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Ionic and Metabolic Requirements for Stimulation of Secretion by Ouabain in Bovine Adrenal Medullary Cells

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SUMMARY

The effect of ouabain on the secretion of catecholamines from isolated bovine adrenal medullary cells was investigated. Ouabain enhances the basal rate of secretion approximately 2-fold, with half-maximal stimulation occurring at a glycoside concentration of around 5×10^{-7} M. Parallel measurements of the release of dopamine β-hydroxylase (EC 1.14.17.1) (an enzyme associated with chromaffin granules) and lactate dehydrogenase (EC 1.1.1.27) (which is confined to the cytosolic compartment) suggest that this increase in secretion occurs as a result of an enhanced rate of exocytosis rather than by any other route. The stimulatory effect of ouabain is dependent on extracellular sodium but is maintained in the nominal absence of calcium and is unaffected by changes in the major external anion. Neither tetrodotoxin nor phenoxybenzamine alters the response to glycoside treatment, but the calcium channel blocker methoxyverapamil reduces the catecholamine secretion evoked by ouabain in a dose-dependent fashion. This study serves to characterize the secretory action of ouabain in isolated chromaffin cells and to provide a foundation for the ion flux studies reported in the following paper [*Mol. Pharmacol.* **23**:681–697 (1983)].

INTRODUCTION

Although the most well-known effect of cardiac glycosides is their ability to increase the force of contraction of a variety of contractile tissues, most especially cardiac muscle, this is by no means their only action. In many tissues in which calcium is thought to bring about secretion by exocytosis, exposure to cardiac glycosides enhances both spontaneous and evoked release of secretory product [see review by Gillis and Quest (1)]. Glycoside-sensitive tissues of this kind include motor neuron terminals (2, 3), pancreatic islets (4), parasympathetic nerves (5), brain cortex (6), and adrenal medulla (7, 8).

All of the various hypotheses advanced to explain these secretory effects of cardiac glycosides make use of their well-documented ability to inhibit the sodium-potassium exchange pump (9–11), and this primary action of glycosides has been linked with secretion both directly and indirectly. Paton and his colleagues (5) have suggested that sodium pump inhibition per se is directly responsible for augmenting the secretion of acetylcholine from parasympathetic nerves by somehow "priming" the cell membrane for exocytosis. Most authors, however, favor the possibility of a rather more indirect link between the two events, possibly mediated by the redistribution of ions across the cell membrane (2, 12).

Despite the fact that a fairly clear picture of the calcium-dependent exocytotic secretory mechanism in the adrenal medulla is now available—indeed the chromaffin cell has often been used as a model for "stimulus-secretion coupling" (13, 14)—uncertainty still exists regarding the explanation for the effect of cardiac glycosides on catecholamine secretion from this tissue. One of the major drawbacks in previous studies concerning the action of ouabain has been the use of either intact gland or tissue slice preparations in which it is extremely difficult to carry out detailed quantitative studies of either secretion or ionic events under conditions in which the environment of the cells is accurately controlled. Many of these problems can be overcome by using a preparation of isolated cells which have been shown to retain many of the features characteristic of the intact gland (15). With this preparation it has been possible to reinvestigate the problem of glycoside action. This and the following paper (16) report the results of detailed studies of both exocytotic release of catecholamine and the ion fluxes which seem to underlie this process.

EXPERIMENTAL PROCEDURES

Materials

Bovine adrenal glands were obtained from a local abattoir. They were removed from the animals as quickly as possible—usually within 30 min of death—and dissected free of perinephric fat and connective tissue. They then received injections, via the adrenal vein, of ice-cold

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sodium-Locke's solution containing 0.5% (w/v) bovine serum albumin before being transported to the laboratory on ice.

Solutions

The primary solution used was 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes)-buffered sodium-Locke's solution containing 144 mM NaCl, 2.7 mM KCl, 1.8 mM $MgCl_2$, 1.8 mM $CaCl_2$, 10 mM Hepes, and 5 mM glucose, buffered to pH 7.3 ± 0.1 using NaOH. This solution was equilibrated with 100% oxygen. Unless otherwise stated, all experiments were carried out at 37°.

In solutions of increased potassium concentration, KCl replaced NaCl on an equimolar basis. Similarly, in sodium-free media, sodium was replaced on an equimolar basis and LiOH was used, instead of NaOH, to adjust the pH. In view of the possibility that choline (frequently used here as a sodium substitute) may possess cholinergic activity, some control experiments were performed to determine whether or not choline stimulated catecholamine secretion from chromaffin cells. Cells suspended in full sodium were mixed with an equal volume of Locke's solution containing 144 mM choline chloride, to give a final choline concentration of 72 mM, in both the presence and absence of 10^{-5} M hexamethonium and 10^{-5} M atropine. Basal catecholamine secretion remained unaltered in each case.

In general, the total divalent cation concentration was 3.6 mM, and calcium replaced magnesium, or vice versa, on an equimolar basis, when necessary.

All inorganic chemicals were obtained from Sigma Chemical Company Ltd. (London) or British Drug Houses Ltd. (London) and were of analytical grade. Quin 2[1] was a gift from Dr. Gerry Smith (Cambridge). A 23187 was obtained from Calbiochem-Behring Corporation (San Diego, Calif.). Unless otherwise stated, all other chemicals were obtained from Sigma Chemical Company. [^{3}H]Noradrenaline was obtained from Amersham International.

Isolation of Adrenal Medullary Cells

The isolation procedure used was similar to that described by Baker and Knight (15). It produces a high yield of functionally viable medullary cells. The glands were bisected and the cortical tissue was removed as completely as possible. Slices (about 0.5 mm thick) were cut from the medulla using single-edged razor blades mounted in a specially designed holder. The slices were rinsed several times in ice-cold Locke's solution (to remove the albumin) and then brought to 37° in a shaking water bath. Routinely, between three and six glands were used for each experiment and the volume of incubation medium was 30–50 ml. Dissociation of the cells was achieved during successive periods of enzymatic digestion. Two 30-min incubations in 0.1% (w/v) protease (Sigma Type XIV) were followed by an additional 30- to 45-min incubation in medium containing 0.1% (w/v) hyaluronidase (Sigma Type 1-s), 0.1% (w/v) collagenase (Sigma Type II), and 0.2% (w/v) bovine serum albumin. As digestion advanced, the softened slices could be pipetted gently to and from a 5-ml automatic pipette whose plastic tip had been cut to increase its minimal bore width to approximately 2 mm. As the tissue softened still further, the bore was gradually reduced. In this way, large numbers of dissociated cells could be obtained. These were then separated from large pieces of debris by straining them several times through muslin or a similar material. Finally, the cells were washed by centrifugation (3 min at 100 × *g*) and resuspended in fresh, albumin-free solution at 37°. Contaminating red blood cells, visible as a thin red band lying above the cream-colored chromaffin cells, were removed by careful aspiration. After three or four washes, the suspension was virtually free of debris, and more than 95% of the total catecholamine present was associated with the cells.

Routinely, about 10^7 cells could be obtained from each gland, as assessed by standard hemocytometry; in most experiments, each experimental point represents data from 10^6 cells. Cortical cells were the major contaminants of the final suspension, amounting in some preparations to as much as 15% of the total cell count.

Measurement of Secretory Activity

In cell suspensions. After resuspension of the cells in an appropriate volume of the experimental solution, the suspensions were divided into equal-sized aliquots in plastic test tubes. Catecholamine secretion was measured by centrifuging samples (1500 × *g* for 10 sec) at different times and assaying the catecholamine present in the supernatant. Samples of total suspension (lysed with 0.1% Triton X-100) were also assayed to enable expression of the secretion as a percentage of the total cellular catecholamine. Where appropriate, dopamine β-hydroxylase (EC 1.14.17.1) and lactate dehydrogenase (EC 1.1.1.27) secretion were expressed in a similar manner.

In cells embedded in agar. Occasionally, catecholamine secretion was investigated using cells which had been embedded in agar. A very dense suspension of cells was mixed with agar [2%(w/v) in Locke's solution] in a small piece of plastic tubing sealed at one end with Parafilm, just before the agar began to solidify (39°). After complete solidification (37°) the column of gelled cells could be extruded from the tube and cut into slices (approximately 1 mm thick). Routinely, cells from three or four glands (i.e., 3–4 × 10^7 cells) were mixed with 4 ml of agar solution to give a final cell density in the agar of about 10^7 cells/ml.

This technique had two advantages. It enabled rapid change of the solution bathing the cells, without centrifugation. This was confirmed by control experiments in which the rates of disappearance of ^{22}Na and ^{45}Ca were monitored using cell-free agar slices, previously "loaded" with isotope and subsequently incubated in tracer-free solution. Continuous superfusion of the slices, at a flow rate of 5 ml/min, using a peristaltic pump, showed that under these conditions both isotopes disappeared from the agar with a half-time of less than 30 sec.

The agar technique also permitted continuous oxygenation without the risk of damaging the cells, thus ensuring that all cells received a plentiful supply of O_2 despite their high density.

In intact adrenal glands. A small number of experiments were carried out using intact adrenal glands perfused retrogradely via the adrenal vein according to the method of Banks (17), as modified by Baker and Rink (18). The perfusion rate was always between 5 and 10 ml/min.

Assays

Catecholamine. Total catecholamine (adrenaline and noradrenaline) was measured against an adrenaline standard (adrenaline bitartrate in distilled water) by the trihydroxyindole technique (19) using a Perkin-Elmer fluorescence spectrophotometer (Model 204).

Dopamine β-hydroxylase. The assay was similar to that described by Aunis *et al.* (20), which utilizes the ability of the enzyme dopamine β-hydroxylase to catalyze the conversion of tyramine to octopamine. The final reaction product, PHB, has a sharp absorbance peak at 330–333 nm, in the presence of alkali, and its concentration was assayed using a Pye-Unicam UV spectrophotometer (Model SP6-500). The concentration of PHB in the final reaction mixture was calculated using a value for the extinction coefficient of this substance of 30,032 absorbance units/mole of PHB (determined using standards). The amount of dopamine β-hydroxylase was calculated using the units defined by Aunis *et al.* (20). One unit of dopamine β-hydroxylase is the amount of enzyme that produces 1 μmole of octopamine per minute at 37°, under the assay conditions used.

Lactate dehydrogenase. The assay was that described by Bergmeyer *et al.* (21), in which lactate dehydrogenase activity was measured spectrophotometrically, by the rate of consumption of pyruvate and NADH. The rate of decrease of optical density (at 340 nm and light path 1 cm, at 25°) was recorded over a 5-min period against a water blank, using an Aminco spectrophotometer (Model DW-2 UV/VIS) and recorder.

[1] The abbreviations used are: Quin 2, fluorescent quinoline analogue; PHB, *p*-hydroxybenzaldehyde; TTX, tetrodotoxin; EGTA, ethylene glycol bis(B-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; D-600, methoxyverapamil.

RESULTS

Effects of Ouabain on Catecholamine Secretion from Isolated Chromaffin Cells

The response of isolated adrenal medullary cells to ouabain closely resembles that of the intact perfused gland (7). Figure 1A illustrates the effect of exposing a suspension of cells to a high concentration (10^{-4} M) of glycoside. The rate of catecholamine release is approximately doubled in the presence of this level of drug (Table 1). Of particular interest is the finding that the increase in secretory rate is established within a very short time of resuspending the cells in solution containing glycoside, and is then sustained without further change for the entire experimental period—often for as long as 100 min. The secretory effect of ouabain was not readily reversible. Secretion remained high even 60 min after the removal of glycoside from the incubation medium.

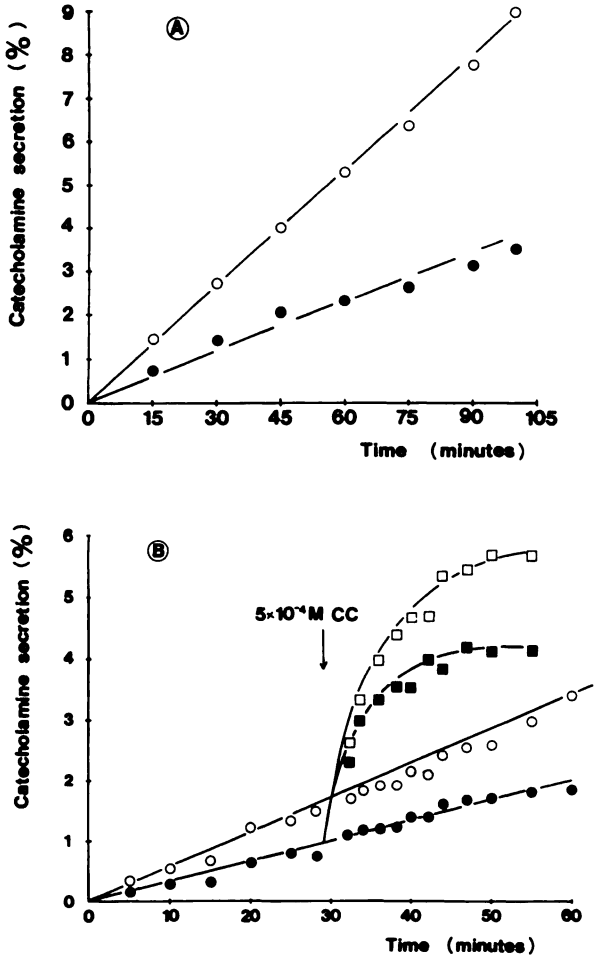

FIG. 1. *Effect of ouabain on catecholamine secretion from isolated bovine adrenal medullary cells*

In both cases the glycoside was added to the cell suspension at zero time.

A. Basal catecholamine secretion from chromaffin cells in the presence (○) and absence (●) of 10^{-4} M ouabain. Stimulation of secretion was established virtually immediately following the addition of glycoside.

B. Catecholamine secretion from chromaffin cells in response to a carbamylcholine challenge (*cc*) in the presence (○, □) and absence (●, ■) of 10^{-4} M ouabain. Ouabain does not inhibit the response of cells to a challenge. Indeed, in some experiments, the initial rate of secretion following the addition of the nicotinic agonist was slightly enhanced in cells pretreated with the cardiac glycoside.

TABLE 1

Stimulation by ouabain of basal catecholamine secretion from chromaffin cells

In each experiment, basal catecholamine secretion was measured for 60–100 min after the addition of ouabain to half of the cells, by assaying samples of supernatant at 5- or 10-min intervals. Secretion was stimulated immediately in each case (see Fig. 1A) and the rates of catecholamine secretion were calculated from the slopes of the lines obtained. Collective values are means ± standard error of the mean.

Catecholamine secretion		Stimulation of secretion
Control	10^{-4} M Ouabain	
%/hr	*%/hr*	*%*
2.7	4.5	67
1.9	4.9	158
1.6	3.5	119
1.5	5.4	260
3.6	7.5	108
1.9	4.0	111
1.0	2.0	100
1.8	4.4	144
3.5	6.6	89
3.2	7.4	131
4.1	8.0	95
1.3	3.0	131
2.34 ± 1.03	5.1 ± 1.92	126 ± 46.9

Figure 1B illustrates that the catecholamine release evoked by exposure of the cells to the nicotinic agonist carbamylcholine (5×10^{-4} M) persists in the presence of ouabain. Indeed, in some experiments, the initial rate of catecholamine release, resulting from the addition of carbamylcholine, was slightly elevated in cells which had previously been exposed to ouabain.

Does Ouabain Prevent the Reuptake of Catecholamine?

A possible explanation for the increased release of catecholamine in the presence of ouabain is that the glycoside may block the reuptake of catecholamine by the medullary cells. This seems unlikely for two reasons. First, 30 μM phenoxybenzamine—a potent and irreversible antagonist of catecholamine uptake (22)—had no effect on the rate of secretion from either control or ouabain-treated cells. In a single experiment, basal secretion was 5.2%/hr in control cells and 5.1%/hr in cells treated with phenoxybenzamine. In both sets of cells, secretion was stimulated by 65% when 10^{-4} M ouabain was added to the incubation medium.

Second, ouabain had no effect on the rate of [^{3}H] noradrenaline uptake by the cells. Figure 2 shows the results of a single experiment in which the accumulation of labeled (±)-noradrenaline by control and ouabain-treated cells was investigated. The total amount of catecholamine in the vicinity of the cells (i.e., that secreted by the cells in addition to that included as radioactive noradrenaline) was measured at each time point, using the fluorimetric assay described previously. This enabled corrections to be made for isotopic dilution in the extracellular fluid.

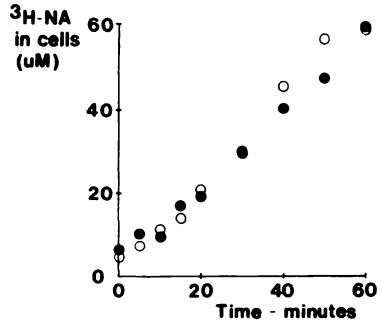

FIG. 2. *Uptake of [^{3}H]noradrenaline (^{3}H-NA) by isolated chromaffin cells in the presence (○) and absence (●) of 10^{-4} M ouabain*

Tracer noradrenaline was added to all the cells (to give a final concentration of 0.66 μM) at zero time, 10 min after the addition of ouabain to half of the cells. At the times indicated, samples of suspension were centrifuged through 300 μl of an oil mixture containing 10 parts di-*n*-butylphthalate to 1 part light liquid paraffin, in order to separate the cells from the extracellular fluid. Samples of the supernatant were assayed fluorimetrically for catecholamine content and the pellets were lysed in 500 μl of 0.1% Triton X-100 prior to liquid scintillation counting. The amount of [^{3}H]noradrenaline associated with the cells at zero time probably represents extracellular fluid centrifuged down with the cells. These results were obtained from a single experiment.

Does Ouabain Stimulate Exocytosis?

It is very important to establish whether the ouabain-induced release of catecholamine reflects an increased incidence of exocytosis or whether it is due to the loss of catecholamine by some other route. Judged by their ability to exclude the dye trypan blue (M_r 961), ouabain-treated cells show no sign of damage. However, the most convincing evidence that ouabain is stimulating exocytosis comes from measurements of the release of intragranular materials other than adrenaline and noradrenaline. Figure 3 shows that ouabain increases the release of the enzyme dopamine β-hydroxylase, which is present with catecholamine in the chromaffin granules (23), but does not alter the release of lactate dehydrogenase, an enzyme which is confined to the cytosol. These findings are strongly suggestive of exocytotic release and argue against the possibility of cell damage. This view is supported by a quantitative comparison of the ratio of dopamine β-hydroxylase to catecholamine released. The total dopamine β-hydroxylase to catecholamine ratio measured in aliquots of cell suspension was 0.2–0.6 milliunits/μmole, which compares favorably with other quoted values (15). Since about 50% of the total cellular content of dopamine β-hydroxylase is membrane-bound within the granules and, therefore, unavailable for secretion by exocytosis (24), one might expect 0.1–0.3 milliunit of dopamine β-hydroxylase to be released with each micromole of catecholamine, from both control and ouabain-treated cells. The measured value was 0.12–0.14 milliunit/μmole, which is sufficiently close to the theoretical value to suggest that the increased rate of catecholamine secretion observed with ouabain reflects true exocytosis.

Does Ouabain Increase the Frequency of Action Potentials?

Stimulus-secretion coupling in adrenal medullary cells may involve an increase in the frequency of TTX-sensitive action potentials (15, 25). It is possible that ouabain also acts in this way, but such a mechanism is rendered unlikely by the observation that treatment of isolated cells with a concentration (10 μM) of TTX high enough to block the sodium channels which can be opened by veratridine (15) had no effect on the catecholamine secretion induced by ouabain (Fig. 4).

Dose-Response Characteristics of Secretion in Response to Ouabain.

Figure 5A shows the results of a series of experiments in which cells were treated with different concentrations

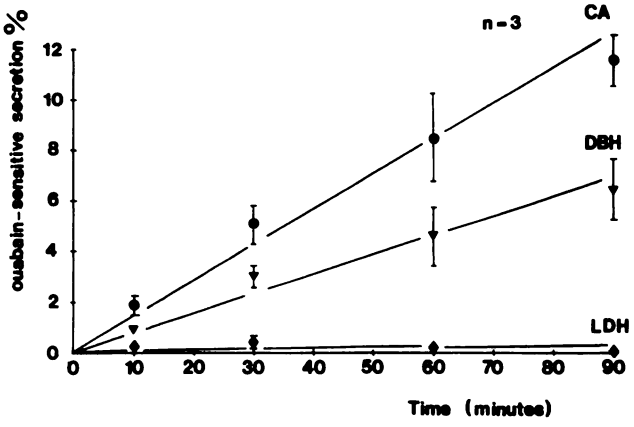

FIG. 3. *Evidence that the catecholamine secretion seen in response to 10^{-4} M ouabain represents exocytosis*

Ouabain-induced secretion of catecholamine (*CA*), dopamine β-hydroxylase (*DBH*), and lactate dehydrogenase (*LDH*) is illustrated. Although lactate dehydrogenase release appeared to be completely unaltered by the presence of ouabain, even after 90 min, release of dopamine β-hydroxylase was stimulated in parallel with that of catecholamine. The glycoside was added to the cells at zero time.

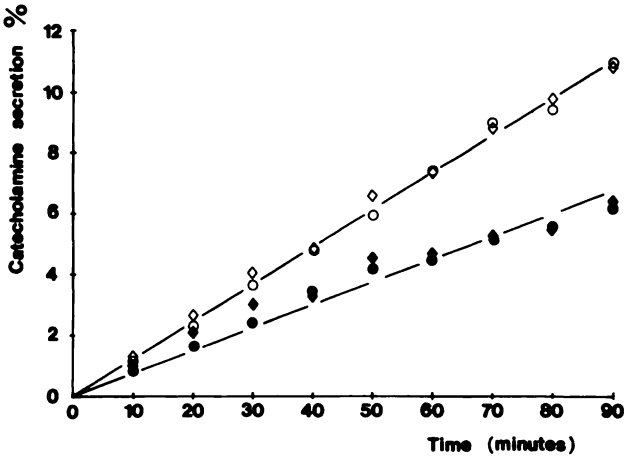

FIG. 4. *Insensitivity of the secretory effect of ouabain to TTX*

Closed symbols represent control cells and *open symbols* represent cells treated with 10^{-4} M ouabain. The presence of toxin had no apparent effect on basal secretion in either control or ouabain-treated cells, arguing against a role for increased action potential frequency in the secretory action of ouabain. Ouabain was added to the cells at zero time. Cells were pretreated with TTX for 10 min prior to the start of the experiment. ●, ○, No TTX; ♦, ◇, 10 μM TTX.

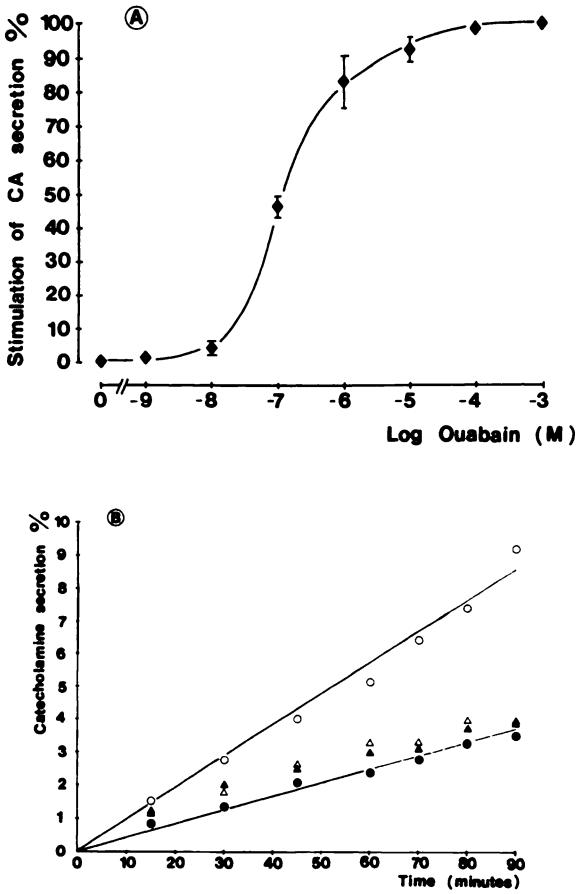

FIG. 5. *Ouabain-stimulated catecholamine secretion*

A. Dose-response curve for the stimulation of chromaffin cell catecholamine (*CA*) secretion (basal) by ouabain. The means and standard errors of the means of six separate experiments are shown; the results have been normalized so that for each of the six determinations the stimulation produced by 10^{-3} M ouabain is assigned a value of 100%. The degree of stimulation was calculated from the rates of catecholamine secretion measured over a period of 60 min.

B. Dependence on external sodium of the secretory effect of ouabain. Basal catecholamine secretion was monitored using cells which had been washed and resuspended in either sodium-Locke's solution or lithium-Locke's solution 10 min before the start of the experimental period. Ouabain was added at zero time. *Closed symbols* represent untreated cells; *open symbols* represent cells treated with 10^{-4} M ouabain. ●, ○, sodium-Locke's solution; ▲, △, lithium-Locke's solution. Ouabain was ineffective in the absence of extracellular sodium over the entire 90-min experimental period. The results of a single experiment are shown. Further details of the effects of sodium removal are given in Table 2.

of ouabain. The concentration of glycoside which produces half-maximal stimulation of spontaneous catecholamine release is approximately 5×10^{-7} M.

Ionic Requirements of the Secretory Effect of Ouabain

Sodium. Ouabain has been shown to require sodium extracellularly for both its positive inotropic action in heart (26) and its effects on secretion in several tissues (2, 4, 12). Figure 5B shows that, under conditions in which extracellular sodium is totally replaced by lithium, the stimulatory effect of ouabain on release of medullary cell catecholamine is lost. Other sodium substitutes such as choline, sucrose, and magnesium were found to be equally unsuccessful in supporting ouabain-stimulated catecholamine secretion. The secretory response to challenge with acetylcholine or carbamylcholine was also blocked in cells suspended in sodium-free media (data not shown).

Figure 6 presents the results of a series of experiments which investigate in more detail the role of extracellular sodium in the secretory action of ouabain. Figure 6A and Table 2 show the effects of partial replacement of extracellular sodium. The activation curve in Fig. 6A indicates that the mechanism is far from being saturated even at 150 mM external sodium, a concentration which is slightly above the physiological level. Table 2 presents data from the same three experiments in which basal catecholamine secretion was monitored at different levels of extracellular sodium in both control and ouabain-treated cells. As well as illustrating the sodium dependence of the secretory effect of ouabain, these results also demonstrate that there is no significant alteration in the rate of secretion following the removal of sodium ions. Previous reports of the effects of sodium withdrawal in medullary tissue have been conflicitng. Whereas Douglas and Rubin (13) and Lastowecka and Trifaro (27) have noted a sharp (although transient) increase in secretion from perfused glands, Rink (28), using adrenal medullary slices, reported little effect of sodium deprivation on spontaneous catecholamine secretion. The present findings in cells resemble most closely those reported for slices.

Figure 6B shows that, once the secretory effect of ouabain has been established in a medium containing sodium, subsequent replacement of this ion with choline does not reduce the response appreciably. Moreover, Fig. 6C shows that the readdition of sodium to cells preexposed to ouabain in a sodium-free medium still produces a large increase in basal catecholamine secretion, even when ouabain is no longer included in the solution. Reintroduction of sodium to cells not previously treated with glycoside produces no change in basal catecholamine secretion.

Calcium. It has been reported that the stimulatory effect of ouabain on catecholamine secretion from perfused glands requires extracellular calcium (7). Figure 7A confirms this finding. Interestingly, however, isolated cells do not appear to possess this absolute requirement for calcium. Figure 7B shows that varying the level of extracellular calcium between nominally zero and 3.6 mM has no apparent effect on either basal catecholamine secretion or the stimulatory effect of ouabain. The addition of 1 mM EGTA to the solution bathing the cells, however, does appear to reduce (but not abolish) catecholamine secretion occurring in response to glycoside treatment. Table 3 presents the data obtained from several experiments of this kind.

Figure 7A also illustrates another interesting feature of the effects of ouabain on the secretion from perfused glands. Acetylcholine challenges were given at intervals in order to demonstrate the viability of the organ. Secretion was enhanced approximately 5- to 6-fold within 2 min of exposure to secretogogue. However, this response was transient, and decayed with a half-time of 2–3 min. This finding is in agreement with other work performed using this preparation (18). The responses to successive

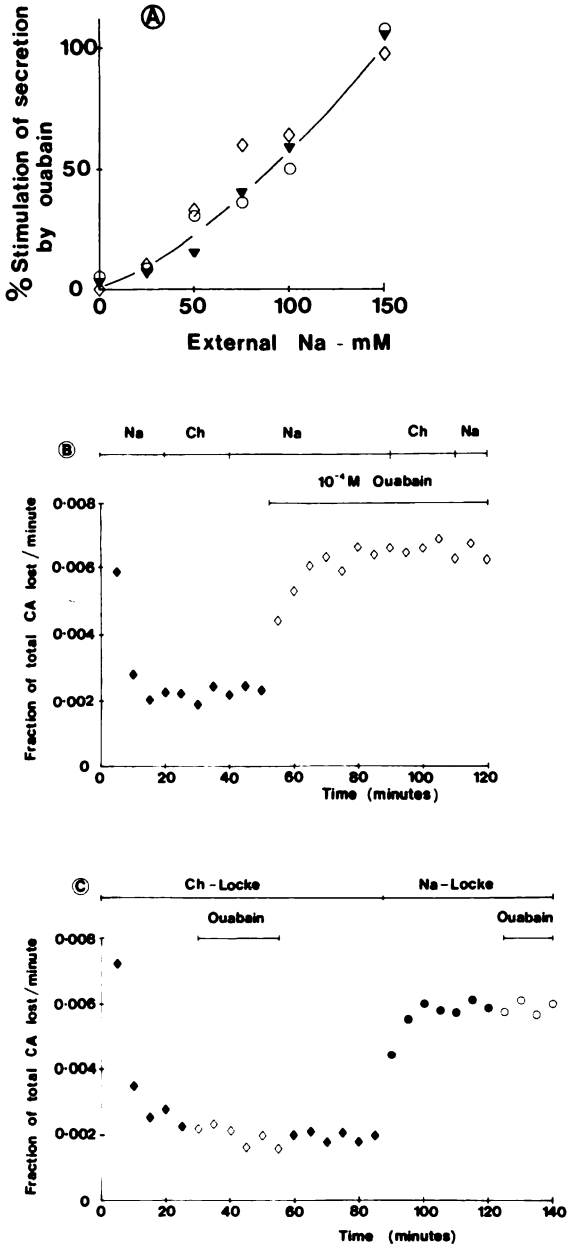

FIG. 6. *Requirement of the secretory effect of ouabain for extracellular sodium*

A. Activation of the secretory effect of ouabain by extracellular sodium. The results of three separate experiments are shown. Basal catecholamine secretion was measured over the period of 1 hr in both control and ouabain (10^{-4} M)-treated cells suspended in various sodium concentrations. Percentage stimulation, as calculated from the rates of secretion, is plotted against the extracellular sodium concentration.

B. Effect of replacing external sodium with choline after the onset of the secretory effect of ouabain. Cells embedded in agar at a density of approximately 10^7 cells/ml were used to monitor secretion. Solution was flowed past the slices, by means of a peristaltic pump, at a rate of 5 ml/min and its composition was altered as indicated. (It is unlikely that the effect is due to the presence of residual quantities of ouabain in the perfusate since experiments with [^{3}H]ouabain have shown that all measurable glycoside has disappeared within 10 min at this perfusion rate.)

C. Effect of adding back sodium to cells pre-exposed to ouabain (10^{-4} M) in the absence of sodium ions. Cells embedded in agar slices at a density of approximately 10^7 cells/ml and superfused with solution at a rate of 5 ml/min were used to monitor secretion. *CA*, Catecholamine.

TABLE 2

Sodium dependence of the secretory effect of ouabain

The results of three separate experiments are shown so that the effects of external sodium on both basal and ouabain-stimulated catecholamine secretion may be compared. In each case, cells were washed and resuspended in the appropriate concentration of sodium 5 min before the addition of glycoside.

Extracellular sodium	Secretion		Stimulation
	Control	10^{-4} M Ouabain	
mM	*%/hr*	*%/hr*	*%*
0	0.96	1.01	5.5
	1.8	1.78	0
	3.2	3.31	3.4
25	1.27	1.39	9.4
	1.8	1.98	10.0
	3.77	4.1	8.7
50	1.63	2.12	30
	1.43	1.9	33
	3.01	3.48	15
75	1.47	2.01	37
	1.92	3.07	60
	4.4	6.18	40
100	1.05	1.58	50
	1.71	2.81	64
	3.01	4.8	59
150	1.91	3.77	97
	1.91	3.97	108
	3.52	7.23	105

challenges always diminish with time, as the gland ages; this can be seen by comparing the first two responses in Fig. 7A. The final challenge was given during exposure of the tissue to ouabain and was, if anything, slightly greater than the previous response. It was certainly higher than might be expected if ouabain had not been administered. Of course, it would be inappropriate to conclude from this that ouabain enhances the response to a challenge, since it is not possible to show a control response in the same gland, but this result (obtained reproducibly in several other experiments) supports the observation mentioned earlier that ouabain often appears to improve the secretory response of isolated cells to secretogogues.

Potassium. Figure 8 illustrates the effects on catecholamine secretion of varying the external potassium concentration in the incubation media of both control and ouabain-treated cells. Figure 8A shows that the complete removal of extracellular potassium results in a stimulation of secretion which has a time course similar to that provoked by ouabain and which is not further enhanced by the addition of glycoside. Figure 8B shows that when external potassium is raised the dose-response curve for the stimulation of secretion by ouabain is shifted to the right, suggesting that potassium and ouabain compete for the same membrane sites—presumably the sodium pump. Although this finding, coupled with the apparently identical effects of potassium removal and ouabain, may point to a role for sodium pump inhibition in the secretory responses to these agents, the additional finding that the secretory effects of low potassium concentrations (but not those of ouabain) require external calcium sug-

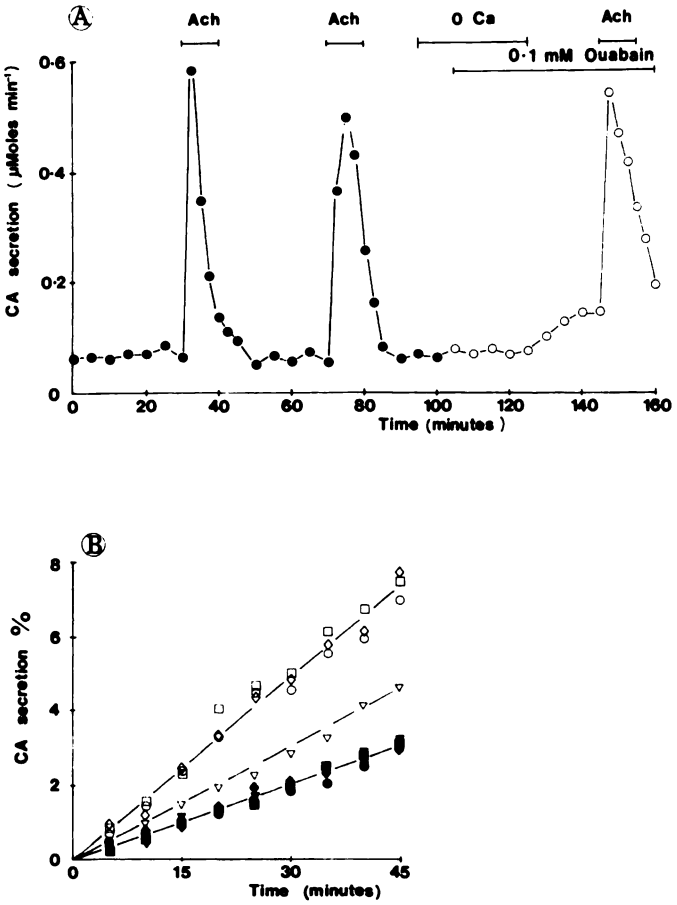

FIG. 7. *Calcium dependence of the secretory effect of ouabain*

A. Effect of 10^{-4} M ouabain (*open symbols*) on catecholamine (*CA*) secretion from a perfused adrenal gland in the presence and absence of extracellular calcium (1.8 mM). Catecholamine secretion was enhanced only when calcium ions were returned to the perfusion fluid. Actylcholine (*Ach*) challenges were given at intervals to check the viability of the gland. The perfusion rate was 6–7 ml/min. Results are from a single experiment.

B. Effect of ouabain on basal chromaffin cell catecholamine (*CA*) secretion in a variety of media. *Closed symbols* represent untreated cells; *open symbols* represent cells treated with 10^{-4} M ouabain from zero time. In each case cells were resuspended in solution containing the appropriate calcium concentrations 10 min before the start of the experiment. ●, ○, 0 mM calcium; ♦, ◇, 1.8 mM calcium; ■, □, 3.6 mM calcium; ▼, ▽, 0 mM calcium + 1 mM EGTA.

gests that the two agents may not act in an identical fashion.

Is the Nature of the External Anion Important?

Although the basal secretion of catecholamines from adrenal medullary cells is largely independent of external anions, it has been shown (15, 29) using cells rendered "leaky" by exposure to high-voltage discharges that, when allowed access to the interior of the cell, certain anions are capable of altering exocytotic properties. Increases in the internal concentrations of Cl^-, I^-, and SCN^- ions appear to increase the rate of calcium-independent secretion, whereas glutamate and acetate have no effect. If ouabain exerts its secretory effect by raising intracellular Cl^-, perhaps as a result of sodium pump inhibition, the glycoside effect should be reduced if external chloride is replaced by glutamate or acetate ions. Table 4 summarizes the results of an experiment in which the importance of the external anion was investigated. None of the ions examined significantly altered the secretory response to ouabain.

TABLE 3

Effect of variations in extracellular calcium on the stimulation of catecholamine secretion by 10^{-4} M ouabain in isolated medullary cells

The errors appear large since the cells used in each separate experiment exhibited rather different basal rates of secretion (see also Table 1), but if individual experiments are considered, the differences between the secretion rates of control and ouabain-treated cells are statistically significant ($p < 0.01\%$, Student's *t*-test). Values are means ± standard error of the mean.

Calcium	Catecholamine secretion		Stimulation by ouabain
	Control cells	Ouabain-treated cells	
mM	*%/hr*	*%/hr*	*%*
0 (+ 1 mM EGTA) ($n = 4$)	2.5 ± 0.53	3.9 ± 0.84	54 ± 11.2
0 (nominally) ($n = 4$)	2.6 ± 0.81	5.4 ± 0.92	110 ± 32.1
1.8 ($n = 12$)	2.34 ± 1.03	5.1 ± 1.92	126 ± 46.9
3.6 ($n = 4$)	2.71 ± 0.94	5.5 ± 1.01	115 ± 24.2

Effects of Some Other Agents on Catecholamine Secretion from Isolated Medullary Cells

Anoxia. If sodium pump inhibition per se or the subsequent rise in intracellular sodium enhances basal catecholamine secretion when cells are exposed to ouabain, then one might expect to see similar enhancement under other conditions in which the pump is blocked. Anoxic cells are likely to lose their ability to maintain the activity of their sodium pumps [indeed, this is shown conclusively in figure 5 of the following paper (16)], and for this reason the effects on catecholamine secretion of oxygen and glucose deprivation were tested. Cells were washed and resuspended in glucose-free solution saturated with 100% oxygen-free nitrogen, and, to reduce further entry of oxygen to the medium during the incubation period, the surface of the cell suspension was covered with a layer (2–3 cm deep) of light liquid paraffin. Figure 9A shows that even after a prolonged period of anoxia (80 min) the basal rate of catecholamine secretion remained at control levels, whereas ouabain (10^{-4} M) stimulated basal secretion by similar amounts in both control and anoxic cells. Furthermore, anoxic cells gave an apparently normal secretory response to a carbamylcholine challenge given after 60 min of metabolic deprivation in both the presence and absence of ouabain (Fig. 9B).

Other metabolic poisons. In view of the discovery that the secretory behavior of anoxic cells is apparently normal, the effects of a variety of other well-known metabolic inhibitors were examined. Figure 9B illustrates the effects of these agents on both basal secretion and secretion evoked by the presence of carbamylcholine. The secretory properties of metabolically deprived cells are compared with those of both control (untreated) cells and cells exposed to ouabain. Interestingly, of the poisons tried, only the combination of 2-deoxyglucose and cyanide produced any real alteration in the secretory activity of chromaffin cells. Basal release was not significantly

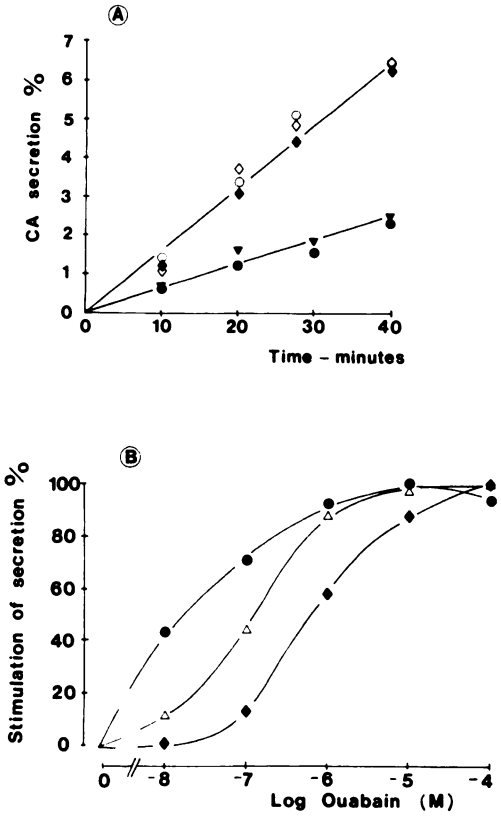

FIG. 8. *Effects of extracellular potassium on the secretory effect of ouabain*

A. Basal catecholamine (*CA*) secretion from isolated cells under a variety of conditions of external potassium and calcium. Cells were resuspended in the appropriate ionic environment 5 min before the start of the experiment. Ouabain was added, where necessary, at zero time. *Open symbols* represent cells treated with 10^{-4} M ouabain and *closed symbols* represent untreated cells. ●, ○, 2.7 mM potassium, 1.8 mM calcium; ♦, ◇, 0 mM potassium, 1.8 mM calcium; ▼, 0 mM potassium, 0 mM calcium. Although the removal of extracellular potassium stimulated basal secretion in a manner similar to ouabain, this stimulation required extracellular calcium. These results represent the means from two experiments.

B. Effect of varying external potassium on the dose-response curves for the stimulation of basal catecholamine secretion by ouabain. The results have been normalized so that the maximal degree of stimulation is assigned a value of 100% for each potassium concentration. The degree of stimulation of secretion in each case has been calculated from the rate of secretion measured over a period of 40 min. The graph suggests some degree of competition between potassium and ouabain, since increasing the level of external potassium shifts the dose-response curve for stimulation of secretion by ouabain to the right. The curves were drawn by eye. ●, 1 mM potassium; △, 2.7 mM potassium; ♦, 10 mM potassium. One hundred per cent stimulation of secretion was obtained with 10^{-4} M ouabain at all potassium values.

changed but, under these conditions, the secretory response to a carbamylcholine challenge was almost completely blocked. These findings are discussed more fully in the next paper (16) in conjunction with the effects of these inhibitors on sodium pump activity.

D-600. D-600 is known to block voltage-sensitive calcium channels. Figure 10A shows that, at doses within the range over which it is also found to inhibit the secretory response to acetylcholine (10^{-6}–10^{-4} M), D-600

TABLE 4

Lack of importance of the major external anion in the stimulation of basal catecholamine secretion by ouabain

Cells were washed and resuspended in the appropriate anion 10 min before the addition of ouabain at zero time. Basal catecholamine release was monitored for the next 60 min. These results were obtained from two experiments.

Anion	Basal secretion		Stimulation of secretion
	Control cells	10^{-4} M Ouabain	
	%/hr	%/hr	%
Glutamate	3.1	5.76	86
Acetate	3.1	5.86	89
Chloride	2.8	5.1	82
Iodide	3.4	5.68	67
Thiocyanate	3.2	5.15	61

is able to inhibit partially the action of ouabain. This suggests that calcium entry through these channels may play a role in the secretory action of the glycoside, although this is hard to reconcile with the apparent calcium independence of the effect in isolated cells.

Quin 2. Chromaffin cells were loaded with the acetoxymethyl ester of Quin 2 (30). The ester enters cells very readily but, once inside, is hydrolyzed to form free Quin 2, which may be used as a calcium-specific indicator (31, 32) and is known to chelate calcium with a high selectivity over Mg^{2+} and protons. By using different concentrations of ester in the external medium to achieve varying levels of Quin 2 inside the cells, it was shown that the secretory response to an acetylcholine challenge was reduced in a dose-dependent manner (although the absolute intracellular concentration of chelator was not known) (Fig. 10B). This indicates that the ester does indeed enter chromaffin cells and is hydrolyzed successfully. Free Quin 2 is capable of blunting calcium-dependent responses, presumably because of its calcium-chelating properties. If ouabain produces its effect by raising free intracellular calcium in some way, one might expect Quin 2 to prevent the increase in catecholamine secretion normally associated with glycoside treatment. Figure 10C shows that this is indeed the case.

DISCUSSION

This study describes in some depth the characteristics of the secretory effect of ouabain on adrenal medullary cells. Isolated cells, like intact adrenal glands, secrete when they are exposed to this cardiac glycoside. The biochemical evidence that this secretion reflects exocytosis, coupled with a failure to demonstrate ouabain-sensitive reuptake of catecholamine, argues in favor of a real effect of the glycoside on the exocytotic secretory mechanism of the chromaffin cell.

Ouabain stimulates secretion from isolated cells even in the nominal absence of calcium ions, a finding which deserves some comment since it is in sharp contrast with both past (7) and present studies carried out using perfused adrenal glands. In intact preparations, extracellular calcium seems to be an absolute requirement for the stimulatory effect of ouabain (Fig. 7A). One possible explanation for this discrepancy may concern differences

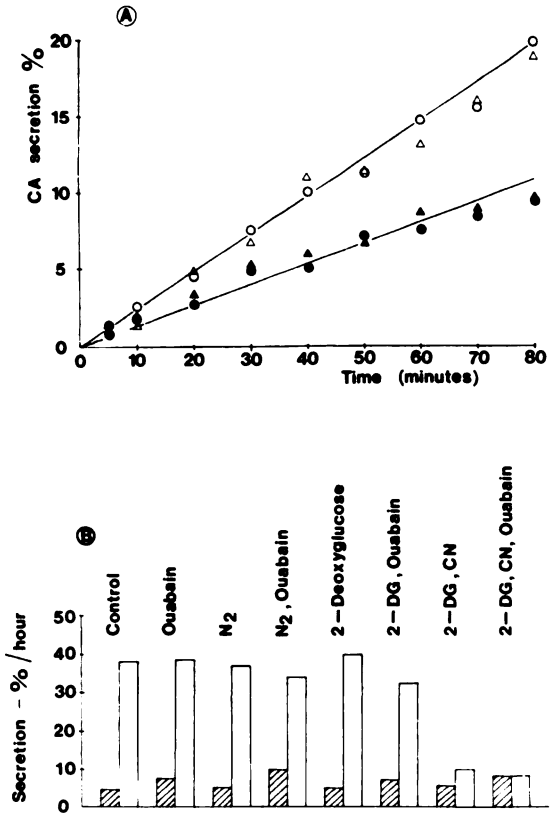

FIG. 9. *Effects of metabolic deprivation on basal and evoked catecholamine (CA) secretion from isolated medullary cells*

A. Secretory response of both healthy (●, ○) and anoxic (▲, △) cells to ouabain (10^{-4} M). In this experiment, the anoxic cells had been incubated in a glucose-free solution saturated with 100% oxygen-free nitrogen for 15 min before the start of the experiment, and were kept in this solution for the subsequent 80-minute experimental period. Where appropriate, ouabain was added at zero time. The anoxic cells exhibited an apparently normal basal rate of secretion, and responded to ouabain in the usual way. *Closed symbols* represent glycoside-free cells; *open symbols* represent cells exposed to 10^{-4} M ouabain. These results are from a single experiment.

B. Effects of a variety of metabolic inhibitors on both basal secretion (*hatched bars*) and secretion evoked by the addition of 5×10^{-4} M carbamylcholine 60 minutes after resuspension of the cells in the appropriate medium (*open bars*). The rates of secretion evoked by carbamylcholine were calculated from the rates observed over the first 3 min of the transient responses. In each case, cells were washed and resuspended in the appropriate solution 15 min prior to the start of the experiment, except that ouabain (10^{-4} M) was always added at zero time (i.e., the start of the experimental period). Cells labeled *Control* were suspended in solution containing 5 mM glucose and saturated with 100% oxygen. The conditions labeled *N_2 Ouabain* refer to cells suspended in glucose-free medium saturated with 100% O_2-free N_2. The concentration of 2-deoxyglucose (*2-DG*) used was 3 mM, and the concentration of sodium cyanide (*CN*) used was 2 mM. These results represent the means from three experiments.

in the timing of experiments. Whereas in intact glands rather prolonged perfusion with calcium-free solution prior to the addition of ouabain is necessary to ensure complete washout of calcium, isolated cells can be washed thoroughly within a few minutes. Prolonged exposure to a low-calcium environment is likely to bring

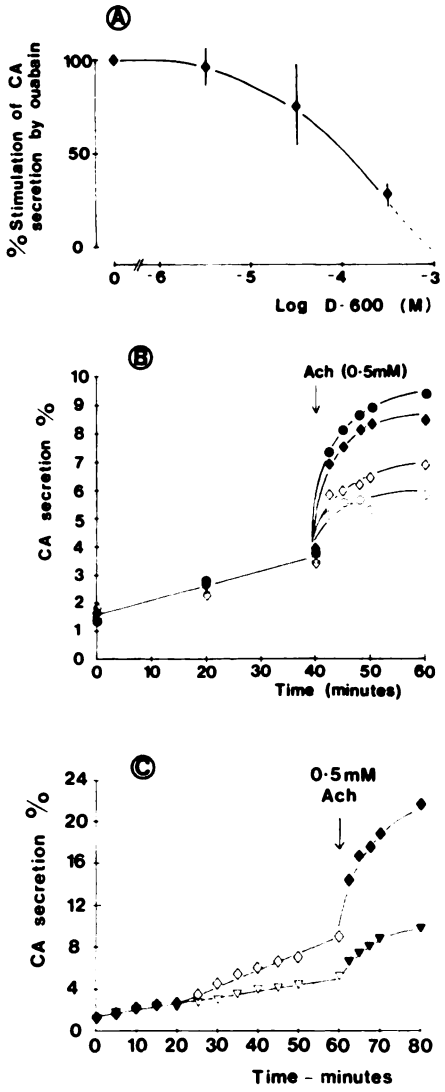

FIG. 10. *Effects of various agents and conditions on chromaffin cell secretion*

A. Inhibition of ouabain-induced catecholamine (*CA*) secretion by D-600. Half-maximal inhibition of the catecholamine secretion produced by 10^{-4} M ouabain is brought about by a concentration of D-600 of approximately 10^{-4} M. These results are representative of three experiments.

B. Inhibition, by the presence inside the cells of the calcium chelator Quin 2, of the secretory response of chromaffin cells to acetylcholine (*Ach*). Cells were previously incubated for 45 min with 0 (●), 1 (♦), 10 (◇), or 100 (○) μM Quin 2 (ester). Basal secretion was monitored for 40 min prior to the addition of 0.5 mM acetylcholine to all cells. There appeared to be an inhibition of the secretory response which was related to the level of ester to which the cells had been exposed. This inhibition presumably reflects the intracellular concentration of Quin 2.

C. Inhibition of the secretory response to ouabain by pretreatment with Quin 2. Catecholamine (*CA*) secretion from untreated control cells (♦, ◇) is compared with that from cells previously incubated with 70 μM Quin 2 (ester) (▼, ▽). Ouabain (10^{-4} M) was added to both sets of cells after 25 min as indicated by the *open symbols*. Secretion was stimulated in the control cells but not in those exposed to the chelating agent. At 60 min, both sets of cells were challenged with 0.5 mM acetylcholine (*Ach*). The resultant secretory response was markedly reduced in the Quin 2-treated cells. These results are from a single experiment.

about cellular calcium depletion, thereby rendering the cells less able to show normal responses involving subtle changes in calcium balance. Indeed, the discovery that the presence of EGTA (which might be expected to hasten the process of calcium depletion) reduces the secretory response to ouabain even in isolated cells supports this idea.

Furthermore, although ouabain does appear to require calcium for its secretory effects in some tissues [for example, pancreatic insulin release (4)], the chromaffin cell is by no means unique in displaying calcium independence. Acetylcholine release at both the frog neuromuscular junction (3) and rat diaphragm (33) in response to ouabain continues in the nominal absence of this ion.

The secretory effect of ouabain has a specific requirement for extracellular sodium; no other ion studied here can replace it. However, the characteristics of this requirement are somewhat complex. Once secretion in response to ouabain has been initiated, the continued presence of sodium is not necessary to sustain it, at least for short periods. Furthermore, the readdition of sodium to cells previously exposed to ouabain in a sodium-free medium and subsequently washed free of glycoside results in enhancement of catecholamine secretion (Fig. 6). This clearly indicates that ouabain is able to bind to chromaffin cell membranes in the absence of sodium ions [this is confirmed by measurements of sodium pump activity described in the following paper (16)] even though sodium is required for the manifestation of the secretory effect. This finding seems to argue rather strongly against a direct link between sodium pump inhibition per se and enhanced catecholamine secretion. The behavior of anoxic cells lends further support to this view, although none of these experiments can give much insight into the part played by sodium ions in this phenomenon.

We can, however, draw some conclusions from these studies of catecholamine secretion alone. Despite the sodium requirement of the secretory effect of cardiac glycosides in chromaffin cells, the increase in exocytotic activity is not likely to result from increased action potential frequency since it is insensitive to tetrodotoxin. Similarly, external anions appear to be without a major mediatory role.

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