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CORRELATION BETWEEN CATECHOLAMINE SECRETION FROM BOVINE ISOLATED CHROMAFFIN CELLS AND [^{3}H]-OUABAIN BINDING TO PLASMA MEMBRANES

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1 Secretion of catecholamines (CA) evoked by ouabain, chlormadinone acetate (CMA), phenoxybenzamine (Pbz) and vanadate, four agents known to inhibit Na^+, K^+-dependent Mg^{2+}-activated adenosine triphosphatase (ATPase) activity has been studied in suspensions of bovine isolated adrenal medullary cells.

2 Acetylcholine (ACh) evoked a 5 fold increase of the basal CA secretion from isolated cells suspended in oxygenated Krebs-bicarbonate solution kept at 27°C. Secretion was antagonized by Ca^{2+}-deprivation or hexamethonium, indicating good functional viability of the cells.

3 Ouabain (10^{-7} to 10^{-4} M) evoked a progressive, dose-dependent release of CA from cell suspensions. Study of the time course of the secretory response for 2 h allowed the separation of two components in the secretory response at all doses studied: a slow initial component (0.011 pg/min CA) and a second faster component (0.032 pg/min CA).

4 CMA evoked a clear-cut CA secretory response. The ED_{50} for CMA was 10^{-4} M, as compared to 3×10^{-6} M for ouabain. Pbz and vanadate did not induce CA release.

5 [^{3}H]-ouabain was taken up and bound to intact isolated cells by a non-saturable binding process. However, in semi-purified plasma membranes from bovine adrenal medulla a saturable specific [^{3}H]-ouabain binding process was observed with a K_D of 8.1 nM. Binding to the membranes was ATP-dependent and antagonized by K^+.

6 [^{3}H]-ouabain specific binding to membranes was antagonized by ouabain and CMA, but not by Pbz or vanadate; the ID_{50} for ouabain and CMA were 10^{-6} and 10^{-5} M respectively.

7 Ouabain partially inhibited, in a dose-dependent manner, Na^+, K^+-Mg^{2+} ATPase activity of the semi-purified plasma membranes.

8 The results demonstrate a good correlation between the ability of different drugs, known to inhibit ATPase activity, to displace [^{3}H]-ouabain binding to adreno–medullary plasma membranes and their capacity to evoke a CA secretory response from isolated chromaffin cells. The data also suggest that the CA secretory effects of ouabain may not be due simply to inhibition of the Na^+ pump and the subsequent ionic redistribution across the plasma membrane; a second mechanism may also be involved.

Introduction

The cardiac glycoside, ouabain, favours the release of catecholamines (CA) in the perfused bovine (Banks, 1967) and cat (García, Hernández, Horga & Sánchez-García, 1980) adrenal gland. The secretory effect of ouabain is not secondary to the release of acetylcholine (ACh) from cholinergic nerve terminals present in the adrenal gland but due to a direct action on the chromaffin cell itself. This action is exerted through redistribution of monovalent cations secondary to the inhibition by the glycoside of the sodium pump, which will ultimately lead to a rise of intracellular ionized calcium levels (García *et al.*, 1980).

Even though the secretory effects of cardiac glycosides in different organ systems have generally been directly or indirectly associated with the membrane Na^+,K^+-dependent Mg^{2+}-activated adenosine triphosphatase (subsequently referred to as ATPase; Birks & Cohen, 1968; Vizi, 1972; García & Kirpekar, 1973a, b; 1975a, b; Baker & Crawford, 1975; Duncan, 1977), studies on the direct correlation between inhi-

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bition of the enzyme, ouabain-receptor interactions and CA secretory response to ouabain and other agents known to inhibit the enzyme are lacking. This type of study has been hampered by lack of preparations in which adequate experimental designs could be performed.

We now present evidence for a positive correlation between the degree of CA secretion evoked by ouabain, chlormadinone acetate (CMA), phenoxybenzamine (Pbz) and sodium orthovanadate (Na_3VO_4), from bovine isolated adrenal medulla chromaffin cells and their ability to displace [^{3}H]-ouabain binding to semi-purified chromaffin cell plasma membranes.

Methods

Preparation of isolated adrenal medullary cells

Chromaffin cells were isolated essentially as described by Fenwick, Fajdiga, Howe & Livett (1978). Bovine adrenal glands were obtained fresh from the local slaughter house; a cannula was inserted in the adrenal vein through which 10 ml Ca^{2+}-free Krebs solution was injected gently, after making small incisions in the cortex with a scalpel blade. Once in the laboratory, the cortex was carefully dissected out and the intact medulla, with the inserted cannula, was mounted in a perfusion system adapted for the simultaneous processing of six glands. After washing the glands with 50 ml of fresh Ca^{2+}-free Krebs solution to remove red blood cells, a solution of Ca^{2+}-free Krebs containing 5 mg/ml of bovine serum albumin (BSA) and 0.5 mg/ml collagenase (from *Clostridium histolyticum*, E.C. 3.4.23.3; Boehringer, Mannheim) was recirculated through the adrenals at 37°C, with continuous bubbling with 95% O_2 and 5% CO_2 for 30 min. Then the medullae were minced finely with a scalpel blade in a solution containing fresh collagenase and BSA and the chopped tissue was incubated for a further 15 min at 37°C.

The suspension was filtered twice through 80 μm nylon mesh to remove undigested material. The mixture was centrifuged at room temperature for 10 min at 100 *g*, the supernatant removed and the pellet gently resuspended in 2 ml of Ca^{2+}-free Krebs solution containing BSA but not collagenase. All incubation solutions subsequently used contained BSA. The suspension was centrifuged and resuspended three times more to give a final preparation of isolated chromaffin cells. An aliquot of the final suspension was then counted with an haemacytometer and viability was estimated with 0.4% trypan blue.

Catecholamine release

Aliquots of cell suspension (2×10^5 cells per tube) were then transferred to tubes which contained 500 μl of oxygenated normal Krebs solution of the following composition (mM): NaCl 119, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4 . 7\ H_2O$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2, glucose 11 and BSA 0.5%. This solution was equilibrated with 95% O_2 and 5% CO_2, the final pH being 7.4 to 7.5. In some experiments, the Ca^{2+} concentration of the incubation medium was changed by removal or addition of appropriate amounts of $CaCl_2$; no osmotic adjustments were made.

Spontaneous CA release, or release of CA evoked by ACh, ouabain, CMA, Pbz or vanadate were measured by incubating the cells for different time periods at 27°C in a water bath with continuous shaking and stopping the reaction by immersion of the tubes in ice water. Cells were quickly separated from the medium by centrifugation at 10,000 *g* at 2°C for 10 min. The supernatant was collected, acidified with perchloric acid (PCA) to a final concentration of 0.4 N, and centrifuged at 10,000 *g* for 10 min in order to precipitate BSA and other proteins. The pellet was vigorously resuspended in 500 μl of 0.4 N PCA, and centrifuged at 10,000 *g* for 15 min. The total CA (noradrenaline plus adrenaline) content of media and cells was then estimated in 50 μl aliquots according to Anton & Sayre (1962) without further purification on alumina. CA values are expressed as pg/cell. Net CA release was calculated by subtracting spontaneously released from drug-evoked release. None of the drugs tested, at the final concentrations used in our experiments interfered with CA fluorescence.

Electron microscopy

Isolated cells washed (twice) with Ca^{2+}-free Krebs solution were centrifuged at 100 *g* for 10 min and resuspended in 500 μl of 5% glutaraldehyde in 0.1 M phosphate buffer pH 7.4 at 28°C. The preparation was kept at room temperature for 30 min, centrifuged and resuspended in 500 μl of 0.1 M phosphate buffer containing 7.5% sucrose. Cells were kept 15 min at room temperature, centrifuged and again resuspended in the same medium. Cells were finally centrifuged at 3000 *g* for 20 min and processed for electron microscopy after post-fixation with osmium tetroxide, by dehydration in graded alcohols (50 to 100%) and embedding in Araldite. Ultra-thin sections stained with uranylacetate and lead citrate were examined in a Philips EM 300 electron microscope at 60 kV.

Purification of bovine adrenal medulla plasma membranes

Plasma membranes were partially purified following a recent procedure (Wilson & Kirshner, 1977). The procedure has been described in detail elsewhere (Aunis, Pescheloche & Zwiller, 1978). The membranes were

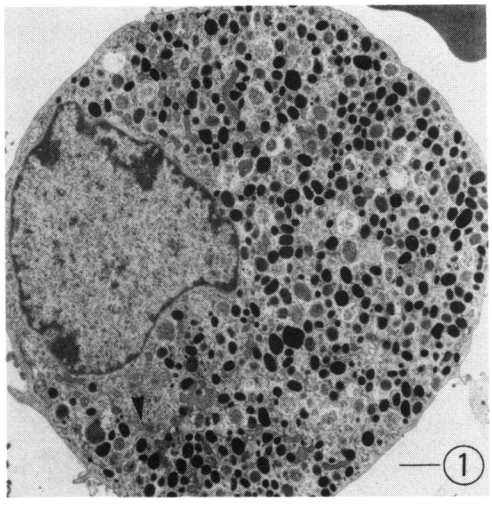

Figure 1 Electron micrograph of an isolated adrenal medullary cell showing numerous electron-dense granules of about 200 nm mean diameter. The granules are located at one end of the cell and the nucleus toward the other end. Note the intact plasma membrane and well-preserved subcellular structures. Arrow head indicates Golgi apparatus. Magnification: ×5,400; Bar, 1 μm.

washed and resuspended in 50 mM Tris–HCl buffer, pH 7.4, and frozen at −20°C until use.

Protein assay

Proteins were measured according to Lowry, Rosebrough, Farr & Randall (1951), with bovine serum albumin used as standard.

[^{3}H]-ouabain binding to purified plasma membranes

Competing test substance or unlabelled ouabain was incubated in a tube containing 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1.25 mM ATP (freshly added), 10 mM [^{3}H]-ouabain (New England Nuclear, specific activity 19.5 Ci/mmol) in a final volume of 1 ml. The tubes were pre-incubated at 37°C for 5 min with continuous shaking and the binding reaction was initiated by adding 10 µl aliquots of membrane suspension (200 µg protein). Incubation was stopped by rapid cooling of the assay tubes in an ice bath and centrifuging at 10,000 *g* for 20 min. The supernatant containing unbound [^{3}H]-ouabain was removed by aspiration and discarded. The membrane pellet was resuspended in 1 ml cold reaction mixture lacking ATP and [^{3}H]-ouabain and recentrifuged. The pellet was then dissolved in 0.3 ml 0.1 N KOH by heating in a 70°C water bath for 10 min and 0.2 ml was transferred to a vial containing 10 ml of scintillation medium (Eurofluor, New England Nuclear). Radioactivity was counted in a LS-9000 Beckman Scintillation Counter. Specific [^{3}H]-ouabain binding was calculated by subtraction of the [^{3}H]-ouabain bound to membranes in the presence of 10^{-4} M unlabelled ouabain. [^{3}H]-ouabain binding was expressed as pmol/mg protein.

Na^+-K^+ dependent Mg^{2+}-activated ATPase assay

ATPase activity was determined by measuring the release of inorganic phosphate from ATP. The reaction mixture contained (mM): NaCl 114, KCl 5.68, $MgCl_2$ 6.82, EGTA 1.14, Tris–HCl (pH 7.4) 34; plus enzyme, 200 µl of membrane suspension (20 µg protein) in a final volume of 2.5 ml. The reaction was initiated by addition of 100 µl ATP solution (3 mM final concentration in the reaction mixture). The reaction was run at 37°C for 30 min and stopped by the addition of 10% perchloric acid/8% silicotungstic acid (v/w) in water. Inorganic phosphate was extracted into ethylacetate as the phosphomolybdate complex and measured spectrophotometrically at 310 nm (Naylor, Dick, Dick, Le Poidevin & Whyte, 1973).

Ouabain and Na_3VO_4 were dissolved in water; CMA and Pbz in ethanol; when ethanol was used as a solvent, controls had the same concentration of ethanol. At the concentrations used, ethanol did not modify spontaneous CA secretion or [^{3}H]-ouabain binding to membranes.

Results

The number of isolated cells obtained varied every day between 10 and 22 × 10^6/six adrenal medullae. Under the light microscope, the cell suspension contained few erythrocytes and isolated spherical intact chromaffin cells (phase-bright, granular aspect, smooth contour, 20 µm in diameter). Practically 100% of the cells appeared to be viable by exclusion of trypan blue.

The appearance of a typical cell is shown in Figure 1. The cell contains a large number of vesicles with electron-dense contents (mean diameter, about 200 nm). The cells exhibited polarity with the vesicles located towards one end of the cell and the nucleus and other structures distributed towards the other end.

Catecholamine secretion evoked by acetylcholine

In the intact perfused adrenal gland, spontaneous CA release was lower at room temperature than at 37°C; also, ACh-evoked release (Dixon, García & Kirpekar, 1975) and ouabain-evoked release (García *et al.*, 1980) were more reproducible at room temperature than at 37°C; therefore we decided to carry out these experiments at 27°C.

Figure 2 shows that ACh (10^{-4} M for 10 min)-evoked CA release in the presence of physostigmine (10^{-5} M) was dependent on the extracellular Ca^{2+} concentration and was linear up to 1 mM Ca^{2+}. The peak secretory response was 4.9 times the basal release. Cell CA content at the end of the incubation period was declining in parallel with the secretory response indicating that synthesis of new CA is not keeping pace with release.

Increasing doses of hexamethonium progressively inhibited CA release evoked by ACh. Thus, with hexamethonium 10^{-6} M, CA secretion was 99% of control, at 10^{-5} M, 74% and at 10^{-4} M only 38%. These results indicate that nicotinic receptors are important in mediating the release of CA evoked by ACh in these isolated cells.

Effect of ouabain on the secretion of catecholamines

The presence of ouabain increased in a dose-dependent manner the spontaneous release of CA. Figure 3 shows the time course of the CA secretory response to different concentrations of ouabain. The presence of ouabain increased in a dose-dependent manner the release of CA into the cell suspension media. The secretory response was gradually increasing at the begin-

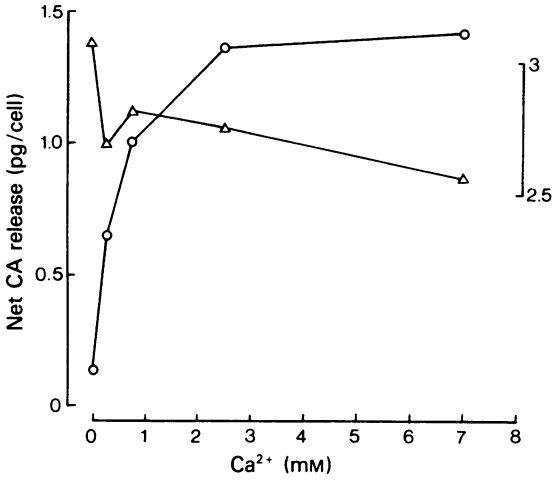

Figure 2 Catecholamine (CA) secretion evoked by acetylcholine (ACh) and its dependence on Ca^{2+} in isolated chromaffin cells. Cell suspensions (2×10^5) were incubated for 10 min in oxygenated Krebs solution containing different Ca^{2+} concentrations, physostigmine (10^{-5} M) and acetylcholine (ACh, 10^{-4} M). Left ordinate scale represents the net CA release (○) (peak ACh-evoked release was 4.9 times the spontaneous release). Right ordinate scale shows the cell content of CA (△) at the end of the experiment.

ning of ouabain exposure. The ouabain threshold concentration evoking a clear secretory response was approximately 10^{-7} to 10^{-6} M and the maximal secretory response was reached at 10^{-5} M. Greater ouabain concentrations (up to 10^{-4} M) did not further enhance amine release.

When CA release versus time is plotted on semilogarithmic paper (Figure 4), straight lines are obtained with two apparent components at all doses studied: a first, slow, secretory phase (0.011 pg/min at 10^{-6} M ouabain and 0.019 pg/min at 10^{-5} and 10^{-4} M ouabain) and a second, faster, secretory phase (0.032 pg/min at 10^{-6} M ouabain and 0.048 pg/min at 10^{-5} and 10^{-4} M ouabain). It is interesting to note that the lines are quite parallel at all doses studied. The existence of two phases suggests that two mechanisms may be involved in the CA secretory response to ouabain. Depletion of the CA content of the cell is unlikely since the endogenous CA levels in the cell are very high at the end of the experiment.

Comparative catecholamine secretory effects of ouabain, chlormadinone, phenoxybenzamine and vanadate

Since these four drugs are known to inhibit ATPase activity in membrane preparations from different biological systems, it was interesting to compare their ability to initiate the CA secretory process in isolated

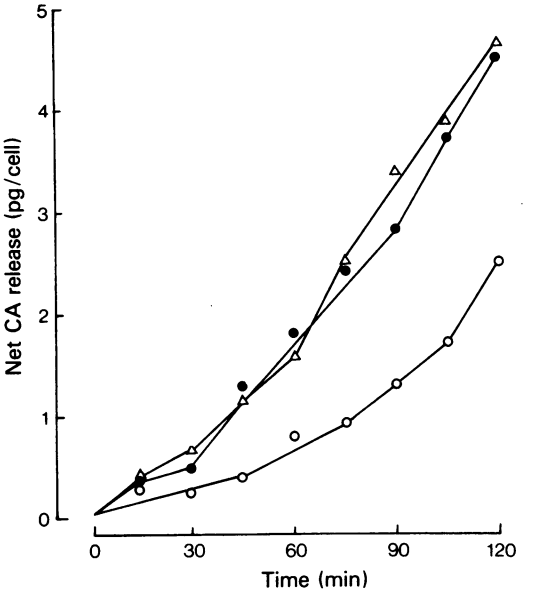

Figure 3 Release of catecholamines (CA) induced by ouabain in isolated chromaffin cells. Cell suspensions (2×10^5 cells) were incubated with 0.5 ml of normal Krebs solution containing ouabain at different concentrations (△) 10^{-4} M; (●) 10^{-5} M; (○) 10^{-6} M. The ordinate scale shows the net CA release (release in the presence of ouabain minus spontaneous release in the absence of the drug for each time of incubation).

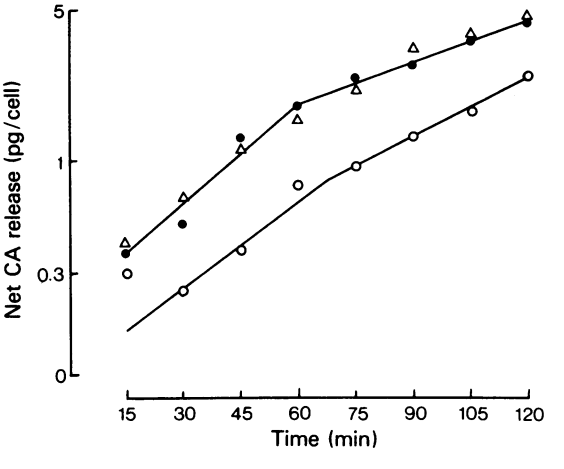

Figure 4 Time course of the release of catecholamines (CA) evoked by ouabain: (○) 10^{-6} M; (●) 10^{-5} M; (△) 10^{-4} M. Ordinate shows the net CA release plotted on logarithmic scale. This graph was plotted with data from Figure 3.

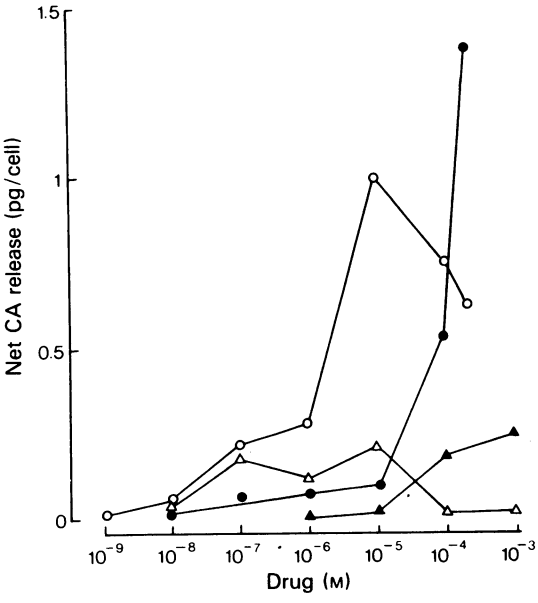

Figure 5 The effects of ouabain (○), chlormadinone acetate (●, CMA), phenoxybenzamine (▲) and sodium orthovanadate (△) on catecholamine (CA) release from isolated chromaffin cells. ED_{50} for ouabain was calculated taking the peak release as 100% and it was 3×10^{-6} M. With CMA, because of solubility problems, a plateau could not be reached and the approximate ED_{50} for CMA was about 10^{-4} M.

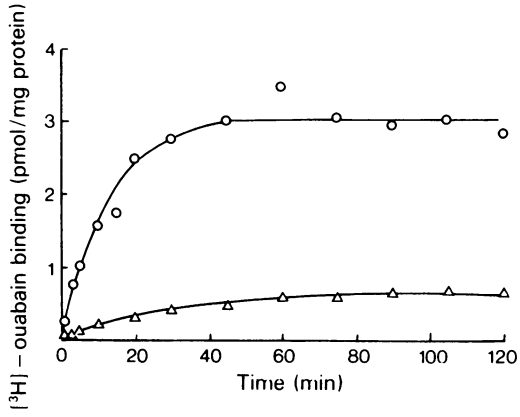

Figure 6 Time course of [^{3}H]-ouabain binding to purified adrenal medullary plasma membranes in the absence (○) and in the presence (△) of K^+. Membranes (200 μg protein) were incubated for indicated time periods at 37°C in the presence of 10 nM [^{3}H]-ouabain. Bound and free [^{3}H]-ouabain were separated by centrifugation. Specific [^{3}H]-ouabain binding was calculated by substraction of the [^{3}H]-ouabain bound in the presence of 10^{-4} M cold ouabain (72 ct/min in the presence of 10^{-4} M ouabain versus 6.140 ct/min in the absence of cold ouabain). Points are the mean of 4 determinations. Initial rates of binding were 0.15 and 0.021 pmol mg^{-1} protein min^{-1} in the absence and the presence of K^+, respectively.

chromaffin cells. Results of these experiments are depicted in Figure 5. Cells were incubated for 30 min in the presence of different concentrations of ouabain, CMA, Pbz and Na_3VO_4. Taking as 100% the peak CA secretory response for each drug, approximate ED_{50}s were calculated, even though, in the case of CMA, a plateau could not be reached because of solubility limitations of CMA. The ED_{50}s for ouabain and CMA, were 3×10^{-6} and 10^{-4} M, respectively. Therefore, the CA secretory response to CMA was about 33 times lower than that to ouabain. The secretory response to Pbz and vanadate was very small and no attempts to calculate ED_{50}s were made.

Binding of [^{3}H]-ouabain to intact chromaffin cells and to semi-purified plasma membranes

In order to see whether intact cells had a saturable specific binding for [^{3}H]-ouabain, we carried out experiments in the presence of increasing concentrations of ouabain. The binding of ouabain to intact, isolated chromaffin cells was linear at concentrations ranging from 10^{-8} to 10^{-3} M, indicating that a saturable binding process was not present in intact cells. Uptake-binding of ouabain to intact cells at concentrations of 10^{-8} to 10^{-3} M ouabain ranged from 0.002 to as much as 100 fmol/cell.

Therefore, we decided to perform these binding studies in semi-purified adrenal medulla plasma membranes. Figure 6 shows the time course of the binding process in the absence of K^+, which is linear during the first 10 min of incubation to reach a plateau at around 30 min. When the membranes were incubated in the presence of K^+ (5.9 mM), [^{3}H]-ouabain binding was almost completely abolished. The initial rates of binding, calculated from the slope of the linear part of the curves, were 0.15 and 0.21 pmol mg^{-1} protein . min^{-1} in the absence and presence of K^+, respectively. In the absence of ATP, the binding reaction was decreased by 80%.

A Scatchard plot of the data in the absence of K^+ ions allowed calculation of the dissociation constant of [^{3}H]-ouabain specific binding to membranes which was 8.1 nM (Figure 7).

In the next series of experiments, competition of drugs for [^{3}H]-ouabain binding to membranes was assessed. Non-labelled ouabain very effectively displaced [^{3}H]-ouabain binding indicating that the binding sites are highly specific (Figure 8). Although less potent than ouabain, CMA also competed for [^{3}H]-ouabain binding sites. The ID_{50}s for ouabain and CMA were 10^{-7} and 10^{-6} M, respectively. Pbz

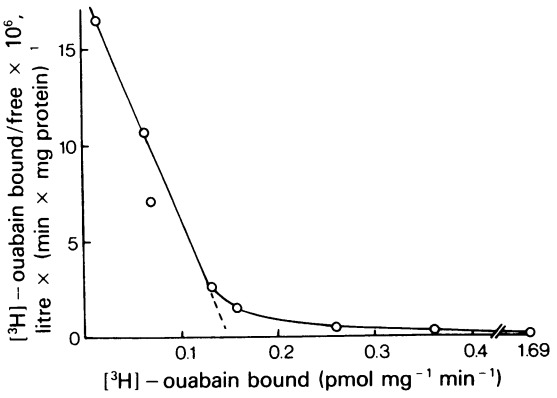

Figure 7 Scatchard plot of [^{3}H]-ouabain binding to purified plasma membranes. Data were calculated from the experiment of Figure 6. Calculated K_D is 8.1 nM.

and vanadate did not modify [^{3}H]-ouabain binding to membranes.

Na^+,K^+-dependent Mg^{2+}-activated ATPase activity of semi-purified plasma membranes

The membrane ATPase seems to be the receptor for cardiac glycosides, at least as far as the cardiac sarcolemma is concerned (Schwartz, 1976; Akera, 1977). Since ouabain has a measurable CA secretory effect in the chromaffin cell and the membranes of these cells possess specific, ligand-recognition sites with a high affinity for [^{3}H]-ouabain, it was of interest to determine the specific activity of Na, K-ATPase and its inhibition by ouabain in purified membranes. In our conditions, the specific activity of ATPase was 10 μmol h^{-1} mg^{-1} protein. Ouabain inhibited the enzyme activity by 18% at 10^{-6} M, 45% at 10^{-5} M and 52% at 10^{-4} M (Figure 9). The enzyme was not totally inhibited by ouabain probably because other ouabain-resistant ATPases, as the Mg-ATPase in membranes from lysed chromaffin granules, were contaminating the plasma membrane preparation.

Discussion

Isolated chromaffin cells from bovine adrenal medulla are a suitable preparation to carry out studies such as the one presented here. CA were released by ACh in a Ca^{2+}-dependent manner; the release was associated with the secretion of the vesicular enzyme dopamine β-hydroxylase and secretion was inhibited by hexamethonium. Thus the isolated cell behaves very much like the intact perfused gland as far as the physiological CA secretory mechanism is concerned.

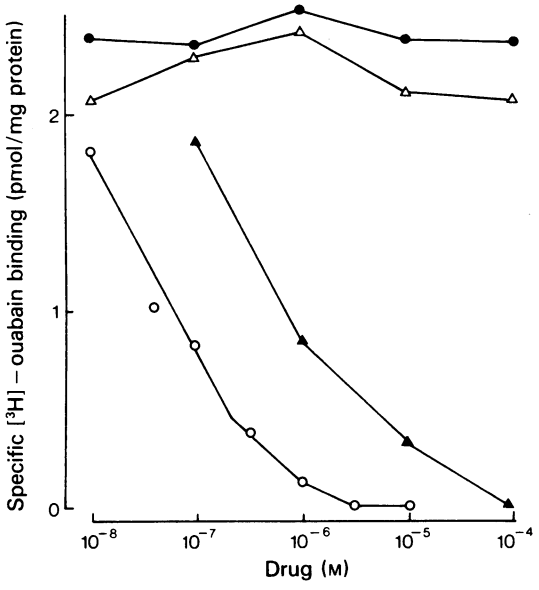

Figure 8 Effects of ouabain (○), chlormadinone acetate (▲), phenoxybenzamine (△) and vanadate (●) on the binding of [^{3}H]-ouabain to semi-purified plasma membranes. Membranes (200 μg protein) were incubated with 10 nM [^{3}H]-ouabain and various concentrations of drugs at 37°C for 10 min. Data are expressed as specific [^{3}H]-ouabain binding (calculated as in Figure 6) versus drug concentration.

Ouabain markedly increased CA release from isolated chromaffin cells into the incubation medium. These data are in accordance with previously reported data in intact adrenergically innervated tissues (Kirpekar, Prat & Yamamoto, 1970; García & Kirpekar, 1973a, b; Lindmar & Löffelhölz, 1974;

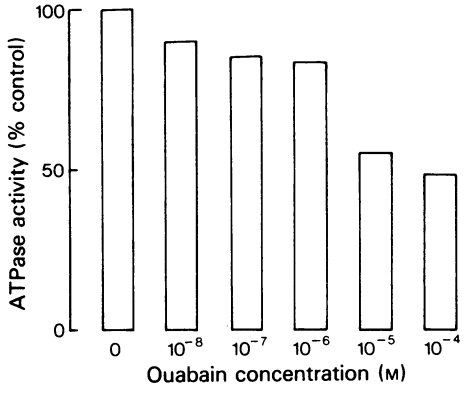

Figure 9 Na^+, K^+-ATPase activity of semipurified adrenomedullary plasma membranes and its inhibition by ouabain. Data are expressed as the percentage of ATPase activity obtained in the absence of the drug, which was 10 μmol Pi h^{-1} mg^{-1} protein.

Weiner & Lee, 1975; Nakazato, Ohga & Onoda, 1978) and perfused adrenal gland (Banks, 1967; García *et al.*, 1979).

It is interesting to note that when isolated chromaffin cells were incubated for up to 2 h in the presence of ouabain, there were two clear phases in the secretory response curve. The rate of CA secretion is slower during phase I than during phase II (note logarithmic ordinate scale of Figure 4); at 10^{-6} M ouabain the ratio, phase II/phase I, was as much as 2.91. These data would suggest that the CA secretory effects of ouabain might involve two mechanisms. One mechanism could be related to ATPase inhibition itself. Since inhibition of the enzyme is quite fast (see Akera & Brody, 1978), it is reasonable to assume that inhibition of the enzyme might be responsible for phase I of the secretory response curve. Late in the ouabain exposure, progressive ionic redistribution secondary to inhibition of the Na^+ pump would lead to acceleration of the rate of CA secretion, probably by activation of a Na^+-Ca^{2+} exchange system located at the chromaffin cell membrane (García *et al.*, 1980). Even though we have no definitive evidence for or against it, this hypothesis seems plausible and attractive in the light of recent experiments which have shown a dissociation between the ability of certain cardioactive steroids to displace [^{3}H]-ouabain binding, inhibition of the Na^+ pump and heart contractile effects (LaBella, Bihler & Kim, 1979; Ghysel-Burton & Godfraind, 1979).

As previously mentioned, the ability of cardiac glycosides to increase the release of CA and other neurotransmitters from a variety of tissues is well documented (see Vizi, 1978). However, a possible correlation between [^{3}H]-ouabain binding and CA secretion in the same tissue has not been established.

It seems that Na^+, K^+-ATPase is the receptor for digitalis in the heart. This assumption is based on the fact that the enzyme is distributed in cardiac sarcolemma, it binds ^{3}H-labelled cardiac glycosides, the binding is characterized by high affinities and glycosides may be displaced by pharmacologically active but not by inactive, congeners (see Schwartz, 1976; Akera, 1977).

In this paper, we have studied the inhibition of ATPase activity by ouabain, its binding to isolated chromaffin plasma membranes, its binding displacement by cold ouabain and other known ATPase inhibitors and the CA secretory response of isolated chromaffin cells to these drugs, in an attempt to establish a relationship among these events.

ATPase activity was inhibited by increasing doses of ouabain only by 52%. The procedure used in this study to isolate adrenal medullary plasma membranes was that of Wilson & Kirshner (1977). It is possible that the ouabain–insensitive portion of the enzyme is associated with intracellular membranes which contaminated the plasma membrane fraction obtained on the continuous sucrose density gradient.

The [^{3}H]-ouabain binding studies demonstrated the presence of a high affinity saturable binding process in these semi-purified plasma membranes, with a K_D of 8 nM. The rate constant of binding was greatly decreased in the presence of K^+ (5.9 mM), indicating that ouabain interacts with a potassium–sensitive region probably located on the external surface of the intact ATPase system.

[^{3}H]-ouabain binding was efficiently inhibited by cold ouabain and CMA, but not by vanadate or Pbz. The ID_{50} for ouabain was 0.09 μM and that for CMA 0.9 μM. CMA is a digitalis-related steroid which was about 1/50th as potent as ouabain in displacing specifically bound [^{3}H]-ouabain in crude dog heart membrane preparations, markedly increased internal Na^+ in guinea-pig atria but did not have any contractile effect on beating atria (La Bella *et al.*, 1979). In contrast, our experiments showed a functional correlation between the ability of CMA to displace [^{3}H]-ouabain bound to chromaffin cell membranes (about 10 times less potent than ouabain) and its capacity to evoke CA secretion from isolated chromaffin cells (about 30 times less potent than ouabain). However, the discrepancy between the concentrations of CMA required to displace [^{3}H]-ouabain and to evoke CA secretion, again suggests that inhibition of the sodium pump (and/or binding of [^{3}H]-ouabain) might not be the only mechanism responsible for the CA secretory effects of ouabain.

Pbz inhibits ATPase from beef cerebral cortex (Hexum, 1978). However, in isolated chromaffin cells, Pbz did not evoke a CA secretory response (up to 1 mM). In addition, Pbz did not inhibit [^{3}H]-ouabain binding to semi-purified plasma membranes, indicating again a good correlation between these two parameters.

Finally, vanadate has been shown recently to be a potent inhibitor of dog kidney ATPase (Cantley, Josephson, Warner, Yanagisawa, Lechene & Guidotti, 1977) and to stimulate [^{3}H]-ouabain binding in cat heart membrane preparations (Erdmann, Krawietz, Philipp, Hackbarth, Schmitz & Scholz, 1979). In our experiments, vanadate did not stimulate CA secretion and did not modify [^{3}H]-ouabain binding.

Since vanadate and Pbz are good inhibitors of ATPase activity and the subsequent Na^+ pumping, the lack of secretory effects of these two agents would provide additional evidence in favour of the idea that ouabain secretory effects are not the simple result of Na^+ pump inhibition.

Two main conclusions seem to arise from these experiments: (a) there is a good correlation between [^{3}H]-ouabain binding to plasma membranes of the chromaffin cell and the secretion of CA evoked by ouabain; however, direct evidence for a causal re-

lationship between these two parameters is still lacking. (b) The ouabain secretory effect might not be due simply to inhibition of Na^+ pumping and to redistribution of monovalent and divalent cations across the plasma membrane.

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