J. Membrane Biol. 94, 205–215 (1986)

The Journal of
Membrane Biology
© Springer-Verlag New York Inc. 1986

Cyclic AMP-Dependent Stimulation of Na,K-ATPase in Shark Rectal Gland

D. Marver, S. Lear, L.T. Marver, P. Silva, and F.H. Epstein
Mt. Desert Island Biological Laboratory, Salsbury Cove, Maine 04672

Summary. Scatchard analysis of ^{3}H ouabain bound to isolated rectal gland cells as a function of increasing ouabain concentrations produced a concave curvilinear plot that was resolved into two specific sites with either a high (I) or low (II) affinity for ouabain. Cyclic cAMP/theophylline (± furosemide, 10^{-4} M) increased the amount of ^{3}H ouabain bound to the high-affinity site I. Vanadate, a phosphate congener which promotes formation of the ouabain-binding state of the enzyme, mimicked the effects of cAMP/theophylline at low concentrations of ouabain, suggesting that cAMP/theophylline increases binding to site I by enhancing the rate of turnover of resident enzyme. Enhanced ^{86}Rb uptake seen following cAMP/theophylline administration was primarily associated with increased flux through the high-affinity ouabain site, and this stimulation was not obliterated by the co-administration of furosemide. A model was presented which suggested the presence of two noninteracting pools of enzyme or isozymes which exhibit either a high or low affinity for ouabain. Cyclic AMP both stimulated turnover via site I, and modified the kinetics of binding of ^{3}H ouabain to site II. The (ave) K_d of ^{3}H ouabain for site II was increased from 3.6 μM (controls) to 0.5 μM (cAMP/theophylline) and the Hill coefficient was modified from 0.45 (controls) to 1.12 (cAMP/theophylline), suggesting a transition from a negative- to a noncooperative binding state. While furosemide reversed the effects of cAMP/theophylline on site II kinetics, it did not obliterate cAMP/theophylline effects on site I. This suggests that cAMP may alter the intrinsic turnover rate of this particular pool of Na,K-ATPase in shark rectal gland.

Key Words Na,K-ATPase · vanadate · rubidium · shark rectal gland · ouabain · cAMP · theophylline · furosemide

Introduction

Cyclic AMP stimulates chloride secretion in the shark rectal gland, a process inhibitable by serosal furosemide. This response has been shown to be associated with enhanced steady-state binding of ^{3}H ouabain to intact rectal glands (Shuttleworth & Thompson, 1980; Silva, Stoff & Epstein, 1979*b*, 1983). Shuttleworth and Thompson (1980) suggested that the enhanced binding of ouabain to rectal gland tissue + cAMP was secondary to stimulation of transport, as furosemide prevented cAMP-dependent ^{3}H ouabain labeling in their studies. In contrast, data from Silva and co-workers (1979*a*,*b*, 1983) showed that cAMP-dependent ^{3}H ouabain binding was not abolished by co-addition of either furosemide or bumetanide, both potent inhibitors of the Na-K-Cl cotransporter and chloride secretion in this tissue. They suggested that cAMP altered the intrinsic performance of the Na pump. A major difference between the two studies was that the former group concentrated on binding seen following addition of μM levels of ^{3}H ouabain while the latter group focused on binding in the presence of nM levels of ^{3}H ouabain. This is an important issue, since analysis of ^{3}H ouabain data in rectal gland slices indicated a concave curvilinear plot, suggesting multiple binding sites.

The present studies were undertaken to determine a) if the sites previously defined in the shark rectal gland with both low and high concentrations of ^{3}H ouabain were associated with the Na pump as evidenced by ouabain-sensitive ^{86}Rb/K uptake rates, b) if paired studies using isolated rectal gland cells ± cAMP/furosemide would support the suggestion of an intrinsic modification of the pump in the presence of cAMP, and c) if the aggregate ^{3}H ouabain binding and ^{86}Rb uptake data would support a two-site model for Na,K-ATPase.

The suggestion of a two-site model (or Na,K-ATPase isozymes) was based on the work of Resh, Nemenoff and Guidotti (1980) and Lytton, Lin and Guidotti (1985) who characterized two forms of Na,K-ATPase in the rat adipocyte with varying affinities for ouabain. There was evidence for both the existence of an α and α (+) subunit [the latter as defined by earlier studies with brain enzyme (Taniguchi & Yoda, 1972)], and that insulin stimulated turnover of the α (+)-containing enzyme. Thus, it is conceivable that curvilinear Scatchard plots of ^{3}H ouabain binding seen in shark rectal gland may reflect the interaction of ouabain with Na,K-ATPase isozymes, and cAMP, in turn, may

uniquely influence the high-affinity binding site or form of the enzyme.

Indeed, the data obtained support a model consisting of two noninteracting pools of Na,K-ATPase, demonstrable in part by their varying affinity for ^{3}H ouabain. Further, cAMP enhances Rb flux primarily via the high-affinity site, an effect only partially blocked by furosemide.

Abbreviations

Na,K-ATPase, sodium and potassium-dependent adenosine triphosphatase; cAMP, cyclic adenosine monophosphate; EDTA, ethylenediamine tetraacetic acid; HEPES, [4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid]; TCA, trichloroacetic acid; TMAO, trimethylamine oxide; K_d, equilibrium dissociation constant; N_{max}, maximum number of binding sites.

Materials and Methods

Materials

^{3}H Ouabain (20 Ci/mmol) and ^{86}Rb (20 Ci/mmol) were obtained from New England Nuclear Corp., Boston, Mass. Collagenase was purchased from Cooper Biomedical (CLS II, 155 U/mg), Malvern, Penn., dibutyl phthalate from Aldrich Chem. Co., Milwaukee, Wisc., sodium orthovanadate from Fisher Scientific, and dibutyryl cAMP, ouabain, furosemide, and pyruvic acid from Sigma Corp., St. Louis, Mo. General biochemicals were all of analytical grade.

Isolated Cell Preparation

Male or female spiny dogfish sharks (*Squalus acanthius*) were sacrificed by multiple rapid transsections of the spinal cord. The rectal gland and its immediate artery were removed, and each gland perfused with 75 ml of a shark Ringer's composed of the following (in mM): Na, 280; K, 5; Cl, 295; Mg, 3; Ca, 2.5; SO_4, 0.5; PO_4, 1; urea, 350; HEPES, 40; TMAO, 70; glucose, 5; acetate, 2.5; pyruvic acid, 10 (pH 7.6). The gravity perfusion was carried out over a period of 15 min, and was followed by infusion of 5 ml of shark Ringer's containing collagenase (200 mg%), fraction V bovine serum albumin (400 mg%), and fetal calf serum (5%). To increase access of enzyme to the tubule cells in the subsequent incubation step, the gland was slit along the longitudinal axis, and the mucosal epithelium cut at ~1-mm intervals with a razor blade. Tissue from 1 to 2 glands was then incubated in a shaking water bath at room temperature (45 min) in 100 ml of Ringer's supplemented with collagenase, albumin and fetal calf serum. During this time, the solution was continuously bubbled with 100% O_2.

Following incubation, the mixture was centrifuged at 600 × *g* for 1 min in a Beckman table-top refrigerated centrifuge (4°C) to remove undigested material. Supernatants were centrifuged twice (600 × *g* for 5 min) and the pellets combined, using a total of 1 to 1.5 ml of Ringer's. The harvested cells were then centrifuged in an Eppendorf centrifuge for 20 sec, the supernatant discarded and the pellet resuspended in an equivalent volume of Ringer's. This mixture was centrifuged twice, each for 10 sec, resuspending the pellet in 1 ml of Ringer's between centrifugations. The final pellet was resuspended in approximately 0.8 ml of Ringer's/gland dissected, and filtered through Nytex. This mixture contained 2.35 ± 0.36 mg TCA-precipitable protein/ml.

Ouabain Binding

Incubations were carried out in 1.5-ml Eppendorf centrifuge tubes maintained at 15°C with circulating seawater. Total volume was 0.5 ml, using shark Ringer's as diluent. Incubation times varied from 0 to 4 hr, and at times included the addition of either furosemide (10^{-4} M), dibutyryl cAMP (1 mM) plus theophylline (1 mM), sodium orthovanadate (1 mM), or variable K concentrations (0.3 or 5 mM). Total protein added per sample was 63 ± 7 μg. Ouabain concentrations are given in the text. At the end of the incubation period, the tubes were centrifuged at 600 × *g* for 10 min (15°C) to recover the cells. The radioactive supernatants were discarded and the cells rapidly suspended in 1.5 ml of ice-cold Ringer's lacking both ouabain and K, and these mixtures poured onto 2.4 cm Whatman DE 81 filters overlaying Whatman GFA filters under suction in an iced 10-place manifold (Hoefer Scientific Instruments, San Francisco, Calif.). Another 0.5 ml of K- and ouabain-free Ringer's was used to rinse each tube. Each filter unit was then washed with 5 × 1 ml of ice-cold Ringer's, and the filters for each sample added to 10 ml of Aquasol® (New England Nuclear Corp., Boston, Mass.). A Packard liquid scintillation counter was used to evaluate labeling of fractions. Protein was determined by precipitating duplicate aliquots of cell samples in 10% trichloroacetic acid. The 10,000 × *g* pellets obtained were resolubilized in 1 N NaOH and protein analyses carried out using the method of Lowry et al. (1951).

^{86}Rb Uptake

All incubations with ^{86}Rb were carried out at 15°C for periods of time ranging from 5 to 30 min. Approximately 0.6 μCi of ^{86}Rb was added per 100 μl of sample. Duplicate incubations were terminated by centrifuging 100-μl aliquots of the labeled sample through 200 μl of dibutyl phthalate (d = 1.043). To accomplish this, the samples were centrifuged 30 sec in an Eppendorf microfuge at room temperature (400 μl Eppendorf tubes). The tubes were immediately frozen in a dry-ice acetone mixture, and the pellet of cells at the bottom removed by slicing across the tube with a razor blade. The base of each tube was added to Aquasol and counted as above. Pilot studies indicated that with the number of cells involved, it was not necessary to use SDS to obtain maximal counts, as has been detailed by some investigators (Resh et al., 1980).

Na,K-ATPase Assays

Following incubation for 3 hr at 15°C in shark Ringer's, cells were pelleted at 600 × *g* as above, resuspended in 200 μl 0.05 M Tris buffer, pH 7.4, and rapidly freeze-thawed three times, using a dry-ice acetone bath. The appropriate enzyme substrates ± ouabain or vanadate were then added to the cell-free mixture. The assay was initiated at room temperature by the addition of ATP and terminated after 30 min with TCA precipitation (Philipson &

Edelman, 1977). Some samples also contained vanadate. Final concentrations of the reactants were as follows: NaCl (100 mM), KCl (5 mM), $MgCl_2$ (3 mM), EDTA (1 mM), Na_2ATP (3 mM), Tris-HCl, pH 7.4 (100 mM) ± ouabain (1 mM) and/or vanadate (1 mM). Activities were judged by measuring ouabain-sensitive P_i liberation into the harvested TCA supernatants, and were corrected for sample protein using the Lowry assay (Fiske & SubbaRow, 1925; Lowry et al., 1951).

Statistical analyses were made by Student's unpaired *t*-test or by the Wilcoxon paired *t*-test (Wilcoxon, 1945), as appropriate. Finally, estimation of the K_d in certain studies lacking apparent cooperative kinetics was made by the LIGAND computer program (Munson & Rodbard, 1980).

Results

Ouabain

Ouabain binding to the α subunit of Na,K-ATPase is facilitated by the presence of the E_2-P configuration of the enzyme, a state which is promoted by a combination of either intracellular [Na + Mg + ATP], [P_i + Mg] or [vanadate + Mg]. It is antagonized, in a noncompetitive manner, by extracellular K^+. Vanadate both promotes ouabain binding, and inhibits Na,K-ATPase activity (Cantley et al., 1977; Hansen, 1984; Huang & Askari, 1984). Of importance to the model, is that K can compete with ouabain for the complex promoted by vanadate (Hansen, 1982).

Given this, the enhanced binding of low concentrations of ^{3}H ouabain plus cAMP reported earlier (Silva et al., 1983) could be attributable entirely, or only in part, to an increased rate of formation of the E_2-P complex coupled to a rise in transport rates and increased cell Na ± ATP levels (Joiner & Lauf, 1978). It is also possible that cAMP could alter the intrinsic turnover of the enzyme independent of changes in cell Na/Mg/ATP concentrations. As discussed in the Introduction, furosemide was seen in one instance to block the effects of cAMP on ouabain binding, and not to block the effect in another (Shuttleworth & Thompson, 1980; Silva et al., 1983). Whether or not binding equilibrium was achieved may account for some of the differences noted.

To validate that the binding reaction was at equilibrium in the present studies, isolated rectal gland cells were incubated for up to 4 hr at 15°C with or without 1 mM vanadate and with 3×10^{-9} to 10^{-5} M ^{3}H ouabain (±1 mM unlabeled ouabain to judge nonspecific binding of ligand). Although the absolute level of ^{3}H ouabain bound/mg protein varied as a function of the presence or absence of vanadate and the ouabain concentration added, the fractional increase in binding, relative to that achieved in 3 hr, did not (Fig. 1). Maximal binding was seen at 3 hr; the ~10% fall in binding activity at 4 hr, was not significantly reduced from 3-hr values. Cyclic AMP was not added to these time controls, since earlier studies reported that in this group, equilibrium was reached at a time ≦ than that required for controls (Silva et al., 1983). Thus, in all subsequent experiments involving analysis of ^{3}H- or unlabeled-ouabain binding, a 3-hr incubation period was used.

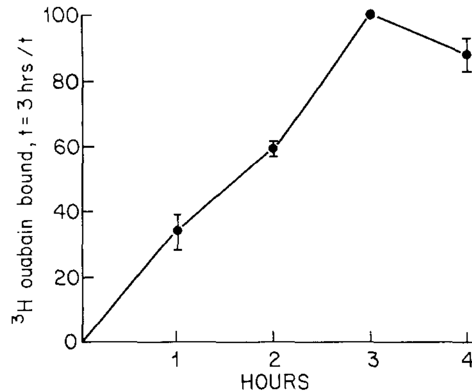

Fig. 1. Time course of ^{3}H ouabain binding at 15°C. In a series of four experiments, rectal gland cells were incubated for periods of time up to 4 hr ± 1 mM vanadate, and at concentrations of ^{3}H ouabain ranging from 3 nM to 10 μM. Binding was corrected for that seen in the presence of 1 mM unlabeled ouabain. Shown is the fractional ^{3}H ouabain bound, relative to the 3-hr value for each respective group (± SE)

Scatchard analyses were carried out on cell fractions incubated at 15°C using a wide range of ^{3}H ouabain concentrations (1 nM to 10 μM) ± 1 mM unlabeled ouabain. The data presented in Figs. 2 and 3 have been corrected for nonspecific binding, which was found to be essentially equivalent to those counts trapped by the filters in the absence of cells. Four major groups were analyzed: controls, cells incubated with dibutyryl cAMP and theophylline at concentrations which have been shown to stimulate Cl secretion and oxygen consumption in these cells (Silva et al., 1979*b*, 1983); cells incubated with dibutyryl cAMP, theophylline and furosemide, the latter at a concentration (10^{-4} M) which has been shown to block that fraction of Cl secretion and the oxygen consumption rate stimulated by cAMP (Silva et al., 1977, 1979*b*, 1983); and vanadate. Vanadate was added to illustrate the effect of an increased steady-state level of E_2-P on binding and to determine if vanadate reproduced the effects of cAMP. Two of the groups (controls, cAMP/theophylline) were reexamined after reducing media K.

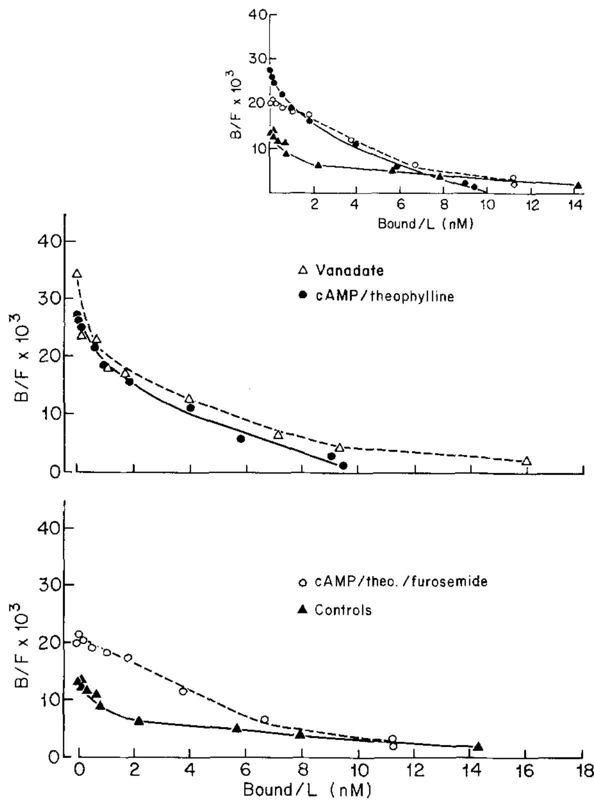

Fig. 2. Scatchard analyses of ^{3}H ouabain binding to intact rectal gland cells (15°C). Groups included controls (—▲—), or cells treated with vanadate (--△--), cAMP/theophylline (—●—), or furosemide plus cAMP/theophylline (--○--). Values shown are the averages from 5 to 6 separate studies, each involving a pool of cells from 3 to 4 rectal glands. The first 5 points at the left of each binding curve represent concentrations ranging from 1 to 60 nM and those 5 points to the right 0.1 to 10 μM. Binding was corrected for that seen in the presence of 1 mM unlabeled ouabain. The inset contrasts controls and samples treated + cAMP/theophylline ± furosemide

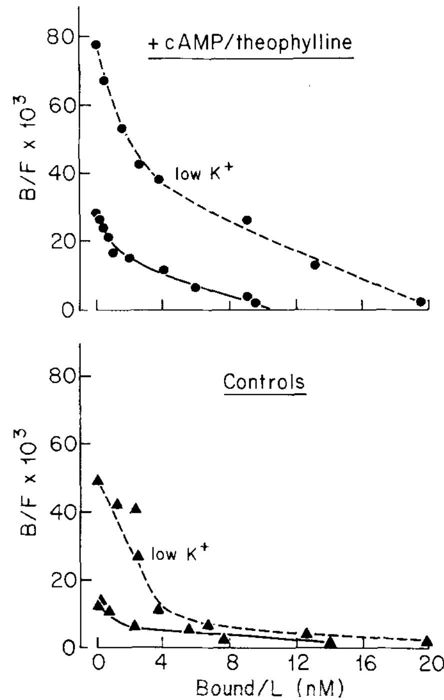

Fig. 3. Influence of K^+ on ^{3}H ouabain binding as determined by Scatchard analysis. Cells were incubated for 3 hr ± cAMP/theophylline in shark Ringer's containing either 5 mM (——) or 0.3 mM K^+ (----). Values have been corrected for nonspecific binding in the presence of 1 mM ouabain (n = 3 to 6)

Data from all groups generated curvilinear Scatchard plots. Further, the overall pattern for control *vs.* cAMP-stimulated ^{3}H ouabain binding in these studies at 15°C, followed that determined in rectal gland slices at 25°C by Silva et al. (1983). Namely, cAMP/theophylline resulted in an increase in the B/F (bound/free) ratio at low doses of ouabain (<0.3 μM), while decreasing the B/F ratio at higher doses of ouabain (>0.3 μM), the latter suggesting a decrease in the number of binding sites (Fig. 2). Vanadate, like cAMP, increased ouabain binding at low concentrations of ouabain, but did not decrease the apparent number of total binding sites. If cells were incubated with both vanadate and cAMP/theophylline, binding was not significantly different from that in cells treated with vanadate alone (*data not shown*). The similarity between certain of the vanadate and cAMP effects leaves open the possibility that the increase in ouabain binding seen at low concentrations of ligand plus cAMP reflects an increased turnover of the enzyme as opposed to an increase in the total number of functional enzyme units.

The addition of furosemide to cAMP-containing incubations reduced the ^{3}H ouabain bound compared to cAMP/theophylline alone (low-dose range, $10^{9} \rightarrow 10^{-8}$ M). Furosemide also reversed the apparent loss of total binding sites seen with cAMP/theophylline. More importantly, however, if labeling was examined in cells exposed to dibutyryl cAMP/theophylline + furosemide,—over a 300-fold range of concentrations (1 nM $\rightarrow 3 \times 10^{-7}$ M),—there was still a significant increase in the amount of ^{3}H ouabain bound to the cells relative to controls. Binding values for two low and two high concentrations of ^{3}H ouabain are given in Table 1.

When ambient K^+ was reduced from 5 to 0.3 mM and ^{3}H ouabain Scatchard analyses repeated, the response depended on the presence or absence of cAMP/theophylline (Fig. 3). In controls, reducing bath K^+ stimulated binding at low relative to high concentrations of ouabain as might be expected, without a significant change in the projected N_{max} value. This same effect was reported previ-

Table 1. Representative ^{3}H ouabain binding values

Group	Concentration of ^{3}H ouabain			
	1 nM	10 nM	1 μM	10 μM
	nmol bound (specific)/100 mg protein ± SE			
Controls	0.013 ± 0.002	0.111 ± 0.020	5.670 ± 1.100	14.210 ± 1.480
cAMP/theophylline	0.024 ± 0.005[a]	0.237 ± 0.044[a]	5.835 ± 1.334	9.426 ± 0.987[a]
+ furosemide	0.027 ± 0.006[a]	0.192 ± 0.033[a]	6.681 ± 0.583	11.588 ± 1.681
Vanadate	0.022 ± 0.005[a]	0.225 ± 0.058[a]	7.424 ± 1.294[b]	17.215 ± 1.704[b]
+ cAMP/theo.	0.034 ± 0.003[a]	0.249 ± 0.032[a]	7.242 ± 0.929[b]	15.030 ± 2.420[b]

[a] $P < 0.05$ relative to controls.
[b] $P < 0.05$ relative to cAMP/theophylline, Wilcoxon paired t-test (n = 10 to 12/group).

Table 2. Hill coefficients and equilibrium dissociation constants (K_d) derived from Hill plots of log fractional ouabain binding versus log ouabain concentration

Site	Group	Hill coefficient	P Values		Average K_d (μM)	P Values	
			vs. C	*vs.* cAMP (II)		*vs.* C	*vs.* cAMP (II)
II	Controls (C)	0.45 ± 0.03[a]	—		3.63 ± 1.08	—	—
	cAMP	1.12 ± 0.10	<0.001	—	0.50 ± 0.02	<0.02	
	+ furosemide	0.49 ± 0.08	NS	<0.001	2.20 ± 0.27	NS	<0.001
	+ vanadate	0.45 ± 0.06	NS	<0.001	1.93 ± 0.43	NS	<0.02
	Vanadate	0.50 ± 0.03	NS	<0.001	2.11 ± 0.37	NS	<0.001
I	cAMP	1.05 ± 0.03	<0.001	NS	0.02 ± 0.01	<0.001	<0.001

[a] ± SE.

ously in slice studies carried out using either high or moderate bath K (Eveloff et al., 1979). Low bath K also accentuated the curvature of the plot in controls, lending credence to a two-site hypothesis. If bath K^+ was reduced to 0.3 mM in cAMP/theophylline-treated cells [the K_m for K in shark rectal gland was reported to be ≅1 mM (Bonting, 1966)], there was essentially a doubling in the apparent number of low-affinity sites with little change in the slopes of the two-component curve, indicating minimal or no change in the K_d for each putative site.

The curves would be resolved into two, or in the case of vanadate three, independent components or possible binding sites, ranging from a K_d of $0.02 \rightarrow 3$ μM, and a combined N_{max} from 10 to 23 nmol ^{3}H ouabain bound/100 mg protein. Since it was suspected that the low-affinity site exhibited negative cooperativity, the estimated N_{max} and K_d of the high-affinity site for ouabain in studies − cAMP are not provided. This is because the estimates of site I parameters are highly dependent on whether cooperative or noncooperative kinetics are involved in the binding reactions. However, we did subject the data to analysis by Hill plots to help determine the presence/absence of cooperative kinetics. The linear slopes derived from log-log plots by necessity focused solely on the low-affinity site(s) (termed site II), since the high-affinity site (termed site I) usually represented ≦10% of total binding (Hill plots limit interpretation to 10 to 90% saturation levels). Table 2 gives the results of such Hill plots for the various groups shown in Fig. 2, as well as for an additional group (cAMP/theophylline + vanadate) alluded to in a preceding paragraph. The Hill coefficient for the binding reaction between ^{3}H ouabain and control cells was 0.45 ± 0.03, while binding data from cells treated with cAMP/theophylline generated a Hill coefficient of 1.12 ± 0.10. Cyclic cAMP/theophylline also reduced the apparent average K_d for this site from 3.5 to 0.5 μM. Both effects were reversed if either furosemide or vanadate were also present, i.e. the Hill coefficient and K_d return to values insignificantly different from controls.

Finally, when ^{3}H ouabain binding data accumulated + cAMP/theophylline were corrected for the noncooperative low-affinity site, and the residual high-affinity site I data subjected to a separate Hill

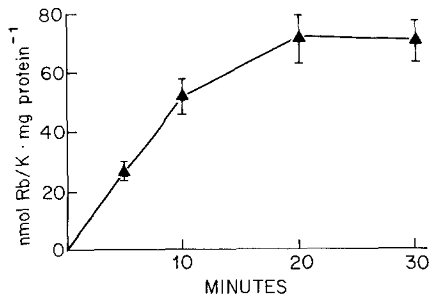

Fig. 4. Uptake of ^{36}Rb/K as a function of time (15°C). Rectal gland cells pretreated ± 1 mM ouabain were incubated with ^{86}Rb for 5 to 30 min. Values shown represent ouabain-sensitive uptake (±SE, n = 3)

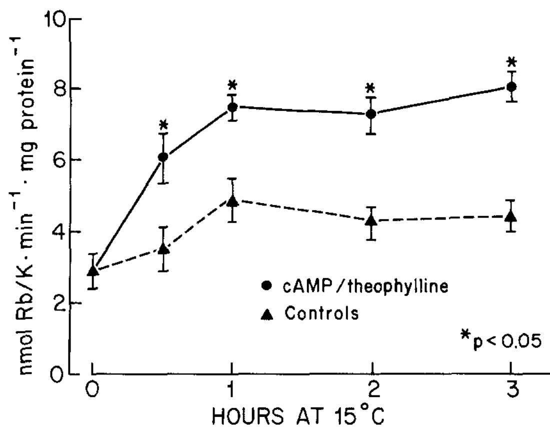

Fig. 5. Uptake of ^{86}Rb/K as a function of hr of pre-incubation ± cAMP/theophylline at 15°C. Rectal gland cells were incubated with either dibutyryl cAMP/theophylline (—●—) or diluent (--▲--) for up to 3 hr. At the times noted, ^{86}Rb was added and uptake rates monitored over 8 min. Values given (± SE) have been corrected for uptake in the presence of 1 mM ouabain (n = 3)

plot analysis, the Hill coefficient for site I was 1.05 ± 0.03, and the K_d, 0.02 ± 0.01 μM. Therefore, in the presence of cAMP/theophylline the kinetics of binding are compatible with two noninteracting sites, each individually generating noncooperative kinetics.

Rubidium Uptake

Several questions arose upon review of the ouabain binding analyses. One was, are both site I and II 'specific' in the sense that they are associated with sodium pump activity, and second, would ion fluxes support the proposed effects of cAMP, furosemide and vanadate on the pump? ^{86}Rb uptake measurements were used to help address these issues.

Rb/K uptake by the cells was first monitored as

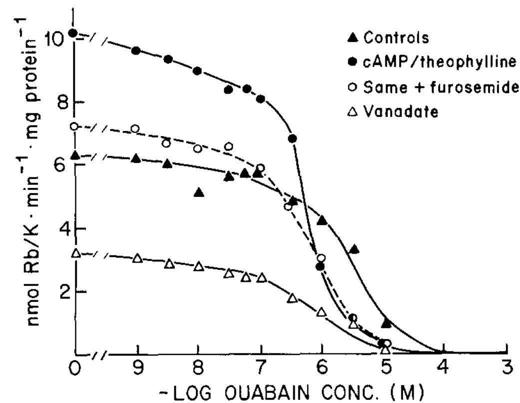

Fig. 6. Uptake of ^{86}Rb/K as a function of ambient ouabain. Isolated shark rectal gland cells were incubated for 3 hr (15°C) with 0 → 10 μM ouabain. Samples included either diluent (—▲—), vanadate (—△—), dibutyryl cAMP/theophylline (—●—), or dibutyryl cAMP/theophylline and furosemide (--○--). Values shown have been corrected for uptake in the presence of 1 mM ouabain. Each point is the average of four separate paired experiments, each utilizing pooled cells from 3 to 4 shark rectal glands

a function of time of incubation with ^{86}Rb (Fig. 4). As uptake was linear over the first 10 min of incubation at 15°C, subsequent analyses were carried out within this time frame. A second control was performed in cells that were pulsed with ^{86}Rb as a function of time incubated at 15°C ± cAMP/theophylline (Fig. 5). Cyclic AMP produced a significant increase in ^{86}Rb uptake at the earliest time point examined (30 min), as expected from the rapid effect of cAMP on Cl secretory rates (Silva et al., 1983). Although there was a significant increase in ^{86}Rb uptake rates at 1 to 3 hr in controls relative to the initial 0 time values ($P < 0.02$ to 0.05), control rates at all time points from 30 min to 3 hr did not significantly vary from each other. The viability of the preparation was inferred from the lack of a decline in uptake rates in controls over the 3 hr of incubation.

^{86}Rb was then used to estimate uptake of K by Na,K-ATPase, assuming equivalency of interaction of Rb and K with the Na pump, as shown previously in this tissue by Hilden and Hokin (1975). The same control and experimental groups represented in the Scatchard analyses in Fig. 2 were reevaluated monitoring acute ^{86}Rb uptake rates after 3 hr of incubation at 15°C. It is important to note that preliminary studies indicated that furosemide alone (3 hr) did not alter ^{86}Rb/K uptake at 3 hr relative to paired controls (6.33 ± 0.59 nmol Rb-K/min/mg protein + furosemide *vs.* 6.34 ± 0.87 in controls, P = NS).

Aggregate findings are given in Fig. 6. In the absence of ouabain, cAMP/theophylline increased the rate of ^{86}Rb uptake by >50% relative to control

rates ($P < 0.01$), and this enhanced flux was in large part inhibitable by furosemide. However, the residual flux in the presence of furosemide (7.09 ± 0.35 nmol Rb-K/min/mg protein) was increased relative to paired controls (6.26 ± 0.51 nmol Rb-K/min/mg protein, $P < 0.02$). Vanadate did not completely inhibit Rb uptake as originally expected, but reduced it to about 50% that of control values. This may reflect the modest intracellular level of inhibitor achieved, since 1 mM vanadate added to cell-free Na,K-ATPase assays of shark rectal gland cells inhibited activity to levels achieved with 1 mM ouabain [total ATPase (μmol/hr/mg protein) = 10.62 ± 0.31; residual ATPase + 1 mM vanadate = 3.67 ± 0.20; residual ATPase + 1 mM ouabain = 3.97 ± 0.12, 15°C, n = 3].

A biphasic pattern was observed when the sites were titrated with varying concentrations of ouabain. Between 10^{-9} and 10^{-7} M ouabain (site I), there was a modest decrease in ^{86}Rb uptake, while concentrations $\geqq 3 \times 10^{-7}$ (site II) promoted a marked inhibition of uptake, in keeping with both the relative fraction of enzyme associated with each proposed site, and the relative affinity of ouabain for each site. The ^{86}Rb data showed that both the low- as well as the high-affinity ouabain binding site was associated with ion flux, and thus 'specific sites' by this criterion.

If the complex ^{3}H ouabain binding and $^{86}Rb/K$ uptake curves generated with increasing concentration of labeled or unlabeled ouabain represent separate functional states of the same enzyme or separate isozymes, then these two functional states within a single group might be readily defined if the rate of ^{86}Rb uptake were plotted as a function of sites unoccupied by ouabain (i.e. the Rb/K transporting sites). This analysis appears in Fig. 7. The curves were generated assuming that the total number of specific ^{3}H ouabain binding sites was 200 pmol/mg protein, or the average N_{max} derived from previous Scatchard analyses of controls/vanadate-treated samples. Bound ouabain, at any given concentration of ouabain, was known because of the previous data represented in Fig. 2. Thus $^{86}Rb/K$ uptake was plotted as a function of the unbound sites [total (200 pmol/mg protein) minus ouabain-bound] at any given concentration of ouabain. The slopes of these lines are indicative of the turnover rates of the enzyme. Values between 180 and 200 pmol/mg protein roughly approximate the high-affinity ouabain binding site, and those below, the low-affinity site. It is interesting that the more the turnover rate, the less the apparent number of total ouabain binding sites or available α subunits. For instance, the cells treated with cAMP/theophylline have the highest enzyme turnover rate/site, and the

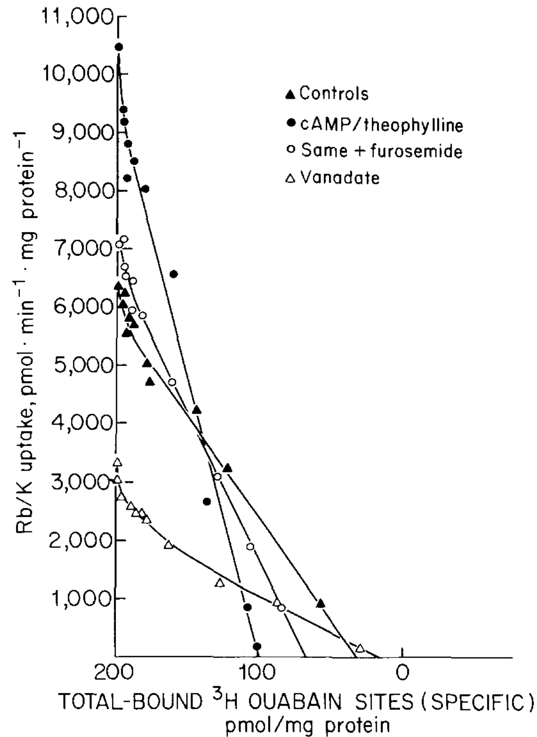

Fig. 7. $^{86}Rb/K$ uptake as a function of unoccupied ^{3}H ouabain binding sites. The data from Figs. 2 and 6 were replotted as indicated, assuming the total number of available ouabain binding sites was 200 pmol/mg protein

lowest number of apparent ^{3}H ouabain binding sites (100 *vs.* 200 pmol/mg protein). It is not readily apparent why this association should hold true.

In addition, the high-affinity ouabain binding sites exhibit a higher flux-rate per site than the low-affinity sites. Flux through each of these putative sites can be estimated from Fig. 7; these values are shown in Table 3. As indicated in Table 3, absolute Rb/K flux through site I was enhanced 3.6-fold by cAMP/theophylline while flux via site II was 1.3× higher than basal levels. In the presence of furosemide, $^{86}Rb/K$ uptake via site I remained 1.5× that of controls, while absolute flux associated with the putative site II returned to control values. If we consider fluxes + cAMP ± furosemide, furosemide reduced ouabain-sensitive site I Rb uptake by 2100 nmol and site II uptake by 1400 nmol. These particular Rb fluxes are directly linked to the Na pump and indirectly linked to the cotransporter, since a) they are entirely inhibited by high concentrations of ouabain and b) blocking Na,K-ATPase with ouabain would presumably influence the driving force for cotransporter action. Thus, the furosemide data in Table 3 cannot be used to argue for or against a parallel action of cAMP on the intrinsic performance of the cotransporter. Vanadate did not no-

Table 3. Estimation of ouabain-sensitive Rb/K flux associated with site I versus II Na,K-ATPase (nmol · min^{-1} · mg total $protein^{-1}$)

	Site I	Ratio flux/C	Site II	Ratio flux/C	I/(I + II)
Controls (C)	1000	1.00	5400	1.00	16%
+ cAMP/theo. + furosemide	1500	1.50	5600	1.04	21%
+ cAMP/theophylline	3600	3.60	7000	1.30	34%
+ vanadate	1000	1.00	2400	0.44	29%

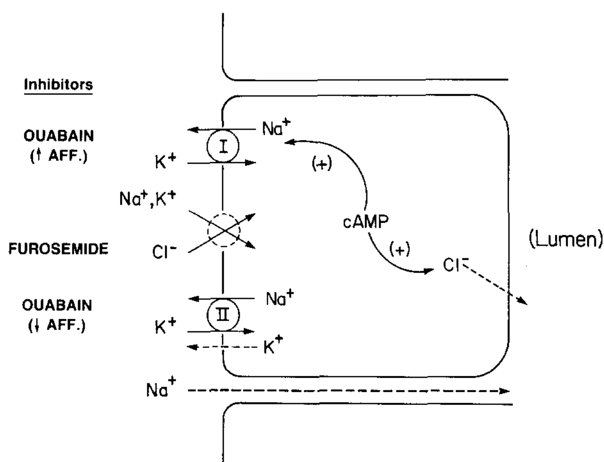

Fig. 8. Hypothetical model of cAMP effects in shark rectal gland. Two noninteracting pools of Na,K-ATPase are shown (I, II), only one of which may be directly influenced by cAMP (I)

ticeably alter the flux through the high-affinity sites, but reduced flux through low-affinity sites by >50%. This may represent variable vanadate sensitivities as has been reported previously in cardiac preparations (Clough, 1984).

Figure 8 schematically portrays the postulated direct action of cAMP on site I Na,K-ATPase, or the high-affinity ouabain-binding enzyme. Thus, it is suggested that among the cellular actions of this hormone-mediator is an augmentation of the intrinsic turnover properties of this form of the enzyme. The mechanism by which cAMP brings about such changes is unclear.

Discussion

The aggregate findings suggest that binding reactions using intact shark rectal gland cells and various concentrations of ^{3}H ouabain define two populations of binding sites, termed I and II (high *vs.* low affinity). In the control state, $\leqq$10% of the total ^{3}H ouabain bound at steady state is associated with the high-affinity site, while about 16% of the total ^{86}Rb/K flux is associated with this site. Further, the low-affinity ouabain-binding site shows cooperative kinetics (negative) in control cells, thus accounting for the wide range of ouabain concentrations required to obtain minimal *vs.* maximal inhibition of the pump. Cyclic AMP actions were examined both with respect to its effect on ouabain binding to site I *vs.* II, and to its effect on ^{86}Rb/K flux through each of these proposed sites. First, the compound stimulated ^{3}H ouabain binding to site I and depressed total ouabain binding capacity of the cells. There was also an apparent loss of the cooperative kinetics associated with site II and a shift in the average affinity of site II from about 3 to 0.5 μM in the presence of cAMP/theophylline. Ouabain binding data in controls ± cAMP also were determined following reduction of bath K to 0.3 mM. While the degree of site II cooperativity was unaltered by this maneuver (i.e. controls still exhibited negative cooperativity and cells + cAMP lacked cooperative kinetics), the apparent loss of total binding sites seen with cAMP (*vs.* controls) was reversed. This suggests that extracellular K and/or the rate of K influx modulates the apparent number of ouabain type II sites in cAMP-treated cells. However, the binding reaction seen + cAMP ± low K^{+} is itself complex and could be resolved into either two separate binding sites as described above (i.e., site I + site II) or another negative cooperative model. [It has been suggested, for instance, that Na,K-ATPase exists as a tetramer which exhibits cooperative kinetics between pairs and a separate degree of cooperative kinetics within pairs of protomers (Hansen et al., 1979).]

The analysis of ^{86}Rb flux via Type I or Type II sites supports a two-site model, as cAMP preferentially enhanced ^{86}Rb/K flux via the Type I site. While furosemide reduced the magnitude of this flux, there was still enhanced ^{86}Rb/K flux through this site relative to controls (as was there enhanced ^{3}H ouabain binding relative to controls in Scatchard analyses). One could argue that a higher concentration of furosemide might have deleted this modest stimulatory effect. The present data cannot speak to this caveat in the isolated cell experiments, as only

a single concentration of 10^{-4} M was used. However, Silva et al. (1983) previously reported significant elevations of ^{3}H ouabain binding to the high-affinity site with cAMP plus 10^{-4} M of the more potent analogue, bumetanide, as well as in experiments in which transport activity was inhibited by omitting Cl^- or Na^+ from the bathing medium. This leaves open the possibility that cAMP alters the intrinsic turnover of this isozyme (or noninteracting pool of common enzyme) in intact cells. The earlier finding that steady-state cell Na was reduced and that of K enhanced by cAMP, supports this contention (Silva et al., 1979*b*).

The alteration in kinetics associated with the low-affinity site ± cAMP/theophylline appears most likely secondary to changes in Na_i as has been proposed by Shuttleworth and Thompson (1980). Doses of ouabain high enough to bring about a significant inhibition of Na,K-ATPase would result in a rise in Na_i in cells treated with cAMP, to a level that would exceed both controls or cell fractions containing both cAMP and furosemide. The rise in Na_i may result in both an increase in the average affinity of ^{3}H ouabain for the site and the apparent loss of cooperative kinetics. It is unclear if the decrease in the number of low-affinity sites as a function of increased turnover rates represents a true down-regulation (i.e., loss of enzyme from the ouabain-binding pool), the enhanced capacity of K to compete for these sites and thus reduce the apparent amount of ^{3}H ouabain bound at steady state, or the ability of cAMP/K to alter accessibility of ouabain sites in a noncompetitive manner. While Lindenmayer and Schwartz (1970) have argued that K influences the rate but not the capacity of the Na pump for ^{3}H ouabain, it has been noted that if K altered the ouabain association rate constant > dissociation rate constant, K would modify the steady-state level of ^{3}H ouabain bound to the enzyme (Choi & Akera, 1977). It is doubtful, however, that K could make such a large difference in the apparent ouabain N_{max} parameter if the effect were directed at the association *vs.* dissociation constants. Rather it would seem plausible that either a true down-regulation of enzyme occurred (during a time when maximal transport rates were extant), or a mechanism was evoked that modified access of ouabain to approximately half of the sites. Of these two possibilities, the latter seems more reasonable.

Since ouabain-sensitive Na,K-ATPase activities were determined in permeabilized cell samples in these studies, it is possible to estimate the *in situ* turnover ± cAMP relative to maximal turnover rates at 15°C. It was found that *in situ* turnover in controls was about 3% maximal levels and about 13% in the presence of cAMP/theophylline. These relatively low fractional rates are similar to those observed by Lytton (1985) in adipocytes ± insulin (15 → 28%, 37°C), taking into account temperature differences. Various models have been invoked to explain low *in-situ* turnover rates, such as half-site reactivity, or the presence of inhibitory proteins in the intact cell (Askari & Huang, 1980; Geny et al., 1982). Maximal rates of ATP hydrolysis at 15°C was also low in permeabilized control cells (≅584 molecules ATP/min/ouabain binding site). Again, temperature in large part explains this low turnover rate, as Hokin et al. (1973) reported that activity at 15°C was only 17% that seen at 37°C in microsomal fractions of shark rectal gland.

Vanadate was used as a probe in these analyses. Its partial inhibition of ^{86}Rb flux may be the results of the reduced free intracellular level of vandate achieved in intact preparations. Sequestration of vanadate by intracellular proteins or the conversion of vanadate to the less active vanadyl ion could account for the reduced vanadate sensitivity of intact preparations (Searle et al., 1983). Nonetheless, about 50% inhibition of Rb flux was achieved. As projected, it enhanced steady-state binding of ^{3}H ouabain, thus reproducing the effects of cAMP on site I kinetics. It, however, did not reduce the apparent cooperativity of the low-affinity site, did not increase the average affinity of the low-affinity site, nor significantly decrease the apparent number of low-affinity sites, as did cAMP. Thus, actions of cAMP/theophylline on ^{3}H ouabain binding kinetics cannot be explained solely by an increase in steady-state levels of E_2-P.

That two noninteracting pools of Na,K-ATPase or Na,K-ATPase isozymes exist in rectal gland are inferred from two lines of evidence. First, ^{3}H ouabain binding data could not be reduced to a rectilinear binding site on Scatchard analysis under any of the experimental conditions employed. Second, cAMP enhanced ^{86}RB/K uptake by site I and ^{3}H ouabain binding to site I ± furosemide. The two-site model also is supported by the recent work of Esmann and Norby (1985) on inactivation of purified rectal gland (*Squalus acanthius*) Na,K-ATPase by N-ethylmaleimide. They have found that approximately 80% of enzyme activity disappears 40 to 1000× faster than the remaining 20%. They suggest that either inactivation takes place via two routes or a heterogeneous pool of enzyme exists in this tissue.

Whether two isozymes of Na,K-ATPase are involved in the shark rectal gland model requires analysis of the subunits derived from this tissue. With regard to this possibility, Sweadner (1985) and Sweadner and Gilkeson (1985) have recently reported that α (+) enzyme, with a high affinity for

ouabain, has a twofold higher affinity for ATP than α and that α exhibits negative cooperativity and α (+), positive cooperativity in binding reactions. Lytton (1985) has made the astute observation that insulin may modulate turnover via the high-affinity ouabain binding site by decreasing the $K_{0.5}$ for Na. A similar action of cAMP on sodium binding to the "high-affinity Na,K-ATPase" may occur in this preparation. Interestingly, in both rectal gland and adipocyte studies, pretreatment of cells with either cAMP or hormone (insulin), respectively, does not alter apparent Na,K-ATPase activity monitored in the cell-free state.

Against the possible presence of two distinct α subunits in shark rectal gland are the earlier analyses of Hokin et al. (1973) on the purification of shark rectal gland Na,K-ATPase. These investigators reported no evidence of heterogeneity in the α subunit as analyzed on denaturating gels. Equally plausible is the presence of two noninteracting pools of enzyme, both containing α subunit. In this instance, cellular modifiers would alter the properties of one or both pools.

Finally, what of the apparently conflicting findings presented by Shuttleworth and Thompson (1980) and Silva et al. (1983) concerning the furosemide sensitivity of cAMP effects on ^{3}H ouabain binding to rectal gland slices? It is highly likely that two groups were characterizing two separate pools of enzyme, since the former investigators used μM concentrations of ligand and the latter investigators, nM concentrations of ^{3}H ouabain. The reason for the differences in the behavior of the two pools of enzyme, however, remains to be resolved.

This study was carried out during the tenure (D.M.) of a Markey Fellowship. The work was supported by the Markey Fellowship, by NIH grants AM 21576 and AM18078, NSF grants PCM7922665 and BG7581, and a grant from the Maine Affiliate of the American Heart Association.

References

Askari, A., Huang, W. 1980. Na$^+$,K$^+$ATPase: Half-of-the-subunits cross-linking reactivity suggests an oligomeric structure containing a minimum of four catalytic subunits. *Biochem. Biophys. Res. Commun.* **93:**448–453

Bonting, S.L. 1966. Studies on NaK ATPase. The rectal gland of the elasmobranchs. *Comp. Biochem. Physiol.* **17:**953–966

Cantley, L.C., Josephine, L., Warner, R., Yanagisawa, M., Lechene, C., Guidotti, G. 1977. Vanadate is a potent NaK ATPase inhibitor found in ATP derived from muscle. *J. Biol. Chem.* **252:**7421–7423

Choi, Y.R., Akera, T. (1977). Kinetic studies on the interaction between ouabain and (Na$^+$,K$^+$)-ATPase. *Biochim. Biophys. Acta* **481:**648–659

Clough, D.L. 1984. Greater sensitivity to vanadate of rat renal relative to cardiac NaK ATPase. *Life Sci* **35:**1937–1946

Esmann, M., Norby, J.G. 1985. A kinetic model for NEM inhibition of the NaK ATPase from rectal glands of *Squalus acanthius. Biochim. Biophys. Acta* **812:**9–20

Eveloff, J., Karnakz, K.J., Silva, P., Epstein, F.H., Kinter, W.B. 1979. Elasmobranch rectal gland cell. *J. Cell Biol.* **83:**16–32

Fiske, C.H., SubbaRow, Y. 1925. The colorimetric determination of phosphorus. *J. Biol. Chem.* **66:**375–400

Geny, B., Paraf, A., Fedon, Y., Charlemagne, D. 1982. Characterization of a β-actinin-like protein in purified non-muscle cell membranes. Its activity on NaK ATPase. *Biochim. Biophys. Acta* **692:**345–354

Hansen, O. 1982. Studies on ouabain-complexed NaK ATPase carried out with vanadate. *Biochim. Biophys. Acta* **692:**187–195

Hansen, O. 1984. Interaction of cardiac glycosides with (Na$^+$ + K$^+$)-activated ATPase. A biochemical link to digitalis-induced inotropy. *Pharmacol. Rev.* **36:**143–163

Hansen, O., Jensen, J., Norby, J.G., Ottolenghi, P. 1979. A new proposal regarding the subunit composition of NaK ATPase. *Nature (London)* **280:**410–412

Hilden, S., Hokin, L.E. 1975. Active K transport coupled to active Na transport in vesicles reconstituted from purified NaK ATPase from the rectal gland of *Squalus acanthias. J. Biol. Chem.* **250:**6296–6303

Hokin, L.E., Dahl, J.L., Deupree, J.D., Dixon, J.F., Hackney, J.F., Perdue, J.F. 1973. Studies on the characterization of NaK ATPase. Purification of the enzyme from the rectal gland of *Squalus acanthias. J. Biol. Chem.* **248:**2593–2605

Huang, W.H., Askari, A. 1984. Simultaneous bindings of ATP and vanadate to (Na$^+$ + K$^+$)-ATPase. *J. Biol. Chem.* **259:** 13287–13291

Joiner, C.H., Lauf, P.K. 1978. Modulation of ouabain binding and potassium pump fluxes by cellular sodium and potassium in human and sheep erythrocytes. *J. Physiol. (London)* **283:**177–196

Lindenmayer, G.E., Schwartz, A. 1970. Conformational changes induced in NaK ATPase by ouabain through a K-sensitive reaction: Kinetic and spectroscopic studies. *Arch. Biochem. Biophys.* **140:**371–378

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:**265–275

Lytton, J. 1985. Insulin affects the Na affinity of the rat adipocyte NaK ATPase. *J. Biol. Chem.* **260:**10075–10080

Lytton, J., Lin, J.C., Guidotti, G. 1985. Identification of two molecular forms of (Na$^+$,K$^+$)-ATPase in rat adipocytes. *J. Biol. Chem.* **260:**1177–1184

Munson, P.J., Rodbard, D. 1980. LIGAND: A versatile computerized approach for characterization of ligand-binding systems. *Anal. Biochem.* **107:**220–239

Philipson, K.D., Edelman, I.S. 1977. Thyroid hormone control of Na$^+$ + K$^+$-ATPase and K-dependent phosphatase in rat heart. *Am. J. Physiol.* **232:**C196–C201

Resh, M.D., Nemenoff, R.A., Guidotti, G. 1980. Insulin stimulation of (Na$^+$,K$^+$)-adenosine triphosphatase-dependent ^{86}Rb$^+$ uptake in rat adipocytes. *J. Biol. Chem.* **255:**10938–10945

Searle, B.M., Higashiro, H., Khalil, F. Bogden, J.D., Tokushige, A., Tamura, H., Kino, M., Aviv, A. 1983. Vanadate effect on the NaK ATPase and the NaK pump in in vitro-grown rat vascular smooth muscle cells. *Circ. Res.* **53:**186–193

Shuttleworth, T.J., Thompson, J.L. 1980. The mechanism of cyclic AMP stimulation of secretion in the dogfish rectal gland. *J. Comp. Physiol.* **140:**209–216

Silva, P., Epstein, J., Spokes, K., Miller, D., Epstein, F.H. 1979*a*. Ouabain binding in rectal gland of *Squalus acanthias*. *Bull. Mt. Desert Isl. Biol. Lab.* **19:**72–74

Silva, P., Epstein, J.A., Stevens, A., Spokes, K., Epstein, F.H. 1983. Ouabain binding in rectal gland of *Squalus acanthias*. *J. Membrane Biol.* **75:**105–114

Silva, P., Stoff, J., Epstein, F.H. 1979*b*. Indirect evidence for enhancement of Na-K-ATPase activity with stimulation of rectal gland secretion. *Am. J. Physiol.* **237:**F468–F472

Silva, P., Stoff, J., Field, M., Fine, L., Forrest, J.N., Epstein, F.H. 1977. Mechanism of active chloride secretion by shark rectal gland: Role of NaK ATPase in chloride transport. *Am. J. Physiol.* **233:**F298–F306

Sweadner, K.J. 1985. Enzymatic properties of separated isozymes of the NaK ATPase. *J. Biol. Chem.* **260:**11508–11513

Sweadner, K.J., Gilkeson, R.C. 1985. Two isozymes of the NaK ATPase have distinct antigenic determinants. *J. Biol. Chem.* **260:**9016–9022

Taniguchi, K., Yoda, S. 1972. Two apparently different ouabain binding sites of (Na^+-K^+)-ATPase. *Biochim. Biophys. Acta* **288:**98–102

Wilcoxon, F. 1945. Individual comparison by ranking methods. *Biometrics Bull.* **1:**80–83

Received 14 July 1986