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Voltage-independent catecholamine release mediated by the activation of muscarinic receptors in guinea-pig adrenal glands

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1 The differences between the mechanisms of muscarinic and nicotinic receptor-mediated catecholamine secretion with respect to their dependence on voltage changes and extracellular Ca were examined using perfused adrenal glands of the guinea-pig.

2 Acetylcholine (ACh, 10^{-6} to 10^{-3} M) caused a dose-dependent increase in catecholamine secretion. The ED_{50} value for ACh was 7×10^{-5} M. In the presence of atropine (10^{-5} M), the dose-response curve for ACh was shifted to the right. Hexamethonium (5×10^{-4} M) preferentially reduced the responses to higher concentrations of ACh ($> 10^{-5}$ M). Pilocarpine (5×10^{-4} M) and nicotine (3×10^{-5} M) also stimulated catecholamine release.

3 During perfusion with isotonic KCl solution, ACh and pilocarpine, but not nicotine, evoked catecholamine secretion. These responses were abolished by atropine (10^{-6} M). Pilocarpine-stimulated catecholamine secretion was enhanced during perfusion with isotonic KCl solution. Under these conditions, hexamethonium (10^{-3} M) significantly augmented ACh-evoked catecholamine release.

4 During perfusion with either Ca-free isotonic KCl or Ca-free Locke solution, ACh and pilocarpine caused a partial increase in catecholamine secretion whereas nicotine and high K solution (56 mM) did not. The responses to ACh and pilocarpine were completely inhibited by atropine but not by hexamethonium.

5 When guinea-pig adrenal glands were perfused with isotonic KCl solution containing 2.2 mM Ca which was subsequently removed and replaced with EGTA, ACh-induced catecholamine secretion was similar in magnitude to that observed during perfusion with Locke solution.

6 We conclude that both nicotinic and muscarinic receptors are involved in ACh-induced catecholamine secretion from guinea-pig adrenal chromaffin cells. Activation of muscarinic or nicotinic receptors appears to stimulate catecholamine release through different mechanisms with respect to both voltage-dependence and Ca requirements.

Introduction

Acetylcholine (ACh), the transmitter released from greater splanchnic nerve terminals, causes catecholamine secretion from the adrenal gland by increasing Ca entry into adrenal chromaffin cells (Douglas, 1968; Douglas & Poisner, 1961; 1962). Both muscarinic and nicotinic receptors participate in ACh-induced catecholamine secretion from adrenal glands of various species (Douglas, 1975; Ungar & Phillips, 1983; Livett, 1984). It has been proposed that two types of Ca channel participate in the ACh-induced Ca influx into adrenal chromaffin cells; one is opened by depolarization of chromaffin cell membranes and the other is closely related to the cholinoceptor which is independent of voltage changes (Douglas *et al.*, 1967; Brandt *et al.*, 1976; Kidokoro & Ritchie, 1980; Kidokoro *et al.*, 1982). The activation of nicotinic receptors stimulates catecholamine secretion by increasing Ca entry through voltage-dependent Ca channels in both perfused adrenal glands of the rat (Wakade & Wakade, 1983) and bovine isolated adrenal chromaffin cells (Kilpatrick *et al.*, 1981; Knight & Kesteven, 1983). The mechanism of muscarinic receptor-linked catecholamine secretion remains unclear.

Recently, we found that ACh and pilocarpine, but

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not nicotine, caused a partial increase in catecholamine secretion during perfusion with Ca-free Locke solution in cat adrenal glands (Nakazato *et al.*, 1984). Catecholamine release under these conditions was inhibited by atropine, but not by hexamethonium. We hypothesized that activation of muscarinic receptors mobilizes intracellular Ca stores which stimulate catecholamine secretion. It has not been determined whether this mechanism is dependent on voltage changes in the chromaffin cell membrane.

The purpose of the present experiment was to characterize the differences between the mechanisms of muscarinic and nicotinic receptor-mediated catecholamine secretion in perfused adrenal glands of the guinea-pig, with attention to dependence on depolarization and Ca availability. We examined the effects of atropine and/or hexamethonium and of the removal of extracellular Ca on the secretory responses to ACh, pilocarpine, nicotine and high K solution, during perfusion with Locke and isotonic KCl solutions.

Methods

Preparations

Guinea-pigs of either sex, weighing 400 to 800 g, were anaesthetized with sodium pentobarbitone (40 mg kg^{-1}) intraperitoneally. Both adrenal glands were perfused and isolated following the general procedure described previously (Ito *et al.*, 1979). The glands were perfused at a flow rate of 0.5 to 0.6 ml min^{-1} and maintained at room temperature (approximately 25°C). The adrenal effluent was collected continuously in 5 min aliquots into glass tubes kept on ice.

The standard perfusion medium was modified Locke solution of the following composition (mM): NaCl 154, KCl 5.6, $CaCl_2$ 2.2, $MgCl_2$ 1.2, Na_2HPO_4-NaH_2PO_4 buffer (pH 7.0) 3 and glucose 10. For the isotonic KCl solutions (159.6 mM), all NaCl was replaced by a corresponding concentration of KCl. In the Ca-free solutions, $CaCl_2$ was omitted and glycoletherdiaminetetraacetic acid (EGTA) (10^{-4} or 10^{-3} M) was added. All solutions contained physostigmine (2×10^{-7} M) to prevent hydrolysis of ACh and were bubbled with pure O_2. For the high K solution, KCl was increased to 56 mM and NaCl was reduced correspondingly.

Drugs were administered 30 to 40 min after isolation of the adrenal glands. Secretagogues were administered for 2 min, except the high K^+ solution which was perfused for 1 min, beginning 30 s before the 5 min sampling period. Samples were acidified with 8 M perchloric acid to a final concentration of 0.4 M and stored on ice until assayed.

Catecholamine assays

Adrenaline, noradrenaline and dopamine were separated by high performance liquid chromatography (h.p.l.c, Jasco) and native fluorescence detected. The treatment of samples for h.p.l.c. was carried out according to the method described by Salzman & Sellers (1982). Catecholamine values presented are the sum of noradrenaline, adrenaline and dopamine. Total catecholamines were also assayed by the fluorometric method of Anton & Sayre (1962).

Materials

The following drugs were used: acetylcholine chloride (Ovisort; Daiichi), atropine sulphate (K & K), glycoletherdiaminetetraacetic acid (Wako Pure Chem.), hexamethonium chloride dihydrate (Wako Pure Chem.), nicotine bitartrate (Tokyo Kasei), pilocarpine hydrochloride (Tokyo Kasei), and physostigmine sulphate (Wako Pure Chem.). All compounds were dissolved in Locke solution.

Statistics

The data are presented as arithmetic means ± s.e.mean. Significance tests were performed by Student's *t* test. Significance was assumed when $P < 0.05$.

Results

ACh, nicotine, pilocarpine and high K solution-induced catecholamine secretion

The responsiveness of perfused adrenal glands was tested by applying ACh (10^{-5} M) for 2 min repeatedly at 15 min intervals over the course of one hour in 14 preparations. Basal catecholamine secretion was 0.31 ± 0.09 nmol 5 min^{-1}. Each bolus infusion of ACh caused a large increase in catecholamine secretion. There was no significant difference ($P < 0.05$) between the amounts of catecholamines released by four repeated ACh infusions; they were 5.69 ± 0.75, 5.64 ± 0.43, 5.48 ± 0.53 and 5.06 ± 0.48 nmol 5 min^{-1} respectively. A dose-response curve for ACh on catecholamine secretion was constructed by applying 3 or 4 different concentrations of ACh, ranging from 10^{-7} M to 10^{-3} M, at 15 min intervals in increasing concentrations to single preparations. A secretory response to ACh was first evident at 5×10^{-6} M and increased in magnitude as the concentration was increased until it attained a maximum at 5×10^{-4} M. The ED_{50} value for ACh was 7×10^{-5} M (Figure 1). Nicotine (3×10^{-5} M), pilocarpine (5×10^{-4} M) and high K (56 mM) stimulated catecholamine secretion to

8.19 ± 1.13, 2.49 ± 0.18 and 10.11 ± 1.03 nmol $5\,min^{-1}$, respectively.

Effects of atropine and hexamethonium on the dose-dependent increase in ACh-induced catecholamine secretion

In the presence of atropine (10^{-5} M), the secretory response to ACh was first observed at a concentration of 2×10^{-5} M and increased in magnitude as the concentration of ACh was increased until catecholamine secretion attained a maximum at 5×10^{-4} M (Figure 1). The maximum value was 73% of that obtained in the absence of atropine. It appeared that atropine was much more effective in inhibiting the response to lower concentrations of ACh than the responses to higher concentrations of ACh. In contrast, hexamethonium (5×10^{-4} M) preferentially reduced the responses to higher concentrations of ACh. Hexamethonium (5×10^{-4} M) inhibited the response to 10^{-4} M ACh by 18% but blocked the response to 5×10^{-4} M ACh by 43%. Combined application of atropine and hexamethonium caused a marked inhibition of the dose-response curve; the maximum response attained at 5×10^{-4} M was only 18% of the control. The ED_{50} values for ACh were 8×10^{-5} M and 6×10^{-5} M in the presence of atropine and hexamethonium, respectively.

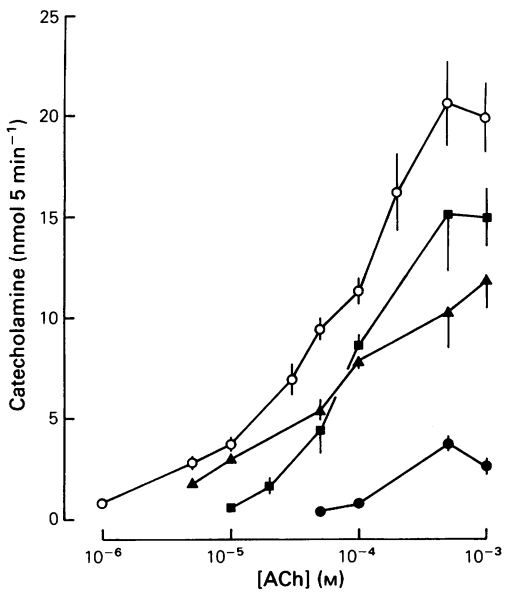

Figure 1 Dose-response curves for acetylcholine (ACh)-induced catecholamine secretion in the presence or absence of atropine and/or hexamethonium. Three or four different concentrations of ACh ranging from 10^{-6} to 10^{-3} M were sequentially applied for 2 min to single preparations of adrenal glands during perfusion with Locke solution in the absence (○) and presence (■) of atropine (10^{-5} M), hexamethonium (5×10^{-4} M) (▲) and both (●). Means, with s.e.mean indicated by vertical lines if it exceeds the size of the symbol, are shown (4 to 21 experiments). The ordinate scale represents the amount of catecholamines released and the abcissa scale the concentration of ACh on a logarithmic scale. Catecholamines were assayed by the fluorometric method of Anton & Sayre (1962).

Catecholamine secretion evoked by ACh, pilocarpine and nicotine during perfusion with isotonic KCl solution

When the perfusion medium was switched to isotonic KCl solution, a significant increase in catecholamine secretion occurred (Figure 2). This response quickly attenuated to a level which was about 5 fold higher than that observed during perfusion with Locke solution. Under the new steady-state condition, ACh (10^{-5} M) caused a secretory response similar to that obtained during perfusion with Locke solution. Nicotine (3×10^{-5} M) failed to stimulate secretion during perfusion with the isotonic KCl solution. The response to nicotine was partially restored after the perfusion medium was switched back to Locke solution. The secretory response to pilocarpine (5×10^{-4} M) during exposure to isotonic KCl was 2.8 times larger than that seen during perfusion with Locke solution (Figure 3).

Effects of atropine, hexamethonium and nicotine on ACh and pilocarpine-stimulated catecholamine secretion during exposure to isotonic KCl solution

Atropine (10^{-6} M) completely inhibited, while hexamethonium (10^{-3} M) significantly enhanced, catecholamine secretion evoked by ACh (10^{-5} to 10^{-3} M) during perfusion with isotonic KCl solution (Table 1). These findings indicate that ACh causes an increase in catecholamine secretion from completely depolarized chromaffin cells through activation of muscarinic receptors. They further suggest that nicotinic receptor activation may have an inhibitory influence on muscarinic receptor-mediated catecholamine secretion under depolarizing conditions when nicotinic stimulation is not effective in releasing catecholamines.

Next, we determined whether nicotine influenced the secretory response to pilocarpine during exposure to isotonic KCl solution. When pilocarpine (5×10^{-4} M) was infused with nicotine (10^{-5} M) during exposure to isotonic KCl, pilocarpine stimulated catecholamine secretion by 1.2 fold (Figure 4a). However, when hexamethonium (10^{-3} M) was added with nicotine during exposure to isotonic KCl solu-

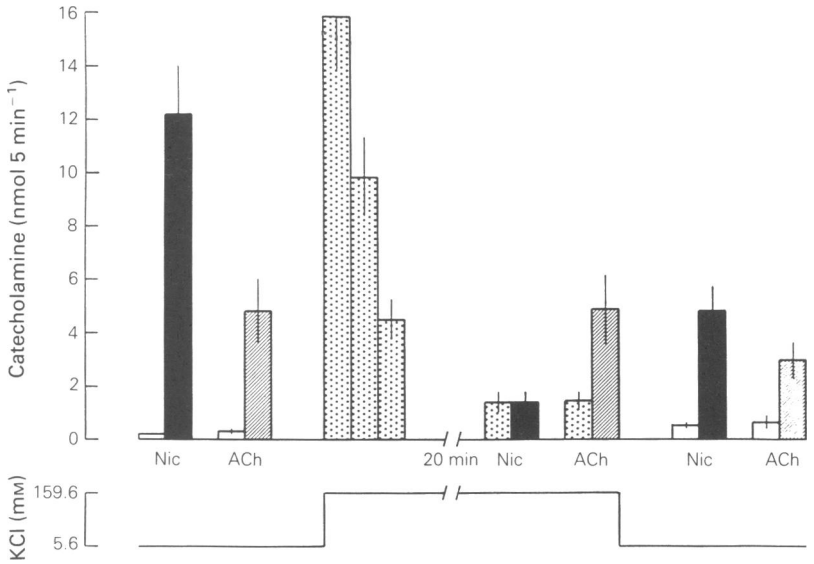

Figure 2 Catecholamine secretion induced by nicotine (Nic) and acetylcholine (ACh) during perfusion with isotonic KCl solution. Nicotine (3×10^{-5} M) and ACh (10^{-5} M) were sequentially applied for 2 min at 15 min intervals during perfusion with Locke solution, 30 min after switching the perfusion medium to isotonic KCl (159.6 mM) solution and 15 min after switching back to Locke solution. Columns represent the mean (and vertical lines s.e.) of catecholamine secretion evoked by nicotine (solid columns), ACh (hatched columns) and isotonic KCl (stippled columns) obtained from 3 experiments. In this and the following figures, except for Figure 5, the catecholamines represent the sum of adrenaline, noradrenaline and dopamine assayed by h.p.l.c.

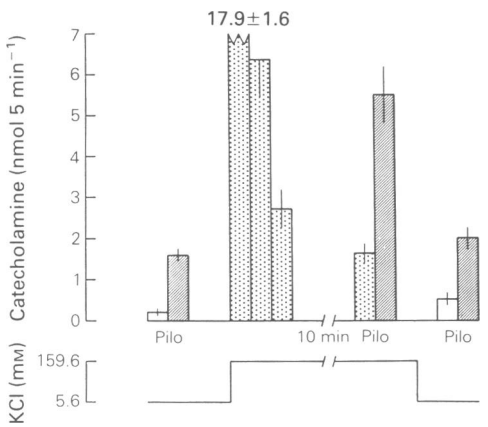

Figure 3 Catecholamine secretion induced by pilocarpine (Pilo) before, during and after exposure to isotonic KCl solution. Pilocarpine (5×10^{-4} M) was applied for 2 min before, 35 min after the start of exposure to isotonic KCl (159.6 mM) solution and 15 min after switching back to Locke solution. Columns represent the mean (and vertical lines s.e.) of catecholamine secretion evoked by pilocarpine (hatched columns) and isotonic KCl solution (stippled columns) in nmol $5\,min^{-1}$ obtained from 5 experiments.

tion, pilocarpine stimulated catecholamine secretion by 3.2 fold (Figure 4b), which was comparable to the stimulation resulting from the application of pilocarpine alone (Figure 3).

Effect of removal of extracellular Ca on secretagogue-stimulated catecholamine secretion during exposure to isotonic KCl solution

We have previously demonstrated that ACh and pilocarpine, but not nicotine and high K, are partially effective in increasing catecholamine secretion in the absence of extracellular Ca in cat perfused adrenal glands (Nakazato *et al.*, 1984). The present experiment analysed the results of similar treatments in perfused adrenal glands of the guinea-pig. Nicotine (3×10^{-5} M), high K solution, ACh (10^{-5} M) and pilocarpine (5×10^{-4} M) were alternatively or independently applied before and during perfusion with Ca-free Locke solution containing EGTA. As shown in Figure 5a, in the presence of Ca, all secretagogues caused increases in catecholamine secretion. Removal of extracellular Ca decreased basal catecholamine secretion to about one fifth of normal levels. In the absence of Ca, nicotine and high K solution were

Table 1 Catecholamine secretion from adrenal glands perfused with Locke and isotonic KCl (159.6 mM) solutions

	A				B		
	Locke	*Isotonic KCl solution*			*Locke*	*Ca-free isotonic KCl*	
			Atropine (10^{-6} M)	C_6 (10^{-3} M)	C_6 (10^{-3} M)	C_6 (10^{-3} M)	*Ca loading* C_6 (10^{-3} M)
No. of tests	4	4	1	4	5	5	5
Resting release	0.3 ± 0.0	1.8 ± 0.1	2.0	1.8 ± 0.1	0.4 ± 0.1	0.5 ± 0.2	0.2 ± 0.0
Acetylcholine (M)							
10^{-5}	3.1 ± 0.6	5.1 ± 1.0	2.0	10.0 ± 1.2	1.6 ± 0.2	0.7 ± 0.2	1.7 ± 0.2
5×10^{-5}	10.4 ± 1.1	6.1 ± 0.9	2.0	—	—	—	—
10^{-4}	14.3 ± 1.9†	7.5 ± 1.1†	2.0	13.5 ± 1.8	4.9 ± 0.6	0.8 ± 0.1	6.8 ± 1.2
5×10^{-4}	20.2 ± 1.3	8.6 ± 1.1	—	—	—	—	—
10^{-3}	29.4 ± 2.8	8.7 ± 0.9	—	15.1–± 2.0	7.9 ± 0.8	1.3 ± 0.2	6.3 ± 0.6

Numbers indicate the mean (± s.e.) of catecholamines (sum of noradrenaline, adrenaline and dopamine) released in nmol 5 min^{-1} in the various conditions indicated. † No. of tests is 8. Abbreviation, C_6: hexamethonium.

ineffective, but ACh still caused a partial increase in catecholamine secretion (Figure 5b). The responses to ACh attenuated on repeated application, but were partially restored after the reintroduction of Ca for 25 min before re-exposure to Ca-free medium (Figure 5c). Similar results were seen for pilocarpine (data not shown). Atropine (10^{-6} M) completely inhibited these responses while hexamethonium (10^{-3} M) was ineffective.

The effect of the removal of extracellular Ca during exposure to isotonic KCl solution was examined in the presence of hexamethonium (10^{-3} M). After the res-

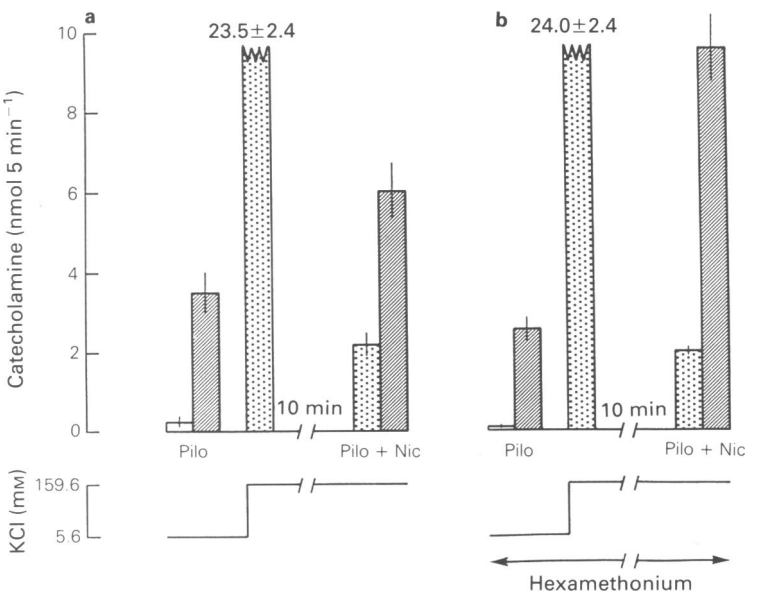

Figure 4 Catecholamine secretion induced by pilocarpine (pilo) plus nicotine (Nic) during exposure to isotonic KCl solution in the absence and presence of hexamethonium. Pilocarpine (5×10^{-4} M) and pilocarpine (5×10^{-4} M) plus nicotine (10^{-5} M) were applied for 2 min respectively before and during exposure to isotonic KCl (159.6 mM) solution in the absence (a) and presence (b) of hexamethonium (10^{-3} M). Columns represent the mean (and vertical lines s.e.) of catecholamine secretion evoked by pilocarpine (hatched columns) and pilocarpine plus nicotine (hatched columns) and isotonic KCl (stippled columns) obtained from 4 respective experiments.

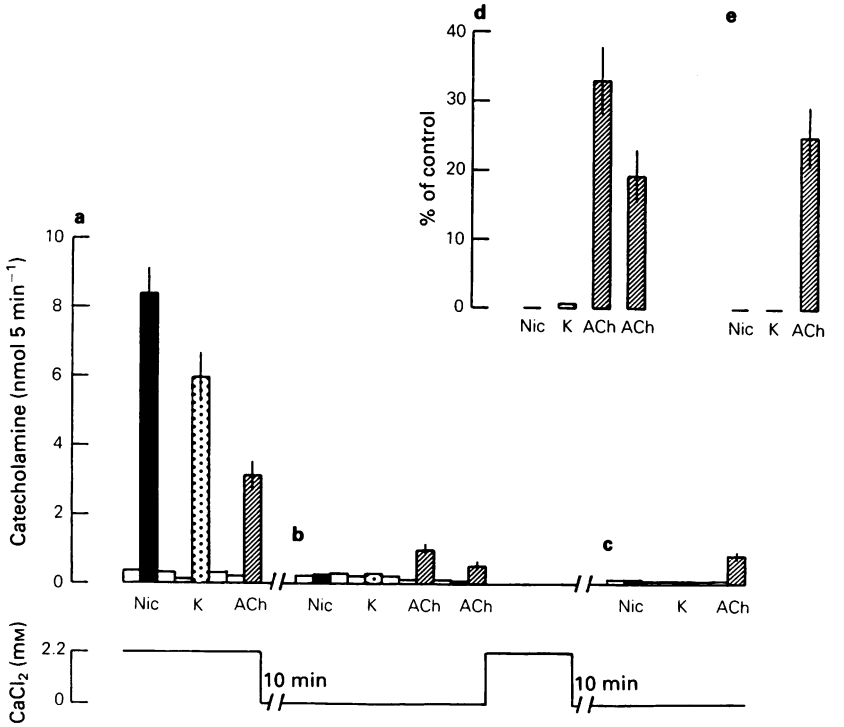

Figure 5 Effect of Ca deprivation on catecholamine secretion induced by nicotine (Nic), acetylcholine (ACh) and excess K. Nicotine (3×10^{-5} M) and ACh (10^{-5} M) for 2 min, and high K solution (56 mM) for 1 min were sequentially applied at 15 min intervals: (a) during perfusion with Locke solution; (b) 15 min after the removal of Ca in the presence of EGTA (10^{-4} M) and (c) 15 min after the 25 min period of readdition of Ca. Columns represent the means (and vertical lines s.e.) of catecholamine secretion evoked by nicotine (solid columns), high K (stippled columns) and ACh (hatched columns) obtained from 4 experiments. In (d) and (e), the responses appearing in (b) and (c) respectively are expressed as a percentage of those in the presence of Ca. The calculations were made after the basal secretion was subtracted from the evoked secretion. Catecholamines were assayed by the fluorometric method of Anton & Sayre (1962).

ponse to ACh was obtained during perfusion with Locke solution, the perfusion medium was switched to Ca-free Locke for 5 min and then to Ca-free isotonic KCl solution. No secretory response was evoked by exposure to isotonic KCl solution in the absence of Ca (Figure 6). Under these conditions, ACh caused a small increase in catecholamine secretion, but there was no comparable dose-dependent increment of ACh-induced secretory response compared to that observed during perfusion with Locke solution (Table 1). The Ca-free isotonic KCl solution was subsequently changed to Locke solution for 25 min and then to isotonic KCl solution, which caused a significant increase in catecholamine secretion. After this response attenuated and attained a new steady-state level, extracellular Ca was removed and the glands were again stimulated with ACh. ACh caused an increase in catecholamine secretion which was similar to that obtained during perfusion with Locke solution. The dose-dependency of the ACh-induced response was also restored (Table 1). However, repeated application of ACh failed to induce the response (Figure 6). Qualitatively, the same result was obtained when pilocarpine (5×10^{-4} M) was used. When the glands were exposed to isotonic KCl solution after removal of extracellular Ca, pilocarpine caused only a small increase in catecholamine secretion (Figure 7a). When extracellular Ca was removed after the start of exposure to isotonic KCl solution, pilocarpine caused a substantial increase in catecholamine secretion which was not present on repeated application (Figure 7b).

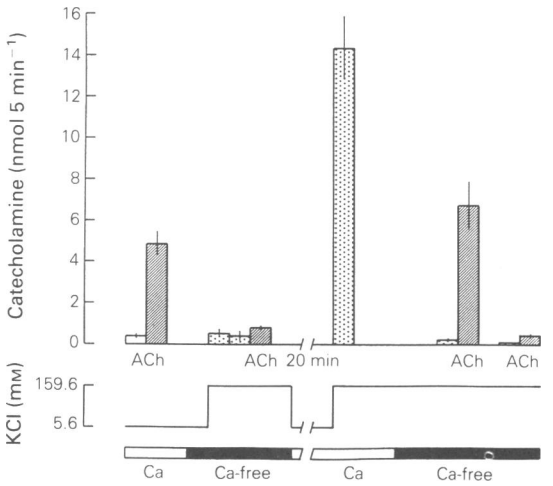

Figure 6 Catecholamine secretion evoked by acetylcholine (ACh) during exposure to isotonic KCl solution in the absence of extracellular Ca before and after Ca loading. ACh (10^{-4} M) was applied for 2 min during perfusion with Locke solution (□). Then, the perfusion medium was switched to Ca-free Locke solution for 5 min, followed by a Ca-free, isotonic KCl (159.6 mM) solution for 20 min (no Ca loading) (■); 10 min after the start of exposure to the KCl solution, the gland was stimulated by ACh. Then the medium was switched back to Locke solution for 25 min, followed by isotonic KCl solution (Ca loading) (□). After another 25 min, Ca-free isotonic KCl solution was substituted (■) and 15 min later ACh was again applied. Columns represent the mean (and vertical lines s.e.) of catecholamine secretion evoked by isotonic KCl (stippled columns) and ACh (hatched columns) from 5 experiments. Hexamethonium (10^{-3} M) was present throughout.

Discussion

Nicotinic receptors on adrenal chromaffin cells have been shown to be associated with voltage-dependent Ca channels in the ox (Kilpatrick *et al.*, 1981; Knight & Baker, 1983; Knight & Kesteven, 1983) and the rat (Wakade & Wakade, 1983). This voltage-dependent Ca channel is first activated and later inactivated by high K (Baker & Rink, 1975; Knight & Kesteven, 1983). Consistent with previous results, we found in the present experiments that nicotine failed to stimulate catecholamine secretion from adrenal chromaffin cells of the guinea-pig under the depolarizing conditions of perfusion with isotonic KCl. Under these conditions, ACh stimulated catecholamine secretion and this stimulated release was inhibited by atropine and unexpectedly enhanced by hexamethonium. In addition, pilocarpine was not only effective, but more potent, in increasing catecholamine secretion during exposure to isotonic KCl solution than during perfusion with Locke solution. Nicotine inhibited the potent effect of pilocarpine during exposure to isotonic KCl solution and hexamethonium reversed this action of nicotine. These results indicate that ACh and pilocarpine are able to release catecholamines through activation of muscarinic receptors without any apparent changes in membrane potential. Furthermore, activation of nicotinic receptors appears to inhibit the enhanced muscarinic receptor-mediated secretory action in depolarized chromaffin cells.

Similar to our findings in perfused cat adrenal glands (Nakazato *et al.*, 1984), ACh and pilocarpine, but not nicotine, caused a partial increase in catecholamine secretion from guinea-pig adrenal glands perfused with Ca-free Locke solution. The responses to ACh and pilocarpine in the absence of extracellular Ca may be mediated by Ca mobilized from an intracellular pool linked to muscarinic receptors. The evidence supporting this is: (1) ACh and pilocarpine stimulated catecholamine secretion in the absence of extracellular Ca which was inhibited by atropine but not by hexamethonium; (2) catecholamine secretion was absent upon repetitive stimulation with ACh or pilocarpine in the absence of extracellular Ca; (3) the reduced secretory response upon repetitive application of the agonist was restored by brief exposure to extracellular Ca; and (4) if the entry of Ca is facilitated by exposing the chromaffin cells to isotonic KCl containing Ca, the intracellular Ca pool appears to be able to store sufficient Ca to release an amount of catecholamine equal to that released in the presence of extracellular Ca.

Several groups have found that the activation of muscarinic receptors increases cytoplasmic free Ca in bovine isolated chromaffin cells, though there was no associated catecholamine secretion (Misbahuddin *et al.*, 1985; Cheek & Burgoyne; 1985, Kao & Schneider, 1985; 1986). Recently, it has been demonstrated that muscarine-evoked catecholamine secretion from perfused adrenal glands of the rat can occur in the absence of extracellular calcium (Harish *et al.*, 1987). Our work confirms and extends these findings. It appears that neither voltage changes of chromaffin cell membranes nor extracellular Ca are essential for muscarinic agonist-stimulated catecholamine release from guinea-pig adrenal glands as long as there is an intracellular source of Ca.

Muscarinic agonists not only evoke catecholamine release in adrenal chromaffin cells but appear to potentiate nicotinic receptor-stimulated catecholamine secretion (Forsberg *et al.*, 1987; Nakazato & Oleshansky, unpublished observations). Nicotinic receptors appear to exert an inhibitory influence on

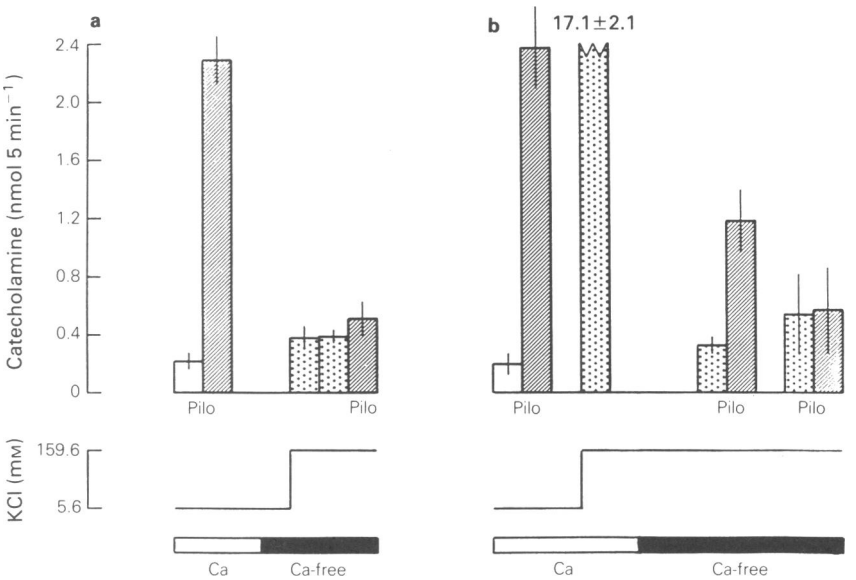

Figure 7 Catecholamine secretion evoked by pilocarpine (Pilo) during exposure to isotonic KCl solution in the absence of extracellular Ca before and after Ca loading. Pilocarpine (5×10^{-4} M) was applied for 2 min during perfusion with Locke and isotonic KCl (159.6 mM) solutions in the absence of extracellular Ca. Different groups of animals were used for the experiments in which Ca was not loaded (a) and was loaded (b) respectively. For further details, see the legend for Figure 6. Columns represent the mean (and vertical lines s.e.) of catecholamine secretion induced by pilocarpine (hatched columns) and isotonic KCl (stippled columns) from 4 experiments.

the muscarinic receptor-stimulated catecholamine secretion in guinea-pig adrenal glands which have been depolarized by perfusion with isotonic KCl solution. The mechanisms and physiological roles of these receptor interactions are currently under investigation.

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In conducting the research described in this paper, the investigator(s) adhered to the 'Guide for the Care and Use of Laboratory Animals' as promulgated by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council.

The views of the author(s) do not purport to reflect the position of the Department of the Army or the Department of Defense (para 4-3, AR 360-5).

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