J. Membrane Biol 75, 105–114 (1983)

The Journal of
Membrane Biology

Ouabain Binding in Rectal Gland of *Squalus acanthias*

Patricio Silva, Jonathan A. Epstein, Arthur Stevens, Katherine Spokes and Franklin H. Epstein
Charles A. Dana Research Institute, Harvard-Thorndike Laboratory of Beth Israel Hospital, and
Department of Medicine, Harvard Medical School and Beth Israel Hospital, Boston, Massachusetts 02215

Summary. In an attempt to examine the mechanisms of activation of (Na, K)-ATPase when epithelial transport is stimulated, the binding of ouabain to rectal gland tissue was measured before and after stimulation with dibutyryl cAMP and theophylline. Stimulation significantly altered the characteristics of ouabain binding to slices of *Squalus acanthias* rectal gland and to isolated rectal gland cells, accelerating the rate of binding and increasing the amount of ouabain bound at equilibrium when low concentrations of ouabain (10^{-9} to 10^{-7} M) were present in the medium. Scatchard plots of ouabain binding were nonlinear, suggesting at least two classes of binding sites, one of higher and one of lower affinity. Stimulation with cAMP and theophylline appeared to increase the affinity of the high-affinity site. Ouabain binding was increased by cAMP and theophylline even in the presence of furosemide (10^{-4} M) or bumetanide (10^{-5} M), and when Li^+ was substituted for Na^+, or NO_3^- for Cl^--maneuvers known to inhibit rectal gland secretion. The changes in ouabain binding induced by cAMP and theophylline do not appear, therefore, to be secondary to secretory activity but may reflect a change in the configuration, environment or location of existing enzyme so as to enhance its activity. Stimulation of ouabain binding cannot be demonstrated in whole homogenates of rectal gland, indicating that intact cells are necessary for the cyclic AMP-induced increase in ouabain binding to become manifest.

Key Words shark rectal gland · ouabain binding · (Na, K)-ATPase · cyclic AMP · slices · isolated cells

Introduction

The rectal gland of the spiny dogfish, *Squalus acanthias,* secretes chloride against an electrochemical gradient. The process requires the expenditure of energy (Silva et al., 1980), depends on the activity of (Na, K)-ATPase located in basolateral cell membrane of the cells lining the tubules (Silva et al., 1977), and involves the cotransport of sodium and chloride into the cell across the basolateral cell membrane (Silva et al., 1977; Eveloff et al., 1978). The secretion of chloride by the rectal gland can be greatly stimulated by vasoactive intestinal peptide (Stoff et al., 1979) or its putative second messenger, cyclic AMP (Stoff et al., 1977). When chloride secretion is stimulated by cyclic AMP in isolated perfused rectal glands, the activity of (Na, K)-ATPase appears to be increased, as indicated by the following observations. First, intracellular concentration of sodium falls and that of potassium may rise. Second, ouabain-inhibitable oxygen consumption increases sevenfold (Silva, Stoff & Epstein, 1979*b*).

The purpose of the present studies was to explore the mechanism of activation of (Na, K)-AT'Pase in the rectal gland of *Squalus acanthias* by investigating the changes in ouabain binding after stimulation.

Materials and Methods

Dogfish of either sex were taken by gill nets or by hook and line from Frenchman Bay, Maine, and kept in marine live cars until used, usually within three days of capture. Dogfish were killed by segmental transection of the cord and the rectal glands removed via an abdominal incision.

Ouabain Binding Studies

The rectal glands were perfused by gravity with 100 ml of shark-Ringer's with or without theophylline 2.5×10^{-5} M and dibutyryl cyclic AMP 5×10^{-5} M. The addition of theophylline and cAMP regularly increased rectal gland secretion, as previously reported (Silva et al., 1977). Coronal slices of rectal gland were prepared using a Stadie-Riggs microtome. The slices were kept in ice-cold shark-Ringer's until used, usually within fifteen minutes. The binding of ouabain to slices of rectal gland was measured in a solution of the following composition (in mM): Na 280; K 5; Cl 295; Mg 3; Ca 2.5; SO_4 0.5; phosphate 1; urea 350; glucose 5; bicarbonate 8; pH 7.6 with a 99% O_2/1% CO_2 gas phase; containing 10^{-9} M carrier-free ^{3}H-ouabain. The temperature of incubation was 25 °C. The time necessary to achieve steady-state binding was determined first and all other incubations were run for that length of time. A parallel incubation containing 10^{-3} M theophylline and 10^{-3} M dibutyryl cyclic AMP was run to determine the effect of stimulation of rectal gland cells on ouabain binding. Additional incubations were done with varying concentrations of unlabeled ouabain in order to determine the kinetics of ouabain binding. Nonspecific bind-

ing of ouabain to the slices was estimated by measuring the binding of tritiated ouabain in the presence of 10^{-4} M unlabeled ouabain. Extracellular space was estimated using ^{14}C-inulin, 0.1 μCi/ml. After varying times of incubation, the slices were removed from the incubation medium and transferred through three successive washes in ice-cold ouabain-free solutions of the same composition as the incubation medium to remove all unbound ouabain. As found previously, there was no loss of bound ^{3}H-ouabain from rectal gland slices washed at 0–4 °C (Eveloff et al., 1979). Adequacy of removal of the free ouabain was assessed by measuring the amount of ^{14}C-inulin remaining in the slices after the three washes. In all experiments ^{14}C counts returned to background. The slices were then lightly blotted on a piece of filter paper, weighed, and dissolved in NCS®. The dissolved slices were counted for radioactivity in a scintillation counter using Spectrafluor®. Specific binding to the slices was calculated from the radioactivity remaining in the tissue minus that remaining in the presence of 10^{-4} M ouabain, divided by the specific activity of the ouabain in the medium. Results are expressed as picomoles of ouabain bound per unit of wet weight.

Cells were isolated from rectal glands using enzymatic digestion. After perfusion with 100 ml of shark-Ringer's solution of the following composition (in mM): Na 280; K 5; Cl 295; Mg 3; Ca 2.5; SO_4 0.5; phosphate 1; urea 350; glucose 5; pyruvate 10; acetate 2; bicarbonate 8; pH 7.6 with a gas phase of 99% O_2/1% CO_2; the rectal glands were perfused with 10 ml of a shark-Ringer's containing in addition 0.2% collagenase, 0.25% hyaluronidase, and 10% fetal calf serum. The glands were then minced with a razor blade and the minced tissue digested in the same solution for 45 min at room temperature. The tissue digest was centrifuged at 500 rpm for 1 min a refrigerated centrifuge to remove undigested tubules and the supernatant was then centrifuged at 1500 rpm 3 min to harvest the cells. The cells were washed twice and suspended in a final volume of approximately 1 ml in a buffer of the following composition (in mM): Na 280; K 5; Cl 295; Mg 3; Ca 2.5; SO_4 0.5; phosphate 1; urea 350; HEPES 40; pH 7.6. Binding of ouabain to isolated rectal gland cells was measured using a filtration technique. The cells were incubated in a solution of the same composition as the one used to resuspended them containing in addition 10^{-9} M ^{3}H-ouabain and various concentrations of unlabeled ouabain. The temperature of incubation was 25 °C. The time course of incubation required to achieve steady-state binding was determined first and all subsequent incubations run for the length of time necessary to obtain steady-state binding. Incubations were stopped by dilution of the cells, still in the incubation medium, in ten times the volume of ouabain-free incubation solution followed by rapid filtration through HA 0.45 micron Millipore filters. The filters were then washed with 4 ml of incubation solution without ouabain. ^{14}C-inulin, 0.1 μCi/ml, was used as a marker of extracellular space. The filters were dissolved in 1 ml of ethylacetate and radioactivity counted in a scintillation counter using Hydrofluor®. Nonspecific binding to the filters was measured by filtering incubation solution containing labeled and unlabeled ouabain and labeled inulin without cells. Specific binding of ^{3}H-ouabain to cells was determined by displacement of the labeled ouabain with 10^{-4} M unlabeled ouabain. Specific binding to the cells was calculated from the total ouabain binding minus the ^{3}H-ouabain binding after incubation with 10^{-4} M ouabain and minus the binding to the filter blanks. Results are expressed as picomoles of ouabain bound per milligram of protein (Lowry et al., 1951).

Homogenates were prepared from perfused rectal glands. Prior to the preparation of the homogenates, rectal glands were perfused for 30 min with shark-Ringer's with and without theophylline 2.5×10^{-4} M and dibutyryl cyclic AMP 5×10^{-5} M. The glands were homogenized in a buffer of the following composition (in mM): Na 280; K 5; Cl 295; Mg 3; Ca 2.5; SO_4 0.5; phosphate 1; urea 350; HEPES 40; pH 7.6, containing in addition an ATP regenerating system (phosphoenol pyruvate 2×10^{-2} M, and pyruvate kinase 16 units/ml). Binding of ouabain was measured using a filtration technique. ^{14}C-Inulin, 0.1 μCi/ml, was used as a label for the incubation solution. The homogenate was incubated in a solution of the same composition as the homogenizing solution in the presence and absence of 10^{-3} M theophylline and 10^{-3} M dibutyryl cyclic AMP. Incubations were stopped by dilution of a measured amount of homogenate, still in the incubation medium, in ten times the volume of ouabain-free incubation solution followed by rapid filtration through HA 0.45 micron Millipore filters. The filters were dissolved in 1 ml of ethylacetate and radioactivity counted in a scintillation counter using Hydrofluor®. Nonspecific binding of ^{3}H-ouabain was determined by displacement of the labeled ouabain with 10^{-4} M unlabeled ouabain. Nonspecific binding to the filters was measured by filtering incubation solution containing labeled and unlabeled ouabain and labeled inulin but without homogenate. Specific binding to the homogenate was calculated from the total binding minus the nonspecific binding after incubation with 10^{-4} M ouabain and minus the nonspecific binding to the filter blanks. Results are expressed as picomoles of ouabain bound per milligram of protein.

Rectal Gland Perfusion

The rectal gland artery, vein and duct were cannulated with PE-90 tubing. The glands were kept at 15 °C in a glass perfusion chamber. Perfusion was done by gravity at a pressure of 40 mm Hg and a temperature of 15 °C in a glass perfusion chamber. The concentration of the perfusate was (in mM): Na 280; Cl 290; K 5; HCO_3 8; Ca 2.5; Mg 1.2; phosphate 2.0; SO_4 1; urea 350; pH 7.6 when gassed with 99/1 O_2/CO_2. Glucose was the sole exogenous substrate. Secretion from the duct was collected at timed intervals and its volume measured. The perfusate was also collected over the same time interval and its volume measured.

Oxygen Consumption Measurements

In isolated perfused rectal glands oxygen tension was measured using a polarographic oxygen electrode in both arterial and venous perfusate. The perfusate was sampled anaerobically through a self-sealing connector placed in the arterial line immediately before the gland and directly from a connector at the end of a short venous catheter. Oxygen content in the perfusate was calculated from the solubility coefficient of oxygen adjusted for temperature and solute content. Oxygen consumption was calculated from the arteriovenous oxygen difference and the timed flow of the perfusate. Results were expressed as micromoles of oxygen consumed per hr per gram wet weight.

Oxygen consumption of slices and isolated cells was measured using a YSI oxygen meter and connected to a recorder equipped with a constant temperature (37 °C) cell that was stirred constantly. The measuring chamber contained in both cases 1.5 ml of a solution of the following composition (in mM): Na 280; K 5; Cl 295; Mg 3; Ca 2.5; SO_4 0.5; phosphate 1; urea 350; HEPES 40; pH 7.6; glucose 5; acetate 5. The oxygen consumption was calculated from the rate of decrease in the oxygen tension, the solubility coefficient of oxygen, the wet weight of the cells or slices, and the volume of solution in the chamber. The results were expressed as micromoles of oxygen consumed per hr per gram wet weight.

Materials

Bovine serum albumin, HEPES, dibutyryl cyclic AMP and colchicine were obtained from Sigma Chemical Co., St. Louis, Mo., ouabain from K & K, Plainview, N.Y., collagenase from Worthington, Freehold, N.J., hyaluronidase from Boehringer-Mannheim, Indianapolis, Ind., fetal calf serum from Gibco, Grand Island, N.Y. Hydrofluor®, ^{3}H-ouabain, and ^{14}C-inulin from New England Nuclear, Boston, Mass., NCS® and Spectrafluor® were obtained from Amersham, Arlington Heights, Ill. All other chemicals were obtained from regular suppliers and were reagent grade.

All values are expressed as mean ± SEM. Statistical significance was determined using Student's "t" test or "paired t" test wherever appropriate.

Animals used in this study were maintained in accordance with the guidelines of the Animal Care Committee of the Beth Israel Hospital and those prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW publication No. (NIH) 78-23, revised 1978).

Results

Time Course of Ouabain Binding

Binding of 10^{-9} M ^{3}H-ouabain to slices of rectal gland required approximately 4 hr to reach a steady state at 25 °C (Fig. 1). Binding of ^{3}H-ouabain was 50% greater in the presence of theophylline and cyclic AMP than in unstimulated control slices. The rate of binding was temperature dependent; at a temperature of incubation of 15 °C, binding did not reach steady state by 5 hr of incubation; at 37 °C (*data not shown*) binding reached a peak at 120 min and then steadily declined. The rate of binding also depended on the concentration of ouabain in the incubation medium. As the concentration of ouabain increased, the time required to attain the steady state shortened. At 10^{-4} M, steady-state binding was achieved by 120 min (Fig. 2). The rate of labeling of the extracellular space with ^{14}C-inulin was measured in a separate set of slices to determine whether the slow binding of ouabain was due to poor permeation of the extracellular space by the ouabain. Accumulation of ^{14}C-inulin by the slices reached a maximum between 30 and 60 min of incubation and remained stable thereafter, indicating that there was rapid equilibration between the extracellular space of the slices and the incubation solution.

The time course of binding was also measured in isolated rectal gland cells (Fig. 3) which showed a pattern similar to that seen in slices.

In both slices and isolated rectal gland cells, dibutyryl cyclic AMP and theophylline stimulated oxygen consumption by three- to fivefold (Table 1), presumably a reflection of the increased energy demand of ion transport.

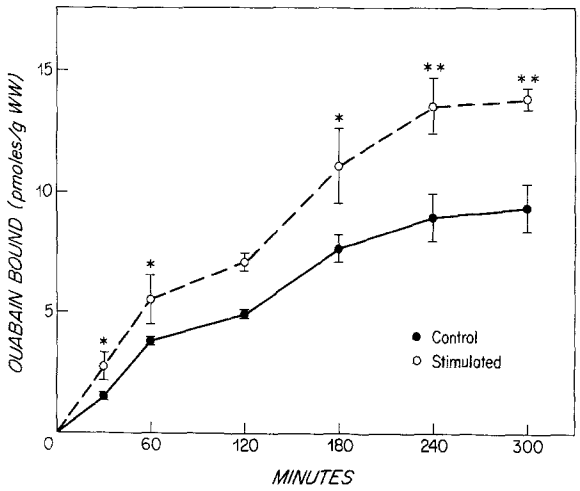

Fig. 1. Time course of ^{3}H-ouabain binding (10^{-9} M) to slices of rectal gland at 25 °C. Each point represent duplicate determinations in six experiments. Steady-state binding was reached after 4 hr of incubation. Binding was 50% greater in the presence of theophylline 10^{-3} M and dibutyryl cyclic AMP 10^{-3} M. Symbols are mean ± SEM. $^*P<0.05$, $^{**}P<0.01$

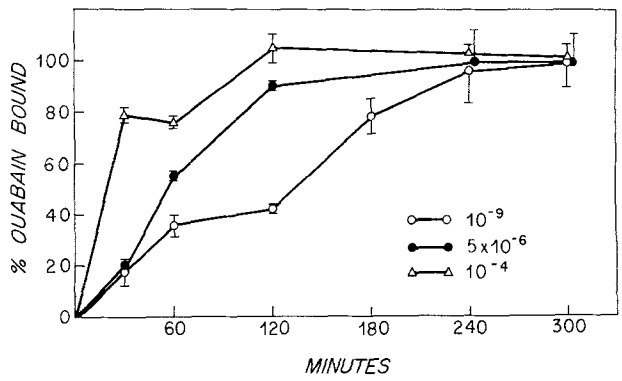

Fig. 2. Time course of the binding of ouabain at 25 °C to slices of rectal gland at different concentrations of ouabain in the incubation solution. The time required to reach a steady state decreases as the ambient concentration of ouabain increases. The concentration of ^{3}H-ouabain used as tracer was 10^{-9} M in all experiments. Symbols are mean ± SEM, $n=6$ for 10^{-9} M, 4 for 5×10^{-6} M and 6 for 10^{-4} M

Binding of ^{3}H-Ouabain to Rectal Gland at Varying Ouabain Concentrations

Stimulation with cAMP and theophylline affected ouabain binding in a way dependent on ouabain concentration. The effect of varying the concentration of unlabeled ouabain on the binding of ^{3}H-ouabain to rectal gland slices after 300 min of incubation at 25 °C is shown in Fig. 4. At concentrations of unlabeled ouabain lower than 2×10^{-7} M, stimulation with dibutyryl cyclic AMP and the-

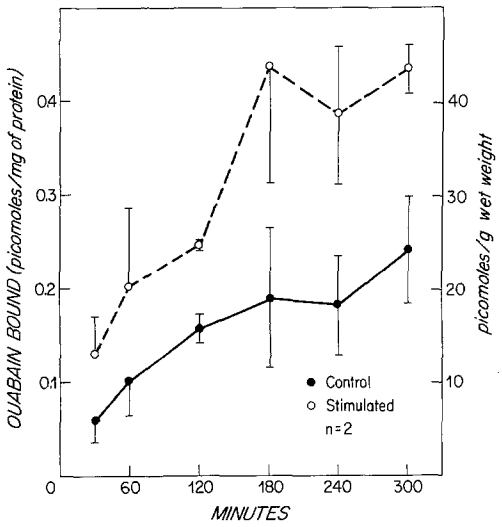

Fig. 3. Time course of ^{3}H-ouabain binding (10^{-9} M) to isolated rectal gland cells at 25 °C. Symbols are mean ± SEM, $n=2$

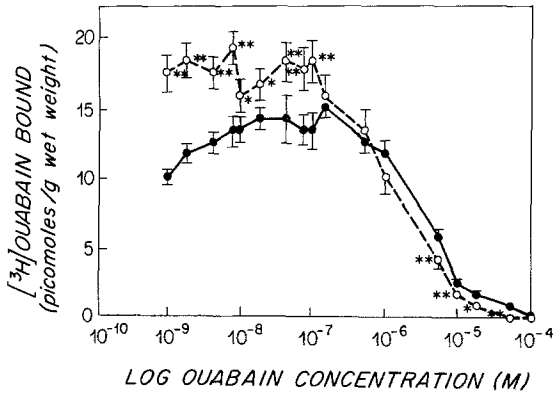

Fig. 4. Relation between ouabain concentration in the incubation solution and ^{3}H-ouabain bound to slices of rectal gland. Symbols as in Fig. 1. Each point represents the average of eight experiments performed in duplicate. Dibutyryl cyclic AMP 10^{-3} M and theophylline 10^{-3} M significantly increased the amount of ^{3}H-ouabain bound up to and including a ouabain concentration in the incubation solution of 10^{-7} M. Symbols are mean ± SEM, $*P<0.05$, $**P<0.01$

Table 1. Effect of dibutyryl cyclic AMP and theophylline on oxygen consumption in rectal gland slices and isolated cells

Experimental conditions	Control	Dibutyryl cyclic AMP and theophylline
Rectal gland slices ($T=15$ °C, db cAMP 0.5×10^{-3} M, theophylline 2.5×10^{-3} M)	16 ± 5 (4)	85 ± 27 (4)[a]
Isolated rectal gland cells ($T=20$ °C, db cAMP 2×10^{-3} M, theophylline 2×10^{-3} M)	19 ± 4 (6)	65 ± 5 (6)[b]

Units are micromoles of O_2 utilized per hr per gram wet weight. Values are mean ± SEM, number of observations in parentheses.

[a] $P<0.05$. [b] $P<0.005$.

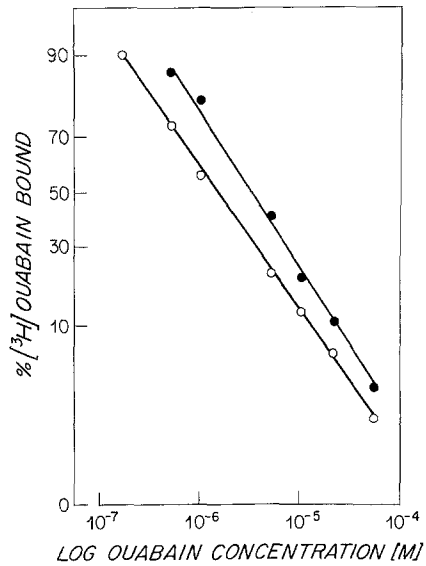

Fig. 5. Probit transformation of the data shown in Fig. 4. The average of the ^{3}H-ouabain bound at a concentration of ouabain in the incubation medium from 10^{-9} to 10^{-7} M was used as the 100% value for the ^{3}H-ouabain bound in the presence of dibutyryl cyclic AMP and theophylline. The average of only the four highest points were used in the control experiments as the 100% value because the amount of ^{3}H-ouabain bound increased as ouabain in the incubation medium was added up to a concentration of 10^{-8} M. The half-maximal concentration values calculated from these lines are therefore weighted against the finding of a difference between control and experimental groups

ophylline clearly increased the binding of ^{3}H-ouabain, whereas at ouabain concentrations above 10^{-7} M, the curves of ^{3}H-ouabain binding in the presence and absence of stimulation resembled one another.

A probit transformation of these curves (Fig. 5) (Akera & Cheng, 1977), permits the calculation of an apparent K_d of 1.4×10^{-6} M ouabain per gram wet weight in stimulated slices, and 3.0×10^{-6} M per gram wet weight in basal controls. Thus, the addition of cAMP and theophylline appeared to decrease the dissociation constant, K_d, i.e., to increase the affinity of rectal gland slices for tritiated ouabain.

Stimulation produced a similar change in the binding of ^{3}H-ouabain to isolated cells (Fig. 6). The probit transformation plot of the data shown in Fig. 6 indicates that cAMP and theophylline decreased the apparent K_d from 2.3×10^{-7} M in basal controls to 1.2×10^{-7} M, reflecting an increase in affinity with stimulation (Fig. 7).

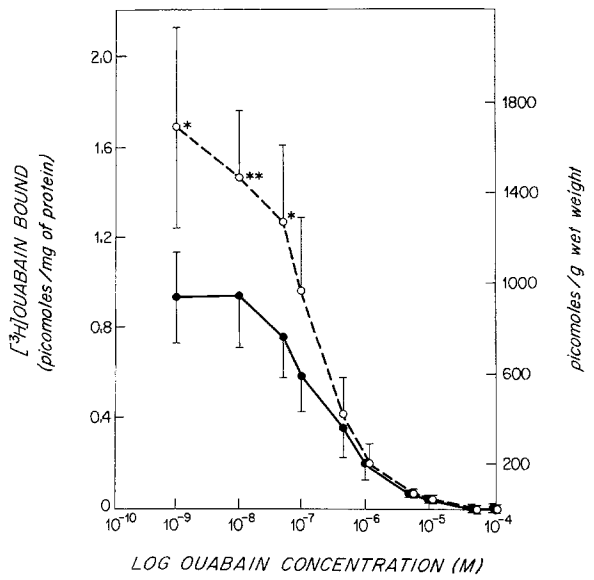

Fig. 6. Relation between ouabain concentration in the incubation solution and ^{3}H-ouabain bound to isolated rectal gland cells. Each point represents the average of single determinations in six different experiments. Dibutyryl cyclic AMP 10^{-3} M and theophylline 10^{-3} M significantly increased the amount of ^{3}H-ouabain bound up to and including a ouabain concentration in the incubation solution of 5×10^{-8} M. Symbols are mean ± SEM. $*P<0.05$, $**P<0.01$

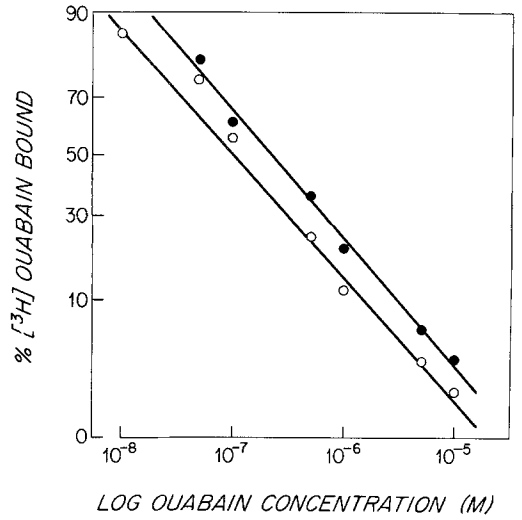

Fig. 7. Probit transformation of data shown in Fig. 6. Symbols are the mean of six experiments

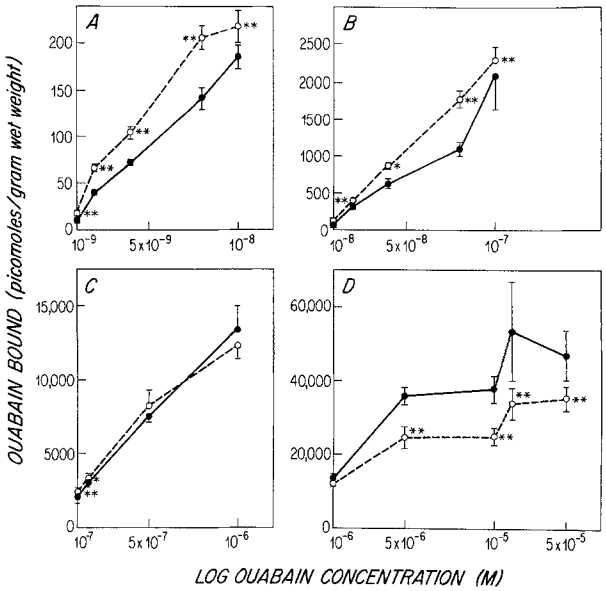

Fig. 8. Relation between ouabain concentration in the incubation solution and total ouabain bound to slices of rectal gland. Symbols as in Fig. 1. Each point represents the average of eight experiments done at least in duplicate. With the exception of the points at a ouabain concentration in the incubation solution of 5×10^{-7} M and 10^{-6} M, stimulation with theophylline 10^{-3} M and dibutyryl cyclic AMP 10^{-3} M significantly changed ouabain binding to slices of rectal gland. Below 10^{-6} M it increased binding; above 10^{-6} M it reduced it. Symbols are mean ± SEM. $*P<0.05$, $**P<0.01$

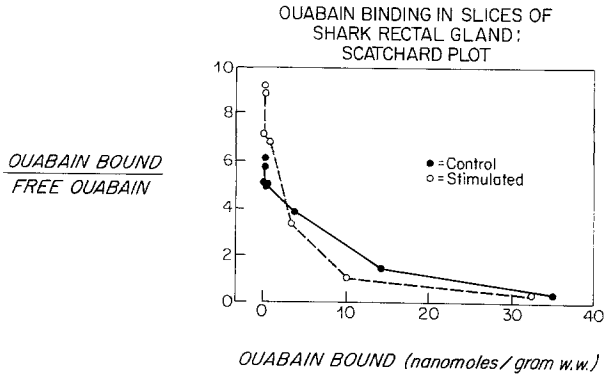

Fig. 9. Scatchard plot of the data shown in Fig. 8. The relation between bound ouabain/free ouabain *vs.* bound ouabain is curvilinear both in the presence and absence of theophylline 10^{-3} M and dibutyryl cyclic AMP 10^{-3} M

The total quantity of ouabain bound to rectal gland slices at various ouabain concentrations in the incubation solution is shown graphically in Fig. 8. Binding reaches saturation at a ouabain concentration of 2×10^{-5} M both in the presence and in the absence of theophylline and cyclic AMP (Fig. 8*D*). At concentrations of ouabain greater than 10^{-6} M the binding of ouabain is significantly depressed by theophylline and cyclic AMP (Fig. 8*D*). At concentrations of ouabain lower than 5×10^{-7} M, however, theophylline and cyclic AMP significantly increased ouabain binding above that of controls (Fig. 8*A–C*). The findings in six separate experiments using isolated rectal gland cells were similar.

When these data are visualized in a Scatchard

Table 2. Calculated kinetic parameters of ouabain binding to rectal gland slices

	Number of sites (nanomoles)		Apparent K_d ($M \times 10^{-7}$)	
	N_1	N_2	K_{d_1}	K_{d_2}
Control	0.32	1.18	2.44	50
Stimulated	0.22	1.41	0.6	50

Values are calculated from the experimental data of Fig. 7, using the equation for binding given in the text. N_1 and N_2 represent the number of high-affinity and low-affinity sites, respectively. K_{d_1} and K_{d_2} are the dissociation constants at the high-affinity and low-affinity sites.

plot (Fig. 9), the relation between bound/free ouabain and bound ouabain is curvilinear both in the presence and absence of theophylline and cyclic AMP. Curvilinearity in the Scatchard plots speaks against a simple binding relation for ouabain to the rectal gland cell and suggests either that there is more than one binding site or that "negative cooperativity" alters the characteristics of binding. Calculation of the "average affinity" according to DeMeyts and Roth (1975), indicates that this is increased by cyclic AMP and theophylline by 50% from 0.12 liter/micromole to 0.18 liter/micromole.

A mathematical model can be developed to fit the experimental data of Fig. 8, using the law of mass action for analysis of binding, as follows:

$$\text{Binding} = \frac{n_1 K_1 F}{1 + K_1 F} + \frac{n_2 K_2 F}{1 + K_2 F}$$

where n is the number of class 1 (high affinity) or 2 (low affinity) binding sites, K is the affinity constant for class 1 or 2 binding sites, and F is a weight factor different for the two classes of sites. Using this function, values for the number of putative binding sites and their apparent affinities can be calculated (Table 2). Stimulation with dibutyryl cAMP and theophylline increased the apparent affinity of the high affinity binding site K_1 by 400%, from 41 liter/nmol ($K_d = 2.44 \times 10^{-7}$ M) to 168 liter/nmol ($K_d = 0.6 \times 10^{-7}$ M). The apparent affinity of K_2, the low-affinity binding site, was not altered and the number of binding sites were not modified by stimulation.

Comparison of Ouabain Binding with Oxygen Consumption and Chloride Secretion of Intact Rectal Glands

In two isolated perfused rectal glands, fifty percent inhibition of oxygen consumption (K_i) was seen at a ouabain concentration of 5.5×10^{-7} M while

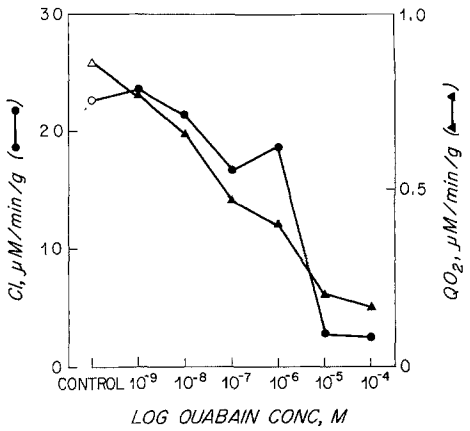

Fig. 10. Inhibition by ouabain of the ratio of oxygen consumption (▲—▲) and chloride secretion (●—●) in two isolated perfused rectal glands. Oxygen consumption and chloride secretion were measured over a time span of 240 min. Ouabain was added sequentially every 30 min to increase the concentration in the perfusate in steps. Three 10-min collections were made during each 30-min interval and the results of the last two collections averaged

maximal inhibition was seen at 10^{-5} M (Fig. 10). Inhibition of the rate of chloride secretion, also shown in this Figure, roughly paralleled that of oxygen consumption. This K_i may be compared to the half-maximal binding concentration (dissociation constant) of ouabain binding in rectal gland slices (Table 2) as well as to the level of ouabain concentration in the medium at which the effects of cAMP stimulation on binding were observed. As in HeLa and kidney cells (Baker & Willis, 1972) the concentration of ouabain necessary to inhibit transport appears to be slightly greater than that necessary to saturate high-affinity binding sites. Part or all of the difference seen in the present experiments, however, may have been due to differences in the time of ouabain exposure. The data shown in Fig. 10 were obtained at 30-min intervals after sequential addition of ouabain, whereas in the binding experiments slices were exposed to ouabain for 300 min in order to attain binding equilibrium.

Effect of Stimulation on Ouabain Binding when Rectal Gland Secretion is Inhibited (Table 3)

An important question is whether the change in ouabain binding induced by cAMP and theophylline is a direct result of the cellular action of cAMP or an indirect consequence of ion secretion by rectal gland cells. For example, a plausible mechanism for activation of (Na, K)-ATPase might be that an increase in the movement of sodi-

Table 3. Effect of furosemide, bumetanide and sodium or chloride removal on the stimulation of ouabain binding with dibutyryl cyclic AMP (10^{-3} M) and theophylline (10^{-3} M) in rectal gland slices

	10^{-9} M ouabain		10^{-8} M ouabain		Mean % increase with stimulation
	Basal	Stimulated	Basal	Stimulated	
Control	12.3±0.9 (4)	21.6±2.5 (4)			78±20 (4)[b]
Furosemide 10^{-4} M	11.8±1.4 (4)	24.7±2.9 (4)			111±18 (4)[c]
Control	14.3 (1)	26.9 (1)	154.4±16.4 (3)	226.4±20.5 (3)	58±11 (4)[c]
Bumetanide 10^{-5} M	15.2 (1)	19.8 (1)	186.2±32.1 (3)	278.3±32.1 (3)	46±16 (4)[a]
Control			113.3±10.9 (6)	173.0±20.8 (6)	55±12 (6)[c]
Bumetanide 10^{-4} M			139.0±14.2 (3)	200.7±35.5 (6)	42±16 (6)[b]
Solvent			144.3±19.8	179.8± 8.5 (4)	30±13 (6)[a]
Control			215.6±14.1 (3)	290.3±12.4 (3)	37±14 (3)[a]
Lithium chloride (no sodium)			203.0± 9.6 (3)	260.1±15.1 (3)	29± 9 (3)[b]
Control	14.3 (1)	26.9 (1)	154.4±16.4 (3)	226.4±20.5 (3)	58±11 (4)[c]
Sodium nitrate (no chloride)		20.9 (1)	181.6±22.8 (3)	291.1±37.6 (3)	61±11 (3)[c]

Units are picomoles of ouabain bound per gram wet weight. Values are mean ± SEM (n).

[a] $P<0.05$. [b] $P<0.025$. [c] $P<0.01$.

um into the cell, or a change in transmembrane potential evoked by the secretory process, might directly activate the enzyme. Secretion can be inhibited in the rectal gland by "loop diuretics" like furosemide or bumetanide, as well as by incubating or perfusing in the absence of Na^+ or Cl^- (Silva et al., 1977; Palfrey et al., 1979). These maneuvers also block sodium-chloride cotransport across isolated basolateral membrane vesicles derived from rectal gland homogenates (Eveloff et al., 1978). Furosemide has also been found to inhibit the downhill movement of sodium into intact gland cells that is stimulated by cAMP and theophylline (Silva et al., 1979*a*). High-affinity ouabain binding (at 10^{-9} M and 10^{-8} M) was therefore measured in slices incubated with and without theophylline and cyclic AMP in the presence of furosemide (10^{-4} M) and bumetanide (10^{-4} and 10^{-5} M). Additional experiments were carried out in which sodium was replaced by lithium, or chloride was replaced by nitrate, maneuvers that inhibit secretion by interfering with sodium chloride cotransport (Solomon et al., 1977; Silva et al., 1980). Table 3 illustrates that theophylline and cyclic AMP stimulated ouabain binding even in the presence of furosemide or bumetanide and whether or not sodium or chloride were present in the incubation solution.

Effect of Colchicine on Ouabain Binding

The increase in ouabain binding evoked by dibutyryl cyclic AMP and theophylline could be the expression of an increase in the number of membrane binding sites as a result of transfer to the cell membrane of already formed binding sites available in the cytoplasm. If such is the case, a role for membrane bound or cytoplasmic microtubules might be envisioned as the mechanism for the transfer of the preformed sites to the cell membrane. Colchicine at a concentration of 10^{-4} M had no effect on the increased ouabain binding brought about by dibutyryl cyclic AMP and theophylline. Dibutyryl cyclic AMP and theophylline increased ouabain binding by 44±12% ($n=6$) in the presence of colchicine, from 138.0±7.9 picomoles/g wet weight to 200.7±35.5, $P<0.01$, while in parallel controls in the absence of colchicine binding increased by 46±12% ($n=6$) from 127±6.6 to 187.6±19.9. This experiment suggests that tubulin, as far as can be determined by the lack of effect of colchicine, does not appear to be involved in the process of stimulation of ouabain binding by dibutyryl cyclic AMP and theophylline.

Effect of Stimulation on Ouabain Binding by Whole Homogenates of Rectal Gland

The stimulation of ouabain binding induced by dibutyryl cyclic AMP and theophylline has been observed both in rectal gland slices and in isolated cells. To test whether an intact cellular architecture is necessary to discern this effect, we examined the effect of prior stimulation by dibutyryl cyclic AMP and theophylline on ouabain binding by rectal gland homogenates.

Table 4. Ouabain binding by whole homogenates of basal and stimulated rectal glands

Time (min)	10^{-8} M ^{3}H-ouabain	
	Basal	Stimulated
0	0.06±0.02 (4)	0.04±0.02 (5)
15	0.88±0.13 (4)	0.63±0.32 (5)
30	1.82±0.15 (5)	1.15±0.30 (5)[a]
60	3.36±0.26 (5)	2.04±0.25 (5)[a]
120	5.72±0.56 (5)	3.87±0.32 (5)[a]
180	7.74±1.04 (5)	5.23±0.54 (5)[a]
240	9.33±1.11 (5)	6.50±0.76 (5)[a]
300	10.41±1.30 (5)	7.23±0.86 (5)[a]

Units are picomoles of ouabain bound per milligram of protein. Values are mean±SEM (*n*).

[a] $P<0.05$.

Table 4 shows the time course of ouabain binding to homogenates of basal and stimulated rectal glands. Ouabain binding was not increased in homogenates of stimulated glands but rather decreased, indicating that disruption of cells eliminates the enhancing effect of cAMP stimulation on high-affinity binding.

Discussion

The way in which membrane (Na, K)-ATPase is activated when transepithelial transport in various organs is stimulated remains an interesting and unsolved question. It can be studied usefully in the rectal gland because transport work can be abruptly increased to such high levels. Under these circumstances, ouabain-inhibitable oxygen consumption increases several-fold, providing strong evidence that the hydrolysis of ATP by (Na, K)-ATPase is accelerated. The demonstration that the ouabain-binding characteristics of intact cells are altered under these circumstances suggests that the process of activation involves a change in the nature of the membrane-bound enzyme.

The binding of ouabain to rectal gland cells is complex. While it must be appreciated that the interpretation of nonlinear Scatchard plots is fraught with difficulties (Klotz & Hunston, 1975; Hansen, 1976), analysis using the common methods of determining kinetic parameters indicates that the relationship between binding and the concentration of ouabain in the incubation medium cannot be expressed as the simple interaction of ouabain with a single binding site, as is the case in some systems (Erdman & Schoner, 1973; Joiner & Lauf, 1978; Schwartz & Adams, 1980). The present findings are reminiscent of other reports (Baker & Willis, 1972; Taniguchi & Ilida, 1972; Erdman & Schoner, 1973; Hansen, 1976). Two populations of ouabain-binding species have been described in cardiac membrane vesicles (Wellsmith & Lindenmayer, 1980) and, based on the nonlinearity of Scatchard plots, Hansen (1976) postulated two or more classes of enzymes with different affinities for substrates and ligands which affect cardiac glycoside binding. A Scatchard plot of our data showed marked curvilinearity, suggesting that the binding of ouabain to rectal gland cells involves more than one site. Alternatively, the phenomenon of "negative cooperativity" might be present; i.e., as occupancy of a single class of binding sites increases there is progressive inhibition of ouabain binding at the remaining available sites (DeMeyts & Roth, 1975). Arithmetical analysis of the data is compatible with the law of mass action for two different binding sites, one of high and one of low affinity, with no interaction between them (Table 2). While no method of mathematical analysis offers a definitive answer, all suggest that exposure of the rectal gland to cAMP and theophylline increases the affinity of the gland for ouabain, while not greatly affecting the total number of binding sites.

That the rate of ouabain-binding is accelerated when membrane transport is increased has been reported for erythrocytes (Joiner & Lauf, 1978), frog skin (Cala, Cogswell & Mandel, 1978), skeletal muscle (Erlij & Grinstein, 1976; Clausen & Hansen, 1977; Erlij & Schoen, 1981), cultured toad bladder epithelial cells (Handler et al., 1981) and cultured pig kidney epithelial cells (Mills et al., 1981). An increase in the rate of binding need not, however, be associated with an increase in the amount of ouabain bound at equilibrium. In the present experiments, incubation was carried out over a long enough time for equilibrium to be reached. The results are noteworthy in that, at its high-affinity site (e.g., at concentrations of ouabain from 10^{-9} to 10^{-7}), stimulation with cAMP not only accelerated the rate of ouabain binding but also increased the amount bound at equilibrium after 5 hr of incubation. On the other hand, at its low-affinity site (e.g. at concentrations higher than 10^{-6} M), the binding of ouabain was actually less in the presence of cAMP and theophylline than in control experiments.

These experiments extend to the spiny dogfish, *Squalus acanthias*, the observation by Shuttleworth and Thompson in the European dogfish *Scyliorrhinus canicola* that cAMP and theophylline increase ouabain binding by slices of rectal gland tissue (Shuttleworth & Thompson, 1978, 1980).

The latter authors did not distinguish between high-affinity and low-affinity binding, and conducted most of their experiments at 10^{-6} M ouabain; it is not clear from their published data whether equilibrium was reached. Our results differ from those of Shuttleworth and Thompson in that in our experiments the changes in ouabain binding produced by cAMP stimulation were not prevented by maneuvers that block secretion in the perfused gland. At the concentrations used in these experiments, furosemide and bumetanide inhibit rectal gland secretion by 60 to 70% (Palfrey et al., 1979; Silva et al., 1980). Replacement of Na^+ with Li^+ or of Cl^- with NO_3^- in the perfusate inhibits stimulated secretion completely (Solomon et al., 1977; Silva et al., 1980). However, neither incubation with furosemide or bumetanide, nor exposure to an incubation solution low in sodium or chloride, altered the increase in high-affinity ouabain binding produced by dibutyryl cAMP and theophylline. These experiments therefore suggest that in *Squalus acanthias* the effect of dibutyryl cyclic AMP and theophylline to stimulate ouabain binding does not depend on the entry of sodium and chloride into the cell. Ouabain binding is increased by cAMP stimulation at a time when passage of sodium and chloride into the cell is either absent or substantially slowed.

The experiments with whole homogenates of rectal gland indicate that intact cells are required for the cyclic AMP-induced increase in ouabain binding to become manifest. It is interesting, in this connection, that the (Na, K)-ATPase activity of whole homogenates of rectal gland is also not altered by prior stimulation with cAMP and theophylline (Silva et al., 1979*b*). Cell homogenization may expose all enzymatic sites within the cell to binding by ouabain, whereas in intact cells ouabain binds chiefly those on the surface of the cell. The amount of ouabain bound by whole homogenates of rectal gland was, on a weight basis assuming a protein content of the cell of 10%, three to ten times greater than the amount bound by either slices or isolated cells. The greater binding capacity of whole homogenates suggests that homogenization may uncover or activate binding sites that are not normally available on intact cells. It also may provide an explanation for the absence of a stimulatory effect of dibutyryl cyclic AMP and theophylline; the large increase in binding capacity involving both cell surface and cellular binding sites would abolish the difference in binding by sites on the cell surface found in unstimulated and stimulated glands.

These results suggest that an increase in the secretory activity of the gland or in the rate of sodium entry into the cell are not prerequisites for the alterations in ouabain binding evoked by cyclic AMP. The changes in ouabain binding may therefore reflect some change in configuration of the enzyme or its distribution within cell membranes that results directly from the cascade of cellular events initiated by cAMP rather than secondarily from ion movements in the course of stimulated secretion. It is attractive to suppose that this change is related to the activation of (Na, K)-ATPase that occurs when the gland is stimulated by cAMP. It is not yet clear, however, in what way the change in affinity for ouabain that we have demonstrated might relate to an increase in enzyme activity. The absence of a stimulatory effect of cAMP and theophylline on ouabain binding or (Na, K)-ATPase activity in broken-cell homogenates suggest that the changes in (Na, K)-ATPase produced by cAMP stimulation do not involve an increase in the amount of enzyme in rectal gland cells but rather reflect a change in the configuration, environment, or location of existing cellular enzyme so as to enhance its activity.

This work was aided by grants from the National Institutes of Health (AM18078), the National Science Foundation (PCM7922665) and the Maine Affiliate of the American Heart Association.

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Received 21 January 1983; revised 21 February 1983