CYCLIC AMP EFFECTS ON ^{3}H OUABAIN BINDING AND ^{86}RUBIDIUM UPTAKE RATES IN CELLS ISOLATED FROM THE RECTAL GLAND OF SQUALUS ACANTHIAS

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The stimulation of chloride transport in the rectal gland of Squalus acanthias by cyclic AMP is associated with an enhanced activity of the sodium pump as evidenced by an increase in the amount of ^{3}H ouabain bound to rectal gland cells or slices (Silva et al J Membr Biol 175:105-144, 1983; Shuttleworth and Thompson J Comp Physiology 140:209-216, 1980) and a rise in ouabain-inhibitable oxygen consumption (Silva et al Am J Physiol 237:F468-F472, 1979). The increase in ouabain binding was found to be sensitive to furosemide in one report (Shuttleworth and Thompson J Comp Physiology 140:209-216, 1980) and independent of furosemide, bumetanide or extracellular sodium in another (Silva et al J Membr Biol 175:105-114, 1983). In the present studies we explored further the question of furosemide sensitivity and examined the possibility that two functionally distinct NaK ATPases extrude sodium out of the rectal gland cell.

Ouabain binding

The methods used for isolation of cells and incubation with ^{3}H ouabain were modified from those reported previously (Silva et al J Membr Biol 75:105-114, 1983). Under the conditions of assay (15°C), the ^{3}H ouabain reaction was at equilibrium following 3 hours of incubation. Therefore, whole cells were incubated for 3 h with either cAMP + theophylline (1 mM each, ± 10^{-4}M furosemide), vanadate (1 mM), or diluent. In some experiments, the ambient K concentration (5mM) was reduced to 0.3 mM to observe how the partial loss of this competitor and NaK ATPase substrate would affect the extent of ^{3}H ouabain binding at equilibrium. Vanadate, an inhibitor of NaK ATPase, was included, since it is a congener of phosphate which stimulates the formation of the E2-P or ouabain-binding configuration of the enzyme. Its use in these protocols was to see if the compound would either aid to differentiate between possible isozymes of NaK ATPase, and/or mimic the effects of cAMP/theophylline on ^{3}H ouabain binding kinetics.

Controls and samples treated with cAMP/theophylline ± furosemide or vanadate produced concave curvilinear plots when ^{3}H ouabain binding data was analyzed by Scatchard plots, suggesting that at least two binding sites were involved, or that the binding reaction involved cooperative kinetics.

The overall effects of cAMP and theophylline as determined by interpretation of the Scatchard plots were a) an increase in the extent of equilibrium binding at low conc. of ^{3}H ouabain, b) a decrease in the apparent number of total ^{3}H ouabain binding sites, and c) a decrease in the apparent (average) Kd for the low affinity site from 3.6 μM to 0.5 μM. Reducing bath K to 0.3 mM in cells treated with cAMP/theophylline altered b), or the apparent number of sites. Thus, in low K media, there was no apparent reduction in the total number of sites + cAMP/theophylline vs controls. Vanadate reproduced the apparent increase in binding at low concentrations of ouabain [or a) above], supporting the concept that this effect of cAMP is in part due to the increased rate of formation of E2-P, secondary to enhanced transport rates. The fact that furosemide did not return binding to control levels suggests that cAMP/theophylline may stimulate the intrinsic turnover of a fraction of the enzyme, independent of

transport rates. Finally, concentrations of ouabain < 3×10^{-7}M appeared to define a high affinity site, while those > 3×10^{-7}M defined a low affinity site or sites. The high affinity sites represented about 10-15% of the total binding sites.

A Hill plot analysis was carried out on the ^{3}H ouabain binding data generated by the low affinity site(s), or concentrations of ouabain > 3×10^{-7}M. In controls (± low media K), in vanadate-treated samples and in samples incubated with cAMP/theophylline and furosemide, the binding data generated a Hill coefficient ≅ 0.5, in keeping with either a two-site model, or a site exhibiting negative cooperativity. On the other hand, data derived from samples incubated + cAMP/theophylline (± low K media) resulted in a Hill coefficient of ≅ 1.0, suggesting that cAMP resulted in a modification of this pool of enzyme from a cooperative to a non-cooperative state. The fact that furosemide reversed the effect was interpreted as indicating that the loss of a cooperative binding plus cAMP/theophylline was dependent on a rise in intracellular Na. To wit, in the presence of cAMP/theophylline, the enhanced Na entry via the NaCl cotransporter would not be adequately balanced by the exit of Na via the ouabain-inhibited Na pump, leading to an increase in Na_i. On the other hand, if furosemide were also present, the rise in intracellular Na concentration would be blunted, since Na entry via the cotransporter would be blocked. Indeed, in the presence of furosemide, this low affinity ouabain-binding site again exhibited negative cooperativity. Thus, the effects of cAMP/theophylline on the kinetics of interaction of ^{3}H ouabain with the low affinity site appear secondary to cAMP + ouabain effects on intracellular Na concentration.

Once having shown that the low affinity ouabain-binding site lacked cooperative kinetics in the presence of cAMP and theophylline, it was possible to correct the Scatchard analysis plot for the low affinity component in this group. The residual high affinity site data had a Hill coefficient of ≅ 1.0. Thus in the presence of cAMP, two independent sites could be characterized, one high-affinity (Kd, 0.08 μM) and one low affinity (Kd, 0.5 μM) each exhibiting non-cooperative kinetics. No experimental manipulation reduced this two component Scatchard binding curve into a single site exhibiting a single Kd. For this reason, it was suggested that the ouabain was titrating two populations of non-interacting monomers/multimers of NaK ATPase, or two isozymes of NaK ATPase.

^{86}Rubidium uptake

Measurements of ^{86}Rb uptake were carried out as described by Resh et al (J Biol Chem 255:10938-10945, 1980). Uptake rates were linear over the first 10 min at 15°C, thus all ^{86}Rb uptake measurements were made within this time frame.

Acute uptake of ^{86}Rb was measured following 3h incubation (as in the ^{3}H ouabain binding assays) ± cAMP/theophylline, vanadate, and cAMP/theophylline plus furosemide, and as a function of varying concentrations of unlabelled ouabain in the incubation medium (range, 1 nM → 10 μM). Cyclic AMP/theophylline stimulated Rb/K uptake relative to controls at low concentrations of ouabain, while depressing that seen at high concentrations of ouabain.

Rb/K uptake rates were plotted as a function of the number of unoccupied NaK ATPase sites at any given ouabain concentration (occupied vs unoc-

cupied sites were determined from the previous ^{3}H ouabain binding analyses). The ^{86}Rb uptake data supported the presence of two different or non-interacting forms of NaK ATPase. The rate of Rb/K flux/site through the pool of enzyme with a high affinity for ouabain (termed site I) exceeded that for the pool with a low affinity for ouabain (termed site II) in all groups. Cyclic AMP/theophylline enhanced Rb/K uptake through site I by approximately 3.6 fold and through site II by 1.3 fold, relative to controls. In the presence of furosemide plus cAMP/theophylline, uptake rates via site I were still 1.5 x that of control values, while uptake rates via site II were returned to control values. Vanadate did not appreciably alter the flux through site I, but reduced flux via site II by > 50%. The fact the 1 mM vanadate did not completely inhibit uptake of Rb/K via NaK ATPase in whole cell preparations is probably due either to sequestration of vanadate by intracellular proteins, thus reducing free levels, or by cellular conversion of vanadate to vanadyl, a less potent inhibitor of the pump. The fact that furosemide did not eliminate the effect of cAMP/theophylline on ^{86}Rb uptake via site I or the enhanced binding of ^{3}H ouabain to this site suggests that cAMP/theophylline may directly alter pump efficiency, independent of effects on transport. The possibility remains, however, that the concentration of furosemide used in these studies was insufficient to completely block chloride secretion stimulated by cAMP. However, earlier experiments showed that neither $10^{-4}M$ furosemide nor $10^{-4}M$ bumetanide, a far more potent analogue, eliminated the effects of cAMP on ouabain binding (Silva et al J Membr Biol 175:105-114, 1983).

The whole of the data suggests that there are two isozymes (or non-interacting pools) of NaK ATPase in the rectal gland. These observations are reminiscent of those reported by Guidotti and co-worders in rat adipocytes (J Biol Chem 255: 10938-10945, 1980; J Biol Chem 260:1177-1184 and 10075-10080, 1985). These authors found a complex binding interaction between ^{3}H ouabain and NaK ATPase and suggested dual sites or a cooperative binding interaction. They ultimately concluded that two separate isozymes were involved, one containing an α and the other an α (+) subunit. These two subunits differed not only with respect to their affinity for ouabain but also with respect to their affinity for sodium and ATP. Further, in the adipocyte, insulin stimulated the turnover of the α (+) enzyme by increasing the affinity of Na for this particular isozyme. The similarities between the studies in adipocytes and the present observations in rectal gland cells suggest that in the latter, there are also two functionally distinct isozymes of NaK ATPase. In such a model, cyclic AMP would directly affect only one of these enzymes. It is equally plausible that a common pool of NaK ATPase exists in shark rectal gland, but that there are cellular modifiers which restrict and alter the properties of a fraction of the total enzyme present. Again, the direct action of cAMP may be focused on a subpopulation of the enzyme. A final point should be made. In order to sustain the cAMP response on transport, theophylline was added to preparations containing cAMP. However, theophylline has multiple sites of action in shark rectal gland. Thus, the possible association of theophylline alone with this pathway deserves attention in future.

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