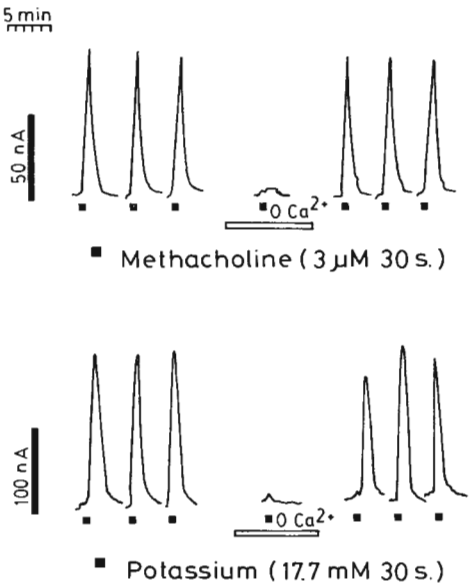

Fig. 2. Profiles of catecholamine secretory responses obtained upon perfusion of cat adrenal glands with high K concentrations or with methacholine. The graphs show the release of total catecholamines evoked by 30-s pulses of methacholine (top) or K (bottom). On Ca deprivation (horizontal bars), the release to methacholine or K was abolished; upon Ca restoration, the response recovered fully. The horizontal top bar reflects the elapsed time and the vertical bar the oxydation current obtained in nanoamperes at the electrochemical detector. The curves were drawn directly from the recording paper and are taken from a typical experiment out of 5.

desensitization; and (3rd) that, as previously shown (20), Ca deprivation abolishes the secretory response. These similarities might lead to the conclusion that both, methacholine and K enhance catecholamine release by a similar mechanism, i.e., activation of voltage-sensitve Ca channels. However, when the secretory responses are carefully analysed, they considerably differ, as the following experiments demonstrate.

Methacholine (3 µM for 30 s) released 207 ± 23 ng/pulse (n = 20) of total catecholamines from which, 80% accounted for adrenaline; in contrast, K (17.7 mM for 30 s) released 310 ± 30 ng/pulse of catecholamines from which 48% was noradrenaline and 52% adrenaline, suggesting that only muscarinic stimulation discriminates between adrenergic and noradrenergic chromaffin cells.

The second important difference concerns the membrane potential; although pilocarpine and acetylcholine depolarize gerbil (3) and rat (6) chromaffin cells and atropine blocks it, it is unlikely that muscarinic depolarization is responsible for the secretory effects of methacholine.

Two facts support this assertion: (1st) methacholine-evoked secretion does not inactivate after prolonged stimulation, but the K response quickly inactivates (21,22); and (2nd) methacholine evokes a full secretory response even in depolarized glands. In 3 experiments, methacholine (30 μM for 30 s) was applied on top of various K concentrations (1.2, 5.9, 17.7, 35, 59 and 125 mM) once the K response was inactivated. It is known that chromaffin cells remain depolarized after long periods of K exposure, in spite of the decline of the rate of release to almost basal levels (23,24). Methacholine evoked a similar secretory response at all levels of depolarization. This finding contrasts with one obtained using nicotine as secretagogue in an otherwise similar protocol: at low K depolarizations, nicotine evoked a healthy secretory response but at higher depolarizations nicotine failed to enhance catecholamine release (A.R. Artalejo and A.G. García; unpublished results). So, the nicotine-secretory effects seem to require cell depolarization but methacholine, no.

The third difference was established using the S(+) enantiomer of the dihydropyridine PN200-110, a potent and selective Ca antagonist in the cat adrenal medulla (25). In 15 glands, 35 mM K pulses given at 30-min intervals released 3.15±0.23 μg of catecholamines (N=94 pulses); cumulative concentration-response curves gave an IC50 for (S)(+)PN200-110 to inhibit K-evoked secretion of 0.9 nM (N=8). At a concentration of 10 nM, that effectively blocks high K-stimulated release, the methacholine response was unaffected (Fig. 3). Furthermore, there was no difference in the ability to inhibit methacholine-stimulated release between the more active (+) and the less active (-) stereoisomers of (+)PN200-110; in contrast, K-evoked release was inhibited by the (-) enantiomer at concentrations 100-fold higher than those required with the (+) enantiomer (25). These results show a high degree of stereospecific inhibition of K-evoked release by (+)PN200-110, but no stereospecific inhibition of methacholine-induced release suggesting a different patway for Ca entry during both types of stimulation. We also performed experiments to test the effects of muscarinic stimulation on ^{45}Ca uptake into adrenomedullary cells (Fig. 3). Methacholine doubled and K tripled the basal ^{45}Ca uptake into adrenomedullary chromaffin cells; while 10 nM (+)PN200-110 inhibited K-evoked uptake by 80%, the methacholine effects were not affected by the dihydropyridine.

An experiment using Sr and Ca as permeant cations established a clear-cut fith difference between K and methacholine. Sr permeates chromaffin cell Ca channels better than Ca, causing a greater secretory

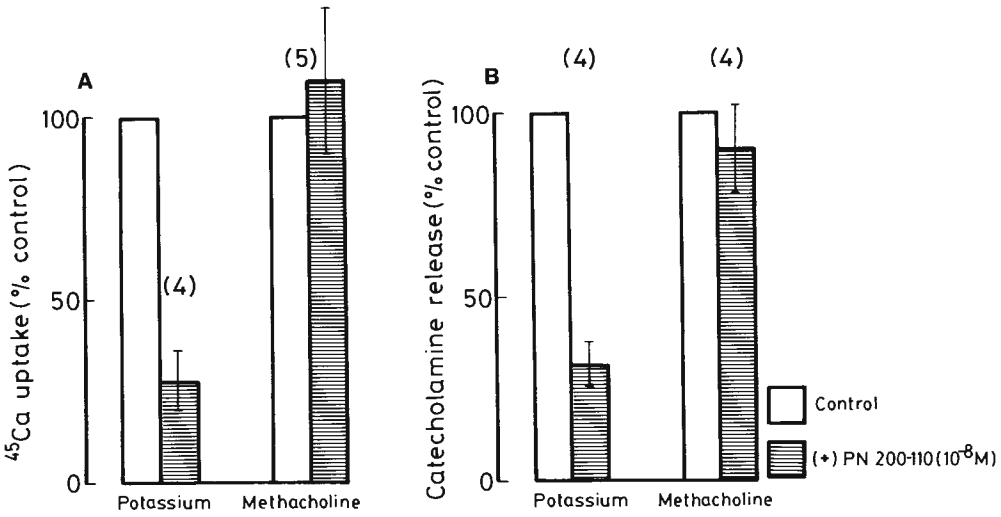

Fig. 3. A. ^{45}Ca uptake into adrenomedullary chromaffin cells upon stimulation with methacholine or K. Basal uptake of ^{45}Ca amounted to 245± 30 cpm/μg protein; net ^{45}Ca uptake evoked by methacholine or K, with or without (+)PN200-110 (10 nM added 10 min before the ^{45}Ca pulse), were calculated by subtracting the basal to the total tissue uptake. Data are means ± s.e. of 4-5 experiments.

B. Effects of (S)(+)PN200-110 on catecholamine release evoked by 30 s pulses of methacholine (30 μM) or K (35 mM). Pulses of methacholine or K were given to parallel glands at 30 min intervals; once the responses were stabilized, cumulative concentrations of (+)PN200-110 were introduced in the perfusion fluid. Data are means of peak catecholamine release ± s.e. of 4 paired experiments.

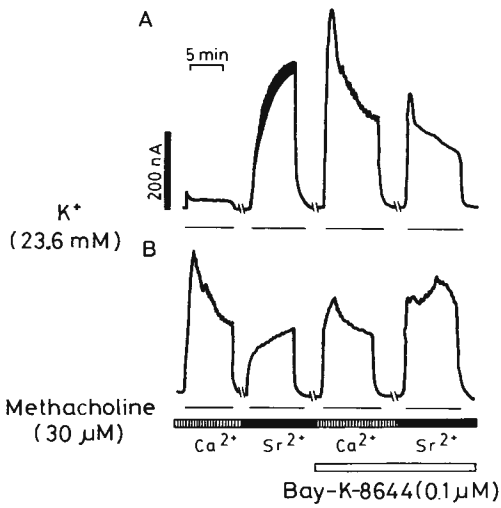

Fig. 4. Effects of Bay-K-8644 on the secretory responses to sustained stimulation with methacholine or high K. In gland B, methacholine (30 μM) was applied first in the presence of 2.5 mM Ca and then in 2.5 mM Sr; these stimulations were repeated in the presence of Bay-K-8644 (10^{-7}M). In gland A, a similar protocol was performed but this time using 17.7 mM K as secretagogue. Results from a typical experiment out of 3. The vertical bar corresponds to the oxidation current in nanoamperes.

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