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The mode of action of caffeine on catecholamine release from perfused adrenal glands of cat

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1 Adrenaline and noradrenaline secretion induced by caffeine was investigated in the perfused cat adrenal glands.

2 Caffeine (10–80 mM) caused a dose-dependent increase in both adrenaline and noradrenaline secretion when applied for 1 min and 10 min after replacing Ca^{2+} with 10^{-5} M EGTA in the perfusion solution. The ratio of adrenaline to noradrenaline was about 1:1. Mg^{2+} and/or Ca^{2+} inhibited the response to caffeine.

3 When caffeine (40 mM) was repeatedly applied in the absence of extracellular Ca^{2+}, the secretory response almost disappeared but only at the second challenge with caffeine. However, the response was partially restored after readmission of Ca^{2+} (2.2 mM) and was augmented after the readmission of Ca^{2+} with ouabain (10^{-5} M).

4 Caffeine-induced secretion of adrenaline and noradrenaline increased with the increase in the preloaded concentration of Ca^{2+} and attained a maximum at 16 mM Ca^{2+}.

5 During perfusion with Ca^{2+}-free Locke solution containing hexamethonium (10^{-3} M), acetylcholine (10^{-4} M) caused increases in both adrenaline and noradrenaline secretions with a ratio of about 1:2. The secretory responses were partially inhibited by preceding stimulation with exposure to caffeine (80 mM).

6 These results suggest that caffeine mobilizes Ca^{2+} from an intracellular storage site that may not be entirely the same as that linked to muscarinic receptors, and causes an increase in both adrenaline and noradrenaline secretion from cat adrenal chromaffin cells.

Introduction

Xanthine derivatives, caffeine and theophylline are known to increase catecholamine secretion from perfused adrenal glands of the cat (Peach, 1972) and ox (Poisner, 1973a), isolated and halved adrenal glands of the rat (Cohen & Gutman, 1979) and cultured bovine adrenal chromaffin cells (Morita *et al.*, 1987a,b). In these papers, the stimulant action of xanthine derivatives has been interpreted as associated with an accumulation of adenosine 3′:5′-cyclic monophosphate (cyclic AMP) resulting from the inhibition of cyclic nucleotide phosphodiesterase, despite the lack of any direct supporting evidence.

According to Poisner (1973b), stimulation by caffeine is as effective in increasing catecholamine secretion in the absence of extracellular Ca^{2+} as in its presence, and the response to caffeine in Ca^{2+}-free medium is increased by the loading of Ca^{2+} stores in perfused bovine adrenal glands. Recently, we found that caffeine was much more effective in increasing catecholamine secretion in the absence of extracellular Ca^{2+} and Mg^{2+} than in their presence, and that the responses to caffeine and muscarinic receptor activation were reversibly inhibited by an intracellular Ca^{2+} antagonist, TMB-8, in perfused cat adrenal glands (Yamada *et al.*, 1988). These findings support the view that caffeine (Poisner, 1973b), as well as muscarinic agonists (Nakazato *et al.*, 1984; 1988), mobilize intracellular Ca^{2+} from some storage sites to stimulate catecholamine secretion from adrenal chromaffin cells.

The present experiments were designed to gain an insight into the properties of the intracellular Ca^{2+} storage site responsible for this agonist-induced catecholamine secretion. This paper describes the effects

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of divalent cations, ouabain and Ca^{2+} loading on caffeine-induced catecholamine secretion in perfused adrenal glands of the cat.

Methods

Isolation and perfusion of cat adrenal glands

Cats (1.5–4.0 kg) of either sex were anaesthetized with sodium pentobarbitone (40 mg kg^{-1}, i.p.). Under artificial respiration, both adrenal glands were perfused through a cannula inserted into the lower aorta below the renal artery at a flow rate of about 0.7 ml min^{-1} at room temperature (approximately 25°C). The adrenal effluent was drained through a cannula inserted into the vena cava at the same level as the arterial cannula. After this procedure was completed, the perfused adrenal glands were isolated and placed on a moistened cork board. The adrenal effluents were collected at 5 min intervals and acidified with 8 M perchloric acid, giving a final concentration of 0.4 M, and stored on ice until assayed. Drugs were administered 40–60 min after the start of perfusion. Secretagogues were administered for 1 min prior to the following 5 min sample-collection period, because of the 1 min dead time of the arterial cannula.

Perfusion media

The standard perfusion medium was modified Locke solution of the following composition (mM): NaCl 154, KCl 5.6, $CaCl_2$ 2.2, Na_2HPO_4-NaH_2PO_4 buffer (pH 7.0) 3 and glucose 10. In Ca^{2+}-free solutions, $CaCl_2$ was replaced with glycoletherdiaminetetraacetic acid (EGTA) (10^{-5} M or 10^{-4} M). The perfusion medium was continuously aerated with O_2.

Catecholamine assay

Catecholamines (adrenaline and noradrenaline) were separated by high performance liquid chromatography (h.p.l.c.) (Jasco) and assayed by electrochemical detector (LC-4B, BAS). The treatment of samples for h.p.l.c. was carried out according to the method described by Salzman & Sellers (1982). Total catecholamine levels were also assayed by a conventional fluorometric method (Anton & Sayre, 1962).

Materials

The following drugs were used: acetylcholine chloride (Ovisort, Daiichi), caffeine monohydrate (Wako Pure Chem.), hexamethonium chloride dihydrate (Wako Pure Chem.), glycoletherdiaminetetraacetic acid (EGTA, Wako Pure Chem.) and g-strophanthin (ouabain, Tokyo Kasei). All compounds were dissolved in Locke solution.

Results

Dose-dependent increase in catecholamine secretion induced by caffeine

Various concentrations of caffeine were applied to the adrenal glands for 1 min, 10 to 15 min after the replacement of Ca^{2+} with 10^{-5} M EGTA in the perfusion solution. Adrenaline and noradrenaline secretions in response to caffeine started to increase at 10 mM and increased with the increase in the concentration of caffeine until they attained a maximum at 40 mM. At concentrations over 40 mM, the secretory responses declined. As shown in Figure 1, the ratio between adrenaline and noradrenaline in the

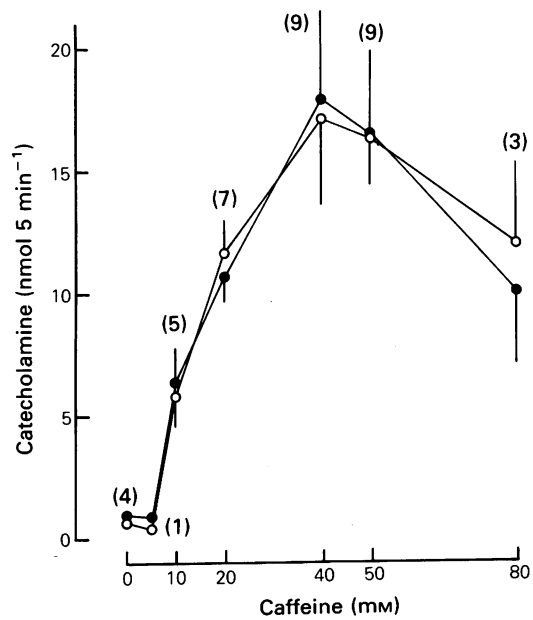

Figure 1 Dose-response curve for caffeine-induced catecholamine secretion in the absence of extracellular Ca^{2+}. A single dose of caffeine ranging from 5 to 80 mM was applied for 1 min to single preparations of perfused adrenal glands 10 to 15 min after replacing Ca^{2+} with 10^{-5} M EGTA in the standard Locke solution. Means, with s.e.means indicated by vertical lines that exceed the size of the symbol, of adrenaline (○) and noradrenaline (●) secretions are shown. The numbers in parentheses represent the number of experiments. The ordinate scale represents the amounts of catecholamines released and the abscissa scale the concentration of caffeine.

response to caffeine was approximately 1:1 with all concentrations of caffeine used. The maximum amounts of adrenaline and noradrenaline released were 17.2 ± 3.5 and 18.0 ± 3.6 nmol 5 min^{-1} respectively.

Effects of Mg^{2+} and Ca^{2+} on caffeine-induced catecholamine secretion

The adrenal glands were sequentially stimulated with caffeine (40 mM) in the presence of various concentrations of Mg^{2+} during perfusion with Ca^{2+}-free Locke solution containing 10^{-5} M EGTA. The concentration of Mg^{2+} was progressively decreased from 1.2 mM to 0.4 mM and 0.1 mM. Before each stimulation with caffeine, the adrenal glands were loaded with Ca^{2+} by exposure to the standard Locke solution for 20 min. To prevent Ca^{2+} from evoking the secretory response by itself when it was added after exposure to Ca^{2+}-free medium, 1.2 mM Mg^{2+} was always included in the standard Locke solution perfusate for Ca^{2+} loading for the first 5 min, after which it was removed. As shown in Figure 2a, the secretory responses of adrenaline and noradrenaline to caffeine started to appear when Mg^{2+} was reduced to 0.1 mM. When Mg^{2+} was totally removed, both adrenaline and noradrenaline secretions were further increased to the same degree.

In a similar fashion, the effect of reducing the concentration of Ca^{2+} on caffeine-induced catecholamine secretion was studied (Figure 2b). Until the concentration of Ca^{2+} was reduced to 0.3 mM, there appeared to be no, or only a trace of, secretory response to caffeine. However, adrenaline and noradrenaline secretions were suddenly increased by caffeine when Ca^{2+} was replaced with 10^{-5} M EGTA.

Dependence of caffeine-induced catecholamine secretion on the concentration of Ca^{2+} preloaded

We examined whether or not the caffeine-induced catecholamine secretion was dependent on the preloaded concentration of Ca^{2+} within the range of 2.2 mM to 32 mM. First, caffeine (40 mM) was applied for 1 min, 10 min after the replacement of Ca^{2+} with EGTA (10^{-4} M) in the standard perfusion solution. Then the adrenal glands were loaded with 8 mM Ca^{2+} for 25 min. After the end of this Ca^{2+} loading, the glands were again stimulated with a second dose of caffeine (40 mM). In a similar fashion, the effects of loading 16 mM and 32 mM Ca^{2+} on the responses to the third and fourth doses of caffeine, respectively, were observed. As shown in Figure 3, caffeine-induced adrenaline and noradrenaline secretions were increased with the increase in the concentration of Ca^{2+} loading until they attained a maximum at 16 mM. The rates of increase were almost the same

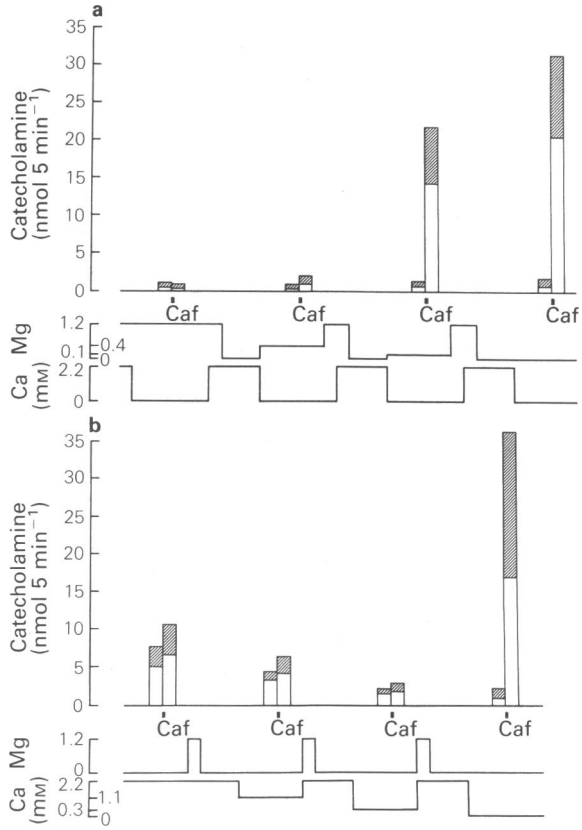

Figure 2 Effects of varying the concentrations of Mg^{2+} and Ca^{2+} on caffeine-induced catecholamine secretion. Caffeine (40 mM) was sequentially applied for 1 min in the presence of 1.2, 0.4 and 0.1 mM Mg^{2+} and in the absence of Mg^{2+} during perfusion with Ca^{2+}-free Locke solution containing 10^{-5} M EGTA in (a), and in the presence of 2.2, 1.1 and 0.3 mM Ca^{2+} and 10^{-5} M EGTA instead of Ca^{2+} during perfusion with Mg^{2+}-free solution in (b). Preceding each challenge with caffeine, the adrenal glands were loaded with Ca^{2+} by exposure to the standard Locke solution for 20 min. To prevent Ca^{2+} from evoking the secretory response by itself, 1.2 mM Mg^{2+} was added during the first 5 min of the Ca^{2+} loading period. Caffeine was applied 15 min after the end of Ca^{2+} loading. Columns represent the amounts of adrenaline (open columns) and noradrenaline (hatched columns). A representative experiment with each is illustrated.

for adrenaline and noradrenaline. Qualitatively similar results were obtained in the three other experiments.

Effects of ouabain on caffeine-induced catecholamine secretion

Previously, we suggested that ouabain increases either the entry of extracellular Ca^{2+} or the concen-

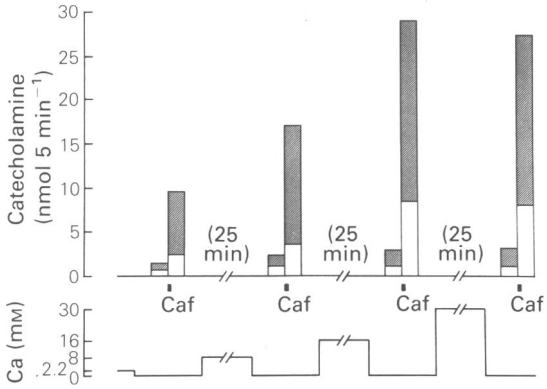

Figure 3 Effect of varying the concentration of preloaded Ca^{2+} on caffeine-induced catecholamine secretion. Caffeine (Caf, 40 mM) was applied for 1 min, 10 min after the removal of Ca^{2+} from the standard Locke solution in the presence of 10^{-4} M EGTA. Then the adrenal glands were loaded with 8 mM Ca^{2+} in the presence of 1.2 mM Mg^{2+} for 25 min and, 10 min after this ended, Ca^{2+} loading took place, after which caffeine was again applied in the presence of 10^{-4} M EGTA instead of both Ca^{2+} and Mg^{2+}. In a similar fashion, the effects of loading 16 and 32 mM Ca^{2+} on caffeine-induced response were observed in a subsequent period. Columns represent the amounts of adrenaline (open columns) and noradrenaline (hatched columns). A representative experiment is illustrated.

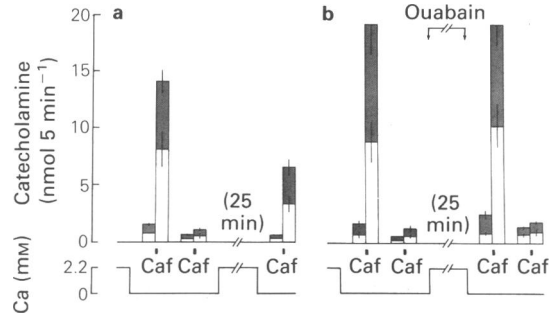

Figure 4 Effect of ouabain on deteriorated caffeine-induced catecholamine secretion. Caffeine (Caf, 40 mM) was applied for 1 min during perfusion with Ca^{2+}-free Locke solution containing 10^{-5} M EGTA before and after readmission of Ca^{2+} without (a) and with (b) ouabain (10^{-5} M). Columns represent the means (and, if vertical lines are present, s.e.) of adrenaline (open columns) and noradrenaline (hatched columns) obtained from 4 experiments with each.

tration of intracellularly stored Ca^{2+} responsible for acetylcholine-receptor mediated catecholamine secretion (Nakazato *et al.*, 1986; Yamada *et al.*, 1989). It was, therefore, of interest to study how ouabain affected caffeine-induced catecholamine secretion.

First, the reproducibility of caffeine-induced catecholamine secretion was determined by repeatedly applying caffeine (40 mM) for 1 min at 15 min intervals in the absence of extracellular Ca^{2+} throughout the course of the experiment. It was found that both adrenaline and noradrenaline secretions induced by caffeine disappeared only on the second challenge with the drug. However, the secretory responses of adrenaline and noradrenaline were restored to about 40 and 50% of the control level after the reintroduction of Ca^{2+} (Figure 4a). In a similar fashion, the glands were challenged with two doses of caffeine (40 mM) at 15 min intervals in the absence of extracellular Ca^{2+}. Then the perfusion solution was changed to the standard Locke solution containing ouabain (10^{-5} M) for 25 min, and the glands were again stimulated with caffeine (40 mM). Caffeine applied after exposure to ouabain caused releases of adrenaline and noradrenaline that were almost the same as or greater in magnitude than those induced by the first challenge with caffeine (Figure 4b).

Effect of caffeine on the subsequent acetylcholine-induced catecholamine secretion

Previously, we assumed that the activation of muscarinic receptors mobilized Ca^{2+} from some intracellular Ca^{2+} pools to release catecholamine in adrenal chromaffin cells (Nakazato *et al.*, 1984; 1988; Yamada *et al.*, 1988; 1989). Thus, it was of interest to examine whether caffeine and muscarinic agonists utilized a common hypothetical intracellular Ca^{2+} pool to release catecholamine.

In the absence of extracellular Ca^{2+} and Mg^{2+}, three doses of acetylcholine (10^{-4} M) were sequentially applied for 1 min at 55 min intervals in the presence of hexamethonium (10^{-3} M) throughout the course of the experiment. Prior to each challenge with acetylcholine, the adrenal glands were loaded with 2.2 mM Ca^{2+} for 25 min. The response to acetylcholine was gradually decreased on repetition of stimulation (Figure 5a). As previously observed (Yamada *et al.*, 1989), acetylcholine caused a preferential release of noradrenaline (about 70% of the total catecholamine released) in the absence of extracellular Ca^{2+}. To determine how caffeine influenced acetylcholine-induced catecholamine secretions, caffeine (80 mM) was applied for 1 min before the second challenge with acetylcholine. As shown in Figure 5b, acetylcholine applied after caffeine still caused increases in both adrenaline and noradrenaline secretions, though the amounts released were partially inhibited. The responses were restored at the third challenge with acetylcholine. The ratio of

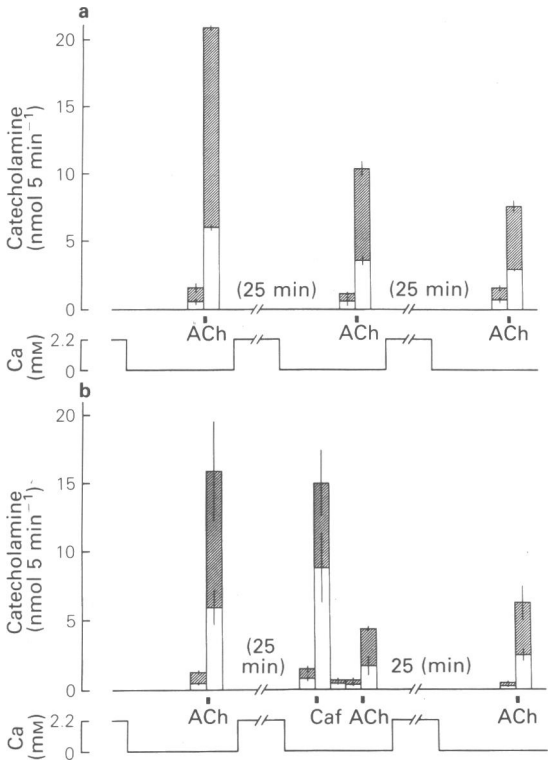

Figure 5 Effect of caffeine on acetylcholine-induced catecholamine secretion. Acetylcholine (ACh, 10^{-4} M) was applied for 1 min, 25 min after the removal of Ca^{2+} from the standard Locke solution in the presence of 10^{-5} M EGTA. Then the adrenal glands were loaded with Ca^{2+} for 25 min by perfusing the Locke solution containing 1.2 mM Mg^{2+} before each challenge with ACh in the following period. The control experiment for ACh-induced responses is shown in (a). In (b), caffeine (Caf, 80 mM) was applied 10 min before the second challenge with ACh. Columns represent the means (and, if vertical lines are present, s.e.) of adrenaline (open columns) and noradrenaline (hatched columns) obtained from 3 experiments.

adrenaline to noradrenaline in the responses to acetylcholine was fairly constant at about 1:2 throughout the experiment.

Discussion

As we previously reported, caffeine was much more effective in releasing catecholamine in the absence of extracellular Ca^{2+} than in its presence (Yamada *et al.*, 1988). In fact, Ca^{2+}, even in lower than normal concentrations, inhibited caffeine from initiating the secretory response. On the other hand, Ca^{2+} loading was indispensible for caffeine to be effective in releasing catecholamine in the subsequent Ca^{2+}-free period. Therefore, it seems likely that extracellular Ca^{2+} plays a dual role in the pharmacological action of caffeine; it both inhibits caffeine-induced catecholamine secretion when it is present outside the chromaffin cells and maintains or augments the action of caffeine when it enters the cells. Mg^{2+} has only an inhibitory action. The normal concentration of Ca^{2+} is known to cause a large increase in catecholamine secretion when added after exposure to Ca^{2+}- and Mg^{2+}-free medium in perfused cat adrenal glands (Douglas & Rubin, 1961). A low concentration of Mg^{2+} such as 2 mM completely blocked this Ca^{2+}-induced catecholamine secretion (Douglas & Rubin, 1963). The most probable action of this Ca^{2+} deprivation was assumed to be an increase in cell membrane permeability that allowed extracellular Ca^{2+} to penetrate into the medullary cells. The reason for this may be that both Ca^{2+} and Mg^{2+} have a stabilizing action on the plasma membrane (Frankenhaeuser & Hodgkin, 1957). Therefore, divalent cations (Ca^{2+} and Mg^{2+}) may decrease the caffeine-induced secretory response through a stabilizing effect on the plasma membrane. In addition, Mg^{2+} may block the catecholamine secretion induced by caffeine by competing with Ca^{2+} for intracellular sites (Aguirre *et al.*, 1977).

As with muscarinic receptor-mediated catecholamine secretion (Nakazato *et al.*, 1984; 1988), the response that deteriorated as a result of repeated application of caffeine in the absence of extracellular Ca^{2+} was partially restored after readmission of Ca^{2+}, depending on its concentration, and was augmented after the readmission of Ca^{2+} with ouabain. In addition, we previously found that an intracellular Ca^{2+} antagonist, TBM-8, reversibly blocked the catecholamine secretions induced both by caffeine and by acetylcholine in the absence of extracellular Ca^{2+} (Yamada *et al.*, 1988). These data fit well with the view that caffeine, as well as muscarinic agonists, possesses an intracellular Ca^{2+} storage site from which the agonists release Ca^{2+} into the cytosol to cause catecholamine secretion (Poisner, 1973b; Nakazato *et al.*, 1984; 1988). However, the process of caffeine-induced catecholamine secretion seems to differ from that of muscarinic agonists, because muscarinic agonists are able to increase catecholamine secretion both in the presence and in the absence of extracellular Ca^{2+} and Mg^{2+} (Yamada *et al.*, 1988; 1989). Recently, it was reported that Ca^{2+} stores in smooth muscle consisted of two classes; one possessing both caffeine (Ca^{2+}-induced Ca^{2+} release) and inositol 1,4,5-triphosphate (IP_3)-induced Ca^{2+} release mechanisms, and the other only an IP_3-induced Ca^{2+} release mechanism (Iino *et al.*, 1988). In our experiments, it was also suggested that Ca^{2+} stores linked to caffeine and muscarinic

agonists do not entirely overlap, because acetylcholine was still effective in releasing catecholamine after a substantial secretory response to caffeine during perfusion with Ca^{2+}-free Locke solution containing hexamethonium (10^{-3} M). The identification of these hypothetical Ca^{2+} storage sites and the mechanism releasing Ca^{2+} from them will certainly be an important subject for further investigation.

Unlike muscarinic agonists, which caused a preferential release of noradrenaline in the absence of extracellular Ca^{2+} (Yamada *et al.*, 1989), caffeine increased adrenaline and noradrenaline secretions in approximately equal ratios throughout the concentrations used. Furthermore, in contrast ouabain enhanced noradrenaline secretion much more than adrenaline secretion in response to muscarinic receptor activation (Yamada *et al.*, 1989), it caused an equal enhancement of the two catecholamines in caffeine-induced secretory response. It appears, therefore, that the population or the capacity of Ca^{2+} storage sites sensitive to caffeine and muscarinic agonists is different between adrenaline and noradrenaline cells. This may partly account for the differential release of adrenaline and noradrenaline by different secretagogues in cat adrenal glands.

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