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CORRELATION BETWEEN CATECHOLAMINE RELEASE AND SODIUM PUMP INHIBITION IN THE PERFUSED ADRENAL GLAND OF THE CAT

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1 Ca^{2+} reintroduction to retrogradely perfused and ouabain (10^{-4} M)-treated cat adrenal glands caused a catecholamine secretory response which was greater the longer the time of exposure to the cardiac glycoside. Such a response was proportional to the external Na^+ concentration $[Na^+]_o$.

2 A qualitatively similar, yet smaller response was observed when glands were perfused with Krebs solution lacking K^+ ions; thus, K^+ deprivation mimicked the secretory effects of ouabain. Catecholamine secretion evoked by Ca^{2+} reintroduction in K^+-free solution (0-K^+) was also proportional to $[Na^+]_o$ and greater the longer the time of exposure of the gland to 0-K^+ solution.

3 The ionophore X537A also mimicked the ouabain effects, since Ca^{2+} reintroduction to glands treated with this agent (25 μM) caused a sharp secretory response. When added together with X537A, ouabain (10^{-4} M) did not modify the response to the ionophore.

4 N-ethylmaleimide (NEM), another Na^+, K^+-ATPase inhibitor, did not evoke the release of catecholamines; on the contrary, NEM (10^{-4} M) inhibited the catecholamine secretory response to high $[K^+]_o$, acetylcholine, Ca^{2+} reintroduction and ouabain.

5 Ouabain (10^{-4} M) inhibited the uptake of ^{86}Rb into adreno-medullary tissue by 60%. Maximal inhibition had already occurred 2 min after adding the drug, indicating a lack of temporal correlation between ATPase inhibition and the ouabain secretory response, which took longer (about 30–40 min) to reach its peak. NEM (10^{-4} M) blocked ^{86}Rb uptake in a similar manner.

6 The results are further evidence in favour of the presence of a Na^+-Ca^{2+} exchange system in the chromaffin cell membrane, probably involved in the control of $[Ca^{2+}]_i$ and in the modulation of catecholamine secretion. This system is activated by increasing $[Na^+]_i$, either directly (ionophore X537A, increased $[Na^+]_o$) or indirectly (Na^+ pump inhibition). However, the simple inhibition of Na^+ pumping does not always lead to a catecholamine secretory response; such is the case for NEM.

Introduction

In addition to their well known inotropic effects on cardiac muscle, cardiac glycosides favour the release of catecholamines from the bovine adrenal gland (Banks, 1967), cat spleen (Kirpekar, Prat & Yamamoto, 1970; Garcia & Kirpekar, 1973a,b), guinea-pig vas deferens (Nakazato, Ohga & Onoda, 1978), rabbit heart (Lindmar & Loffelholz, 1974), cat adrenal gland (García, Hernández, Horga, & Sánchez-García, 1980; Esquerro, García, Hernández. Kirpekar & Pratt, 1980; García, García-Lopez, Horga, Kirpekar, Montiel & Sánchez-García, 1981) and isolated bovine chromaffin cells (Aunis & García, 1981).

Since cardiac glycosides have a clear specific inhibitory action on Na^+-K^+-ATPase (Lee & Klaus, 1971; Schwartz, 1976; Akera & Brody, 1978) it has been postulated that Na^+-K^+-ATPase is the pharmacological receptor for cardiac glycosides. On the basis of this assumption, the catecholamine releasing effects of ouabain have generally been associated with the membrane ATPase (García & Kirpekar, 1973a,b; 1975a,b; Duncan, 1977; Nakazato *et al.*, 1978; García *et al.*, 1980; Esquerro *et al.*, 1980; Aunis & García, 1981).

As a result of ATPase inhibition, a change in the distribution of monovalent cations would lead to an increase in intracellular Na^+ concentration ($[Na]_i$) reducing the Na^+-dependent Ca^{2+} efflux and increasing the Ca^{2+} uptake in exchange for Na^+ loss (Baker, 1972). Evidence favouring this view has recently been obtained by García *et al.* (1980) and Esquerro *et al.* (1980) in the perfused adrenal gland of the cat.

If this were indeed the case then one would expect that all procedures leading to an increase in $[Na]_i$ by non-glycosidic inhibition of Na^+, K^+-ATPase (Ku,

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Akera, Pew & Brody, 1974) or by a more direct enhancement of Na^+ entry by ionophores (Sutko, Besch, Bailey, Zimmerman & Watanabe, 1977; Pascual, Horga, Sánchez-García & García, 1977) would also increase the release of catecholamines from the cat adrenal gland by activation of the Na^+-Ca^{2+} exchange mechanism.

These experiments were undertaken in order to determine whether exposure to N-ethylmaleimide (NEM) or the ionophore X537A, or K^+ deprivation procedures which will allow the accumulation of intracellular Na^+ by different mechanisms, will evoke the release of catecholamines from the perfused adrenal gland of the cat as ouabain does. ^{86}Rb uptake by the chromaffin tissue was also studied in order to have an index of the degree of Na^+, K^+-ATPase inhibition (and of Na^+ pump depression) produced by such procedures.

Methods

Both adrenal glands of the cat were isolated and prepared for retrograde perfusion with Krebs-bicarbonate solution at room temperature as previously described by García *et al.* (1980). The perfusion rate was about 1 ml/min.

Perfusion media

The normal Krebs bicarbonate solution had the following composition (mM): NaCl 119, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4.7H_2O$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2 and glucose 11. The solution was equilibrated with 95% O_2 and 5% CO_2, the final pH being 7.4. Ca^{2+}-free Krebs solution (0-Ca^{2+}) was obtained by omission of $CaCl_2$ without osmotic adjustment being made. Potassium-free solutions were obtained by omission of KCl and replacement of KH_2PO_4 by NaH_2PO_4. Twenty-five mM Na^+ Krebs-solution was prepared by substituting NaCl by iso-osmolar amounts of sucrose or choline chloride. High Na^+ Krebs solution (263 mM) was prepared by adding 119 mM NaCl to normal Krebs solution. In control glands the osmolarity was maintained by the addition of appropriate amounts of sucrose.

Collection of perfusate samples

After 1 h of initial perfusion with normal Krebs solution, the glands were additionally perfused for a 20 min period with Ca^{2+}-free Krebs solution. Samples were collected for 2 min each in chilled tubes to determine the resting secretion of catecholamines. Finally the glands were perfused with a Ca^{2+}-free medium containing 0-K^+, the ionophore X537A, or N-ethylmaleimide for the rest of the experiment. Calcium (2.5 mM) was reintroduced for 4 min, every 10 min in all experimental designs; perfusate samples were continuously collected at 2 min intervals.

Catecholamine assay

Total catecholamine content of the samples (noradrenaline plus adrenaline) was determined according to Shellenberger & Gordon (1971) without further purification on alumina. Catecholamine values are expressed as μg/2 min perfusion period. Net release of catecholamines was calculated by subtracting the basal, spontaneous release from the release evoked by Ca^{2+} reintroduction; these values are expressed as μg/10 min period.

Rubidium (^{86}Rb) uptake measurements

Uptake of K^+ by cardiac tissue provides a useful measure of the activity of the Na^+-K^+-ATPase-mediated monovalent cation active transport system. $^{86}Rb^+$ is a convenient K^+ analogue commonly used as an indicator of potassium transport (Yamamoto, Akera & Brody, 1978). Therefore, changes in ^{86}Rb uptake were used in these experiments to ascertain the degree of ATPase inhibition and Na^+ pump activity, according to the method previously described by Yamamoto *et al.* (1978). Adrenal glands were initially perfused with normal Krebs for a 60 min period, then one gland of each cat was perfused with N-ethylmaleimide (10^{-4} M) or ouabain (10^{-4} M)-containing Krebs solution, for 2 to 30 min. The contralateral gland was kept as a control. At the end of this 30 min perfusion period, still in the presence of NEM or ouabain, traces of ^{86}Rb (800,000 ct min^{-1} ml^{-1}, Amersham), were added to both glands and the perfusion was continued for 5 additional min. The glands were then perfused with fresh Krebs solution for 10 min and finally removed from the perfusion system and prepared for ^{86}Rb analysis. The adrenal medulla was dissected out from the cortex, weighed, homogenized in 1 ml of cold 0.4 N perchloric acid and centrifuged at 27,000 *g* at 2°C for 15 min. Aliquots of the supernatants were then suspended in scintillation fluid and counted in a liquid scintillation spectrometer (Nuclear Chicago, model Mark II). The results are expressed as ct min^{-1} g^{-1} of tissue $\times 10^{-4}$ or as % of control non-treated glands.

Results

The effect of Ca^{2+} reintroduction on catecholamine output from glands perfused with 0-K^+ Krebs solution or ouabain

It has been demonstrated previously that Ca^{2+} rein-

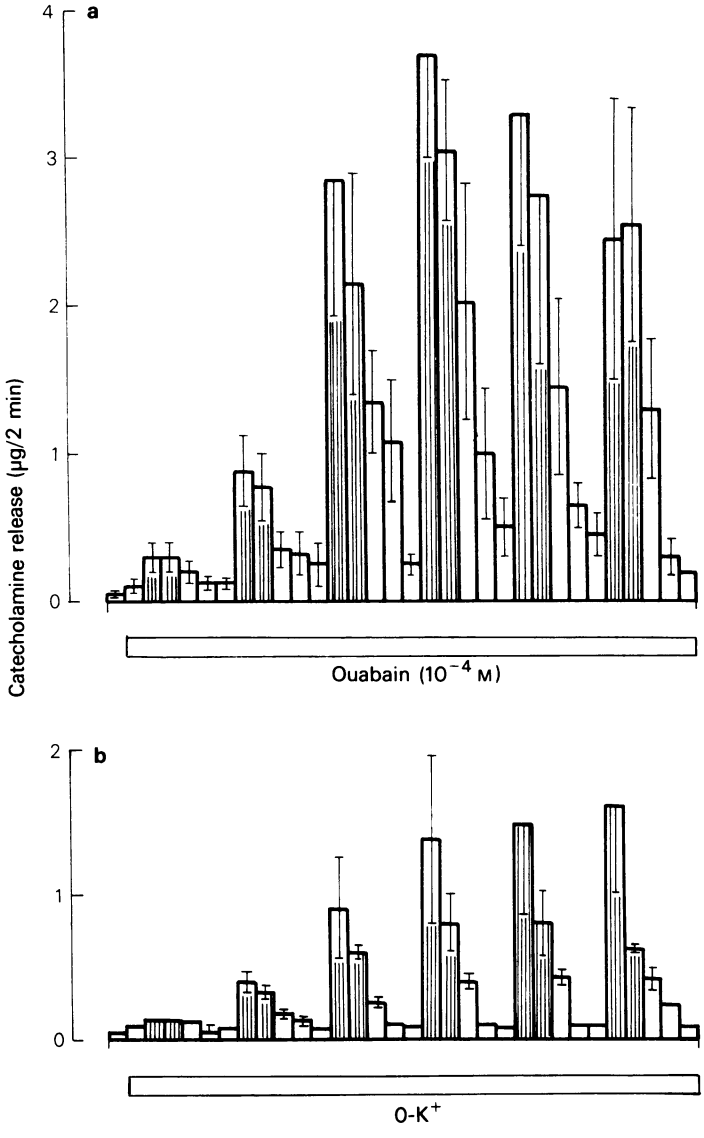

Figure 1 The effect of ouabain (a) and K^+ deprivation (b) on the catecholamine secretory response evoked by Ca^{2+} reintroduction. Adrenal glands of the cat were initially perfused with normal Krebs for 60 min and then with 0-Ca^{2+} solution for 20 min. Ouabain (10^{-4} M) treatment or K^+-deprivation were then initiated. Ca^{2+} was reintroduced every 10 min for 4 min periods (hatched column); 0-Ca^{2+} indicated by open columns. Results are expressed as μg/2 min collection period of total catecholamines released. Vertical lines are s.e.means of 4 experiments.

troduction in a ouabain-treated gland perfused with Ca^{2+}-free Krebs solution, evoked the release of catecholamines (García *et al.*, 1980; Esquerro *et al.*, 1980). However, the time course of such secretory response has not yet been studied. Figure 1 shows that the basal, spontaneous catecholamine release increased from as little as 0.1 μg/2 min to as much as 4 μg/2 min when Ca^{2+} (2.5 mM) was reintroduced in a gland pre-perfused with Ca^{2+}-free solution and treated with ouabain (10^{-4} M). Since K^+ deprivation is known to inhibit ATPase (Skou, 1957), it was of interest to see whether this procedure would increase the release of catecholamines when Ca^{2+} was reintroduced in glands previously perfused with Ca^{2+}-free Krebs solution. Glands were exposed to a K^+-free medium after being perfused for 20 min with

Ca^{2+}-free Krebs; Ca^{2+} (2.5 mM) was then reintroduced at different times after removal of K^+ from the medium. The results are summarized in Figure 1 which shows that Ca^{2+} reintroduction evoked a clear secretory response, qualitatively similar to that found in ouabain-treated glands. The catecholamine output increased progressively with time and reached its maximum at about 30 min. Such secretory response was not observed in control glands perfused with Ca^{2+}-free (but Mg^{2+} containing) Krebs solution.

The effect of external Na^+ concentration on the release of catecholamines evoked by Ca^{2+} reintroduction in glands perfused with 0-K^+ or ouabain

Esquerro *et al.* (1980) and García *et al.* (1980) have demonstrated that the secretory response induced by Ca^{2+} reintroduction in ouabain-treated glands was abolished when perfusion was carried out in low Na^+-Krebs solution (25 mM) and that it was greatly increased when $[Na]_o$ was raised to 263 mM. But the influence of the time of exposure to ouabain on the secretory response evoked by Ca^{2+} reintroduction was not analysed. We now present such experiments (Figure 2). Again, the secretory response was strictly dependent on $[Na^+]_o$. It is worth noting that at 263 mM Na^+, the secretory response was already maximal 2 min after the initiation of ouabain perfusion and then progressively declined.

In view of the results obtained with ouabain, it was of interest to study the effects of $[Na^+]_o$ on the release of catecholamines induced by Ca^{2+} reintroduction in glands perfused with K^+- and Ca^{2+}-free Krebs-solution. Experiments are summarized in Figure 3. Lowering $[Na^+]_o$ to 25 mM practically abolished the output of catecholamines, whereas raising $[Na^+]_o$ to 144 and 263 mM greatly potentiated the release induced by Ca^{2+} reintroduction. In this particular case the release was maximal at the first Ca^{2+} exposure and then declined progressively, reaching values similar to that found in glands perfused with normal Krebs in the third Ca^{2+} stimulus, in spite of the fact that raised $[Na^+]_o$ was maintained throughout the experiment.

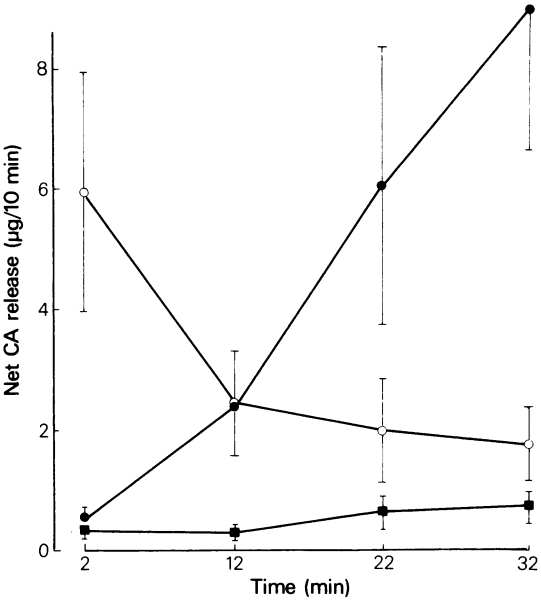

Figure 2 Time course of the catecholamine secretory response to Ca^{2+} reintroduction in ouabain-treated adrenal glands of the cat: effects of the external Na^+ concentration. After the initial perfusion period (60 min with normal Krebs solution) each group of glands was perfused with Krebs solution containing ouabain (10^{-4} M), no Ca^{2+} and one of three different $[Na^+]_o$: (○) 263 mM; (●) 144 mM and (■) 25 mM. Ca^{2+} (2.5 mM) was reintroduced every 10 min for 4 min periods at the time shown on the abscissa scale. Vertical lines are s.e.means of 5 experiments for each Na^+ concentration. Ordinate scale: net catecholamine (CA) release per 10 min perfusion period.

The effect of Ca^{2+} reintroduction on catecholamine release in glands treated with the ionophore X537A

Since the ionophore X537A increases the permeability of biological and artificial membranes to Na^+ and K^+ (Schadt & Haeusler, 1974) treatment of tissue with this drug could eventually lead to an ionic redistribution similar to that found after Na^+-K^+-ATPase inhibition by cardiac glycosides or K^+ deprivation (Pascual *et al.*, 1977). Therefore it was of interest to

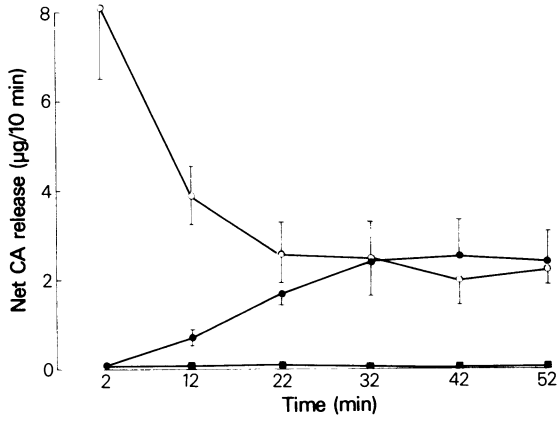

Figure 3 Time course of the catecholamine (CA) secretory response to Ca^{2+} reintroduction in cat adrenal glands perfused with 0-K^+ Krebs solution: effects of the external Na^+ concentration: (○) 263 mM; (●) 144 mM and (■) 25 mM $[Na^+]_o$. Experimental design as in Figure 2, except that K^+ deprivation, instead of ouabain, was used. Vertical lines are s.e. means of 6 experiments.

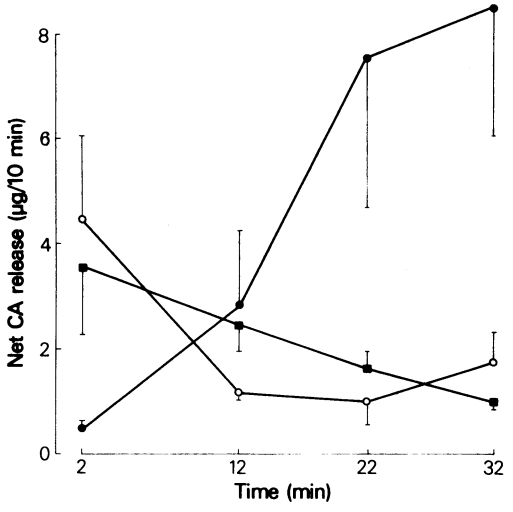

Figure 4 The effect of ionophore X537A, on the catecholamine secretory response evoked by Ca^{2+} reintroduction. Adrenal glands were initially perfused with normal Krebs for 60 min and then with Krebs containing X537A (2.5×10^{-5} M) alone (○), ouabain alone (●) or ouabain (10^{-4} M) plus ionophore (■) for the rest of the experiment. Secretory responses to Ca^{2+} reintroduction were obtained as indicated in Figure 2. Vertical lines are s.e.means of 4 experiments.

explore the effect of Ca^{2+} reintroduction on the release of catecholamines in adrenal glands treated with X537A and perfused with Ca^{2+}-free Krebs solution. Glands were exposed to the ionophore X537A (25 µM) for 20 min and then Ca^{2+} (2.5 mM) was reintroduced for 4 min every 10 min. Figure 4 indicates that Ca^{2+} reintroduction greatly increased the output of catecholamines in X537A-treated glands. The secretory response had already reached a maximum at the first Ca^{2+} reintroduction and then quickly declined. The effects of ouabain (10^{-4} M) develop much more slowly. The combination of ouabain plus X537A did not change the pattern of catecholamine release seen after the ionophore alone.

The effect of N-ethylmaleimide on the release of catecholamines

N-ethylmaleimide (NEM), a sulphydryl blocking agent, is known to inhibit Na^+, K^+-ATPase in a variety of tissues (Skou, 1963; Banerjee, Wong, Khanna & Sen, 1972). Therefore, and since the catecholamine releasing effects of ouabain have been associated to its properties of inhibiting Na^+,K^+-ATPase, it was considered of interest to explore the possibility that NEM would behave in a similar way.

Experiments were carried out on both adrenal glands of the same cat. One of the glands was perfused with Krebs containing NEM (10^{-4} M) and the contralateral gland with ouabain (10^{-4} M). Ouabain caused the usual increase in catecholamine output which seems to reach its maximum at about 30 min; in contrast, NEM-treated glands did not show any change of the basal secretion of catecholamines during the same period of time.

Ca^{2+} reintroduction to ouabain-treated glands caused a progressive catecholamine secretory response. However, Ca^{2+} reintroduction to glands perfused with Ca^{2+}-free Krebs containing NEM (10^{-4} M) did not modify the basal output of catecholamines (Figure 5).

It is important to note that N-ethylmaleimide treated glands, which fail to increase the basal release of catecholamines, or to secrete when Ca^{2+} is reintroduced, also lack the capacity to respond with an increase of catecholamine secretion when they receive additional treatment with ouabain (10^{-4} M), high $[K^+]_o$ (143 mM) or acetylcholine (10^{-4} M).

Effects of ouabain and N-ethylmaleimide on ^{86}Rb uptake by adrenal glands

As previously stated ^{86}Rb uptake is a good indicator of K^+ transport and therefore provides a useful measure of the activity of the Na^+, K^+-ATPase mediated monovalent cation transport (Na^+ pump). On the basis of this assumption and in order to evaluate the extent of ATPase inhibition evoked by ouabain (10^{-4} M) and NEM (10^{-4} M), experiments were carried out to quantitate ^{86}Rb uptake by normal glands and those treated with ouabain or NEM. In all cases, both glands of the same cat were used and the ^{86}Rb uptake was measured in the presence or in the ab-

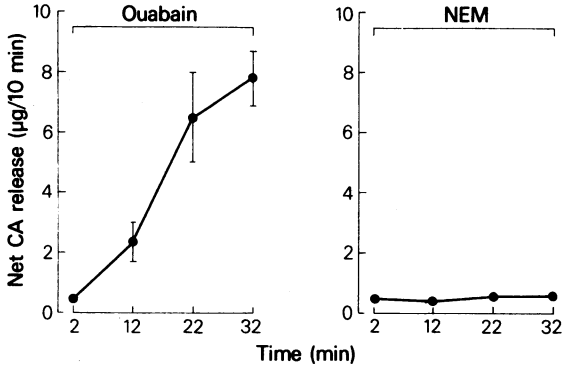

Figure 5 Comparative effects of ouabain and N-ethylmaleimide (NEM) on the release of catecholamines (CA) evoked by Ca^{2+} reintroduction. Cat adrenal glands were perfused with 0-Ca^{2+} Krebs solution in the presence of ouabain (10^{-4} M) or NEM (10^{-4} M). Ca^{2+} (2.5 mM) was reintroduced every 10 min for 4 min periods at the times shown on the abscissa scale. Means of 8 experiments; vertical lines show s.e.mean.

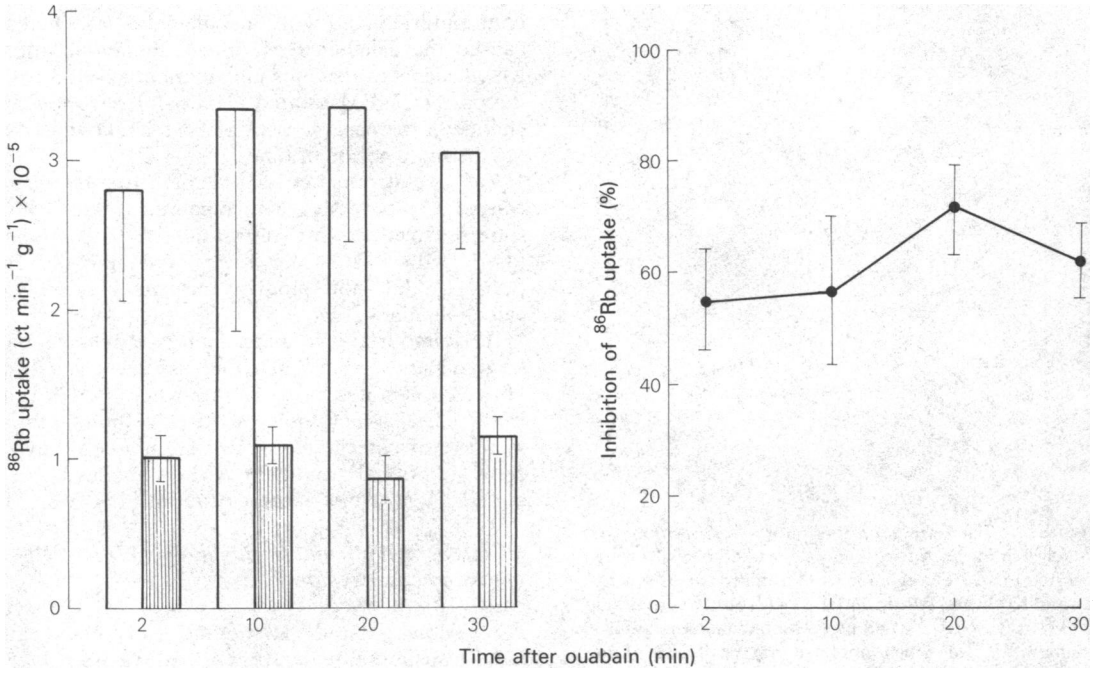

Figure 6 Time course of the blockade of ^{86}Rb uptake evoked by ouabain (10^{-4} M) in the perfused cat adrenal gland. ^{86}Rb was introduced in the perfusion system 2, 10, 20 and 30 min after ouabain, and maintained in contact with the gland for 5 min. A 10 min wash out period with fresh Krebs solution followed. Open columns, control glands; hatched columns, contralateral, ouabain-treated glands. Vertical lines represent s.e.means of 6 experiments.

sence of ouabain or NEM. ^{86}Rb uptake was measured 2 to 30 min after beginning the perfusion with ouabain or NEM; at 30 min, secretion of catecholamines induced by ouabain had usually reached its maximum. ^{86}Rb uptake in the absence and in the presence of ouabain (10^{-4} M) is shown in Figure 6. Initiation of the blockade is very fast, since 2 min after the start of the ouabain perfusion, the inhibition of ^{86}Rb uptake by the medullae was about 60%. The degree of the blockade is similar at different subsequent time periods, up to 30 min. It is, therefore, interesting to note that the time courses of the inhibition of ^{86}Rb uptake and that of the catecholamine secretory response to Ca^{2+} reintroduction in ouabain-treated glands (Figures 1, 2 and 4) are completely different.

The blockade of ^{86}Rb uptake and therefore the extent of ATPase inhibition induced by ouabain and NEM, was similar (Figure 7). This finding excludes the lack of ATPase inhibition as a cause for the inability of NEM to increase the release of catecholamines in the perfused adrenal gland.

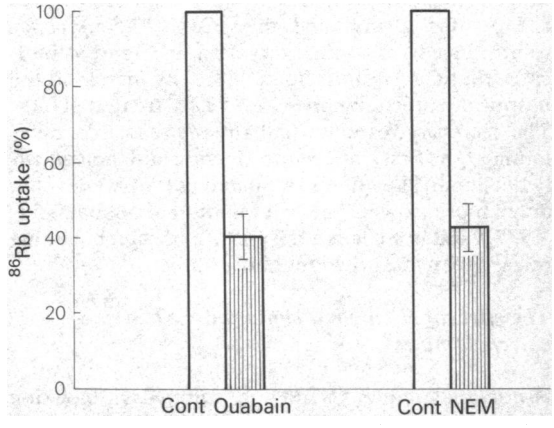

Figure 7 Effect of ouabain (10^{-4} M) and N-ethylmaleimide (NEM, 10^{-4} M) on the ^{86}Rb uptake by adrenal glands. ^{86}Rb was added to perfusion fluid 30 min after ouabain or NEM and was maintained for 5 min. The glands were finally perfused with ouabain or NEM containing normal Krebs and then removed from the perfusion system. ^{86}Rb uptake by the medullae in the presence of ouabain or NEM is expressed as % of controls. Vertical lines indicate s.e.means of 4 experiments.

Discussion

Baker, Blaustein, Hodgkin & Steinhardt (1969) demonstrated that the resting Ca^{2+} efflux in the perfused giant axon of the squid could be augmented by an increase in external Na^+ concentration ($[Na^+]_o$), and presented evidence that movements of Na^+ and Ca^{2+} across the membrane are linked to a Na^+-Ca^{2+} exchange mechanism. Recent evidence from our laboratory (García, *et al.*, 1980; Esquerro *et al.*, 1980; Aunis & García, 1981) indicates that the same Na^+-Ca^{2+} exchange mechanism might also be involved in the control of the intracellular concentration of ionized Ca^{2+} levels ($[Ca^{2+}]_i$) and, therefore, in the modulation of catecholamine release from the chromaffin cell. Any procedure leading to an increase in $[Na^+]_i$ will activate this system. The most widely used procedure has been the inhibition of the membrane Na^+, K^+-ATPase by cardiac glycosides; any other agents (NEM, vanadium compounds) or ionic manipulation (K^+ deprivation) which cause inhibition of the enzyme, or directly affect the asymmetric distribution of Na^+ and K^+ across biological membranes (ionophore X537A), should also cause activation of the Na^+-Ca^{2+} system and enhance catecholamine release.

The results described here support the hypothesis that an increase in $[Na^+]_i$ induced by non-glycoside inhibition of ATPase (i.e., potassium deprivation) or by a direct enhancement of Na^+ entry into the chromaffin cell produced by the ionophore X537A, is related to the ability of Ca^{2+} reintroduction to evoke an increase of the catecholamine output in adrenal glands perfused with Ca^{2+}-free Krebs solution. This idea is supported by the observation that lowering $[Na^+]_o$ almost completely abolished the secretion evoked by Ca^{2+}, whereas increasing $[Na^+]_o$ greatly increased the catecholamine secretion in K^+-free perfused adrenal glands.

The results of our experiments also indicated that ATPase inhibition is not always required to promote an increase in the Na^+-Ca^{2+} exchange mechanism, provided that a similar ionic redistribution across the membrane occurs, as seems to be the case in preparations treated with the ionophore X537A (Pascual *et al.*, 1977).

N-ethylmaleimide, an alkylating agent that binds covalently to sulphydryl groups, inhibits the Na^+,K^+-ATPase in a variety of tissues (Skou, 1963; Banerjee *et al.*, 1972). Our results indicate that NEM (10^{-4} M) and ouabain (10^{-4} M) produce a similar degree of ^{86}Rb uptake blockade in the adrenal gland and therefore of monovalent cation transport and ATPase inhibition. At first sight, the lack of capacity of NEM to increase the basal release of catecholamines, and the inability of Ca^{2+} reintroduction to evoke secretion, seem to oppose the conclusion that an augmented $[Na^+]_i$ increases the entry of Ca^{2+} into the chromaffin cell through the Na^+-Ca^{2+} exchange mechanism, and leads to an increase in catecholamine secretion. However, one has to consider that NEM is not a specific ATPase inhibitor as ouabain is and that many other enzyme systems depend on SH groups for activity (Rothstein, 1970). Therefore, NEM binds to many SH non-ATPase related binding sites, which may be critical for the normal function of the secretory machinery. Under these conditions, the gland will not secrete catecholamines even though the ionic requirements for release are fulfilled. In line with this argument, it is interesting to note that a transient inotropic effect of NEM, followed by a persistent negative inotropic effect, has been reported (Fricke, 1978) and that NEM depressed aortic responses to KCl, noradrenaline, 5-hydroxytryptamine, histamine and angiotensin II (Fleisch, Krzan & Titus, 1973). These effects have been attributed to an interaction of NEM with sulphydryl groups of enzyme systems vital to either membrane function or the contractile apparatus.

In summary, these results add evidence favouring the presence of a Na^+-Ca^{2+} exchange system in the chromaffin cell membrane, probably involved in the control of $[Ca^{2+}]_i$ and in the modulation of catecholamine secretion. This system is activated by increasing $[Na^+]_i$, either directly (ionophore X537A, increased $[Na^+]_o$) or indirectly (Na^+ pump inhibition). However, the simple inhibition of Na^+ pumping does not always lead to a catecholamine secretory response; such is the case for NEM, vanadate or phenoxybenzamine (Aunis & García, 1981).

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