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ON THE RELEASE OF CATECHOLAMINES AND DOPAMINE-β-HYDROXYLASE EVOKED BY OUABAIN IN THE PERFUSED CAT ADRENAL GLAND

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1 Secretion of catecholamines (CA) and dopamine-β-hydroxylase (DBH) activity from the retrogradely perfused cat adrenal gland was studied following ouabain infusion. Perfusion with ouabain (10^{-4} M) for 10 min caused a gradual release of CA in the effluent which reached its peak 30 min after the ouabain pulse, and was maintained constant for at least 1 h. The effect of ouabain seemed to be irreversible.

2 Mecamylamine, while blocking the CA secretory effects of acetylcholine (ACh) perfusion, did not affect the secretion of CA evoked by ouabain. In denervated adrenal glands, ouabain-induced CA secretion was similar to that in the contralateral, innervated gland. However, physostigmine perfusion potentiated the CA secretory effects of ouabain.

3 The release of CA evoked by ouabain was accompanied by a proportional release of DBH activity. The time course of appearance of DBH activity followed the pattern of CA release.

4 The CA and DBH outputs in response to a pulse of ouabain were suppressed in the absence of calcium. Calcium reintroduction to a calcium-free perfused, ouabain-treated gland not only restored but greatly potentiated the release of CA and DBH. The amplitude of the secretory response to calcium reintroduction in ouabain-treated glands was proportional to the extracellular calcium concentration, and was antagonized by an external sodium-deficient medium.

5 These data demonstrate that ouabain releases CA from the perfused cat adrenal gland by a calcium-dependent exocytotic mechanism. The secretory effect of ouabain is not secondary to the release of ACh from cholinergic nerve terminals present in the adrenal gland, but due to a direct action on the chromaffin cell itself. In addition, the results suggest that this action is exerted through redistribution of monovalent cations secondary to the inhibition by the glycoside of the sodium pump. Such monovalent cation redistribution may cause a rise of intracellular ionized calcium levels through the activation of an internal sodium-dependent calcium influx system probably located in the chromaffin cell membrane.

Introduction

Cardiac glycosides favour the release of neurotransmitters and secretory materials from different tissue and organ systems. For example, these drugs induce the release of acetylcholine (ACh) from the rat (Elmqvist & Feldman, 1965) frog (Birks & Cohen, 1968a, b; Baker & Crawford, 1975), and toad (Balnave & Gage, 1974) motor endplates; from guinea-pig ileum parasympathetic nerve terminals (Paton, Vizi & Zar, 1971) and from rat cerebral slices (Vizi, 1972). It has also been demonstrated that cardiac glycosides induce the release of catecholamines (CA) in the perfused bovine adrenal gland (Banks, 1967), guinea-pig vas deferens (Ozawa & Katsuragi, 1974), rabbit heart (Lindmar & Löffelholz, 1974) and cat spleen slices (García & Kirpekar, 1973a, b).

The secretory effects of cardiac glycosides have generally been associated with the membrane sodium, potassium-activated adenosine triphosphatase (ATPase, Birks & Cohen, 1968b; Vizi, 1972; García & Kirpekar, 1973a, b; 1975a, b; Bonaccorsi, Hermsmeyer, Smith & Bohr, 1977; Duncan, 1977) since it is well known that these drugs specifically inhibit this enzyme (Glynn, 1964; Schwartz, 1976; Akera, 1977).

On the other hand, it is not known how the presynaptic cholinergic nerve terminals present in the adrenal gland contribute to the overall CA secretory

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effect of ouabain in the adrenal gland. The aim of the present experiments was to investigate whether the cardiac glycoside ouabain, a specific and well known membrane ATPase inhibitor, evokes the release of CA and dopamine-β-hydroxylase (DBH) from the perfused cat adrenal gland, to study its effects on the presynaptic cholinergic nerve terminals present in the gland and to determine its relation with ions. A preliminary report of some of these findings has been published (Horga, Hernández, García, Esquerro & Sánchez-García, 1978).

Methods

Perfusion of cat isolated adrenal gland

Cats were anaesthetized with ether, followed by chloralose (40 to 60 mg/kg, i.v.) and the abdomen was opened by a mid-line incision. The adrenal glands were removed after insertion of a cannula into the adrenolumbar vein and perfused in a retrograde direction through this vein by means of a perfusion pump. The gland was placed in a glass funnel, and the surface of the gland covered with minute incisions made with a hypodermic needle. The perfusion rate was 1 ml/min. In one group of experiments, 5 mm of the left splanchnic nerve was cut and removed under aseptic conditions. Two weeks after denervation, both glands were perfused as described above.

Perfusion media

All glands were perfused with Krebs-bicarbonate solution with the following composition (mM): NaCl 119, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4.7H_2O$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2 and glucose 11. This solution was equilibrated with 95% O_2 and 5% CO_2, the final pH being 7.4 to 7.5. Low-calcium solutions were made up by removing the appropriate amount of $CaCl_2$; no osmotic adjustments were made. When the sodium concentration was reduced the osmolarity of the solution was maintained with equivalent amounts of sucrose or LiCl. Potassium-rich solution (140 mM) was prepared by the addition of K_2SO_4 and reduction of NaCl to maintain isotonicity.

The glands were perfused at room temperature in a temperature-controlled laboratory (22 to 24°C) since in preliminary experiments CA secretion evoked by ACh or ouabain was more reliable and the spontaneous CA output was lower at room temperature than at 37°C.

Collection of perfusate samples

After 1 h of initial perfusion, a 5 min sample was collected to determine the resting secretion of CA and DBH. Secretion was evoked by perfusion with Krebs solution containing ouabain or, in some cases, ACh or high potassium. Samples were collected at 5 min intervals. The collecting tubes were stored in ice during the collection time. Immediately after the collection period ended 1 ml aliquots were removed and mixed with solid bovine serum albumin (BSA, final concentration, 7‰) which by itself had no DBH activity. These aliquots were used for the assay of DBH activity on the same day of the experiment. The rest of the sample was acidified with perchloric acid to a final concentration of 0.05 N, and frozen until assayed for their total CA content.

Catecholamine assay

Total CA content of the samples (noradrenaline plus adrenaline) was determined according to Shellenberger & Gordon (1971), without further purification on alumina. CA values are expressed as μg/5 min perfusion period. Net release of CA was calculated by subtracting the basal, spontaneous release from the oubain-evoked release.

Dopamine-β-hydroxylase assay

DBH activity of perfusates (0.2 ml aliquots) was assayed according to the procedure described by Molinoff, Weinshilboum & Axelrod (1971). Several concentrations of $CuSO_4$ were tested in order to obtain adequate inactivation of the endogenous inhibitors of the enzyme. Maximum DBH activities were repeatedly found at 2.4 μM (final concentration of $CuSO_4$ in the reaction mixture). For the first step of the reaction the optimal pH was 5 and it was run for 1 h. The second step of the reaction was run for 30 min. DBH activity is expressed as nmol/h in a 5 min collection period of octopamine formed from tyramine. Net release of DBH was calculated by subtracting the basal, spontaneous release from the ouabain-evoked release.

Results

Time course of the release of catecholamines and dopamine-β-hydroxylase evoked by ouabain

Figure 1 shows that ouabain (10^{-4} M) infusion, either as a 10 min pulse or as a constant perfusion, caused a significant increase of the output of CA from the retrogradely perfused cat adrenal gland. The release of CA was accompanied by a parallel release of DBH. The CA release evoked by ouabain was already apparent after 5 min of perfusion with the drug, increased gradually and reached a peak after 30 to 40

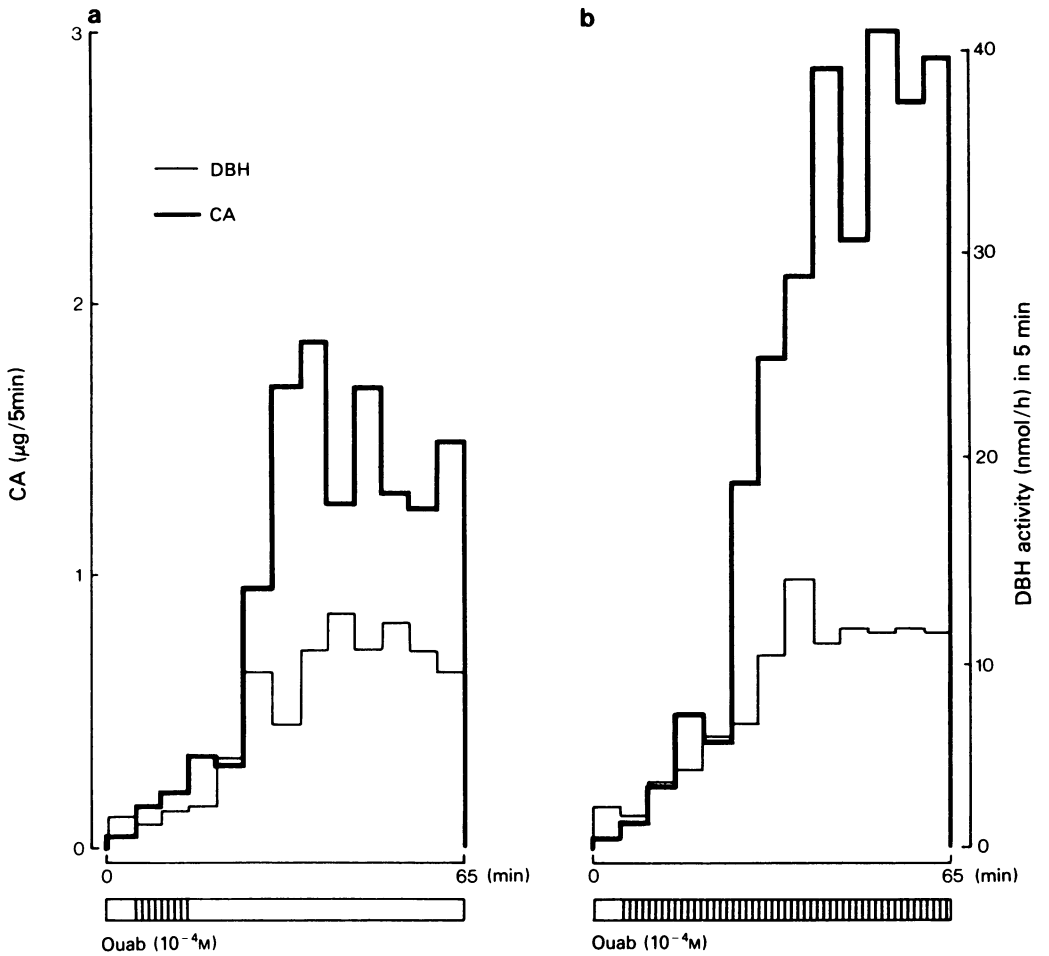

Figure 1 Release of catecholamines (CA) and dopamine-β-hydroxylase (DBH) from retrogradely perfused cat adrenal glands following a 10 min pulse (a) or continuous perfusion with ouabain (b). Heavyline: CA; fine line: DBH. This experiment was carried out on 2 glands from the same animal. Five additional experiments gave similar results. Each bar represents a 5 min collection period.

min. The release was maintained at its high level for at least 1 h.

It is worth noting that while the pattern of release of DBH was similar in the gland perfused with a 10 min ouabain pulse and the gland continuously perfused with the drug, the release of CA was greater in the latter situation. The net CA release during 1 h collection period was, in the case of the 10 min pulse of ouabain 11.5 $\pm$ 2.1 µg/gland, and in the case of the continuous infusion with the drug, the release was 25.6 $\pm$ 5.2 µg/gland of CA ($n = 4$; $P < 0.05$). The net DBH activity recovered after 1 h collection period was 48.7 $\pm$ 8.4 and 48.2 $\pm$ 10.9 nmol/h per gland, respectively for the ouabain pulse or the continuous infusion with the drug. In view of the close parallelism of the release of CA and DBH induced by a pulse of ouabain (10^{-4} M for 10 min), in all subsequent experiments such a ouabain pulse was used.

Effect of mecamylamine on the release of catecholamines induced by ouabain

The CA release evoked by ouabain could be secondary to the release of ACh induced by the drug from the presynaptic cholinergic nerve terminals present in the adrenal medulla. That this is not the case is shown by the experiments summarized in Figure 2. In each of 5 experiments, both glands from the same animal

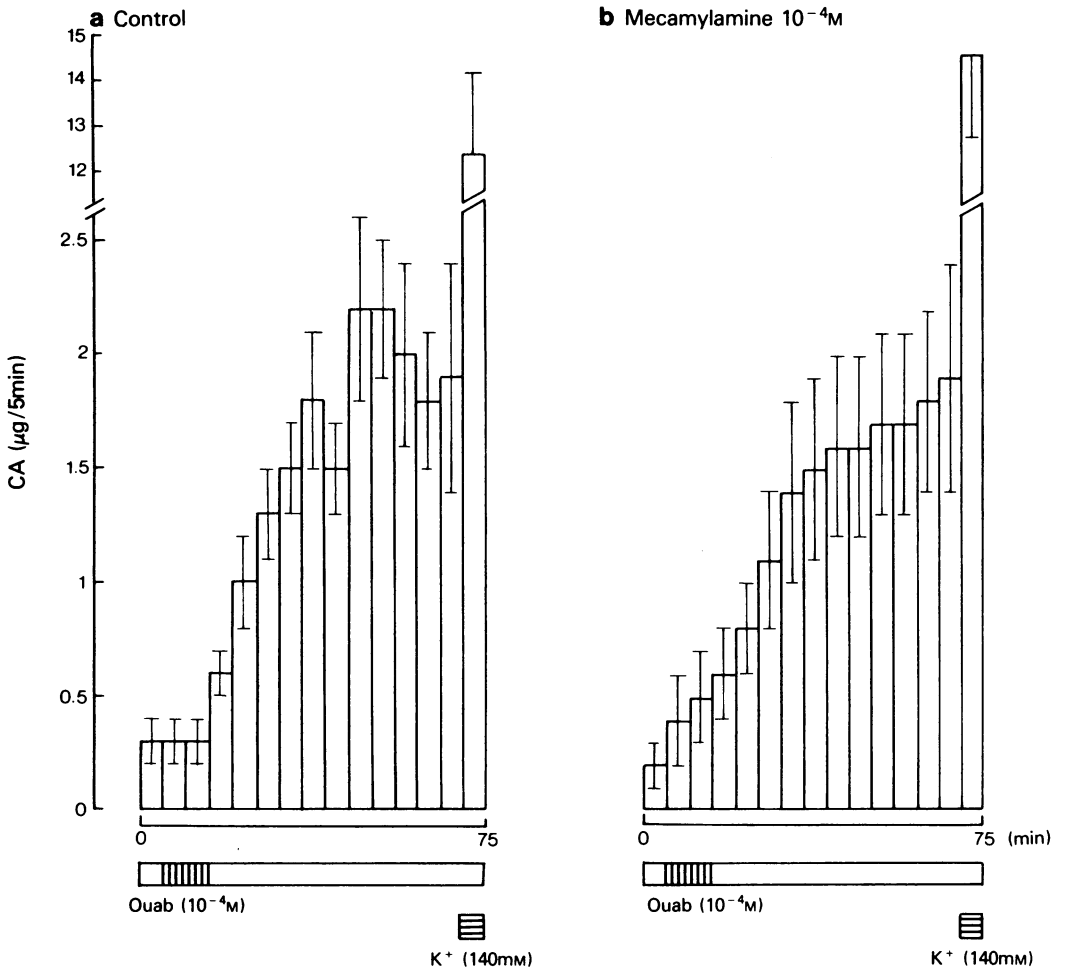

Figure 2 The effect of mecamylamine (b) on the release of catecholamines (CA) induced by ouabain (Ouab); (a) control experiments. Mecamylamine was present 5 min before and during the entire collection period (minutes 0 to 75). Ouabain was given as a 10 min pulse. At the end of the experiment, a high-potassium (K^+, 140 mM) solution was perfused for 5 min. Data are means ± s.e. (vertical lines) of 5 paired experiments.

were perfused with Krebs solution in the presence or absence of the ganglionic blocking agent, mecamylamine (10^{-4} M). In the two cases, the pattern of CA release evoked by a ouabain pulse was identical. Moreover, in a third control gland, perfusion of ACh (10^{-4} M) during a 10 min period induced a massive secretion of CA (9.76 and 12.8 μg/5 min); this ACh secretory response was reduced to 0.76 and 0.34 μg/5 min of CA if mecamylamine (10^{-4} M) was perfused 15 min before and during the administration of ACh. Perfusion with a high potassium (140 mM) solution at the end of the experiment produced a large, further secretory response in both, control and mecamylamine-treated glands.

Effect of physostigmine on the release of catecholamines induced by ouabain

Ouabain could be releasing ACh from presynaptic sites in the adrenal medulla, but the ACh released might be rapidly degraded by acetylcholinesterase (AChE). Therefore, the secretory effect of ouabain was tested in glands pretreated with physostigmine (2×10^{-5} M). Figure 3 shows that the ouabain-

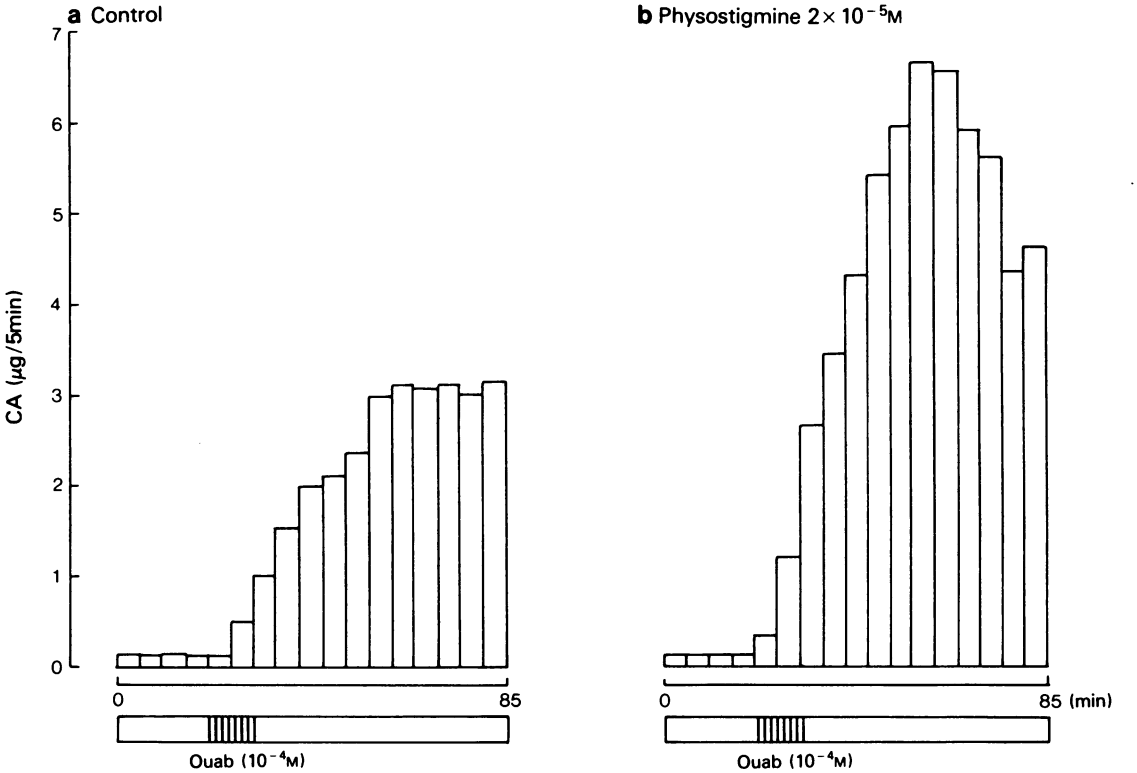

Figure 3 Effect of physostigmine (b) on the release of catecholamines (CA) evoked by ouabain (Ouab); (a) control experiments. Physostigmine was present 5 min before and during the entire collection period (minutes 0 to 85). Ouabain was given as a 10 min pulse. Results are the means of two paired experiments.

evoked CA release was clearly higher in the gland treated with physostigmine than in the control gland.

Secretion of catecholamines evoked by ouabain on intact right and denervated left adrenal

In order to evaluate further the role of ACh release on the overall ouabain CA secretory response, the effect of ouabain was investigated in two paired experiments in which the left adrenals were denervated 2 weeks before the experiment. Figure 4 shows that the secretion of CA evoked by a ouabain pulse was slightly, but not significantly lower in the left adrenal gland when compared to the secretion obtained in the normal innervated gland. Perfusion with a solution containing high potassium (140 mM) concentrations produced a large, further secretory response in both glands; this indicates that both, normal and denervated glands are capable of producing a maximal secretory response.

Effect of calcium ions on the release of catecholamines and dopamine β-hydroxylase induced by ouabain

Since the physiological release of CA and DBH from the perfused cat adrenal gland is dependent on the extracellular calcium concentration (Dixon, García & Kirpekar, 1975), it was of interest to investigate whether the secretory effect induced by ouabain in this preparation was also related to extracellular calcium ions.

Therefore, adrenal glands were perfused with Krebs solution containing 2.5, 0.25 and 0 mM calcium. In all cases a 10 min ouabain pulse was applied. Figure 5 shows that CA and DBH release was identical either at 2.5 or 0.25 mM of calcium; however, the secretory response evoked by the drug was abolished in the absence of extracellular calcium.

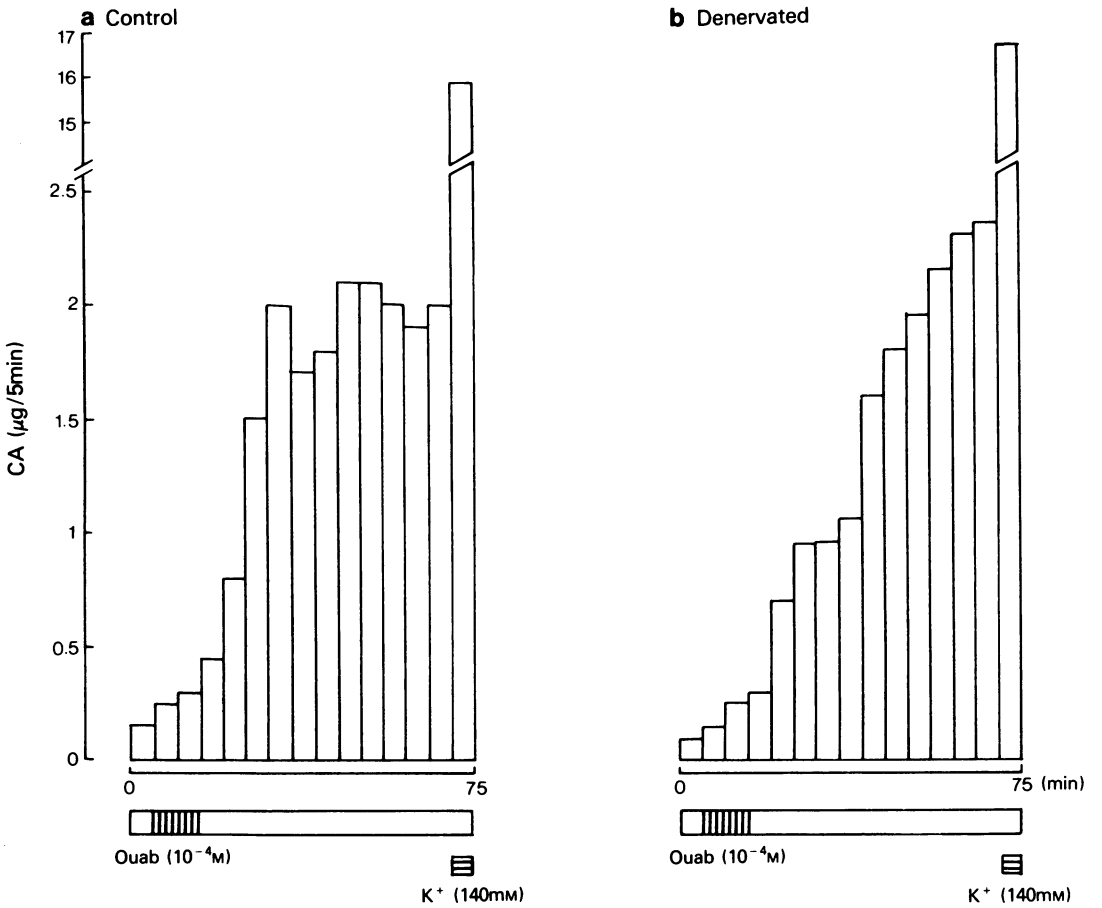

Figure 4 Secretion of catecholamines (CA) evoked by ouabain (Ouab) on intact (a) and denervated adrenal glands (b). The left adrenals were denervated 2 weeks before the experiment. Ouabain was given as a 10 min pulse. At the end of the experiment a high potassium (K^+, 140 mM) solution was perfused for 5 min. Results are the means of two paired experiments.

Effect of calcium reintroduction on the release of catecholamines evoked by ouabain

Since in the absence of calcium ouabain failed to increase the release of CA and DBH, it was of interest to determine whether calcium reintroduction, after a period of calcium deprivation but in the presence of magnesium, would restore the secretory effects of ouabain. In a group of four paired adrenal glands, the following experimental design was followed. Two glands from the same cat were initially perfused with a calcium-free Krebs solution containing magnesium; then one gland was treated with a pulse of ouabain (10^{-4} M for 10 min) in the absence of calcium. Forty-five min later, calcium (2.5 mM) was reintroduced in both glands. Figure 6 shows that calcium reintroduction had no effect in the control gland; however, infusion of ACh still evoked a secretory response, which proved that the gland was functionally intact. It is worth noting that if the glands were pre-perfused with a calcium-magnesium-free Krebs solution for 45 min, when calcium (but not magnesium) was reintroduced, a vigorous but transient CA secretory response was evoked; in contrast, if both calcium and magnesium were simultaneously reintroduced, no secretory response was obtained. These data agree with those obtained by Douglas & Rubin (1963) in the same preparation.

In the gland previously treated with a ouabain pulse, the reintroduction of calcium (after the 45 min of perfusion with magnesium-containing, calcium-free medium) evoked a dramatic increase of the CA out-

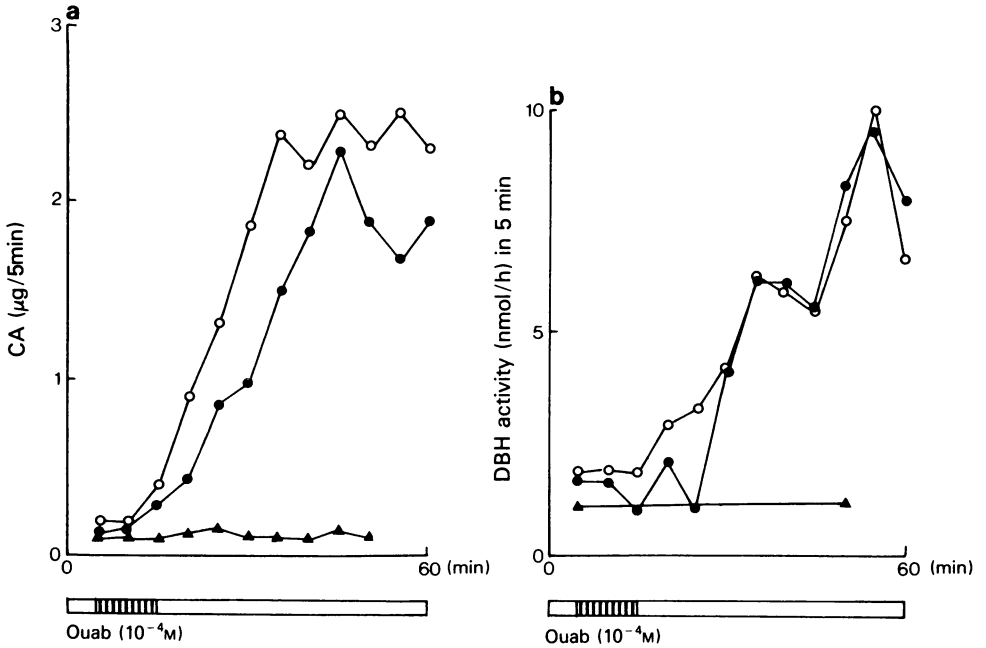

Figure 5 Calcium requirements for the release of catecholamines (CA, a) and dopamine-β-hydroxylase (DBH, b) evoked by a 10 min pulse of ouabain. Before introduction of ouabain in the perfusion system, the glands were perfused for 15 min with Krebs solution containing 0 (▲), 0.25 (●) and 2.5 mM (○) calcium. The figure shows one of three typical experiments.

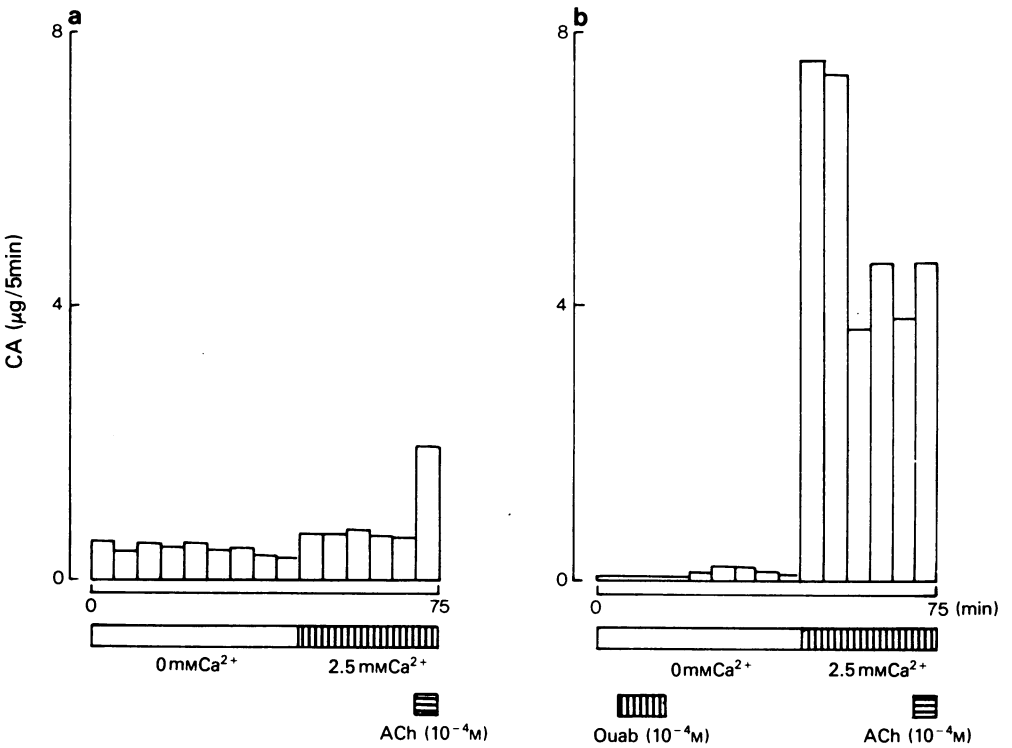

Figure 6 Effect of ouabain on the secretory effects of calcium reintroduction in a gland pre-perfused with calcium-free Krebs solution. The experiment was carried out in 2 glands from the same cat; (a) is control experiment. At the end of the experiment, a solution of acetylcholine (ACh 10^{-4} M) was perfused for 5 min. The data were selected from one of 4 similar experiments.

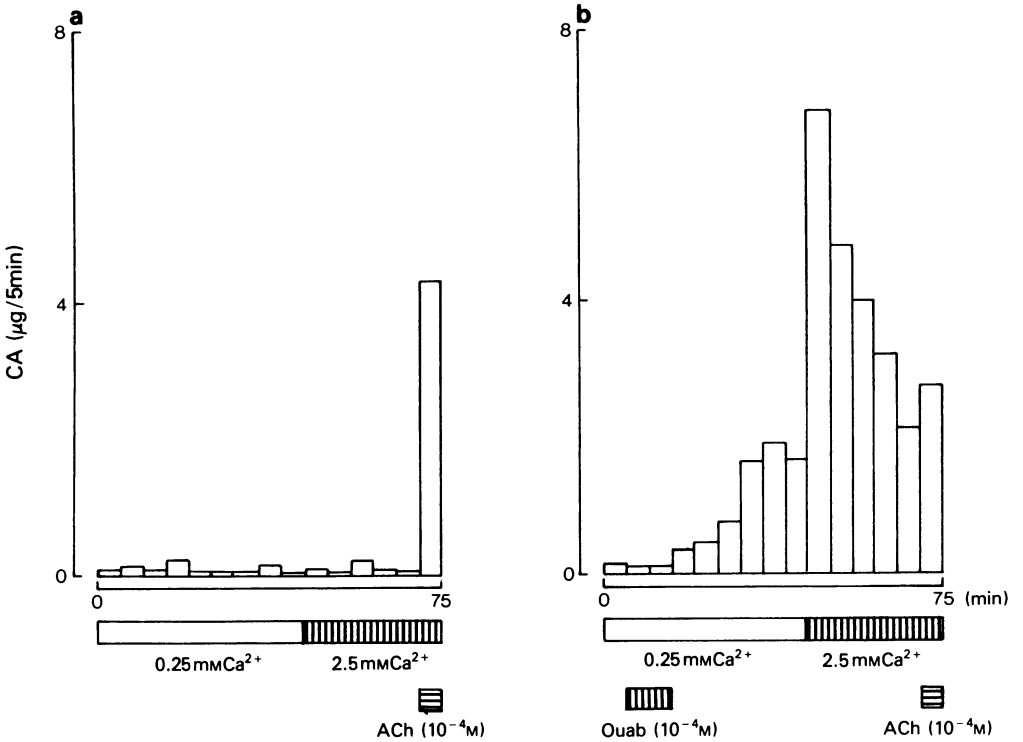

Figure 7 Effect of ouabain on the secretory effects of calcium reintroduction in a gland pre-perfused with 0.25 mM calcium Krebs solution. The experiment was carried out in 2 glands from the same cat; (a) is control experiment. At the end of the experiment a solution of acetylcholine (ACh 10^{-4} M) was perfused for 5 min. The data were selected from one of 4 similar experiments.

put. ACh did not cause any further increase of secretion. When calcium was reintroduced after 2 to 10 min of ouabain perfusion, the secretory response was considerably lower (data not shown). It is interesting to note that the secretory response evoked by calcium reintroduction in the ouabain-treated gland was much faster and larger than the secretion evoked by ouabain when introduced in a gland perfused with normal Krebs solution from the beginning of the experiment (see Figure 1a). In fact, when ouabain was introduced into the perfusion system at the same time as calcium, the peak secretory response was reached 30 min later and the release of CA was about 2 µg/5 min. However, when calcium was reintroduced, in the ouabain pretreated glands, the peak of CA release was 8 µg/5 min; this secretory response was maintained at a high level for an additional 25 min period. This time course of the secretory response sharply contrasts with the transient response obtained with calcium reintroduction in a gland pre-perfused with calcium-magnesium-free Krebs solution in the absence of ouabain (data not shown).

Since the secretory response to ouabain is similar when the pulse is given either in the presence of 0.25 or 2.5 mM calcium (Figure 5), it was of interest to see whether the increase of the calcium concentration from 0.25 to 2.5 mM would increase the release of CA from a ouabain-treated gland. Figure 7 shows that in a control gland, perfused with 0.25 mM calcium, reintroduction of normal Krebs solution did not modify the rate of CA release. It is pertinent to note that in this gland, acetylcholine infusion caused a large secretory response. In another group of glands, a pulse of ouabain was given when the gland was being perfused with 0.25 mM calcium. Thirty min after the pulse, when the ouabain secretory response in 0.25 mM calcium was maximal, reintroduction of normal Krebs caused a large additional CA secretory response (Figure 7). In this case, ACh failed to induce a further secretory response.

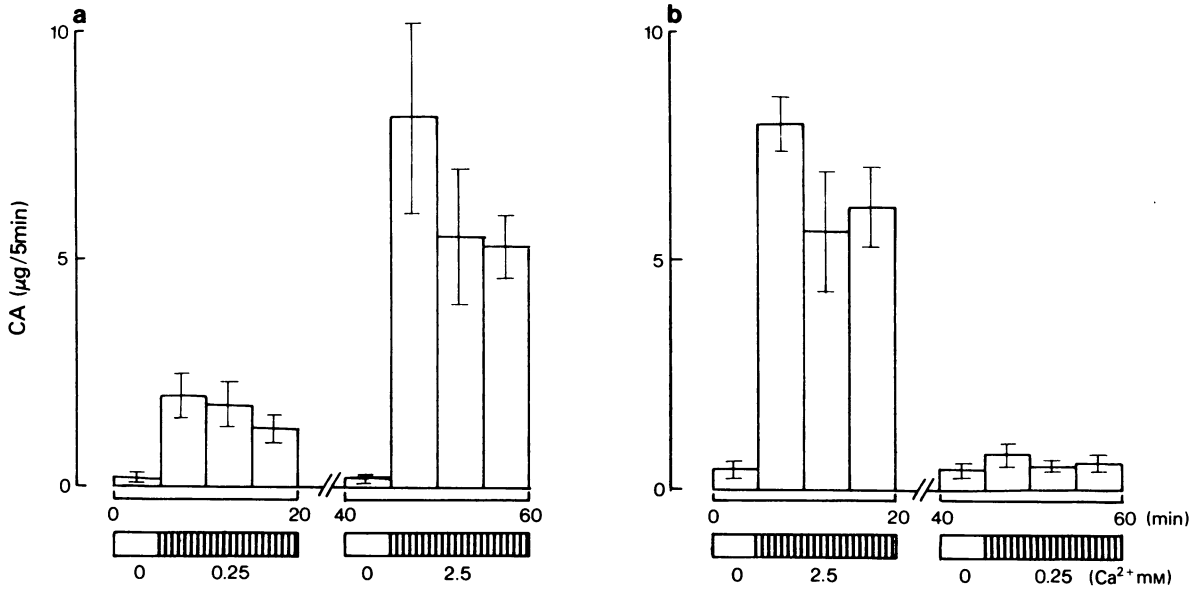

Figure 8 Effect of ouabain on the secretion of catecholamines (CA) evoked by calcium reintroduction. Paired glands (a and b) from the same cat were initially perfused with calcium-free solution, and then a 10 min pulse of ouabain (10^{-4} M) was given; after the ouabain pulse (not shown in the figure), both glands were perfused with calcium-free Krebs solution for an additional 30 min period. Then, 0.25 mM (a) or 2.5 mM (b) calcium was reintroduced. Following a 20 min perfusion period with calcium-free solution, 2.5 mM (a) and 0.25 mM (b) calcium solution were again reintroduced. The figure shows mean results from 4 experiments; vertical lines are s.e. means.

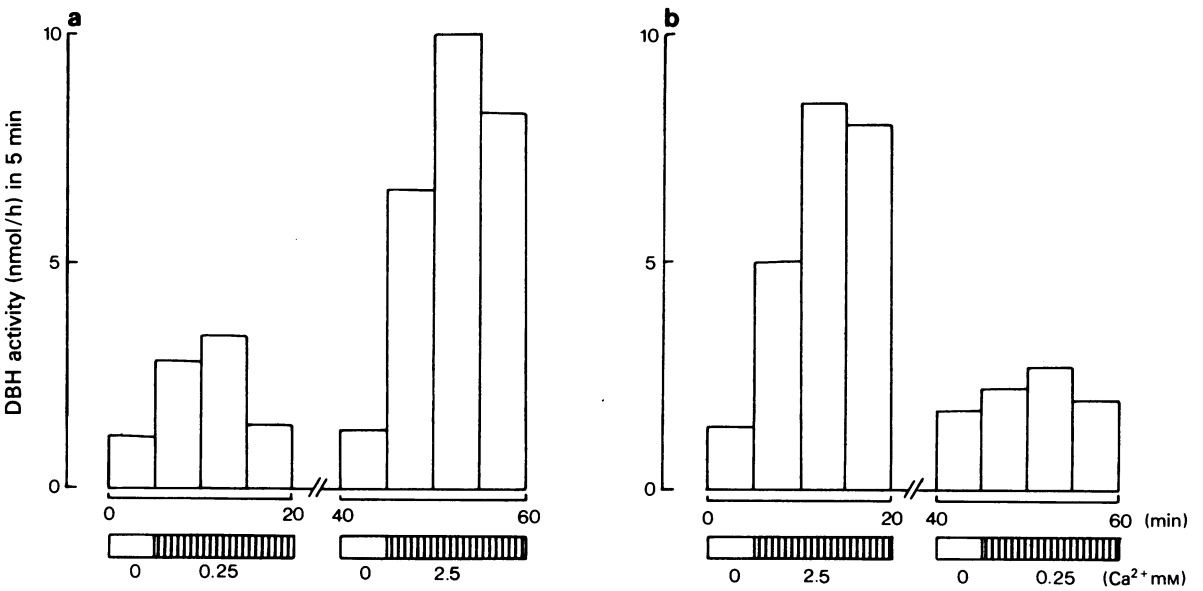

Figure 9 Effect of ouabain on the secretion of dopamine-β-hydroxylase (DBH) evoked by calcium reintroduction. Experimental design as in Figure 8. The figure shows mean results from 4 experiments.

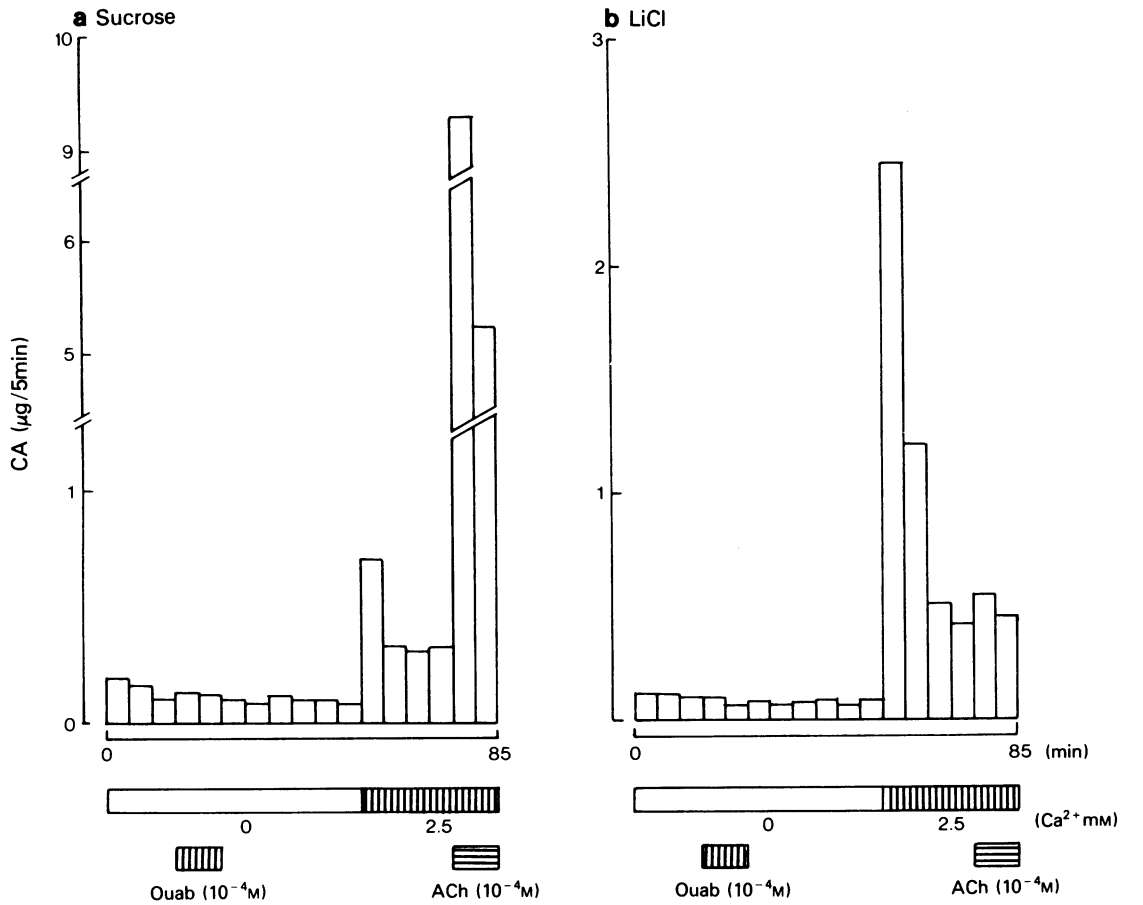

Figure 10 Release of catecholamines (CA) evoked by ouabain (Ouab) in a low (25 mM)-sodium medium. Sodium was substituted by sucrose (a) or LiCl (b). The glands were perfused with the 25 mM sodium medium from time 0 to 85 min. Calcium was reintroduced 30 min after the 10 min pulse of ouabain was given. At the end of the experiment a solution of acetylcholine (ACh 10^{-4} M) was perfused for 5 min

Is the catecholamine and dopamine-β-hydroxylase release evoked by calcium reintroduction in ouabain-treated adrenals related to the external calcium concentration?

It seems that ouabain somehow favours the secretory effects of calcium reintroduction in a gland previously deprived of this ion. If this is true, then the secretory response should be proportional to the external concentration of calcium when this ion is reintroduced in a gland previously perfused with magnesium-containing, calcium-free Krebs solution.

In order to substantiate this point, two ouabain (10^{-4} M for 10 min) treated adrenal glands from the same animal were initially perfused for a 45 min period with magnesium-containing, calcium-free Krebs solution; then in one gland 0.25 mM calcium was reintroduced for 15 min. The gland was reperfused with calcium-free solution for a 20 min period and finally 2.5 mM calcium was reintroduced. In the other gland, the reverse procedure was followed: first 2.5 mM calcium and then 0.25 mM calcium was reintroduced. Figure 8 shows the release of CA and Figure 9 the release of DBH activity into the perfusion medium. In both cases CA and DBH outputs were proportional to the calcium concentration reintroduced after perfusion with calcium-free Krebs. The net CA release in gland A (Figure 8) was 4.28 ± 1.18 and 18.35 ± 3.32 µg after reintroduction of 0.25 mM and 2.5 mM calcium, respectively ($n = 4$, $P < 0.001$). In gland B of the same figure similar differences were found after reintroducing 2.5 mM and 0.25 mM

calcium. The net DBH activity released followed a similar pattern in both cases.

Effect of external sodium ions on the release of catecholamines evoked by calcium reintroduction in ouabain-treated glands

Birks & Cohen (1968a, b) showed that if part of the sodium of the Ringer was replaced by sucrose, the rises in the mean quantal content of the endplate potential (e.p.p.) and the miniature endplate potential (m.e.p.p.) frequency that develop in the presence of cardiac glycosides, did not occur. From this observation they proposed that changes in the intracellular concentrations of monovalent cations initiated the glycoside effects. Since the chromaffin cell treated with ouabain will load with sodium less quickly when external sodium is reduced, the rise in the CA secretion evoked by calcium reintroduction, after a period of calcium deprivation, ought to slow down in media of reduced sodium content. In fact this was seen in the experiment illustrated in Figure 10. In the gland preperfused with 25 mM sodium-sucrose medium, the secretory response to calcium reintroduction was considerably diminished. In the case of low sodium-LiCl solution, calcium reintroduction evoked a larger secretory response, which was, however, still lower than the CA secretion evoked when the extracellular sodium concentration was normal.

Discussion

The present experiments clearly demonstrate that ouabain produces a gradual release of CA and DBH from the perfused cat adrenal gland and that this secretory event is critically dependent on the extracellular calcium concentration.

Elmqvist & Feldman (1965) have found that ouabain caused a calcium-dependent increase in the m.e.p.p. frequency. Similarly, Birks & Cohen (1968b) have also shown that digoxin caused a sodium-dependent increase in e.p.p. size and m.e.p.p. frequency and suggested that these effects arose from an acceleration of calcium influx. In contrast, Baker & Crawford (1975) showed that ouabain did not require external calcium to induce the increase in m.e.p.p. frequency.

With regard to the release of CA, contradictory findings have also been reported. While Vizi (1975) showed that ouabain promotes the release of noradrenaline from isolated vas deferens of the rat even in the absence of calcium, Nakazato, Ohga & Onoda (1978) recently found that the release of noradrenaline from guinea-pig vas deferens evoked by ouabain was exquisitely dependent on extracellular calcium ions. In the perfused bovine (Banks, 1967) and cat adrenal gland (our experiments) the catecholamine secretory effects of ouabain are critically dependent on extracellular calcium. The reason for the discrepancies between different authors' results is not known, but may be due to differences in experimental design, cardiac glycosides or animal species used.

On the site of action of ouabain as a catecholamine secretory agent in the adrenal medulla

ACh, the physiological presynaptic transmitter at the adrenal medulla, releases CA and DBH by a calcium-dependent secretory process (Viveros, Arqueros & Kirshner, 1968; Dixon *et al.*, 1975). Since ouabain also induces the release of ACh from cholinergic nerve terminals in different biological systems (Elmqvist & Feldman, 1965; Birks & Cohen, 1968a, b; Paton *et al.*, 1971; Vizi, 1972; Balnave & Gage, 1974; Baker & Crawford, 1975), the question arises whether secretion of CA and DBH evoked by ouabain in the cat adrenal is secondary to the release of ACh from cholinergic nerve terminals present in the gland.

Since mecamylamine, a well known ganglionic blocking agent, or denervation of the adrenal gland, did not significantly modify the secretory response to ouabain, it is clear that the glycoside effect is due to a direct action on the chromaffin cell. However, inhibition of AChE with physostigmine, before the ouabain pulse, clearly potentiated the drug's secretory effect. This apparent contradictory finding can be explained if ouabain also releases ACh from presynaptic cholinergic sites, as it does in other cholinergic systems but the amounts of ACh being released are probably low and the ACh is quickly degraded by AChE before reaching the nicotinic receptors located on the surface of the chromaffin cell.

Sodium, potassium-ATPase inhibition and catecholamine secretion

Since ouabain specifically inhibits the membrane sodium, potassium-activated ATPase in many biological systems (Schwartz, 1976; Akera, 1977), the question arises whether the binding of digitalis to the chromaffin cell membrane ATPase is causally related to the secretory effect of the drug. Concerning this point, it is reasonable to assume that ouabain binds to the membrane ATPase of the chromaffin cell and inhibits the sodium pump. In fact, Banks (1967) showed that perfusion of bovine adrenal glands with ouabain (10^{-4} M for 30 min) caused a 30% decrease of the potassium content of the medullae, a well known effect of ATPase inhibition in different organ systems.

On the other hand, the slow time course of the secretory effects of ouabain agrees well with the time course of binding and inhibition of ATPase by ouabain in different organ systems. The secretion of CA and DBH evoked by a pulse of ouabain is a long-last-

ing phenomenon, which remains for a long time after the wash out of the glycoside. Also, the binding of digitalis to ATPase is of a slow and very tight or 'pseudoirreversible' type (Schwartz, 1976). In fact, in a highly digitalis-sensitive species, such as the cat, the half-time for dissociation of the digitalis-enzyme complex *in vitro* was about 85 min (Akera, 1977). Considering the difference in experimental conditions, these results indicate a good correlation between the dissociation of digitalis from ATPase and the duration of its secretory effects.

How is the membrane ATPase involved in the secretory effects of ouabain?

Since ouabain promotes the release of CA and DBH by an extracellular calcium-dependent process, the underlying secretory mechanism seems to be similar to the physiological exocytotic mechanism. It therefore seems probable that the action of the glycoside is achieved by a rise in the intracellular ionized calcium concentration. The question arises whether this rise of intracellular calcium is secondary to redistribution of monovalent cation gradients within the chromaffin cell brought about by the inhibition of the sodium pump by ouabain, or rather to the inhibition of the membrane ATPase itself.

Our results are best explained by considering that inhibition of the sodium pump by ouabain will ultimately lead to intracellular sodium accumulation and potassium loss. Such monovalent cation redistribution may cause a rise of intracellular ionized calcium levels first, by depolarization of the chromaffin cell or second, by activation of the sodium-dependent calcium influx system.

It is well established that depolarization of the chromaffin cell produces an increase in calcium uptake (Douglas & Poisner, 1962). But it has also been shown that maintained depolarization of adrenergic neurones and chromaffin cells induces a sharp secretory response which rapidly desensitizes, probably because inactivation of a membrane calcium channel follows a brief period of activation (Baker & Rink, 1975; García, Kirpekar & Sánchez-García, 1976). In our experiments, the secretion of catecholamines evoked by ouabain, or by calcium reintroduction is a long-lasting one and does not apparently desensitize. On the other hand, tetrodotoxin or high magnesium did not antagonize the secretory effects of ouabain (data not shown).

It seems more likely that the explanation of the secretory effects of ouabain can be expressed in terms of the activation of the sodium-calcium counter transport system described in the squid giant axon and other experimental systems (Baker, 1975). In this system, reduction of the electrochemical gradient for sodium, for instance, a rise in internal sodium or a fall in external sodium, will reduce the sodium-dependent calcium efflux and increase calcium uptake in exchange for sodium loss, with the concomitant rise of intracellular ionized calcium concentration. Our experimental findings agree well with this mechanism, since: (a) The secretory effect of ouabain developed gradually probably because the cell was progressively gaining sodium. (b) Ouabain-evoked catecholamine release was dependent on extracellular calcium. (c) In ouabain-treated glands, pre-perfused with calcium-free solution, the reintroduction of calcium produced a dramatic vigorous secretory response, which was proportional to the extracellular calcium concentration. (d) The fact that calcium reintroduction evoked a four times larger secretory response in a ouabain-treated gland pre-perfused with calcium-free solution (Figure 6), as compared to the secretion evoked by ouabain in normal Krebs solution (Figure 1), may be explained by considering that in the absence of calcium the accumulation of sodium seems to be facilitated because of the lack of the stabilizing effect of calcium (Frankenhaeuser & Hodgkin, 1957). (e) In a ouabain-treated gland pre-perfused with a low-sodium (sucrose) medium, the secretory effects of the glycoside were greatly antagonized. (f) Finally, in a gland perfused with calcium-free medium, the reintroduction of calcium (2.5 mM) 2, 10, 20 and 30 min after ouabain, evoked an increasing secretory response which was larger as the time between ouabain treatment and the reintroduction of calcium was increased. The time-course of this secretory response was greatly accelerated in the presence of an excess of extracellular sodium and was antagonized in low-sodium medium (unpublished data). These experiments suggest that late in a ouabain exposure, when the chromaffin cell is sodium loaded, the sodium-dependent calcium influx might be much larger than at the beginning.

Birks & Cohen (1968a, b) for the frog motor endplate and Nakazato *et al.* (1978) for the guinea-pig vas deferens have also explained the changes in transmitter release evoked by cardiac glycosides in terms of changes in the intracellular concentrations of monovalent cations.

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