PROTEIN KINASES IN RECTAL GLANDS OF *Squalus acanthicas*

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The effects of cAMP on many cellular functions are thought to be mediated by activation of cAMP-dependent protein kinases. Activation occurs through dissociation according to the following simplified scheme: R-C + cAMP $\rightleftarrows$ R-cAMP + C. RC represents the inactive holoenzyme consisting of regulatory subunit R (also called cAMP binding protein) and C, the catalytic subunit. Recent work by Stoff et al. (J. Exp. Zool. 199:443-448, 1977) has demonstrated that active chloride secretion by the rectal gland is modulated by cyclic AMP. We therefore examined the distribution and activation of protein kinases in the rectal gland.

Glands were removed and perfused *in vitro* as described by Stoff et al. (J. Exp. Zool. 199:443-448, 1977). At the end of the perfusion experiments transverse slices (approximately 100 mg) were obtained, blotted and frozen in dry ice-acetone. Slices were then either kept in liquid nitrogen or directly processed for determination of protein kinase activity or cAMP binding capacity. The frozen tissue was coarsely pulverized and then homogenized (10 sec at maximum setting with a Polytron) in 2-4 ml of ice cold buffer (10 mM potassium phosphate pH 6.8, 2 mM EDTA and 150 mM KCl). The homogenate was centrifuged for 20 min at 30,000 x g, the supernatant removed and the pellet washed with 0.2 ml of buffer and recentrifuged. The resultant supernatant was combined with the first one. The pellet was resuspended in homogenization buffer in a volume equal to that of the combined supernatants. Protein kinase was determined by a modification of the method of DeLange et al. (J. Biol. Chem. 243:2200-2208, 1968). The reaction contained 20 mM potassium phosphate pH 6.8, 10 mM Mg-acetate, 0.05 mM [γ^{32}P] ATP (1-2 x 10^6 CPM) 80 µg histone F_{2b} and, when used, 2 µM cAMP in a final volume of 0.1 ml. Reactions were carried out for 3 min at 20°C and proceeded linearly with time up to at least 4 min for the homogenate, supernatant and pellet at protein concentrations of 5-100 µg per assay.

Cyclic AMP binding was assayed by a modification of the method of Gilman (PNAS 67:305-312, 1970). Incubations were carried out for 2 hrs at 4°C in 0.2 ml of 25 mM potassium phosphate buffer pH 7.0, 0.2 mg/ml bovine serum albumin, 0.2 µM [^{3}H] cAMP (400,000 CPM) and 20-80 µl of the respective preparation. Protein was determined by the method of Lowry et al. (J. Biol. Chem. 193:265-275, 1951).

As the regulatory subunit of cAMP-dependent protein kinase represents the cAMP binding protein, protein kinase distribution can be evaluated by determining cAMP binding in homogenate, 30,000 x g supernatant and pellet of rectal glands. At 200 nM cAMP in the assay the homogenate bound 1319 ± 166 fmol cAMP/mg protein, the supernatant 1086 ± 166 and the pellet 915 ± 160 (n=6). The sum of cAMP binding in supernatant and pellet accounted for 70% of the binding in the homogenate. The supernatant represented 54% of the binding and the pellet 46%. Further binding studies suggest however, that in contrast to the supernatant, most of the cAMP binding to the pellet represents unspecific binding. For example, while the supernatant showed typical saturation kinetics with half maximal binding occurring at 10-20 nM cAMP, binding to the pellet was nonsaturable. Furthermore, in the supernatant over 90% of bound [^{3}H] cAMP could be displaced by 10 µM cold cAMP, whereas neither 10 µM adenosine nor 5' AMP caused a measurable displacement. In contrast, in the pellet, 10 µM cAMP only displaced 60% of [^{3}H] cAMP bound, whereas adenosine displaced 70%. These results suggest specific binding of cAMP in the supernatant and mostly unspecific binding in the pellet.

Protein kinase ± cAMP was determined in the homogenate, supernatant and pellet of rectal glands. Control glands were perfused (average 1 hr) with dogfish Ringer's solution until basal secretory rates were achieved (mean flow rate 5.1 ± 1.3 µl/min; n=6). Results are shown in Table I A. About 50-60% of total protein kinase (i.e., + cAMP) assayed in the homogenate was present in the 30,000 x g supernatant fraction. Cyclic AMP significantly stimulated protein kinase activity to 131% of basal in the homogenate and to 163% in the supernatant while protein kinase activity in the pellet was only

TABLE I

Protein kinase activity in dogfish rectal glands

pmol P_i/mg protein x 1 min

n=6	A. glands perfused with Ringer's only				
	Basal	+ cAMP	P value compared to basal	Basal + inhibitor	P value compared to basal
Homogenate	156±57	205±57	<0.02	not done	
Supernatant	111±30	181±42	<0.01	68±25	<0.02
Pellet	148±47	164±45	<0.1	137±46	N.S.
n=6	B. glands perfused with Ringer's +0.1mM db cAMP + 0.25mM theophylline				
Homogenate	123±26	170±26	<0.01	not done	
Supernatant	93±30	159±39	<0.01	46±16	<0.05
Pellet	112±29	138±31	<0.05	108±32	N.S.

Inhibitor, when present, was used at 25µg protein per assay, a dose that totally inhibits cAMP stimulation.

minimally stimulated by cAMP. Phosphorylation of homogenate, supernatant or pellet in the absence of exogenous histone was negligible and indistinguishable from blank values. Also using pellet (instead of histone) as substrate for phosphorylation by 30,000 x g supernatant resulted in values indistinguishable from blanks. In order to evaluate how much of the basal protein kinase activity (i.e., in the absence of exogenous cAMP) is cAMP independent and how much is due to preactivation of cAMP-dependent kinase, kinase activities in the supernatants and pellets were determined in the presence of protein kinase inhibitor prepared by the method of Walsh et al. (J. Biol. Chem. 246:1977-1985, 1971). This inhibitor is specific for the catalytic subunit of cAMP-dependent protein kinase and does not affect cAMP independent protein kinases (Walsh and Ashby, Recent Progr. Horm. Res. 29:329-359, 1973). As shown in Table 1 A kinase activity in the supernatant can be significantly inhibited, indicating some dissociation of cAMP-dependent protein kinase under our control conditions. As kinase activity in the presence of inhibitor represents the real baseline activity (i.e., without endogenous cAMP) stimulation by cAMP is about threefold in the supernatant fraction. In contrast protein kinase in the pellet was not inhibited. Together with the minor stimulation of kinase activity by cAMP in the pellet this strongly suggests that the pellet contains mostly cAMP independent protein kinases. These results are in good agreement with those obtained for cAMP-binding. We next evaluated the effect of perfusing the glands with dogfish Ringer's containing 0.1 mM dibutyryl cAMP and 0.25 mM theophylline on subsequent determination of protein kinase activity. As to be expected, this perfusion increased secretory rates 10-fold to 59 ± 7.3 µl/min. Protein kinase results are shown in Table 1 B. In spite of the 10-fold stimulation of the secretory rate the degree of activation by exogenous cAMP was not significantly affected. A slightly higher degree of inhibition (51%) by protein kinase inhibitor was observed in the supernatant of the stimulated gland than in the nonstimulated glands (39%) suggesting a higher degree of kinase dissociation in the experimental glands but this was not statistically significant. The failure to show an effect of perfusion with db cAMP and theophylline on the kinase activity may be due to reassociation of cAMP-dependent protein kinase during the preparation of the tissue.

Our results demonstrate that the dogfish rectal gland contains cAMP-dependent and independent protein kinases. The cAMP-dependent protein kinase activity is cytosolic as demonstrated by the specificity of cAMP binding, the activation by cAMP and the inhibition by specific protein kinase inhibitor. In contrast membrane bound protein kinase activity is mostly cAMP independent, as shown by

the nonspecific binding characteristics for cAMP, the minimal stimulation by cAMP, and the absence of an effect of protein kinase inhibitor. This investigation was supported by U.S. Public Health Service Grant AM-03858 (to Dr. Richard M. Hays).

INCORPORATION OF AN ORGANIC ANION CARRIER FROM WINTER FLOUNDER (*Pseudopleuronectes americanus*) KIDNEY PLASMA MEMBRANES INTO LIPOSOMES.

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Experiments with isolated membrane fractions have provided mechanistic information on transport processes in several specialized transporting epithelia. Recent developments in membrane biology now indicate that membrane proteins can be inserted into defined artificial lipid membrane systems, e.g., liposomes, and that specific transport activity is retained in the reconstituted system (see, e.g., Biochem. J. 168:311-314, 1977). We present here results of preliminary experiments which show reconstitution of p-aminohippuric acid (PAH) transport in a flounder kidney plasma membrane (PM) protein-liposome system.

Kidney membranes were isolated by the procedures of Eveloff et al. (in preparation), membrane proteins were solubilized and incorporated into liposomes using a modification of the procedure of Kinne and Faust (Biochem. J. 168:311-316, 1977). Briefly, membranes were solubilized in KHT buffer (150 mM KCl, 10 mM $MgSO_4$ and 5 mM Tris-HEPES, pH 7.5) with 0.5% Triton X-100 and membrane fragments were removed by ultracentrifugation. The supernatant was passed through six Bio-Bead SM 2 columns to remove the Triton and the Triton-free extract (4 ml) was mixed with an equal volume of a 20 mg/ml suspension of phosphatidyl choline (Sigma type III=E, with hexane removed under vacuum) in KHT. This mixture was sonicated for 2 min in an ice bath and the proteoliposomes were collected by ultracentrifugation. Proteoliposomes were suspended in KHT and used immediately for transport experiments employing the Millipore filtration procedure of Hopfer et al. (J. Biol. Chem. 248:25-32, 1973).

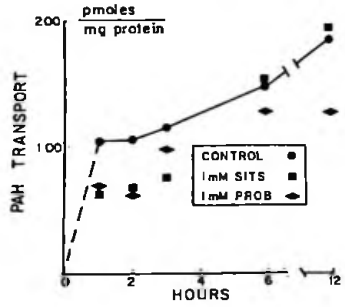

Figure 1. Representative experiment showing time course of PAH uptake by proteoliposomes prepared from flounder kidney PM and phosphatidyl choline. Proteoliposomes, suspended in KHT, were added to an equal volume of KHT containing 20 μM ^{3}H-PAH with or without inhibitor. Fifty μl aliquots were removed at timed intervals, diluted with 1 ml KHT containing 1 mM SITS and filtered through 0.22 Millepore filters. Proteoliposomes on the filters were washed and then counted using standard liquid scintillation procedures. Data given as mean of duplicate determinations from a single proteoliposome preparation (PM from 8 fish kidneys).

Initial experiments with proteoliposomes from flounder kidney brush border membranes showed Na-dependent and phloridzin sensitive transport of D-glucose, but no specific uptake of PAH. With proteoliposomes from PM, PAH transport was inhibited by both SITS and probencid (Figure 1); these chemicals are strong inhibitors of transport in both intact tubules and isolated PM. In addition, PAH uptake was reduced when mannitol was added to the incubation medium (not shown) indicating that substrate was moving into an osmotically active space rather than binding to the proteoliposomes.

The renal organic anion system mediates active excretory transport of xenobiotics and natural metabolites. At present, the driving forces for transport have not been determined and the effects of drugs and chemical pollutants are just beginning to be explored at the subcellular level. Clearly,