MOLECULAR PHARMACOLOGY, 23:681–697

Ion Movements in Isolated Bovine Adrenal Medullary Cells Treated with Ouabain

GILLIAN POCOCK

Department of Physiology, King's College, London, WC2R 2LS, United Kingdom

Received August 24, 1982; Accepted January 3, 1983

SUMMARY

In the previous paper [*Mol. Pharmacol.* **23**:671–680 (1983)] it was shown that ouabain enhances the exocytotic release of catecholamines from isolated bovine adrenal medullary cells. This effect is dependent upon extracellular sodium, but persists in the nominal absence of calcium. In this paper the study has been extended to include an investigation of the effects of ouabain on the fluxes of ^{86}Rb, ^{42}K, ^{24}Na, and ^{45}Ca in these cells. The basic features of the chromaffin cell sodium pump are characterized, and it is shown for the first time that both the pump itself (i.e., the kinetics and properties of transport) and its inhibition by ouabain resemble those of squid axons and erythrocytes. However, serious doubts are cast upon the often-stated possibility that there is a direct link between sodium pump inhibition and exocytotic secretion because parallel measurements of both phenomena have, for example, shown that while the secretory effect of ouabain is sodium-dependent, pump inhibition is not. Instead, an entirely different explanation is suggested by the discovery that ouabain produces a marked decrease in the rate of active calcium extrusion from chromaffin cells, under all conditions in which catecholamine secretion is enhanced. This inhibition seems not to be accompanied by any change in calcium influx, and may therefore provide a direct explanation for the rise in free calcium which is required to stimulate exocytosis in this tissue.

INTRODUCTION

Cardiac glycosides stimulate the exocytotic release of catecholamines from adrenal medullary cells (1), and similar effects have been reported in many other tissues (2–8). The possibility that there is some kind of link, direct or indirect, between these effects and the more familiar ability of these agents to inhibit sodium-potassium exchange in a wide variety of tissues has often been discussed. One of the most popular hypotheses of this kind suggests that the increase in intracellular sodium concentration resulting from sodium pump inhibition may lead to an increased influx of calcium, and reduced efflux of calcium, via a sodium-calcium countertransport system similar to that known to exist in the squid giant axon (9) and heart (10). Such changes could act to bring about an increase in the level of free calcium inside the cells, thereby enhancing the rate of exocytotic release of secretory product. Although there is good evidence for a sodium-calcium exchange system in some tissues, its existence in adrenal medullary tissue is less clear-cut (11). Very few studies of ion fluxes have been made on adrenal medullary tissue, largely because of the severe difficulties of measuring and interpreting ion movements in either whole glands or tissue slices.

This research was supported by the Medical Research Council, the Wellcome Trust, and the British Heart Foundation.

The present study makes use of the isolated cell preparation, described in the previous paper (1), to correlate the changes in spontaneous catecholamine secretion brought about by exposure to ouabain with the actions of this drug on the transmembrane movements of sodium, potassium, rubidium, and calcium ions. The most striking conclusion from the work is that, although increases in catecholamine secretion undoubtedly occur under conditions in which the sodium pump is inhibited, they seem to be related rather more directly to inhibition of the calcium pump.

Preliminary reports of some of this work have appeared previously (12, 13).

EXPERIMENTAL PROCEDURES

Materials

Bovine adrenal glands were obtained from a local abattoir and handled as described in the previous paper (1).

Solutions

The primary solution was a 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-buffered sodium-Locke's solution of composition identical with that described in the previous paper (1). Unless otherwise stated, all solutions contained 5 mM glucose and were saturated with 100% oxygen. All experiments were carried out at 37° unless otherwise stated in the text.

A 23187 was supplied by Calbiochem-Behring Corporation (San

0026-895X/83/030681-17$02.00/0

Diego, Calif.). Quin 2[1] was a gift from Dr. Gerry Smith (Cambridge). All other chemicals were supplied by Sigma Chemical Company Ltd. (London) or British Drug Houses Ltd. (London) and were of analytical grade.

All radioisotopes were supplied by Amersham International.

Isolation of Adrenal Medullary Cells

The method of Baker and Knight (14) was used. This was described in more detail in the previous paper (1).

Measurement of Ion Flux

Influx measurements. The same basic procedure was used to measure the influx rates of ^{24}Na, ^{86}Rb, ^{42}K, and ^{45}Ca. The main problem encountered during influx measurements is to ensure complete removal of extracellular isotope from the cells before counting the radioactivity associated with the pellet. In this series of experiments, the problem was overcome by filtering 0.5- to 1.0-ml aliquots of cell suspension, containing routinely 10^6 cells/ml, through Millipore filters having a pore diameter of 5 μm, and subsequently washing the cells with large volumes (20–30 ml) of ice-cold, tracer-free solution as they lay trapped on the filter. A Millipore manifold with 12 filtering positions was used, and, in the case of the γ-emitting isotopes ^{24}Na, ^{86}Rb, and ^{42}K, the radioactivity remaining on the filter (i.e., that associated with the cells) was counted using a Nuclear Enterprises Model 8311 counter. When ^{45}Ca influx was being measured, the washed cells were solubilized in 0.1% Triton X-100 before liquid scintillation counting in a Packard Tri-Carb spectrometer (Model 3330). In all cases, the amount of radioactivity present in the extracellular fluid was measured by centrifuging a separate aliquot of cells and counting samples of the supernatant. Influx rates were calculated on the assumption that chromaffin cells are spheres having a mean diameter of 18 μm (value obtained using a Coulter counter) and that they are 100% water. Using ^{24}Na as an example, therefore,

$$\text{Concentration of isotope in cells (mM)} = \frac{(\text{counts in pellet}) \times [\text{amount of Na in } y \text{ liters of supernatant (mM)}]}{[\text{counts in } y \text{ liters of supernatant}] \times [\text{pellet volume (liters)}]}$$

This method of measuring influxes could be criticized on the grounds that cells are damaged when subjected to the filtration procedure. For this reason, a small number of experiments were repeated using two other methods of washing the cells. The first of these involved simply centrifuging the cells at low speed and resuspending them several times in 10-ml aliquots of tracer-free Locke's solution; the second involved centrifuging 200-μl aliquots of suspension through 300 μl of an oil mixture consisting of 10 parts di-*n*-butylphthalate to 1 part light liquid paraffin. While the cells will centrifuge down through this mixture, the supernatant remains above the oil and can be aspirated off. Both of the alternative methods gave results which were essentially similar to those obtained using the quicker, less laborious filtration technique.

In each case, the pellet volume was assessed by counting the cells (hemocytometry) and using a value of 3.05×10^{-12} liters for the volume of one cell (assuming a sphere of diameter 18 μm). Rates of influx could then be calculated from the amount of radioactivity in the cells at successive times.

A note on the use of ^{86}Rb. Owing to the extremely short half-life of ^{42}K, ^{86}Rb was used as a tracer for potassium in most of the experiments described. The results shown in Fig. 1A, in which uptake of both ^{86}Rb and ^{42}K are compared and shown to be equal, support the validity of the assumption that ^{86}Rb is an adequate substitute for potassium. Wherever ^{86}Rb was used in this way, the isotope was simply added to cells suspended in a solution containing normal levels of potassium but no "cold" rubidium, and uptake was then measured assuming that ^{86}Rb is treated in a way identical with that for potassium in terms of membrane transport by the cells. The amount of rubidium added to the solution in the form of ^{86}Rb was ignored since it was always much less than 1 μM.

Efflux measurements. 1. Using isolated cells in suspension. A simple centrifugation technique was used to measure efflux rates of ^{24}Na, ^{86}Rb, and ^{45}Ca from medullary cells in suspension. After an initial loading period (45 min for ^{86}Rb and 75 min for ^{24}Na and ^{45}Ca) during which cells, at a density of 3 to 7×10^6/ml, were incubated at 37° in solution containing the appropriate isotope (0.5–1.0 μCi/ml), they were washed free of extracellular isotope. This was accomplished by repeatedly centrifuging (3 min at $100 \times g$) and resuspending the cells in fresh aliquots of tracer-free solution. Routinely the cells were washed and resuspended three times at a cell density of 2 to 6×10^5 cells/ml, so that the entire washing period was complete within 12–15 min. The amount of intracellular isotope lost during this procedure was not measured. After the final centrifugation the cells were resuspended in the appropriate experimental solution to give a final density of approximately 10^6 cells/ml. At various times following resuspension, 300-μl aliquots of cells were centrifuged (at $8000 \times g$ for 20 sec) and the levels of radioactivity were determined in 200 μl samples of supernatant. The activity of the total suspension was also determined and, assuming that this represents the amount of isotope present inside the cells at zero time, rate constants of efflux were calculated from the fraction of tracer remaining in the cells at successive times. True values for efflux could be obtained only by assuming values for the intracellular ion concentrations of the chromaffin cells, and this was done wherever indicated in the text.

2. Using cells embedded in agar. The technique of embedding cells in agar slices was described in some detail in the previous paper (1), and this was used very occasionally to monitor the efflux of ^{24}Na and ^{45}Ca from cells whenever it was desirable to make solution changes without the need for centrifugation. Cells were loaded with isotope and washed thoroughly before being mixed with the agar to give a final cell density of approximately 10^7/ml. Slices of agar (1 mm thick) containing cells were then prepared and incubated in tracer-free solution, and the amount of radioactivity leaving the cells was monitored in the following way. Solution was flowed past the slices at a rate of 4–6 ml/min by means of a peristaltic pump and samples of the superfusate were collected and counted. At the end of the experiment, the total amount of radioactivity remaining in the cells was measured and efflux was then expressed as the fraction lost per unit time.

Solution changes could be achieved rapidly and completely with this method, and the slices could be bubbled continually with gases without damaging the cells.

Measurement of Secretory Activity

Catecholamine secretion from cells in suspension was measured as described in the previous paper (1).

Measurement of Intracellular Ion Levels

The major problem encountered in measurements of this kind is contamination of the cells under investigation by extracellular fluid. Therefore, it is vital to have an accurate measurement of both the pellet volume and the extracellular space volume. In these experiments ^3H_2O was used as a total space marker and [^{14}C]mannitol was used as an extracellular space marker. When sodium or chloride ions were the ions under investigation the extra precaution of washing the cells in either choline-Locke's solution or sodium glutamate (as appropriate) was taken. After washing, ^3H_2O (2 μCi/ml) and [^{14}C]mannitol (0.6 μCi/ml) were added to the cell suspension. After 2 min the cells were centrifuged ($1000 \times g$ for 20 sec), and samples of supernatant were removed for liquid scintillation counting and determination of ion concentrations. The pellets were then extracted using either 0.1% Triton X-100 or 5% (ω/v) trichloroacetic acid; samples of the extract were analyzed for radioactivity and ion content. A Pye-Unicam SP6 atomic absorption spectrophotometer was used for determination of

[1] The abbreviations used are: Quin 2, Fluorescent quinoline analogue; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; D-600, methoxyverapamil.

cellular levels of sodium, potassium, calcium, and magnesium. A Corning-EEL 920 chloride meter was used for chloride determinations.

Measurement of Membrane Potential Using a Fluorescent Dye

The fluorescent dye 3,3′-diethylthiadicarbocyanine iodide was used to measure the change in potential brought about by exposing the cells to ouabain (see 15). Although it is not possible to obtain accurate quantitative measurements of potential by this technique, the changes in potential produced by alterations in the extracellular potassium concentration could easily be measured and compared with those produced by the glycoside. A dye concentration of 0.3 μM was found to give optimal responses with a cell density of 5×10^6 cells/ml, and fluorescence was read using an excitation wavelength of 620 nm and an analyzing wavelength of 660 nm (Perkin-Elmer fluorescence spectrophotometer Model 204).

Catecholamine Assay

Total catecholamine was measured against an adrenaline standard (adrenaline bitartrate in water) by the trihydroxyindole technique of Von Euler and Floding (16).

RESULTS

Sodium and Potassium Transport in Adrenal Medullary Cells

^{86}Rb influx and ^{24}Na efflux. Figure 1A shows the effect of a high concentration of ouabain (10^{-4} M) on the uptake of ^{86}Rb and ^{42}K by isolated adrenal medullary cells. Both of these isotopes appear to be treated identically by the cells, there being in each case two components of uptake—an ouabain-sensitive component and a linear, glycoside-insensitive component. The similarity between the rates of uptake of ^{86}Rb and ^{42}K justifies the use of ^{86}Rb as a tracer for potassium, a point which was discussed earlier.

Table 1A presents data compiled from several experiments in which the effects of ouabain on ^{86}Rb influx were investigated. Uptake is expressed here as a transmembrane flux, calculated from the initial rates of entry of ^{86}Rb into the cells, assuming that chromaffin cells are spheres of diameter 18 μm.

Figure 1B and Table 1B illustrate the ouabain sensitivity of ^{24}Na efflux from medullary cells. The rate of exit of ^{24}Na is reduced by around 70% in the presence of 10^{-4} M glycoside, indicating that chromaffin cells extrude sodium by an ouabain-sensitive route. Table 1B also presents values for efflux rates (picomoles per square centimeter per second) calculated from the rate constants of exit on the assumption of a constant intracellular sodium concentration of 30 mM. This value was chosen on the basis of the intracellular ion levels shown in Fig. 4A. However, it should be emphasized that the total intracellular sodium level measured here may be considerably higher than the exchangeable sodium within the cytosol, as sodium may be sequestered within the chromaffin granules. Indeed, in many tissues examined, cytosolic sodium concentrations appear to be closer to 10 or 15 mM. If this is so, then all of the calculated efflux values are likely to be overestimates.

Stoichiometry of the pump. From the data given in Table 1A and B, the mean values for ouabain-sensitive ^{86}Rb influx and ouabain-sensitive ^{24}Na efflux are 0.66 and 1.07 pmoles/cm^2·sec, respectively. The ratio of ^{86}Rb influx to ^{24}Na efflux, therefore, is 0.677, which corresponds to an exchange of 2.97 sodium ions for 2 rubidium (or presumably potassium) ions—a value which compares very favorably with data obtained for the sodium pumps of other tissues (17, 18).

Dose dependency of sodium pump inhibition by ouabain. The effectiveness of ouabain as an inhibitor of active sodium and potassium transport by chromaffin cells was tested over a range of glycoside concentrations between 10^{-9} M and 10^{-3} M. Figure 1C illustrates the dose-response curves obtained for the inhibition of both ^{86}Rb influx and ^{24}Na efflux. There are two important points to notice here. First, there is no stimulation of the sodium pump by low concentrations of ouabain as has been reported in heart muscle (19, 20). Second, half-maximal inhibition of both ^{86}Rb influx and ^{24}Na efflux occurs at a glycoside concentration of around 4×10^{-7} M. This value is close to that shown in figure 5A of the previous paper (1) for half-maximal stimulation of basal catecholamine secretion by ouabain.

^{24}Na influx and ^{86}Rb efflux. Table 2 shows the results obtained from a small number of experiments in which the effects of ouabain on both ^{86}Rb efflux and ^{24}Na influx were examined. Although ^{24}Na influx remains unaltered when cells are exposed to a concentration of glycoside which would be expected to inhibit their sodium pumps completely, ^{86}Rb efflux appears to be enhanced significantly under the same conditions. This suggests that ouabain increases the permeability of medullary cells to rubidium (and presumably potassium) without affecting the resting sodium permeability. This is interesting in view of the fact that potassium permeability in some cells is partially controlled by the intracellular free calcium concentration (21) and raises the possibility that ouabain enhances rubidium permeability as a consequence of alterations in the free calcium level, a point which is discussed in more detail in a later section.

How is Sodium Pumping Influenced by Extracellular Cations?

Potassium. The stoichiometry of three sodium ions to two potassium ions for the chromaffin cell sodium pump suggests that two potassium ions must interact at external membrane sites to initiate ion translocation. The kinetics of pump activation by extracellular potassium support this suggestion. Figure 2A shows that ouabain-sensitive ^{86}Rb influx is stimulated by increasing the extracellular concentration of potassium; furthermore, the Hanes plots (22)—$K/V^{1/n}$ against K—in Fig. 2B show that the data most closely approaching a straight line are obtained when $n = 2$; i.e., two sites must be occupied by potassium ions to activate the pump. This is confirmed by experiments in which ^{24}Na efflux was measured over a range of concentrations of external potassium. Figure 2C shows the activation of ouabain-sensitive efflux by potassium, and Fig. 2D reinforces the conclusion that the kinetics of activation fit a two-site model.

Finally, Fig. 2E illustrates the effect of varying the extracellular potassium concentration on the dose-response curve for inhibition of ^{86}Rb influx by ouabain. When extracellular potassium is raised, the dose-re-

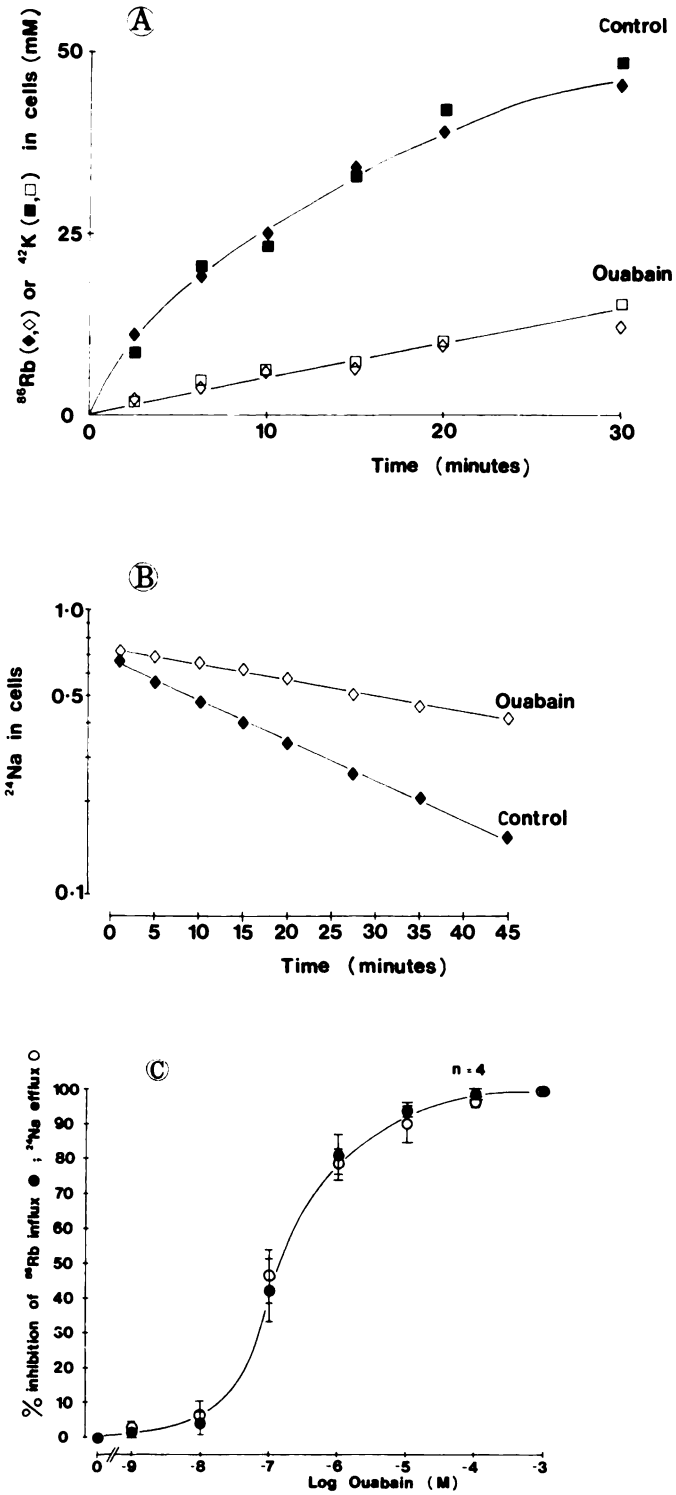

FIG. 1. *Effects of ouabain on the sodium pump activity of isolated adrenal medullary cells*

A. The influx of both ^{86}Rb (♦, ◇) and ^{42}K (■, □) into isolated medullary cells in the presence (*open symbols*) and absence (*closed symbols*) of 10^{-4} M ouabain. In each case the glycoside was added to the cells 5 min before the start of the experiment and the tracer was added at zero time. The amount of ^{86}Rb or ^{42}K in the cells at each time was assessed by filtering the cells through Millipore filters (pore diameter 5 μm) and counting the radioactivity trapped on the filters. The similarity of the results obtained for each of the isotopes supports the use of ^{86}Rb as a tracer for potassium. Results from a single experiment are shown.

B. The efflux of ^{24}Na from preloaded cells in the presence (◇) and absence (♦) of 10^{-4} M ouabain. In this experiment, ouabain was added to the cells 10 min before the start of measurements but the cells were rewashed in the appropriate medium at zero time. The amount of ^{24}Na remaining in the cells at each time is calculated as a fraction of the total radioactivity present (cells plus extracellular fluid) and is plotted on a log scale. Results from a single experiment are shown.

C. Dose-response curves for the inhibition by ouabain of both ^{86}Rb influx (●) and ^{24}Na efflux (○) in isolated adrenal medullary cells. The means ± standard error of the mean of four experiments are shown.

sponse curve is shifted to the right, indicating some degree of competition between potassium and ouabain. This graph should be compared with Fig. 8b in the previous paper (1), which shows that the dose-response curve for the stimulation of catecholamine secretion by ouabain is also shifted to the right by increasing the external potassium concentration. This similarity may support the suggestion that these two effects of the glycoside (stimulation of secretion and sodium pump inhibition) are coupled in some way.

Extracellular sodium. Ouabain appears to have an absolute requirement for external sodium for its secretory action in adrenal medullary cells [see figure 5B in the previous paper (1)]. However, ouabain is still capable of inhibiting the sodium pump in the absence of sodium. Figure 3A presents dose-response curves for the inhibition of ^{86}Rb influx and ^{24}Na efflux in media containing either sodium or lithium (as a sodium substitute). Half-maximal inhibition of active transport occurs at the same glycoside concentration in each case. Magnesium, choline, and (on one occasion) cesium were also used as sodium replacements but none of these had any effect on either sodium pumping itself or pump inhibition by ouabain, although the apparent lack of effect with cesium is perhaps surprising in view of its known potassium-like action on the sodium pumps of other tissues. These experiments would appear to provide strong evidence against a direct link between sodium pump inhibition and catecholamine secretion brought about by the exposure of chromaffin cells to ouabain, although they by no means exclude a mediatory role for changes in the intracellular sodium concentration in these events.

Extracellular calcium. Figure 3B shows the results of a series of experiments in which the effects of varying the extracellular calcium concentration on ouabain-sensitive ^{24}Na efflux were examined. Over the range between nominally zero and 3.6 mM calcium, there appeared to be no change in either the rate of sodium efflux of its inhibition by 10^{-4} M ouabain.

Does Intracellular Sodium Play a Role in the Stimulation of Secretion by Ouabain?

Effect of ouabain on intracellular ion concentrations. Any explanation of the secretory effect of ouabain must be able to account for the striking time course of the increase in catecholamine output by chromaffin cells. The new level of secretion, which is approximately twice that seen in untreated cells, is achieved within a few minutes of exposing cells to glycoside, and is then sustained unchanged for long periods [see figure 1A in the previous paper (1)]. Experiments in which the internal ionic concentrations of medullary cells were measured before and during exposure to ouabain show that, although sodium and potassium levels change progres-

TABLE 1

Sodium pump activity of isolated chromaffin cells

A. ^{86}Rb influx into control and ouabain-treated cells

Expt.	Control	10^{-4} M Ouabain	% Inhibition
	pmoles/cm^2·sec		
1	0.65	0.27	59
2	1.35	0.27	80
3	0.15	0.036	76
4	0.25	0.095	76
5	1.08	0.207	81
6	2.16	0.43	80
7	1.02	0.23	78
8	0.7	0.21	70
9	0.81	0.29	64
10	0.56	0.065	88
Mean ± SEM	0.87 ± 0.58	0.21 ± 0.12	73.8 ± 9.5

B. ^{24}Na efflux from control and ouabain-treated cells

Expt.	Control		10^{-4} M Ouabain		% Inhibition
	Rate constant	Flux	Rate constant	Flux	
	/min	*pmoles/cm^2·sec*	*/min*	*pmoles/cm^2·sec*	
1	0.0099	1.48	0.00358	0.53	65
2	0.0106	1.59	0.003	0.45	72
3	0.0111	1.66	0.0041	0.62	63
4	0.0104	1.56	0.0028	0.42	73
Mean ± SEM	0.0105 ± 0.0004	1.57 ± 0.07	0.0034 ± 0.0005	0.5 ± 0.085	68.2 ± 5.0

sively throughout the entire experimental period, they do so only rather slowly. The results of three such experiments are presented in Fig. 4A. The intracellular concentrations of magnesium, calcium, and chloride have also been investigated in control and ouabain-treated cells. None of these appears to change significantly during a 60-min period of incubation with 10^{-4} M ouabain, the total magnesium content being 11.5 ± 2.3 mM (n = 15), the total calcium being 0.98 ± 0.53 mM (n = 15), and the total chloride being 34 ± 6.5 mM (n = 6).

TABLE 2

Effects of 10^{-4} M ouabain on ^{24}Na influx and ^{86}Rb efflux in isolated adrenal medullary cells

In B, results are expressed as rate constants per minute and as fluxes calculated on the assumption that intracellular potassium is 130 mM and that ^{86}Rb is treated by the cells in the same way as potassium.

A. ^{24}Na influx

Expt.	Control cells	10^{-4} M Ouabain
	pmoles/cm^2·sec	*pmoles/cm^2·sec*
1	0.50	0.48
2	0.43	0.51
3	0.81	0.81
Mean ± SEM	0.58 ± 0.2	0.6 ± 0.18

B. ^{86}Rb efflux

Expt.	Control cells		10^{-4} M Ouabain	
	Rate constant	Flux	Rate constant	Flux
	/min	*pmoles/cm^2·sec*	*/min*	*pmoles/cm^2·sec*
1	0.0061	3.95	0.013	8.4
2	0.0043	2.79	0.0072	4.67
3	0.005	3.24	0.0094	6.09
Mean ± SEM	0.0051 ± 0.0009	3.33 ± 0.48	0.0099 ± 0.0029	6.39 ± 1.54

Effect of ouabain on membrane potential. The somewhat slow and progressive change in sodium and potassium ion concentrations resulting from exposure of medullary cells to ouabain is reflected by the time course of the change in membrane potential recorded in the presence of 10^{-4} M ouabain using a potential-sensitive dye. Figure 4C shows the effect of prolonged incubation with glycoside on the fluorescent signal obtained when cells are incubated with the dye 3,3′-diethylthiadicarbocyanine iodide. The potential appears to alter little for the first 30 min after exposure to glycoside, and then slowly declines. Although this technique gives no accurate information concerning the potential difference across the cell membrane, comparison with the calibration curve in Fig. 4B indicates that the degree of depolarization obtained after 90 min appears to be roughly equivalent to that produced by 15 mM external potassium.

Effect of metabolic poisons on sodium pumping and secretion.

1. Anoxia. Data were presented in the previous paper (see figure 9A in ref. 1) to indicate that cells deprived of both oxygen and glucose by being incubated in glucose-free solutions saturated with 100% nitrogen respond to ouabain in the normal way, i.e., by increasing their basal rate of catecholamine secretion, although their secretion remains unchanged in the absence of glycoside. Cells rendered anoxic by this treatment, however, quickly lose their ability to transport sodium and potassium ions actively. This is illustrated in Fig. 5, in which the effects of anoxia on both ^{86}Rb influx (Fig. 5A) and ^{24}Na efflux (Fig. 5B) are compared with those of ouabain. Active transport is inhibited rapidly and virtually completely by oxygen and glucose deprivation, although Fig. 5B shows that this inhibition is readily reversible as compared with that produced by ouabain. These findings, coupled with

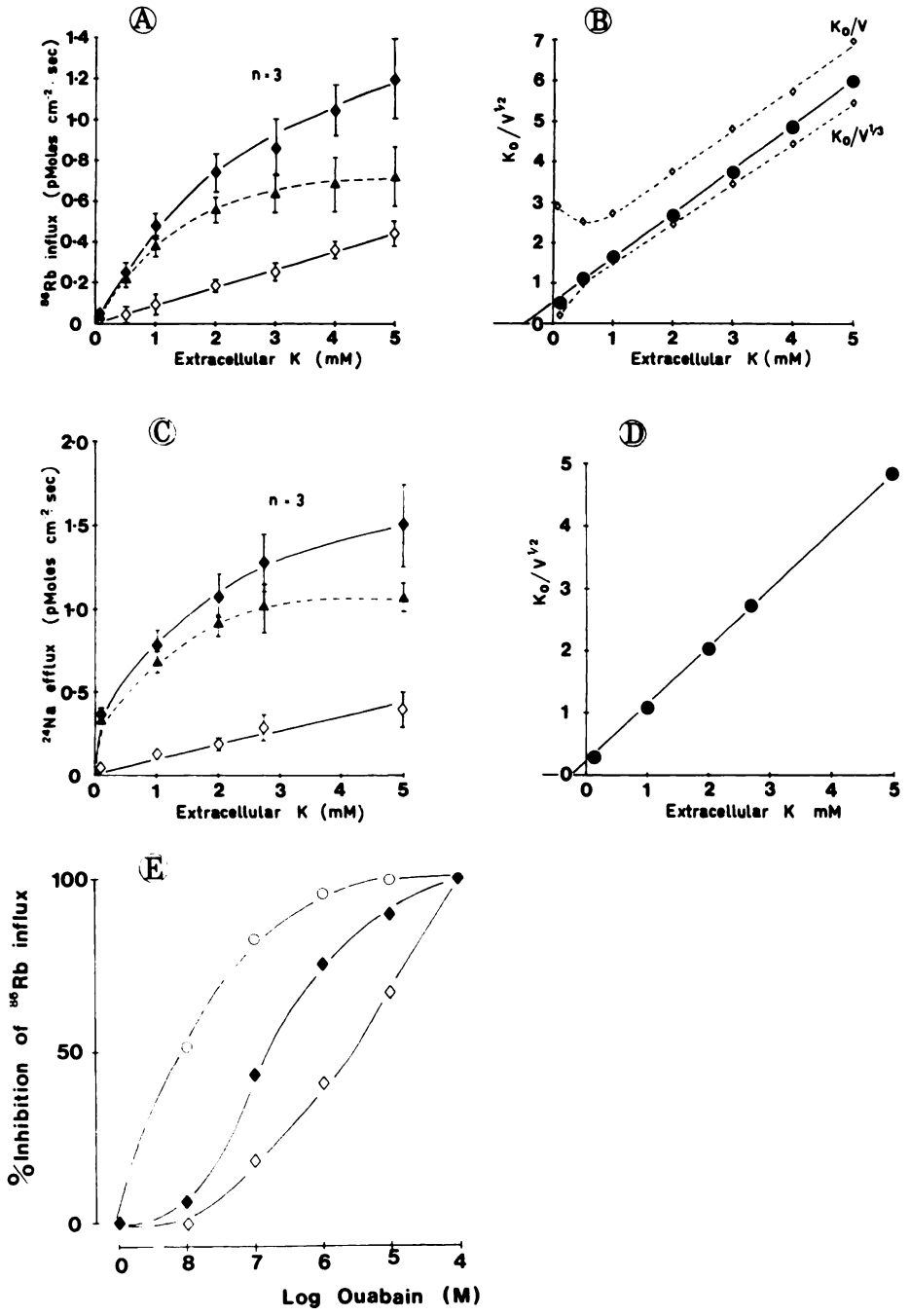

FIG. 2. *Activation of the medullary cell sodium pump by extracellular potassium*

A. The stimulation of ^{86}Rb influx by extracellular potassium. The means ± standard error of the mean of three separate experiments are shown. Influx was measured (calculated assuming a surface area of 1.02×10^{-5} cm^2 for a single cell) in the presence (◇) and absence (◆) of 10^{-4} M ouabain. The ouabain-sensitive component of influx (▲) is also plotted. In each case cells were resuspended in the appropriate potassium-containing solution 10 min before the start of measurements. Ouabain was added at the same time where appropriate, and tracer was added at zero time. The initial rate of influx was then measured, i.e., the influx over the first 10 min after the addition of ^{86}Rb.

B. Hanes Plot (22 ($K_0/V^{1/n}$) against K_0, where K_0 is the extracellular potassium concentration and V is the initial rate of ouabain-sensitive ^{86}Rb influx into isolated cells. The kinetics of activation of influx fit a two-site model: $K_0/V^{1/2}$ is linear (——), and both $K_0/V^{1/3}$ and K_0/V are nonlinear (- - -). The K_m for extracellular potassium = 0.51 ± 0.23 mM (n = 3).

C. The stimulation of ^{24}Na efflux by extracellular potassium. The means ± standard error of the mean of three separate experiments are shown. Efflux from cells preloaded with ^{24}Na was measured (calculated assuming an intracellular sodium concentration of 30 mM) in control cells (◆) and cells treated with 10^{-4} M ouabain (◇). Ouabain-sensitive efflux is also shown (▲). Cells were resuspended in the appropriate solution 10 min before the start of efflux measurements, which were made over 25 min.

D. Hanes plot ($K_0/V^{1/2}$), where K_0 is extracellular potassium and V is efflux of ^{24}Na (picomoles per square centimeter per second) measured over 25 min. The plot is linear, confirming that the activation kinetics fit a two-site model. The K_m for extracellular potassium = 0.41 ± 0.1 mM, which is not significantly different from the value obtained from measurements of ^{86}Rb influx.

E. Dose-response curves for the inhibition of ^{86}Rb influx by ouabain at various concentrations of extracellular potassium. The data have been normalized so that the degree of inhibition achieved with 10^{-4} M ouabain is always assigned a value of 100%. Extracellular concentrations of potassium used were 1 mM (○), 2.7 mM (◆), and 10 mM (◇). The curves were drawn by eye. The degree of inhibition brought about by 10^{-4} M ouabain is very similar at each level of potassium tested.

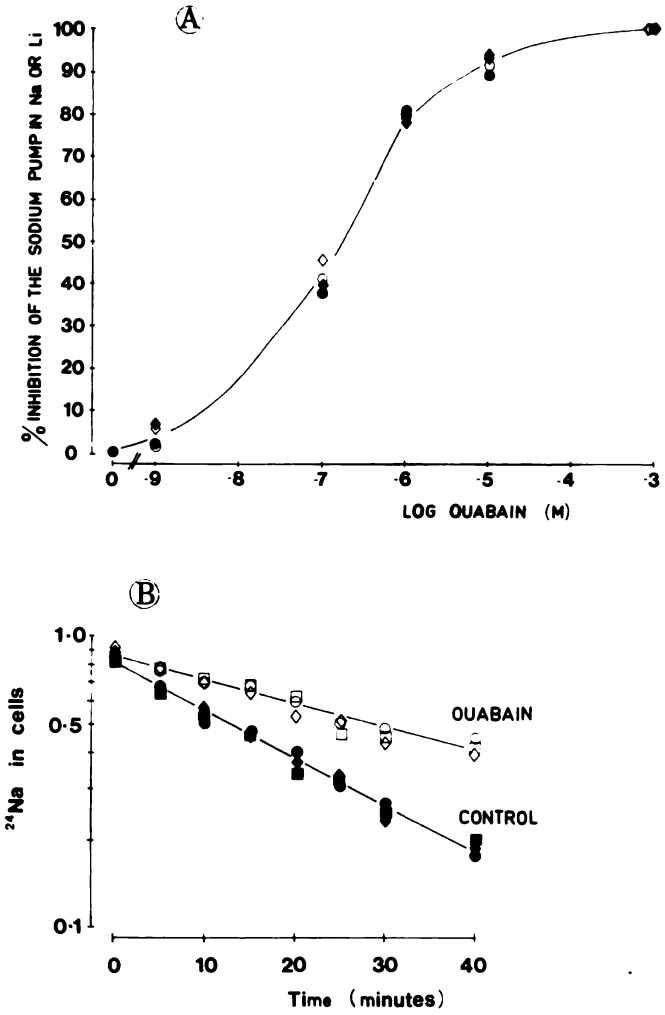

FIG. 3. *Effects of sodium and calcium on sodium pumping in isolated bovine adrenal medullary cells*

A. Dose-response curves for the inhibition of ^{86}Rb influx (●, ○) and ^{24}Na efflux (♦, ◇) in sodium-Locke's solution (*closed symbols*) and a solution in which all of the sodium was replaced by lithium (*open symbols*). The data have been normalized so that the degree of inhibition achieved with 10^{-3} M ouabain is assigned a value of 100%. In these experiments, the cells deprived of sodium were resuspended in the lithium-Locke's solution 10 min before the start of measurements. In the case of ^{86}Rb influx measurements, tracer was added 5 min after the addition of ouabain, where appropriate. In the efflux experiments, ouabain was added 10 min before the start of measurements. The means of three experiments are shown.

B. The effect of variations in the extracellular calcium concentration on ^{24}Na efflux from adrenal medullary cells and its inhibition by ouabain. ^{24}Na efflux from preloaded cells was measured into media containing either nominally zero (♦, ◇), 1.8 mM (●, ○), or 3.6 mM (■, □) calcium in the presence (*open symbols*) and absence (*closed symbols*) of 10^{-4} M ouabain. Cells were resuspended in the appropriate solution 10 min before the start of the experiment. The results of a single experiment are shown.

the results from a single experiment in which anoxia was shown to bring about changes in intracellular ion levels very similar to those seen with ouabain (Fig. 5C), indicate that the intracellular sodium content of isolated chromaffin cells increases when the cells are rendered anoxic. Therefore, the absence of a concomitant increase in the rate of catecholamine secretion argues strongly against a simple role for intracellular sodium in mediating the secretory effect of ouabain. Furthermore, the ability of ouabain to stimulate secretion in cells whose sodium pumps are already fully inhibited favors some alternative mechanism which, although sodium-dependent, is independent of sodium pump inhibition per se.

2. Some other metabolic poisons. The secretory behavior of cells treated with a variety of metabolic inhibitors was described in the previous paper (1). Data are presented here to illustrate the effects of these same agents on active sodium and potassium transport by isolated chromaffin cells. It may be remembered that the only combination of agents found to block the secretory response of medullary cells to carbamylcholine was that of 2 mM sodium cyanide and 3 mM 2-deoxyglucose, whereas none had any significant effect on basal catecholamine secretion. The histogram in Fig. 5D shows that the same combination of poisons brings about complete inhibition of ouabain-sensitive ^{86}Rb influx by the cells. This is consistent with the idea that ATP levels within the cells have fallen below those required to activate both catecholamine secretion and sodium pumping. The lack of effect on basal secretion lends further support to the idea that intracellular sodium levels can rise without stimulating catecholamine secretion. Finally, Fig. 5D shows that, although 2-deoxyglucose alone (like anoxia) partially blocks the sodium pump, it has no effect on evoked secretion [see previous paper (1)], suggesting that it is possible to achieve a situation in which ATP levels have fallen low enough to inhibit or depress the activity of the sodium pump but not low enough to block the secretory response to a nicotinic agonist. While it would certainly be interesting to know how the actual levels of cellular ATP change during metabolic deprivation, such measurements are very difficult to obtain in view of the huge amounts of ATP stored within chromaffin granules (23). Several attempts have been made to obtain values for the free ATP concentration within cells using various methods of lysing the plasma membrane, but none of these has proved to be very satisfactory since lysis of even a very small number of granules during the procedure grossly distorts the results.

Calcium Transport by Isolated Adrenal Medullary Cells

The results presented so far suggest that the stimulation of secretion seen when cells are exposed to the cardiac glycoside ouabain is an inevitable consequence neither of sodium pump inhibition nor of increased intracellular sodium concentration, since both these situations can be invoked without a subsequent rise in the rate of catecholamine release. In the following section results are presented that seem to suggest that ouabain increases secretion as a direct result of an inhibitory effect on the efflux of calcium from medullary cells.

Do chromaffin cells have a calcium pump? Many cell types possess a plasma membrane ATPase which pumps calcium out of the cell, thereby helping to maintain the now familiar low level of intracellular ionized calcium (24). The results shown in Fig. 6A seem to suggest that chromaffin cells also possess such a pump. Figure 6A shows the data obtained from a series of experiments in which the rate of efflux of ^{45}Ca from preloaded cells was measured under a variety of different conditions. In each case, the cells were washed in normal sodium-Locke's

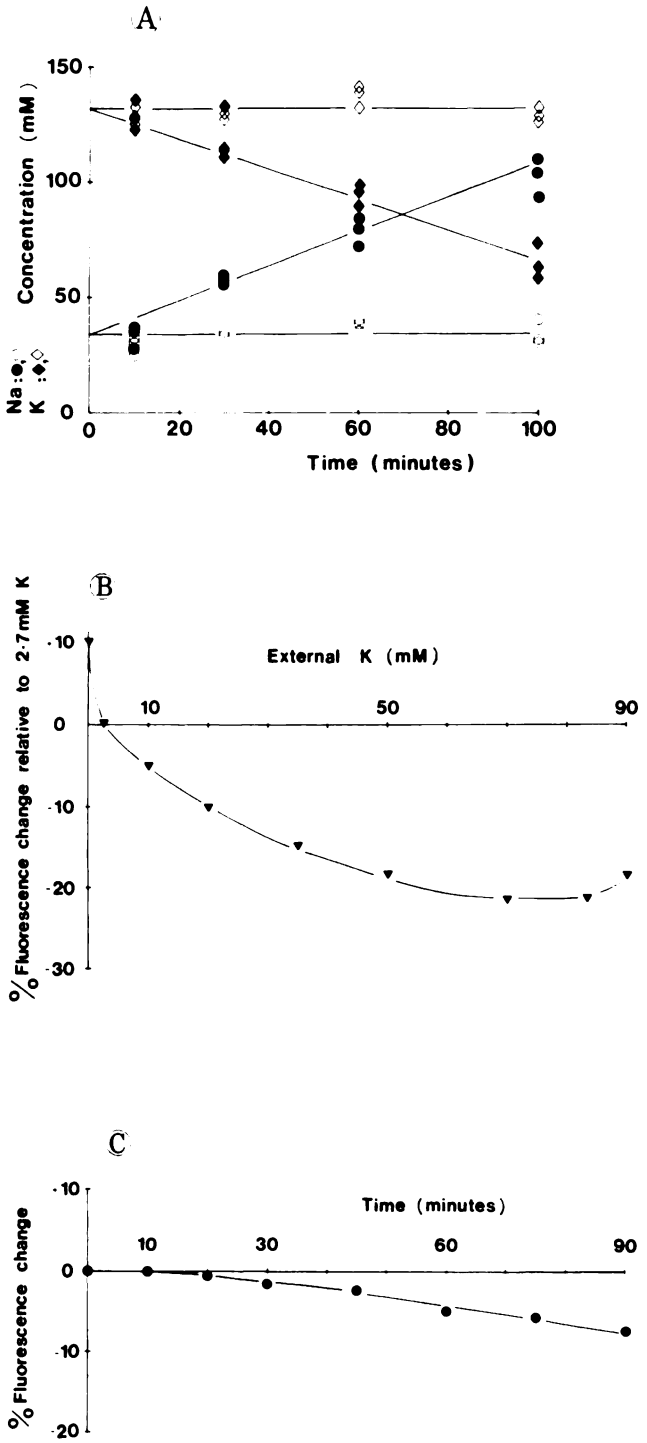

FIG. 4. *Comparison of the effects of prolonged exposure to ouabain on the ion gradients and membrane potential of isolated chromaffin cells*

A. Changes in the intracellular concentrations of sodium (●, ○) and potassium (◆, ◇) in the presence (*closed symbols*) and absence (*open symbols*) of 10^{-4} M ouabain. The results of three separate experiments are illustrated. Although the ion gradients of the ouabain-treated cells did run down during the 100 min of exposure to the glycoside, they did so gradually and continuously. The intracellular ion contents of control cells remained unchanged throughout this period.

B. Use of the dye $DiSC_3$ (6) to monitor membrane potential in chromaffin cells. A calibration experiment is shown in which changes in fluorescence were measured using cells incubated in increasing concentrations of potassium. The fluorescence of a cell-free solution of the dye always decreased when cells were added but this decrease lessened progressively as extracellular potassium was raised and the cells became depolarized. This reduction is plotted as a percentage change against the potassium concentration, the change occurring at 2.7 mM potassium being taken as zero. These results are from a single experiment.

C. The effect of 10^{-4} M ouabain on the membrane potential of chromaffin cells. Glycoside was added at zero time and the fluorescence was monitored continuously for the ensuing 90 min. Although this method cannot give a quantitative estimate of potential, it is possible by comparing this graph with the calibration curve in B to see that depolarization occurs gradually and slowly, so that by 90 min a degree of depolarization equivalent to that seen with 15 mM potassium has been achieved. These results are from a single experiment.

solution at 37° and then resuspended, at zero time, in the appropriate medium. The rates of ^{45}Ca efflux were measured over the ensuing 45 min. The rate of efflux of calcium is markedly reduced by lowering the temperature (to 25° or 0–4°), and enhanced under conditions which might be expected to raise the level of free calcium inside the cells, for example, with cyanide or the proton ionophore FCCP. Resuspension of the cells in a medium containing a depolarizing concentration of potassium (70 mM) also stimulates ^{45}Ca efflux, at least transiently. This may reflect a rise in free intracellular calcium (prior to exocytosis) although it is perhaps more likely to represent a direct effect of high potassium. The calcium dependence of this effect has not been examined. These results, together with some of those in Table 3 (which shows rate constants of ^{45}Ca efflux under several of the conditions just mentioned), seem to argue in favor of the existence of a membrane pump which actively extrudes calcium from chromaffin cells.

Effect of ouabain on calcium extrusion. The effect of ouabain on the efflux of ^{45}Ca from chromaffin cells was examined primarily on the grounds that if it enhances the rate of catecholamine secretion by producing a rise in the level of free calcium inside the cells it may, as a result, increase the rate of efflux of this ion. Figure 6B and Table 3 show that, on the contrary, calcium efflux is strikingly depressed by the glycoside. In nine experiments in which cells were treated with 10^{-4} M ouabain, calcium efflux was inhibited by 52.4 ± 13.1%. This inhibition was apparent within minutes of administering the drug. The value quoted for the degree of inhibition is likely to be an underestimate of the true value for two reasons. First, the increase in catecholamine secretion (i.e., the rate of granule discharge), which occurs at the same time, is almost certain to increase the apparent efflux of any isotope associated with the granule fraction, which is known to contain a large amount of calcium (23). Second, any rise in intracellular calcium which may result from the inhibition of efflux will itself tend to stimulate calcium efflux and hence counteract the inhibition to some extent.

Ionic requirements for the inhibition of ^{45}Ca efflux by ouabain. If the stimulation of secretion by ouabain occurs as a result of inhibition of calcium efflux from adrenal medullary cells, one might expect the ionic requirements of the two effects to be similar. Figure 7A and B shows that this is indeed the case. Inhibition of

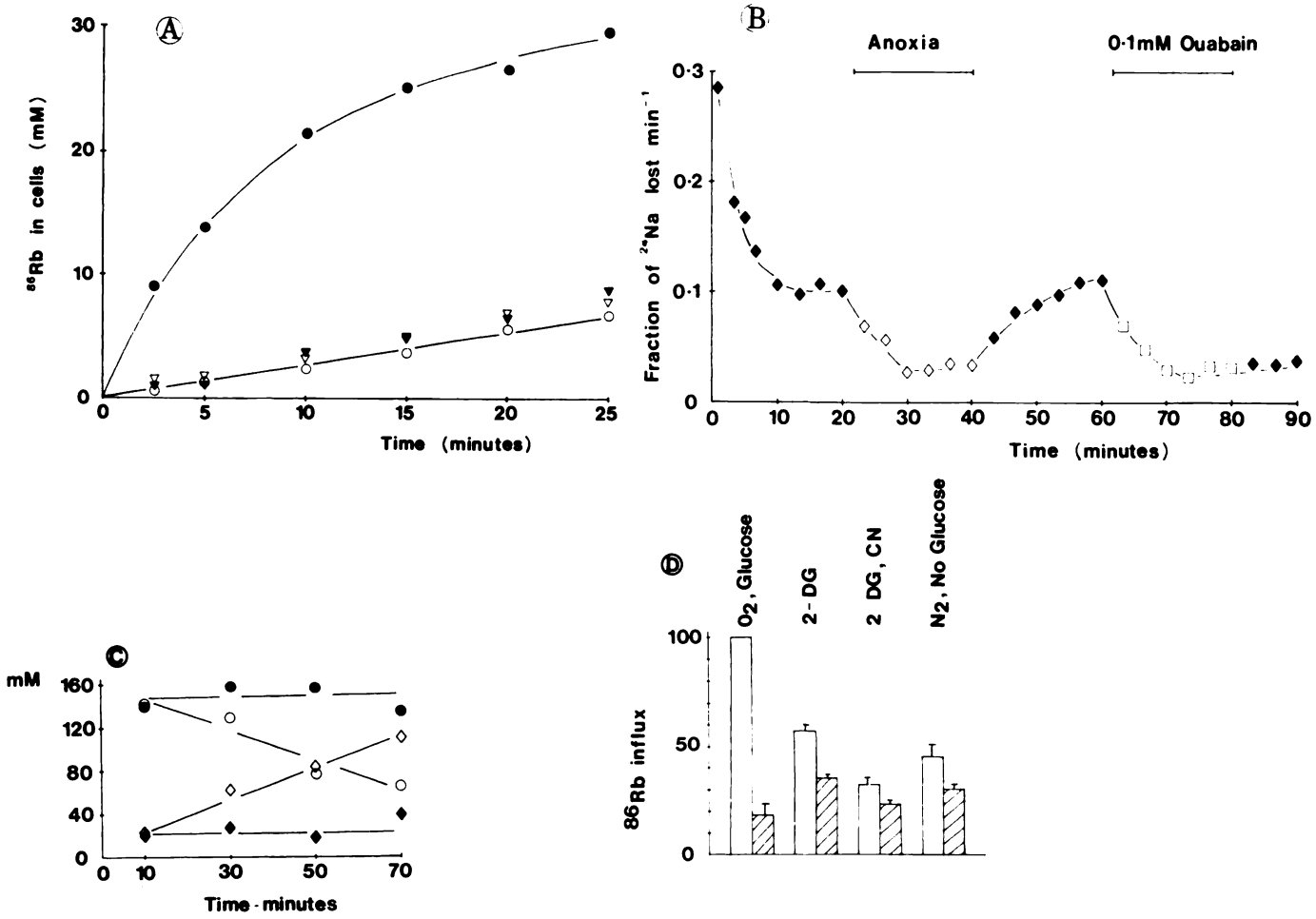

FIG. 5. *Effects of anoxia and a variety of other metabolic poisons on the sodium pump activity of isolated adrenal medullary cells*

A. The effects of oxygen and glucose deprivation on ^{86}Rb uptake by chromaffin cells in the presence (*open symbols*) and absence (*closed symbols*) of 10^{-4} M ouabain. The amount of ^{86}Rb in the cells was examined over a period of 25 min in healthy cells (●, ○) and in cells incubated in glucose-free solution saturated with 100% nitrogen (▼, ▽). Ouabain was added 5 min before the start. Tracer was added at zero time.

B. A comparison of the effects of oxygen and glucose deprivation and of 10^{-4} M ouabain on the efflux of ^{24}Na from cells embedded in agar slices. The cells were preloaded with ^{24}Na and then washed free of isotope before being embedded in a 2% (w/v) agar medium as described under Experimental Procedures. Slices were superfused at a rate of 5 ml/min by means of a peristaltic pump, and samples of superfusate were assayed for radioactivity at the times indicated. During the times indicated (◇), the slices were perfused with glucose-free medium bubbled with 100% nitrogen. Efflux was inhibited rapidly and completely, but this effect was reversible. The degree of inhibition produced by ouabain (10^{-4} M) (□) was similar, but here the effect was not reversible over the period studied. Efflux is expressed as the fraction of total ^{24}Na lost per minute from the cells.

C. The effect of oxygen and glucose deprivation on the intracellular concentrations (◆, ◇) and potassium (●, ○) in isolated adrenal medullary cells. Cells were kept in either normal Locke's solution containing 5 mM glucose and saturated with 100% oxygen (*closed symbols*) or in glucose-free medium saturated with 100% nitrogen (*closed symbols*). Comparison with Fig. 4A shows that anoxia promotes changes in the ion gradients similar to those produced by ouabain, a result which reflects the marked inhibition of sodium pumping by both of these agents. The results represent the mean values obtained from two experiments.

D. The effects of a variety of metabolic poisons on the influx of ^{86}Rb into isolated medullary cells. In each case, the metabolic agents were administered in both the presence (*hatched bars*) and absence (*open bars*) of 10^{-4} M ouabain. The concentration of 2-deoxyglucose (*2-DG*) used was 3 mM and that of sodium cyanide was 2 mM. Inhibitors were added to the cells where appropriate 10 min before the addition of ^{86}Rb. Influx was then measured over the ensuing 30-min period. The data have been normalized so that the influx of ^{86}Rb into untreated cells is assigned a value of 100. Means from three experiments are shown. The statistical significance of the differences between groups has been determined using Student's *t*-test; O_2 and 2-DG (controls), $p < 0.01\%$; O_2 and 2-Dg + CN (controls), $p < 0.01\%$; O_2 and N_2, no glucose (controls), $p < 0.01\%$. The differences measured in ouabain-treated cells from each group are not significantly different from each other.

^{45}Ca efflux by ouabain is unaltered in the nominal absence of extracellular calcium (Fig. 7B) but is completely abolished if extracellular sodium is replaced by lithium (Fig. 7A), findings which lend further support to the possibility that stimulation of basal secretion by ouabain could be the direct result of depressing calcium efflux.

Dose dependency of the inhibition of calcium efflux by ouabain. Further evidence in favor of a direct link between the inhibition of calcium efflux and catecholamine secretion is provided by the results of experiments illustrated in Fig. 7C, in which ^{45}Ca efflux was measured from cells exposed to a range of ouabain concentrations between 10^{-9} M and 10^{-3} M in a variety of external ionic environments. Plotted on the same graph are the dose-

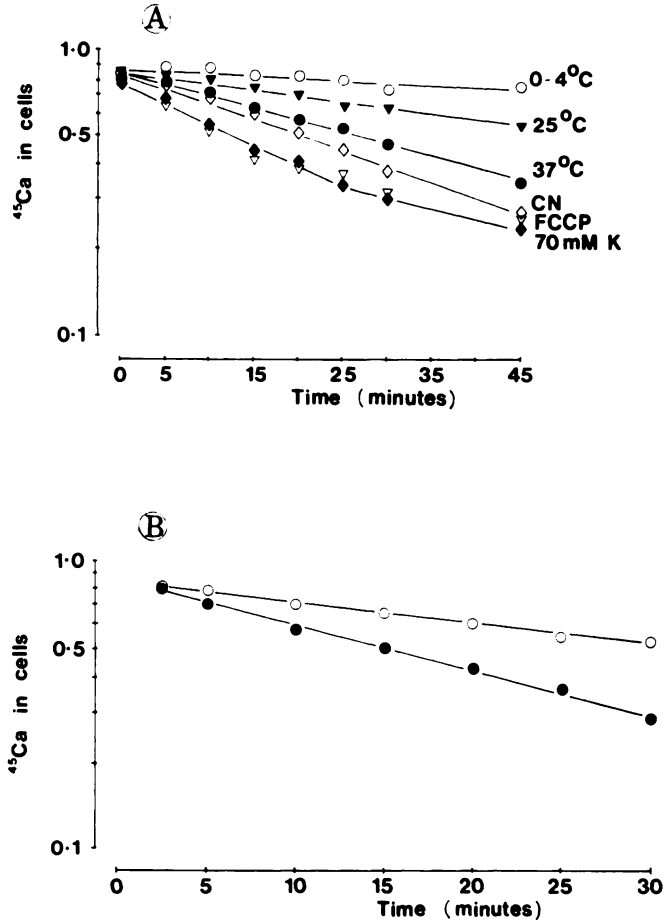

FIG. 6. *Efflux of ^{45}Ca from cells isolated from bovine adrenal medulla*

A. Evidence to suggest that adrenal medullary cells actively extrude calcium. The effects of a variety of agents on the efflux of ^{45}Ca from preloaded cells were examined. In the case of a reduction in temperature, the cells were brought to their new temperature 10 min before the start of measurements (i.e., during the washing period). When drugs were added, they were added 5 min before the start of measurements. Cells were resuspended in 70 mM potassium 5 min before the start of measurements. The fraction of ^{45}Ca remaining in the cells at each time is plotted on a log scale. The following conditions were used: 0–4° (○), 25° (▼), 37° (●), 2 mM sodium cyanide (◇), 2 μM FCCP (▽), and 70 mM potassium (◆). The flux was highly temperature-sensitive and was stimulated by agents likely to raise intracellular calcium.

B. The efflux of ^{45}Ca from medullary cells incubated in the presence (○) and absence (●) of 10^{-4} M ouabain. Glycoside was added to the cells at zero time. Efflux was inhibited virtually immediately by this drug. The fraction of ^{45}Ca remaining in the cells at each time is plotted on a log scale. These results were obtained from a single experiment. Additional data are shown in Table 3.

TABLE 3

Effects of a variety of agents on calcium efflux from isolated adrenal medullary cells

These values were obtained by measuring the rate of loss of labeled calcium from cells loaded with ^{45}Ca over periods ranging between 30 and 60 min. In all cases except that of treatment with ouabain, cells were washed and resuspended in the appropriate solution 10 min before the start of measuring efflux. Ouabain was always added at zero time.

Condition	Rate constant of ^{45}Ca efflux
	/min
Control (144 mM Na, 1.8 mM Ca)	0.0212
	0.0198
	0.0232
	0.0300
	0.0281
	0.0142
	0.0190
	0.0191
	0.0202
Mean ± SEM	0.0216 ± 0.0046
144 mM Li, 0 mM Na, 1.8 mM Ca	0.0200
	0.0196
Mean	0.0198
144 mM choline, 0 mM Na, 1.8 mM Ca	0.0161
	0.0255
	0.0231
Mean ± SEM	0.0216 ± 0.0039
3.6 mM Mg, 0 mM Ca, 144 mM Na	0.0191
	0.0182
	0.0233
	0.0199
Mean ± SEM	0.0201 ± 0.0019
0–4° (ionic medium as in control)	0.0016
	0.0009
Mean	0.00125
Anoxia (100% N_2, no glucose), ionic medium as in controls	0.0251
	0.0173
Mean	0.0212
2 mM sodium cyanide	0.0313
	0.0401
Mean	0.0357
2 μM FCCP	0.0415
	0.0473
Mean	0.0444
10^{-4} M Ouabain	0.0106
	0.0118
	0.0200
	0.0098
	0.0111
	0.0112
	0.0141
	0.0063
	0.0104
Mean ± SEM	0.0117 ± 0.0035

response curves for stimulation of basal catecholamine secretion by ouabain under the same conditions, and it is clear that the two sets of data are very similar. Half-maximal effects occur at the same ouabain dose in each case (just over 10^{-7} M) in both normal and calcium-free media, while there are no effects on either calcium efflux or catecholamine secretion in sodium-free environments.

Further experiments on the role for extracellular sodium. Both the stimulation of secretion and the inhibition of calcium efflux by ouabain are clearly sodium-dependent processes in adrenal medullary cells. Figure 8 shows the results of single experiments which were carried out in order to examine this requirement for sodium in a little more detail. Cells embedded in agar slices were used for these experiments. Figure 6C in the previous paper (1) showed that the readministration of sodium to cells that had previously been exposed to ouabain in choline-Locke's solution and then washed free of glycoside produced a stimulation of basal secretion. Such a

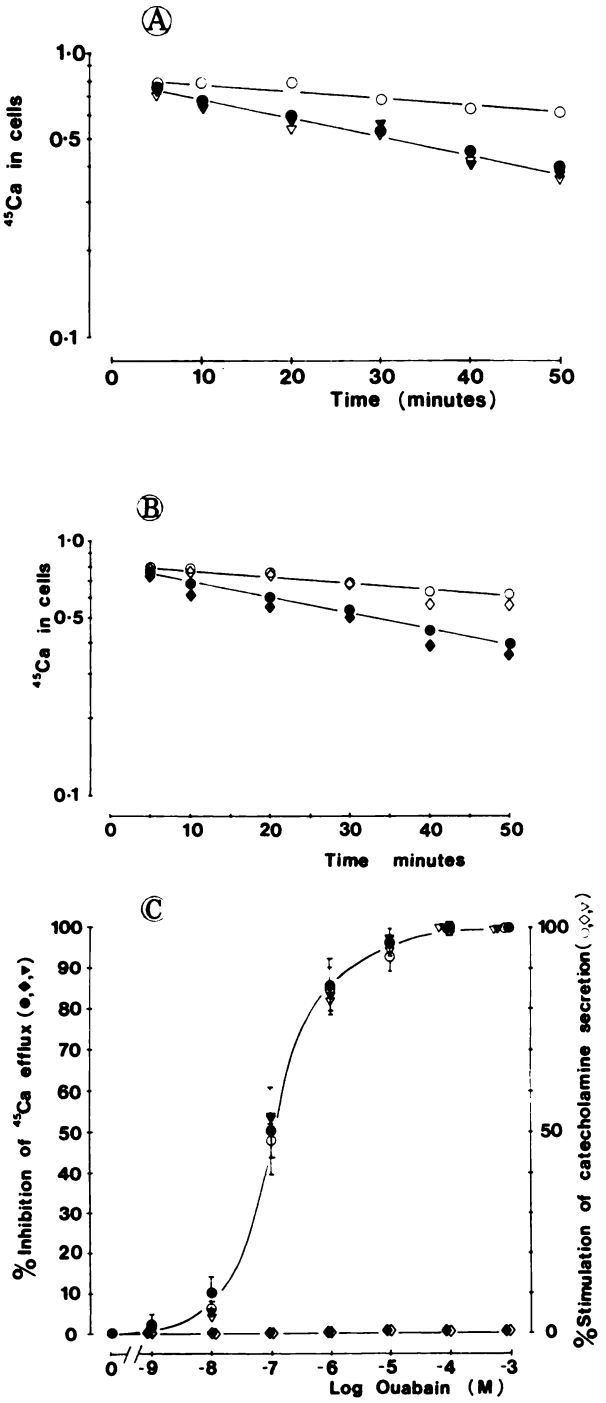

FIG. 7. *Ionic requirements for the inhibition of ^{45}Ca efflux by ouabain*

A. Removal of extracellular sodium prevents the inhibition of ^{45}Ca efflux by ouabain. The fraction of total ^{45}Ca remaining in the cells is plotted on a log scale in cells incubated in sodium-Locke's solution (●, ○) or lithium-Locke's solution (▼, ▽) in the presence (*open symbols*) and absence (*closed symbols*) of 10^{-4} M ouabain. Ouabain was added at zero time. Although efflux was inhibited by ouabain in the sodium medium, it remained unchanged when sodium was replaced by lithium.

B. The inhibition of ^{45}Ca efflux by ouabain is not dependent upon extracellular calcium. ^{45}Ca efflux was monitored using cells incubated in either nominally zero (♦, ◇) or 1.8 mM (●, ○) calcium extracellularly, in the presence (*open symbols*) and absence (*closed symbols*) of 10^{-4} M ouabain (added at zero time). The fraction of ^{45}Ca remaining in the cells at each time is plotted on a log scale.

C. A comparison of the dose-response curves for both the stimulation of catecholamine secretion (*open symbols*) and the inhibition of ^{45}Ca efflux (*closed symbols*) by ouabain in sodium-Locke's solution (●, ○), lithium-Locke's solution (♦, ◇), and nominally calcium-free Locke's solution (▼, ▽). The results have been normalized with both the inhibition of ^{45}Ca efflux and the stimulation of secretion by 10^{-3} M ouabain being assigned values of 100%. In the sodium-Locke's solution and calcium-free Locke's solution the dose-response curves for both effects are identical; in the absence of sodium neither effect is apparent ($n = 4$).

stimulation was never seen in control cells that had not been preexposed to ouabain. Figure 8A of this paper shows that under exactly the same conditions ^{45}Ca efflux is also inhibited as soon as sodium is returned to the extracellular medium. Furthermore, the inhibition of ^{45}Ca efflux by ouabain is not reversed by the removal of extracellular sodium after the onset of the inhibitory effect. This finding is illustrated in Fig. 8B and should be compared with the experiment depicted in figure 6B of the previous paper (1), which shows similar effects on the stimulation of secretion.

Does potassium compete with ouabain in the inhibition of calcium efflux? Increasing the level of potassium outside the cells has the effect of shifting the dose-response curve for stimulation of catecholamine secretion by ouabain to the right, suggesting a degree of competition between the two agents [see figure 8B of the previous paper (1)]. The same effect was seen when the dose-response curve for the inhibition of ^{45}Ca efflux was examined under the same conditions. The results of these experiments, illustrated in Fig. 8C, show that raising the extracellular potassium concentration also shifts this curve to the right. The concentration of ouabain that brings about 50% inhibition of ^{45}Ca efflux is increased from 10^{-8} M in 1 mM potassium to 3×10^{-6} M in 10 mM potassium, a shift which is very similar to that shown for the stimulation of secretion by ouabain.

Does ouabain modify sodium-calcium exchange? The experiments described so far demonstrate that ouabain inhibits the rate of exit of labeled calcium from adrenal medullary cells. However, there are at least two routes by which such inhibition could occur. The first possibility is that ouabain is inhibiting an ATP-dependent calcium pump which normally operates to extrude calcium from the intracellular compartment. The second is that the glycoside modifies a sodium-calcium exchange mechanism similar to that already known to operate in several tissues (for example, in heart muscle and squid giant axon), possibly as a result of raising intracellular sodium, since an absolute requirement for this ion has been established. Evidence which seems to cast some doubt on the existence of such a role for sodium has already been presented, including the finding that ouabain increases intracellular sodium only rather slowly and the demonstration that anoxia, which also raises intracellular sodium (and to the same extent as ouabain), has no effect on catecholamine secretion. In addition to these pieces of information, however, it has also been possible to show that isolated adrenal medullary cells do not appear to possess a significant sodium-calcium countertransport system under any of the conditions of the experiments reported in this paper. Some important criteria for the existence of sodium-calcium exchange may be stated as

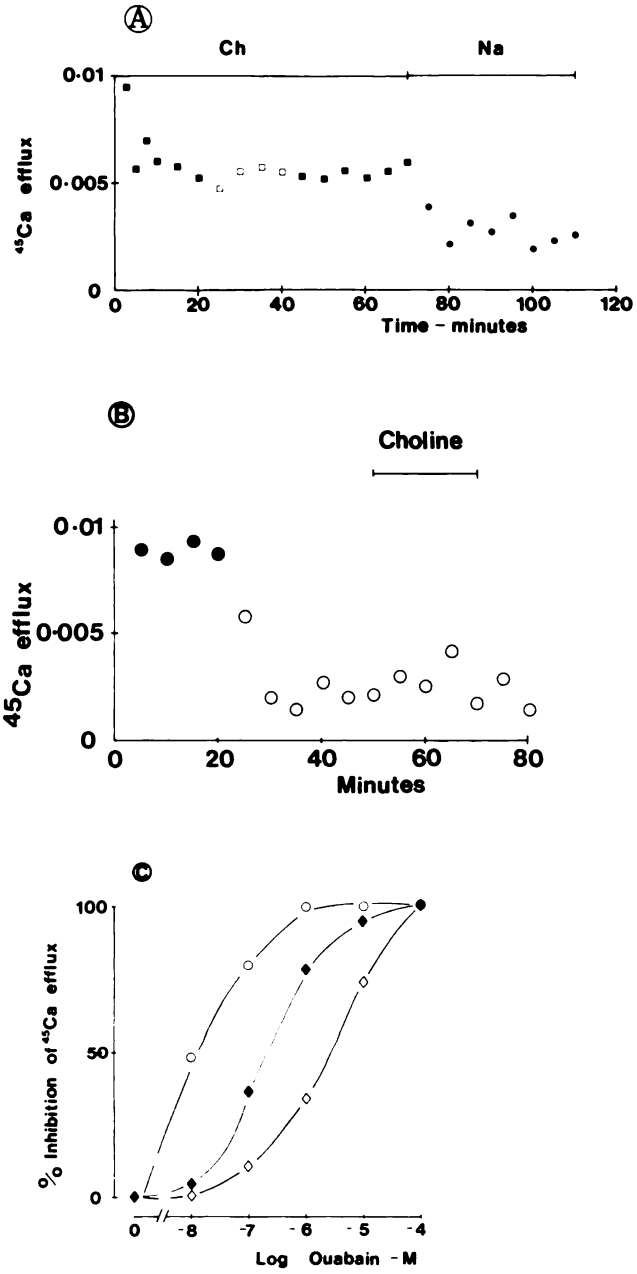

FIG. 8. *Efflux of ^{45}Ca from cells*

A. The efflux of ^{45}Ca from cells embedded in agar and superfused at 5 ml/min using a peristaltic pump. The cells were treated with 10^{-4} M ouabain during the period indicated by □; the superfusing medium was a sodium-free choline-Locke's solution. After washing the cells free of extracellular ouabain, sodium was returned to the bathing medium (●). ^{45}Ca efflux (expressed as the fraction of total ^{45}Ca lost per minute from the cells) was inhibited when sodium was replaced.

B. ^{45}Ca efflux was monitored from cells embedded in agar and superfused with various solutions at 5 ml/min. Ouabain (10^{-4} M) was administered to the cells during the period indicated (○) and efflux (expressed as fraction lost per minute) was inhibited. Subsequent replacement of extracellular sodium by choline had no further effect on the efflux rate.

C. Dose-response curves for the inhibition of ^{45}Ca efflux by ouabain in the presence of increasing concentrations of extracellular potassium. The data have been normalized so that the degree of inhibition achieved with 10^{-4} M ouabain is assigned a value of 100% in all cases. In each case, cells were resuspended in the appropriate medium 10 min before the start of efflux measurements. The potassium concentrations used were 1 mM (○), 2.7 mM (♦), and 10 mM (◇). The curves were drawn by eye. The inhibition obtained with 10^{-4} M ouabain was similar at all potassium concentrations used.

follows (see ref. 9): (a) Sodium withdrawal should stimulate catecholamine secretion, should inhibit calcium efflux, and should stimulate calcium influx. (b) Raising intracellular sodium should mimic the above effects. (c) Calcium removal should inhibit ouabain-insensitive sodium efflux. None of these criteria are adequately met in isolated chromaffin cells.

1. Effects of sodium withdrawal. The effects of sodium withdrawal on secretion were discussed in the previous paper (1). In isolated cells there was apparently no secretory response whatsoever to the replacement of sodium by lithium, choline, sucrose, or magnesium. Similarly, in a single experiment (data not shown) carried out with a perfused intact adrenal gland, no secretory response to sodium withdrawal was seen.

^{45}Ca efflux from isolated cells is also unaffected by the removal of sodium. Figure 7A shows that replacement of sodium by lithium has no inhibitory effect on the rate of efflux of ^{45}Ca. Table 3 gives a series of rate constants determined from experiments in which both lithium and choline were used as sodium substitutes. Neither reduced the rate of efflux to any extent.

^{45}Ca influx was also investigated in a small number of experiments. Figure 9A shows the results obtained. The rate of influx was unaffected by the replacement of external sodium with lithium, and ouabain had no measurable effect on influx under any of the conditions tested.

2. Effect of raising intracellular sodium. Experimental manipulation of the intracellular environment of adrenal cells is very difficult to achieve, but several experiments have been performed which suggest that variations in intracellular sodium do not necessarily influence the movements of calcium in these cells. The most important of these concerns the effects of anoxia. Anoxic cells quickly lose the ability to drive their sodium pumps (Fig. 5A), and consequently gain sodium in a manner similar to ouabain-treated cells (Fig. 5C). Although the latter exhibit a reduced rate of calcium efflux, cells deprived of oxygen and glucose do not. This finding is illustrated in Fig. 9B, which also shows that the calcium channel blocker D-600 is without an inhibitory effect on ^{45}Ca efflux.

The behavior of anoxic cells is interesting for two reasons. First, the discovery that calcium efflux is not depressed in anoxic cells which have an elevated intracellular sodium concentration provides strong evidence against the operation of a sodium-calcium exchange system in these cells. Second, these experiments suggest that, if this calcium extrusion mechanism is indeed ATP-dependent, its affinity for ATP is probably higher than that of the sodium pump, whose activity is inhibited very swiftly under anoxic conditions.

In addition to these results, additional evidence against the possibility that ouabain modifies sodium-calcium exchange by raising intracellular sodium is provided by the experiment (already discussed) in Fig. 8B. This shows that ouabain continues to depress the rate of ^{45}Ca efflux

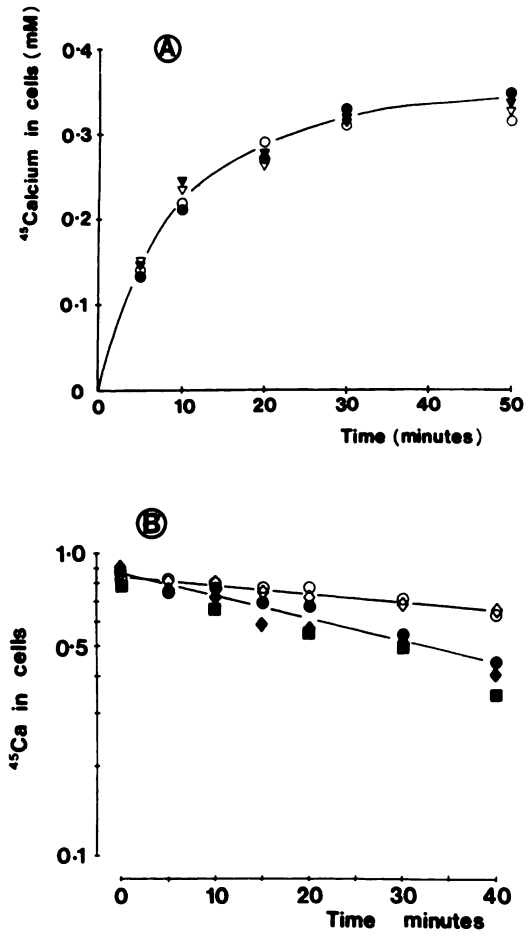

FIG. 9. *Uptake of ^{45}Ca by cells and ^{45}Ca efflux from isolated medullary cells*

A. The uptake of ^{45}Ca into control cells (*closed symbols*) and cells treated with 10^{-4} M ouabain (*open symbols*) in sodium-Locke's solution (●, ○) and lithium-Locke's solution (▼, ▽). The amount of ^{45}Ca in the cells was calculated assuming that cells are spheres 18 μm in diameter and are 100% water. Neither ouabain nor sodium removal per se had any effect on the rate of ^{45}Ca entry. The cells were resuspended in the appropriate solution 10 min before the addition of tracer to the medium. Tracer was added at zero time. The means of two experiments are shown.

B. ^{45}Ca efflux from isolated medullary cells in the presence (*open symbols*) and absence (*closed symbols*) of 10^{-4} M ouabain under a variety of conditions. D-600 (10^{-5} M) had no effect on ^{45}Ca efflux (■), and cells deprived of both glucose and oxygen (◆, ◇) show efflux rates identical with those seen in healthy control cells (●, ○). Cells were resuspended in the appropriate medium 10 min before the start of efflux measurements. The fraction of ^{45}Ca remaining in the cells at each time is plotted on a log scale. The means from three experiments are shown.

even after the withdrawal of external sodium from the cells, a manipulation which will presumably provide a background of falling intracellular sodium upon which ouabain is still effective.

3. Effects of calcium removal on sodium efflux. Figure 3B shows that the ouabain-insensitive efflux of ^{24}Na from isolated chromaffin cells is unaffected by the removal of extracellular calcium. This is further evidence against the operation of sodium-calcium exchange in this preparation.

Does the inhibition of calcium efflux raise intracellular calcium? The experiments described above seem to rule out quite conclusively the suggestion that the stimulatory effect of ouabain on basal catecholamine secretion is mediated by effects on sodium-calcium exchange. It seems likely, therefore, that the inhibition of ^{45}Ca efflux seen when cells are exposed to ouabain represents a direct effect of the glycoside on an extrusion mechanism resembling the calcium pump of other tissues (25). Another possibility must, however, be ruled out: i.e., that the observed depression of efflux is a secondary effect of some other action of ouabain which lowers the free intracellular calcium concentration. The following series of experiments was designed to clarify this point as far as possible.

1. Net loss of calcium from chromaffin cells. If ouabain produces an increase in free intracellular calcium by inhibiting calcium efflux in the absence of any change in influx, then one might expect to be able to detect a reduction in the net loss of calcium from cells incubated with ouabain. Two methods were used to measure net loss of calcium. Both employed nominally calcium-free solutions to ensure a low baseline upon which to monitor changes accurately. These methods were continuous monitoring of net efflux using a calcium electrode in the extracellular solution and atomic absorption spectrophotometric analysis of serial samples of extracellular fluid from centrifuged cells. Both methods revealed a marked reduction in the rate of net calcium loss into calcium-deficient media after exposure to 10^{-4} M ouabain. Table 4 presents the results of several experiments in which net loss of calcium has been calculated in picomoles per square centimeter per second. These values are compared with efflux rates obtained in experiments performed using ^{45}Ca to monitor net loss of calcium into calcium-free

TABLE 4

Net loss of calcium from isolated adrenal medullary cells into solutions containing nominally zero calcium

Values are means ± standard error of the mean

A. Measurements carried out using a calcium electrode in the extracellular medium	
Condition	Net loss of calcium
	pmoles/cm^2·sec
Control	0.054 ± 0.0015 (n = 4)
Treated with 10^{-4} M ouabain	0.019 ± 0.0056[a] (n = 4)
B. Measurements carried out using atomic absorption spectrophotometry	
Condition	Net loss of calcium
	pmoles/cm^2·sec
Control	0.054 ± 0.009 (n = 3)
Treated with 10^{-4} M ouabain	0.023 ± 0.0085[a] (n = 3)
C. Efflux of ^{45}Ca into calcium-free medium[b]	
Condition	Efflux of ^{45}Ca
	pmoles/cm^2·sec
Control	0.0986 ± 0.0093
Treated with 10^{-4} M ouabain	0.0552 ± 0.0165[a]

[a] These differences are statistically significant ($p < 0.01$) according to Student's t-test.

[b] Calculated assuming that intracellular calcium is 1 mM and that ^{45}Ca labels the pool uniformly.

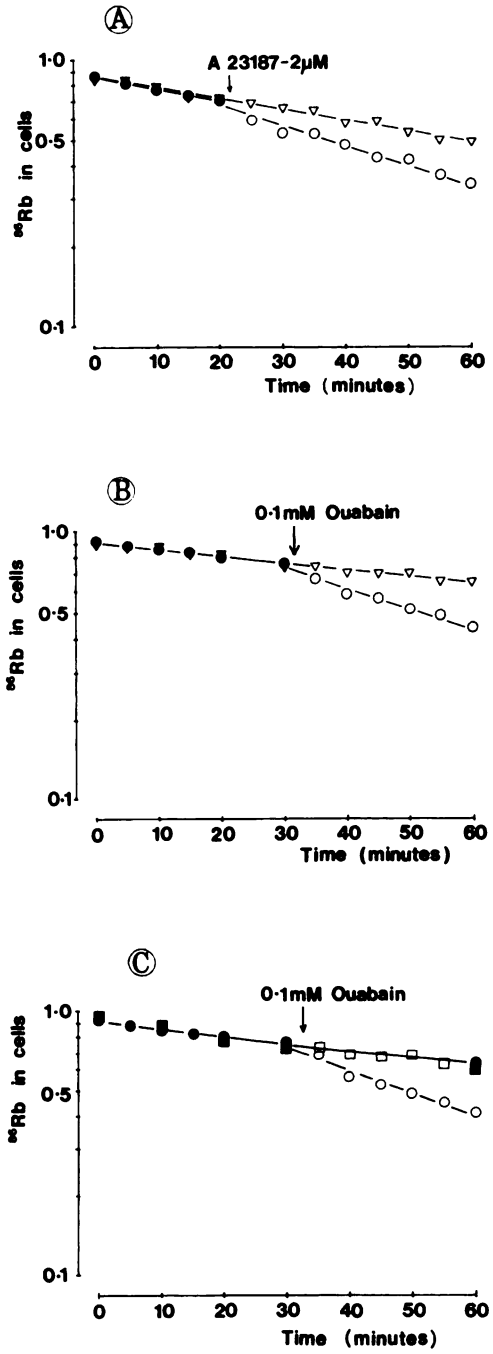

FIG. 10. *Efflux of ^{86}Rb from isolated chromaffin cells under a variety of conditions*

A. Evidence that a rise in intracellular calcium stimulates ^{86}Rb efflux from medullary cells. Cells were resuspended in solution containing either 0.1 mM calcium (●, ○) or 1 mM ethylene glycol bis(β-aminoethyl ether)*N,N,N'N'*-tetracetic acid (EGTA) (▼, ▽) and ^{86}Rb efflux was monitored for the next 20 min. After this time, A 23187 (2 μM) was added to all cells (*open symbols*). Although the rate of ^{86}Rb efflux from cells in EGTA remained unchanged, that from the cells in calcium was increased by 68%.

B. The effects of 10^{-4} M ouabain on the efflux of ^{86}Rb from medullary cells incubated in sodium (●, ○) or lithium (▼, ▽). Cells were resuspended in the appropriate medium and efflux was measured for 30 min. Efflux rates were identical in both sodium and lithium. Ouabain was added after 30 min (*open symbols*) and stimulated the rate of efflux from cells incubated in sodium. There was no change in the rate of efflux from the cells deprived of sodium.

C. The effect of preloading cells with the calcium chelator Quin 2 on the stimulation of ^{86}Rb efflux by ouabain. Cells were prepared in the usual way and then resuspended in either normal sodium-Locke's solution (●, ○) or in sodium-Locke's solution containing 50 μM Quin 2 (■, □). The cells were loaded with ^{86}Rb for 30 min before being washed thoroughly. All cells were subsequently resuspended in control sodium-Locke's solution. ^{86}Rb efflux was monitored during the ensuing 30-min period; efflux was identical in both sets of cells. After 30 min, 10^{-4} M ouabain was added to all the cells (*open symbols*). Efflux of ^{86}Rb was stimulated as before in the control cells, but was unchanged in the cells which had been preexposed to Quin 2.

solutions. Values obtained with the isotope (which are calculated on the assumption that the intracellular calcium concentration of chromaffin cells is 1 mM) are consistently higher than those arrived at by using either of the other methods of monitoring net loss. One possible explanation for this discrepancy could be that much of the calcium inside the cells is bound or sequestered and that during the time of ^{45}Ca loading the isotope does not uniformly label the intracellular pool of bound calcium.

2. Potassium permeability of chromaffin cells. Many animal cells possess a calcium-controlled potassium transport system (21) such that when free intracellular calcium is raised potassium permeability is increased. The system has been studied extensively in red blood cells (26, 27) and evidence for its existence in adrenal medullary cells has recently appeared (28). Mention was made earlier of the finding that ouabain appears to stimulate ^{86}Rb efflux from chromaffin cells (Table 2), and this phenomenon may now be reexamined in the light of the results reported here concerning calcium transport in ouabain-treated cells. Figure 10A presents some data which suggest that chromaffin cells possess a component of potassium permeability which is controlled by the level of cytosolic calcium. ^{86}Rb efflux from preloaded cells was monitored in the presence and absence of extracellular calcium. In both cases, the rate constant for ^{86}Rb exit was 0.0055/min. After 20 min, the calcium ionophore A 23187 (2 μM) was added to both sets of cells. Efflux was stimulated by 68% in the calcium-containing medium but was unaffected in cells deprived of extracellular calcium.

Figure 10B shows the effect of ouabain on the efflux of ^{86}Rb from cells. Efflux from cells exposed to ouabain in the presence of extracellular sodium was stimulated by more than 100% in this experiment. Replacement of sodium by lithium, however, abolished this stimulation completely.

These experiments argue rather forcefully in favor of the idea that ouabain brings about a rise in the free intracellular calcium concentration of adrenal medullary cells. Moreover the sodium dependence of secretion, inhibition of ^{45}Ca efflux, and stimulation of ^{86}Rb efflux indicates that all of these effects of the glycoside may be linked.

3. Experiments with Quin 2. The experiment illustrated in Fig. 10C provides a final piece of evidence to support a rise in free intracellular calcium in response to ouabain. In the previous paper (figure 10 in ref. 1) it was shown that pretreatment of chromaffin cells with the permeable acetoxymethyl ester of the calcium-chelating agent Quin 2(29) partially inhibited the secretory response of chromaffin cells to both ouabain and acetylcholine. Figure 10C shows that the stimulation of ^{86}Rb

efflux produced by ouabain is also inhibited by Quin 2, presumably as a consequence of its calcium-chelating properties.

The experiments described here seem to suggest that ouabain promotes catecholamine secretion from adrenal medullary cells by producing an increase in the resting level of cytosolic ionized calcium. It is probable that this rise is brought about by a direct effect of the glycoside on the calcium-extrusion mechanism of adrenal medullary cells.

DISCUSSION

The two main aims of this research have been to characterize the important features of sodium and calcium pump activity in adrenal medullary cells and to examine the possibility of links between effects on these pumps and the release of catecholamines evoked by the exposure of cells to cardiac glycosides such as ouabain. The use of a preparation of isolated medullary cells has allowed the first detailed studies of ion fluxes to be carried out in this tissue. These have shown that chromaffin cells possess a sodium pump which is highly sensitive to ouabain and which, in general terms, seems to behave in a fashion similar to the sodium pumps of invertebrate nerve and erythrocytes.

From values of ouabain-sensitive fluxes it is possible to show that the sodium pump drives an exchange of roughly three sodium ions to two potassium ions, a stoichiometry similar to that observed in nerve (18), muscle (30), and erythrocytes (17). Further evidence to suggest that two potassium ions interact at the external sites comes from kinetic analysis of the activation of ^{86}Rb influx and ^{24}Na efflux by external potassium. There is no evidence to indicate that very low concentrations of ouabain stimulate sodium pump activity in this tissue, as has been reported in heart by some authors (19, 20).

One of the most important findings to emerge from this study of sodium pump activity is that ouabain inhibits the pump quite normally in the complete absence of extracellular sodium. This finding requires some discussion in the light of certain other work; for instance, the rate of [^3H]ouabain binding to squid axons is sodium-dependent. If this also applied to adrenal medullary cells, one might expect to see impaired pump inhibition in cells exposed to ouabain in sodium-deficient media. The results of Fig. 3A show that this is not the case. However, it is possible that the time course of binding is somewhat altered by the omission of sodium, but this has not been studied in detail because in all of these experiments the cells were preincubated with ouabain for several minutes prior to starting the flux measurements.

The data obtained from the study of glycoside action on the chromaffin cell sodium pump enable us to examine critically some of the hypotheses which have been suggested to explain the enhanced release of secretory product elicited by this drug. It has been suggested that there is a direct link between sodium pump inhibition and secretion (5, 6). In view of the results reported here this now seems highly unlikely because (at least in medullary cells) sodium pump inhibition can be dissociated from catecholamine release by the removal of extracellular sodium.

Doubt is also cast upon the hypothesis that a rise in intracellular sodium is responsible for bringing about secretion, either directly, or by increasing intracellular calcium in accordance with a conventional sodium-calcium exchange system. The time course of the rise in intracellular sodium, which is rather slow and progressive, is difficult to reconcile with the virtually instantaneous and subsequently stable stimulation of secretion. Furthermore, the present study provides no evidence for the operation of sodium-calcium exchange in chromaffin cells. Both ^{45}Ca efflux and catecholamine secretion remain unchanged when either sodium or calcium is removed from the extracellular medium and ^{45}Ca efflux is not inhibited by the rise in intracellular sodium associated with anoxia. ^{45}Ca influx is unaffected by sodium-removal, and ouabain-insensitive sodium efflux is unchanged by the removal of extracellular calcium. These results might suggest not only that Na/Ca exchange is absent but also that Ca/Ca exchange is insignificant under normal conditions in these cells. These results are in sharp contrast with those of Sorimachi *et al.* (32), who report an increase in ^{45}Ca influx in response to ouabain. This discrepancy is difficult to explain at present.

However, the absence of sodium-calcium exchange does not completely rule out a role for intracellular sodium in the mechanism of glycoside action. An increased intracellular sodium concentration might bring about the release of calcium from mitochondria or other intracellular stores, as has been reported by Crompton *et al.* (33). Although it has not been possible to test this directly, the behavior of anoxic cells would seem to argue against a mediatory role of this kind for sodium. In the presence of full extracellular sodium anoxia causes no increase in the resting level of catecholamine secretion even though the sodium pump is completely blocked and intracellular sodium is elevated. Furthermore, the addition of ouabain to anoxic cells stimulates basal secretion in the usual way, although the pump is not inhibited further. In addition to anoxia, the combination of 2-deoxyglucose and cyanide, which inhibits both the sodium pump and the secretory response of the cells to carbamylcholine, is also apparently unable to stimulate basal secretion.

It appears, therefore, that a rise in intracellular sodium may be dissociated from an increase in the rate of catecholamine secretion from chromaffin cells, rendering it extremely unlikely that there is a simple link between pump inhibition (or raised intracellular sodium) and catecholamine secretion.

Instead, there seems to be a good correlation between the effects of ouabain on secretion and the inhibition of calcium efflux by the glycoside. This is illustrated in Fig. 11, which shows data compiled from several of the experiments described in this paper to show the temporal relationships between secretion, ^{45}Ca efflux, membrane potential, and changes in intracellular sodium and potassium concentrations. Although both internal ion levels and potential change slowly and progressively, the inhibition of ^{45}Ca efflux by ouabain has a time course very similar to that of the stimulation of secretion.

Flux data are often difficult to interpret accurately. For example, an increase in the rate of efflux of an ion need not necessarily reflect a rise in its intracellular concentration. Indeed, it may be the result of direct

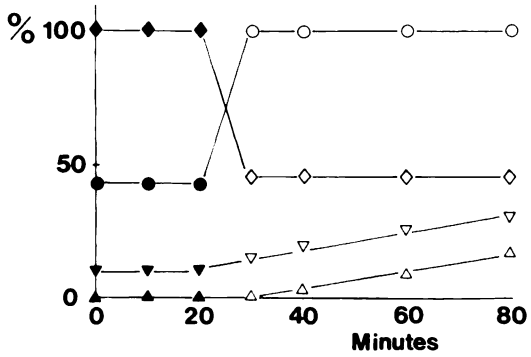

FIG. 11. *Comparison of the time-courses of the effects of ouabain on ^{45}Ca efflux (♦, ◇), catecholamine secretion (●, ○), intracellular sodium (▼, ▽), and the fluorescent signal from the dye $DiSC_3$ (6) (▲, △) in isolated adrenal medullary cells*

The data have been compiled from a number of different experiments and normalized in the following way. The rate of ^{45}Ca efflux under control conditions is assigned a value of 100%. Ouabain reduces the rate to 48% of this value. The rate of catecholamine secretion seen after the addition of ouabain to the cells is assigned a value of 100%. The secretion of untreated cells is 42% of this value. The fluorescence of the cells before the addition of glycoside is assigned a value of zero. Ouabain then induces an increase in the fluorescence corresponding to a gradual depolarization of the cells. After 60 min of ouabain treatment, the fluorescence has increased by 16%. The level of intracellular sodium in untreated control cells has been called 10%. Ouabain then raises internal sodium 2.6-fold over the next 60 min. Glycoside is added at 20 min as indicated by the *open symbols*.

activation of the efflux mechanism which could then reduce the intracellular concentration. Influx rates may also change. Fortunately, the results obtained in the present study are relatively straightforward since, in association with the clear-cut fall in efflux rate following ouabain treatment, there is no corresponding change in influx. We may assume, therefore, that unless ouabain also enhances the sequestration of calcium by intracellular organelles (a possibility rendered unlikely by the experiments on ^{86}Rb efflux and those involving Quin 2) the net result of these changes will be a rise in intracellular calcium (consistent with the observed increase in secretory activity). Moreover, this rise is brought about without the need for extracellular calcium, a discovery which is also consistent with the data on secretion.

The inhibitory action of ouabain seems clearly established and, since sodium-calcium exchange is virtually ruled out, it is reasonable to assume that the glycoside exerts its action on a membrane ATPase system which pumps calcium out of the cell. This is a very surprising finding in view of the apparent absence of glycoside-sensitivity in any other calcium ATPase system previously studied. It must be pointed out that calcium pumping has not generally been the subject of detailed study in mammalian secretory cells (red cells and squid axon being the most popular), and the intriguing possibility that ouabain-sensitive calcium efflux is a property peculiar to such tissue cannot be excluded.

The major problem now lies in elucidating the mechanism of this inhibitory action of ouabain. Any hypothesis must be able to explain the sodium dependence of inhibition. The close parallel between the glycoside concentrations which bring about half-maximal inhibition of both sodium pumping and calcium pumping (as well as enhancement of secretion) raises the possibility that the interaction between ouabain and the sodium-potassium ATPase receptor may somehow influence the calcium ATPase. Perhaps the two enzymes are spatially arranged in the chromaffin cell membrane so as to permit such an interaction when sodium is also present. Interaction of this kind has been reported recently for the (Na^+-K^+)-ATPase and lysolecithin acyltransferase systems of mitogen-stimulated rabbit thymocytes (34).

The findings reported in this paper suggest strongly that there might be a rise in free intracellular calcium when glycosides are administered to adrenal medullary cells. The finding that ouabain stimulates ^{86}Rb efflux in a fashion similar to the calcium ionophore A 23187 and that the calcium chelator Quin 2 blocks this stimulation lends further support to this conclusion. One aspect of the data that is less easily explained is the ability of D-600 (the calcium-channel blocker) to reduce the secretory effect of ouabain. D-600 has no effects of its own on ^{45}Ca efflux but might be expected to reduce any inward leak of calcium ions through D-600-sensitive channels and the secretory effect of ouabain, were this dependent upon a continuous inward leak of calcium. The insensitivity of the ouabain effect to external calcium makes this seem unlikely and we must assume that D-600 is having some other nonspecific effect on either the calcium metabolism or the secretory mechanism of the cells.

Although the findings reported here concern the effects of ouabain on a secretory tissue, it must not be forgotten that the most well-known effect of cardioactive drugs is their ability to increase the force of contraction of failing heart muscle. There has been considerable argument concerning the mechanism of this effect. Although it is unwise to extrapolate too freely between widely differing tissues, the present findings raise the exciting possibility that the positive inotropicity of digitalis-glycosides may also be a consequence of alterations in calcium pump activity either independently of or, as in the present case, in conjunction with sodium pump inhibition. Nevertheless, it is important to remember that although the chromaffin cell appears to lack a significant sodium-calcium exchange system, such a system is well characterized in heart muscle and other tissues. Therefore, it is possible that cardiac glycosides are able to exert their secretory or contractile effects via sodium-calcium exchange in such tissues and that any additional effect on the calcium pump resembling that in the adrenal medulla may be masked or absent.

ACKNOWLEDGMENT

I thank Professor P. F. Baker for helpful discussion.

REFERENCES

1. Pocock, G. Ionic and metabolic requirements for stimulation of secretion by ouabain in bovine adrenal medullary cells. *Mol. Pharmacol.* **23**:671–680 (1983).
2. Birks, R. I., and M.W. Cohen. The action of sodium pump inhibitors on neuromuscular transmission. *Proc. R. Soc. Lond. B Biol. Sci.* **170**:381–399 (1968).
3. Baker, P. F., and A. C. Crawford. A note on the mechanism by which inhibitors of the sodium pump accelerate spontaneous release of transmitter from motor nerve terminals. *J. Physiol. (Lond.)* **247**:209–226 (1975).

4. Milner, R. D. G., and C. N. Hales. The sodium pump and insulin secretion. *Biochim. Biophys. Acta* **135**:375–377 (1967).
5. Paton, W. D. M., E. S. Vizi, and M. Aboo Zar. The mechanism of acetylcholine release from parasympathetic nerves. *J. Physiol. (Lond.)* **215**:819–848 (1971).
6. Vizi, E. S. Stimulation by inhibition of (Na^+-K^+-Mg^{2+})-activated ATPase, of acetylcholine release in cortical slices from rat brain. *J. Physiol. (Lond.)* **226**:95–117 (1972).
7. Ozawa, H., and T. Katsuragi. Ouabain-induced potentiation on the contraction of the guinea-pig vas deferens. *Eur. J. Pharmacol.* **25**:147–154 (1974).
8. Banks, P. The effect of ouabain on the secretion of catecholamines and on the intracellular concentration of potassium. *J. Physiol. (Lond.)* **193**:631–637 (1967).
9. Baker, P. F., M. P. Blaustein, A. L. Hodgkin, and R. A. Steinhardt. The influence of calcium on sodium efflux in squid axons. *J. Physiol. (Lond.)***200**:431–458 (1969).
10. Reuter, H. Exchange of calcium ions in the mammalian myocardium. Mechanisms and physiological significance. *Circ. Res.* **34**:599–605 (1974).
11. Rink, T. J. The influence of sodium on calcium movements and catecholamine release in thin slices of bovine adrenal medulla. *J. Physiol. (Lond.)***266**:297–325 (1977).
12. Pocock, G. The action of ouabain on calcium fluxes and catecholamine secretion in cells isolated from bovine adrenal medulla. *J. Physiol. (Lond.)***307**:38–39P (1980).
13. Pocock, G. Parallel measurement of sodium pump activity and catecholamine release in cells isolated from bovine adrenal medulla. *J. Physiol. (Lond.)* **296**:102–103P (1979).
14. Baker, P. F., and D. E. Knight. Calcium control of exocytosis and endocytosis in bovine adrenal medullary cells. *Philos. Trans. R. Soc. Lond. B* **296**:83–103 (1981).
15. Cohen, L. B., and B. M. Salzburg. Optical measurement of membrane potential. *Rev. Physiol. Biochem. Pharmacol.* **83**:35 (1978).
16. Von Euler, U. S., and T. Floding. A fluorimetric micromethod for differential estimation of adrenaline and noradrenaline. *Acta. Physiol. Scand. Suppl. 118* **33**:45–56 (1955).
17. Sen, A. K., and R. L. Post. Stoichiometry and localization of adenosine triphosphate-dependent sodium and potassium transport in the erythrocyte. *J. Biol. Chem.* **239**:345–352 (1964).
18. Baker, P. F. Phosphorous metabolism of intact crab nerve and its relation to the active transport of ions. *J. Physiol. (Lond.)* **180**:383–423 (1965).
19. Ghysel-Burton, J., and T. Godfraind. Stimulation and inhibition of the sodium pump by cardioactive steroids in relation to their binding sites, and their inotropic effect on guinea pig isolated atria. *Br. J. Pharmacol.* **66**:175–184 (1979).
20. Cohen, I., J. Daut, and D. Noble. An analysis of the action of low concentrations of ouabain on membrane currents in Purkinje fibres. *J. Physiol. (Lond.)* **260**:75–103 (1976).
21. Meech, R. W. Intracellular calcium and the control of membrane permeability. *Symp. Soc. Exp. Biol. Med.* **80**:161–191 (1976).
22. Hanes, C. S. Studies on plant amylases: the effects of starch concentration upon the velocity of hydrolysis by the amylase of germinated barley. *Biochem. J.* **26**:1406–1421 (1932).
23. Winkler, M., and A. D. Smith. The chromaffin granule and the storage of catecholamines, in *Handbook of Physiology*, Sect. 7, Vol. 6: *The Adrenal Gland*. American Physiological Society, *Washington, D. C., Chap. 23*, 321–339 (1975).
24. Baker, P. F. The regulation of intracellular calcium. *Symp. Soc. Exp. Biol. Med.* **30**:67–88 (1976).
25. Schatzmann, H. J. Active calcium transport and Ca^{++}-activated ATPase in human red cells. *Curr. Top. Membr. Transport* **6**:125–168 (1975).
26. Simons, T. J. B. Resealed ghosts used to study the effect of intracellular calcium ions on the potassium permeability of human red cell membranes. *J. Physiol. (Lond.)* **246**:52–53P (1975).
27. Simons, T. J. B. Calcium-dependent potassium exchange in human red cell ghosts. *J. Physiol. (Lond.)* **256**:227–244 (1976).
28. Marty, A. Calcium-dependent potassium channels with large unitary conductance in chromaffin cell membranes. *Nature (Lond.)* **291**:497–500 (1981).
29. Tsien, R. Y. New calcium indicators and buffers with high selectivity against magnesium and protons: design, synthesis and properties of prototype structures. *Biochemistry* **19**:2396–2404 (1980).
30. Dyndynska, M., and E. J. Harris. Consumption of high-energy phosphates during active sodium and potassium interchange in frog muscle. *J. Physiol. (Lond.)* **182**:92–109 (1966).
31. Baker, P. F., and J. S. Willis. Inhibition of the sodium pump in squid giant axons by cardiac glycosides: dependence on extracellular ions and metabolism. *J. Physiol. (Lond.)* **224**:463–475 (1972).
32. Sorimachi, M., S. Nishimura, and K. Yamagami. Possible occurrence of Na^+-dependent Ca^{2+} influx mechanism in isolated bovine chromaffin cells. *Brain Res.* **208**:442–446 (1981).
33. Crompton, M., R. Moser, H. Ludi, and E. Carafoli. The interactions between the transport of sodium and calcium in mitochondria of various mammalian tissues. *Eur. J. Biochem.* **82**:25–31 (1978).
34. Szamel, M., S. Schneider, and K. Resch. Functional interrelationship between (Na^+-K^+)-ATPase and lysolecithin acyltransferase in plasma membranes of mitogen-stimulated rabbit thymocytes. *J. Biol. Chem.* **256**:9198–9204 (1981).

Send reprint requests to: Dr. Gillian Pocock, Department of Physiology, King's College, Strand, London WC2R 2LS, United Kingdom.