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EFFECTS OF THEOPHYLLINE AND PROPRANOLOL ON ACETYLCHOLINE-INDUCED RELEASE OF ADRENAL MEDULLARY CATECHOLAMINES

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Abstract—The effect of theophylline and propranolol on acetylcholine-induced catecholamine release was studied in isolated bovine adrenals perfused *in vitro*. The catecholamine release induced by half maximum dose of acetylcholine, 10^{-4} M, was potentiated about 140 per cent by the presence of 1 mM theophylline in the perfusion medium. Theophylline enhanced the release of adrenaline but had little effect on the release of noradrenaline. The augmentary effect of theophylline on the adrenaline release was partially reduced by 10^{-7} M atropine and by 10^{-6} M propranolol. Propranolol by itself at a concentration of 10^{-7} M, at which it had no membrane-stabilizing effect, reduced the acetylcholine induced secretion about 20 per cent. Propranolol had a greater inhibitory effect on the release of adrenaline than on noradrenaline. It is concluded that theophylline affected the release of adrenaline by mobilization of intracellular calcium stores and by preventing breakdown of cyclic AMP synthesized in response to stimulation of a β-adrenergic receptor located in the adrenaline-storing cells. A possible action of theophylline on cyclic GMP synthesized in response to stimulation of the muscarinic receptor is discussed.

THE SECRETORY processes of various endocrine tissues have been shown to be regulated by adenosine 3',5'-monophosphate (cyclic AMP)[1] and to be closely dependent on calcium.[2] Recent studies on the adrenal medulla indicate that cyclic AMP may also be implicated in the secretory process of this gland. Thus cyclic AMP and its dibutyryl derivative have been reported to evoke release of catecholamines from dog and cat adrenal medulla perfused *in situ*[3,4] and from slices of bovine adrenal medulla.[5] Furthermore, the methylxanthines caffeine and theophylline, known to inhibit phosphodiesterase, both potentiate the cyclic AMP-induced secretion[4,5] and by themselves cause release of catecholamines.[6,7] Extracellular calcium has been shown to be a prerequisite for acetylcholine-induced secretion from the adrenal medulla.[8] Glands deprived of calcium still respond to cyclic AMP, dibutyryl cyclic AMP and theophylline,[4] however, suggesting that the cyclic AMP-evoked release is independent of calcium or that cyclic AMP and theophylline mobilize cellular stores of calcium.

The physiological significance of cyclic AMP in the adrenomedullary secretion is, however, not known. The present investigation was initiated in order to study the effect of cyclic AMP and theophylline on glands stimulated with the physiological transmitter, acetylcholine. Bovine adrenal medullae, perfused in the retrograde manner, were exposed to acetylcholine in the absence and presence of cyclic AMP or

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theophylline. The effect of theophylline was studied also in the presence of atropine and propranolol.

Some of these results have been reported previously.[9]

MATERIALS AND METHODS

Fresh bovine adrenals were obtained at the local slaughter-house. Only one and usually the left gland of each animal was used in each experiment. The glands were perfused in the retrograde manner with Tyrode's buffer as previously described.[10] The perfusion medium was gassed with 95% O_2 + 5% CO_2 and maintained at 37°. The flow rate of the perfusion fluid was adjusted to 18–20 ml/min. In order to obtain low spontaneous secretion the glands were perfused for 30 min before being stimulated. The glands were stimulated every 15 min, 4 ml of a solution of acetylcholine being injected into the tube about 10 cm before it entered the gland. The catecholamines were measured in the effluent collected during periods of 4 min, a control period just prior to stimulation and a period during which stimulation occurred at the initial 2 min. The results presented have been corrected for the spontaneous release.

When testing the effect of theophylline, atropine and propranolol on acetylcholine-induced catecholamine release the drugs were added to the perfusion medium after two periods of stimulation. Twenty min of equilibration were then allowed before restimulating the glands. Dose–response curves for atropine, phentolamine, propranolol, *iso*prenaline, noradrenaline and dopamine were obtained as follows; the glands were stimulated twice with acetylcholine in the absence of drug and then once in the presence of increasing concentrations of drug in the perfusion medium. Ten min of equilibration were allowed after each increase in drug concentration.

Catecholamines were estimated either differentially or as the sum of adrenaline and noradrenaline by the fluorometric method described by Bertler *et al.*[11]

The following chemicals were used: acetylcholine chloride (Hoffman–La Roche); adenosine 3′,5′-cyclic phosphate and N^6,$O^{2'}$-dibutyryladenosine-3′,5′-cyclic phosphate (Calbiochem); L-adrenaline bitartrate and L-noradrenaline bitartrate (Sigma Chemical Co.); dopamine HCl (Koch–Light); *d,l*-propranolol and *d*-propranolol (ICI); and Regitine (Ciba). Theophylline, atropine sulphate, phentolamine HCl and *iso*prenaline sulphate were obtained through Norsk Medisinaldepot.

Statistical significance was determined by Student's *t*-test for dependent groups, each gland serving as its own control

RESULTS

Effect of theophylline on acetylcholine-induced catecholamine release. The release of catecholamines from the perfused glands was induced by administration of 10^{-4} M acetylcholine. This concentration of acetylcholine produced about half maximal output of catecholamines and allowed successive stimulations of the glands without any significant decline in the response. Adrenaline accounted for 87 ± 2 per cent of the amines released spontaneously and 74 ± 1 per cent of the amines released during stimulation. Corrected for the spontaneous release, adrenaline was estimated to make up 69 ± 1 per cent of the amines secreted in response to acetylcholine (mean of 2–3 stimulations, no. of glands = 14).

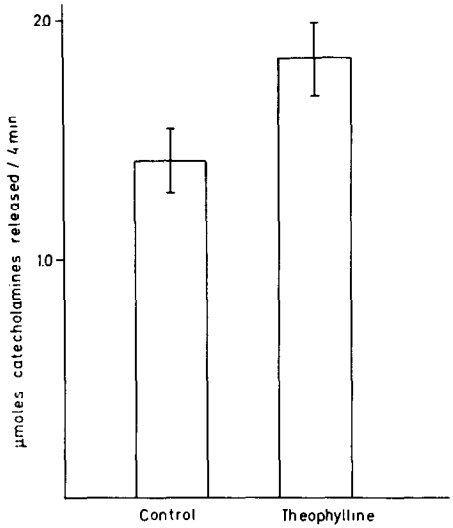

FIG. 1. Effect of theophylline on acetylcholine-induced catecholamine release. Adrenal glands perfused for 30 min with Tyrode's buffer were stimulated twice at 15 min interval by injecting 4 ml of 10^{-4} M acetylcholine over a period of 2 min. Theophylline (1 mM) was then added to the medium and the glands were restimulated in the same manner after 20 min of equilibration. Values represent mean output of amines during periods of stimulation in the absence and presence of theophylline, no. of glands = 11. Vertical bars = S.E.

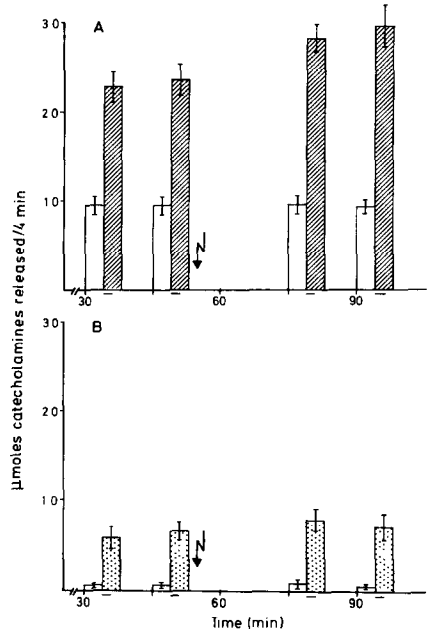

FIG. 2. Effect of theophylline on acetylcholine-induced release of adrenaline and noradrenaline. The glands were stimulated twice in the absence and twice in the presence of theophylline, and the injection of acetylcholine is indicated by the horizontal bars. The arrows indicate the time of addition of theophylline to the medium. The amount of adrenaline released is shown in A, open columns indicating spontaneous release and hatched columns indicating release evoked by infusion of acetylcholine. The amount of noradrenaline released is presented in B, open columns representing spontaneous release and dotted columns representing acetylcholine-induced release. n = 8, vertical bars = S.E.

As shown in Fig. 1 the presence of theophylline in the perfusion fluid potentiated significantly the acetylcholine-evoked release of amines ($n = 11$, $P < 0{\cdot}001$). In the terms of percentage, the output of amines in the presence of theophylline represented 138 ± 5 per cent of that in the absence of the drug. Differential estimations of the amines showed that theophylline enhanced the release of adrenaline whereas it had no significant effect on the release of noradrenaline (Fig. 2, A and B). The spontaneous release of amines was not affected by theophylline as measured 20 min after the addition of the drug (Fig. 2, A and B).

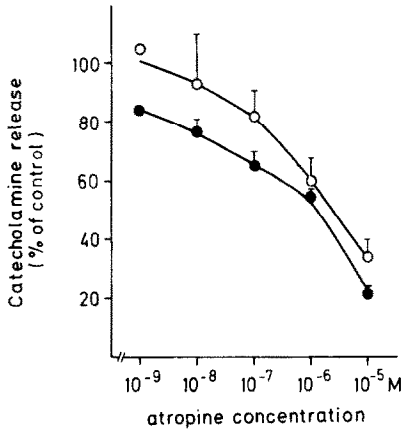

FIG. 3. Effect of increasing concentration of atropine on acetylcholine-induced release of adrenaline and noradrenaline. Adrenals were stimulated twice at 15 min interval with 4 ml of 10^{-4} M acetylcholine. Atropine in increasing concentrations was then added to the medium. 10 min of equilibration was allowed after each raise in drug concentration, and the glands were stimulated once at each concentration. $n = 2$ for 10^{-9} M atropine, otherwise $n = 4$. Adrenaline (●); noradrenaline (○).

Effect of theophylline on atropine-blocked glands. Figure 3 shows the effect of increasing concentrations of atropine on acetylcholine-evoked release of adrenaline and noradrenaline. In agreement with the observations that agonists of the muscarinic type stimulate mainly the release of adrenaline,[12] atropine was found to inhibit the discharge of adrenaline more strongly than that of noradrenaline (see also Table 2). The inhibitory effect of atropine being about 70 per cent at 10^{-5} M, and approaching complete inhibition, suggested, however, an unspecific blocking effect of the drug at high concentrations. A concentration of 10^{-7} M was therefore chosen for the following experiments. The addition of theophylline to glands already blocked with atropine caused an enhancement, although not as great as in the absence of the atropine, of acetylcholine-induced release of adrenaline. Theophylline had no significant effect on the release of noradrenaline (Table 1). Atropine added to the medium after theophylline depressed the output of both amines relative to that in the presence of only theophylline but not relative to that in the absence of both drugs (Table 1). Hence blocking of the muscarinic receptor by 10^{-7} M atropine did not antagonize completely the potentiating effect of theophylline.

Effect of phentolamine and propranolol on acethylcholine-induced catecholamine release. The α-adrenergic blocker phentolamine, applied both as phentolamine-HCl and Regitine, showed no potentiating effect but a slight blocking effect on acetylcholine-induced catecholamine release in concentrations up to 10^{-5} M (Fig. 4). Above

TABLE 1. EFFECT OF THEOPHYLLINE ON ACETYLCHOLINE-INDUCED RELEASE OF ADRENALINE AND NORADRENALINE FROM ATROPINE-BLOCKED GLANDS

No. of expts	Addition to the medium	μmoles Catecholamines released/4 min	% of Control Total catecholamines	Adrenaline	Noradrenaline
4*	None	2·68 ± 0·18	100	100	100
	10^{-7} M Atropine	1·83 ± 0·19	69 ± 6	65 ± 5	82 ± 9
5	None	3·13 ± 0·51	100	100	100
	10^{-7} M Atropine	2·17 ± 0·37	70 ± 2	70 ± 5†	84 ± 17‡
	10^{-7} M Atropine + 10^{-3} M Theophylline	2·51 ± 0·43	81 ± 6	82 ± 5†	89 ± 24‡
4	None	2·00 ± 0·53§	100	100	100
	10^{-3} M Theophylline	2·54 ± 0·62§	141 ± 16	124 ± 6	203 ± 87
	10^{-3} M Theophylline + 10^{-7} M Atropine	1·82 ± 0·39	105 ± 13	93 ± 7	133 ± 53

The experimental conditions were as described in the legend to Fig. 1. Each gland was stimulated twice in the absence of drug, then twice in the presence of either drug and finally twice in the presence of both drugs, the order of addition being as shown in the table. Values are mean ± S.E.

* Same experiments as in Fig. 3.

† Statistical significance of the difference between these figures $P < 0·05$.

‡ Statistical significance of the difference between these figures $P < 0·8$.

§ Statistical significance of the difference between these figures $P < 0·02$.

this concentration a sharp decline in the output of amines occurred approaching almost complete inhibition of the release at 10^{-4} M of phentolamine, indicative of a membrane stabilizing effect rather than a pure α-blocking effect at higher concentrations.

As shown in Fig. 5, the β-adrenergic blocker *d,l*-propranolol inhibited at all concentrations the acetylcholine-evoked catecholamine secretion significantly more than the stereoisomer *d*-propranolol, which exerts mainly a membrane stabilizing effect.[13,14] The effect of propranolol on the release of adrenaline and noradrenaline respectively was tested using 10^{-6} M of the drug. In some of these experiments the inhibitory action of *d*-propranolol on the release of the total catecholamines was as great as that of *d,l*-propranolol (Table 2). However, whereas *d*-propranolol inhibited

TABLE 2. EFFECT OF *d,l*-PROPRANOLOL AND *d*-PROPRANOLOL ON ACETYLCHOLINE-INDUCED SECRETION OF ADRENALINE AND NORADRENALINE

No. of expts	Addition to the medium	μmoles Catecholamines released/4 min	% of Control Total catecholamines	Adrenaline	Noradrenaline
6	None	1·69 ± 0·25	100	100	100
	10^{-6} M *d*-Propranolol	0·92 ± 0·24	53 ± 8	54 ± 9	54 ± 8
5	None	1·14 ± 0·21	100	100	100
	10^{-6} M *d,l*-Propranolol	0·65 ± 0·12	58 ± 7	55 ± 8*	78 ± 9*
8	10^{-3} M Theophylline	2·28 ± 0·16	100	100	100
	10^{-3} M Theophylline + 10^{-6} M *d,l*-Propranolol	1·38 ± 0·11	61 ± 4	59 ± 4†	67 ± 8†

The experimental conditions were as described in the legend to Fig. 1. Each gland was stimulated twice in the absence and twice in the presence of the drug. In the last series of experiments theophylline was present throughout the total period of perfusion. Values are mean ± S.E.

* Statistical significance of the difference between these figures $P < 0·005$.

† Statistical significance of the difference between these figures $P < 0·4$.

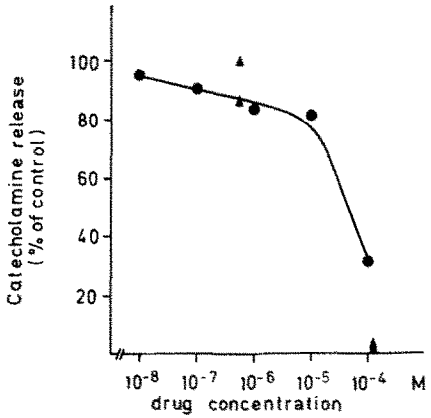

FIG. 4. Effect of increasing concentration of phentolamine and Regitine on acetylcholine-induced catecholamine release. Experimental conditions were as described in the legend to Fig. 3 for phentolamine–HCl (●), n = 2. Experimental conditions were as described in Fig. 1 for Regitine (▲), each symbol representing one gland and each gland being stimulated 3 times in the absence and 3 times in the presence of the drug.

the release of adrenaline and noradrenaline to the same extent, *d,l*-propranolol inhibited the release of adrenaline more strongly than that of noradrenaline (Table 2). In experiments with glands stimulated in the presence of theophylline there was also a tendency for a preferential inhibition of adrenaline discharge by *d,l*-propranolol (Table 2).

Effect of dibutyryl cyclic AMP, cyclic AMP and biogenic amines on catecholamine release. Dibutyryl cyclic AMP was found to induce catecholamine release if injected into the perfusion tube at a concentration of 7·5 mM over a period of 10 min. As 2 ml of the solution of dibutyryl cyclic AMP were injected and the rate of perfusion was adjusted to 8–10 ml/min, the nucleotide reached the glands in a concentration of 0·15 mM. Of the five glands tested, all stimulated twice, only one gland responded to both infusions of dibutyryl cyclic AMP whereas the rest responded only

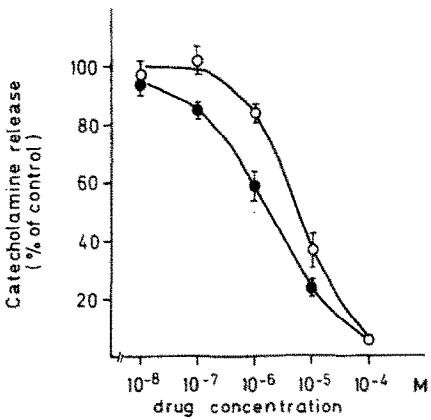

FIG. 5. Effect of increasing concentrations of *d,l*-propranolol and *d*-propranolol on acetylcholine-induced catecholamine release. Experimental conditions were as described in the legend to Fig. 3. n = 4, *d,l*-propranolol (●); *d*-propranolol (○).

to the first infusion. The mean output of amines was 0·08 ± 0·02 μmoles/10 min compared with the increment of 0·51 ± 0·06 μmoles/4 min in the acetylcholine-induced catecholamine release produced by theophylline (Fig. 1). Infusion of 7·5 mM cyclic AMP caused the release of 0·13 μmoles catecholamines per 10 min in one gland tested. Dibutyryl cyclic AMP, if infused in a concentration of 0·5–4·0 mM just prior to or/and during stimulation with acetylcholine, enhanced the catecholamine release by an increment of 0·23 μmoles/3 min (mean of six stimulations in two glands, range 0·05–0·40 μmoles/3 min). Hence the secretory responses to the cyclic nucleotides were of the same magnitude as that reported previously.[3,4] Due to the small output of amines no differential estimation of adrenaline and noradrenaline was undertaken.

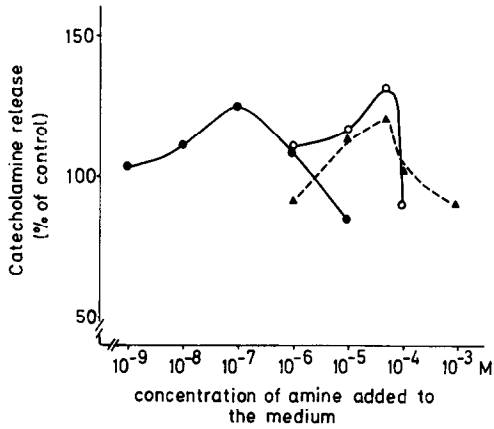

FIG. 6. Effect of exogenous amines on acetylcholine-induced catecholamine release. The experimental conditions were as described in the legend to Fig. 3. Each curve represents the result obtained with one gland. Amine added to the medium: (●) *iso*prenaline; (○) noradrenaline; (▲) dopamine.

Attempts to stimulate the glands by infusion of biogenic amines were unsuccessful and no responses were obtained with concentrations up to 5×10^{-6} M of *iso*prenaline (three glands), 10^{-5} M of adrenaline (one gland), 10^{-5} M of noradrenaline (two glands) and 10^{-4} M of dopamine (two glands). Infusion of amines in higher concentrations was not undertaken due to interference with the fluorometric analytical estimations. The acetylcholine-induced release of catecholamines was, however, augmented by the presence of either *iso*prenaline (two glands), noradrenaline (four glands) or dopamine (four glands) in the perfusion medium. *Iso*prenaline was the most and dopamine the least potent in this respect (Fig. 6). Differential estimation of the amines revealed that *iso*prenaline enhanced the release of adrenaline whereas that of noradrenaline was unaffected. Noradrenaline also augmented the release of adrenaline, exhibiting a maximal stimulatory effect at 10^{-5}–5×10^{-5} M. The course of noradrenaline release varied in a seemingly unspecific way and it was not possible to draw any conclusion to whether exogenous applied noradrenaline affected the acetylcholine-induced noradrenaline release.

High concentrations of exogenous amines inhibited the release of catecholamines elicited by acetylcholine (Fig. 6). Dopamine was also found to inhibit the acetylcholine-induced catecholamine release at a concentration of 10^{-6} or 10^{-5} M (Fig. 6).

DISCUSSION

Histochemical methods in combination with light and electron-microscopical studies have revealed the presence of two populations of cells in the adrenal medulla, each containing either noradrenaline or adrenaline (for review, see ref. 15). In accordance with the *N*-methylation of noradrenaline to form adrenaline being controlled by the cortical steroids,[16] the medullary cells in close proximity to the cortex contain predominantly adrenaline.[15,17] The differential release of adrenaline and noradrenaline, which can be induced *in vivo*[18] as well as *in vitro*[12,19–21] suggests that each type of cell is innervated by separate fibers controlled by different sites centrally. However, the presence of catecholamines has been shown to modulate the transmission in superior cervical ganglia,[22,23] probably by acting on α- and β-receptors located postsynaptically.[24,25] Adrenaline in physiological concentrations also augments adrenal medullary secretion.[23,26] These observations thus indicate the presence of regulatory mechanisms also at the sites of transmission and secretion.

The present investigation showed that the presence of theophylline enhanced the acetylcholine-induced release of adrenaline whereas that of noradrenaline was not affected. In view of the already mentioned anatomical location of the adrenaline storing cells in the adrenal medulla,[15,17] and the glands being perfused in the retrograde manner, this discriminating effect of theophylline cannot be attributed to the adrenaline storing cells being more exposed to the drug. The fact that acetylcholine evoked the release of the two amines in a ratio close to that in which they are found in the intact gland[18] strengthened the assumption that drugs infused into the glands reached both types of cells equally well. Furthermore, *in vivo* administration of caffeine evokes preferentially the release of adrenaline.[27] Thus, theophylline seems to affect a release mechanism either specific for the adrenaline storing cells or although common to both adrenaline and noradrenaline storing cells being more sensitive in the former cells.

The acetylcholine-induced secretion from the adrenal medulla shows an absolute dependency on extracellular calcium, and the amount of catecholamines released is a function of the extracellular concentration of calcium.[8] The ability of theophylline to elicit catecholamine secretion in this gland even in the absence of extracellular calcium illustrates, however, that the intracellular calcium stores also have a function in the secretory process.[4,28,29] The present results showing that theophylline potentiates the acetylcholine-induced catecholamine release are consistent with the ability of the drug to raise the cellular concentration of free calcium.[30] However, it is not known at the moment whether the effect of theophylline on the intracellular calcium stores is related to its inhibitory action on phosphodiesterase.

Earlier assumptions that cyclic AMP is not implicated in the release of adrenal medullary catecholamines have recently been contradicted by the work of several groups, showing that both cyclic AMP and dibutyryl cyclic AMP evoke the release of catecholamines.[3–5] These observations. also confirmed by the present work, may seem somewhat inconsistent with the emerging idea that the muscarinic action of acetylcholine is mediated by an increase in the cellular content of cyclic GMP.[31,32] The adrenal medulla seems to be no exception in this respect as cyclic GMP has been reported to evoke catecholamine release in this tissue.[33] The presently observed ability of atropine to depress the potentiating effect of theophylline on acetylcholine-

induced catecholamine release also supports this concept. The effect of β-adrenergic agents is, however, usually mediated by an increase in the cellular concentration of cyclic AMP. The presence of a β-adrenergic system in the adrenal medulla has indeed been suggested by the present experiments. This system seemed to be confined mainly to the adrenaline storing cells as judged from the ability of propranolol to inhibit the release of adrenaline more strongly than that of noradrenaline, and by the observation that *iso*prenaline and noradrenaline applied externally enhanced the release mainly of adrenaline.

The rapid physiological responses, and hence adrenomedullary catecholamine secretion, produced by stimulation of the nicotinic receptor are probably mediated independently of a complex series of biochemical reactions.[31] The adrenomedullary secretion mediated by the β-adrenergic system, however, is probably dependent on the synthesis of cyclic AMP. Different cellular mechanisms may therefore underly the secretion in response to acetylcholine and the catecholamines.

In this connection it is of interest to consider the model for catecholamine secretion suggested by Poisner and Trifaró[34] to account for the ability of ATP and Mg^{2+} to release the amines from isolated granules, particularly in view of an augmentary effect of cyclic AMP on this reaction.[5] On the assumption that calcium is indispensable in the events leading to discharge of the catecholamines, the ability of cyclic AMP to elicit catecholamine release from calcium deprived glands outlasting that of theophylline,[4] suggests that the secretion elicited by cyclic AMP and theophylline respectively relies on different pools of intracellular calcium. The pool mobilized by cyclic AMP may in fact be the granular pool of calcium, which we have previously shown not to be stationary as calcium accumulates in the granules during secretion.[35] According to these considerations the β-adrenergic mediated release of catecholamines may take place during a phase of the secretion when the intracellular concentration of free calcium becomes a limiting factor to the cholinergic induced secretion.

In conclusion the present experiments taken together with earlier observations[23,26] indicate that the adrenomedullary secretion is influenced by catecholamines, exhibiting both inhibitory and facilitating actions. The opposing effects are probably obtained by stimulation of α- and β-adrenergic receptors. The action of the catecholamines on the secretion is dependent on a simultaneous cholinergic stimulation of the cell or application of acetylcholine, suggesting that amines released locally may have a physiological role in modulating the secretion from the adrenal medulla.

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