inosine which is inactive at adenosine receptors. ADA has no effect on ATP. Thus, ADA should not affect a pure ATP response. In three glands, 8 BrATP (10 μM) had no effect on chloride secretion while ATP (10 μM) increased s secretion significantly ($p < 0.05$, paired t). ADA (0.6-1 U/ml) significantly reduced this ATP response ($p < 0.05$, paired t). A typical experiment showing the effect of 8 BrATP and ADA on chloride secretion is shown in Figure 2. Note that 8 BrATP is entirely ineffective in stimulating secretion and that ADA reversibly inhibits the effect of ATP on chloride secretion.

While ATP stimulates chloride secretion in the rectal gland, prior hydrolysis to adenosine appears necessary. Three lines of evidence support the hypothesis that the effect of ATP is mediated by adenosine: 1) theophylline, a specific adenosine receptor antagonist blocked the ATP response, 2) 8 BrATP, an active ATP analogue that upon hydrolysis yields 8 BrADO, an inactive adenosine analogue, had no effect on chloride secretion in the gland, and 3) ADA, an enzyme that deaminates adenosine to inosine (which is inactive at adenosine receptors) substantially reduced the ATP response. Thus, it is unlikely that the rectal gland has an ATP receptor. Rather, the ATP response is mediated by adenosine.

STIMULATION AND INHIBITION OF ADENYLATE CYCLASE IN THE RECTAL GLAND OF SQUALUS ACANTHIAS

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Forrest et al., have provided evidence for two external adenosine receptors on the shark rectal gland: one that stimulates (R_a) chloride secretion (Forrest et al., Bull. MDIBL 20:152-155, 1980 and Kidney Int. 21:253, 1982) and one that inhibits (R_i) chloride secretion (Poeschla et al., Bull. MDIBL 22:S19-S23, 1982). Both receptors probably act by affecting changes in cAMP levels (Poeschla et al., Bull. MDIBL 22:S15-S18, 1982). The simplest mechanism to explain this change in cAMP content would be by a direct effect on adenylate cyclase activity; however, there are other possible mechanisms including a change in cAMP fluxes or phosphodiesterase activity. To further characterize adenosine receptors on the shark rectal gland, and to determine the possible mechanism(s) by which adenosine affects cAMP levels and chloride secretion in the gland, an adenylate cyclase assay was performed. Preliminary data are presented showing stimulation and inhibition of cyclase activity by adenosine analogues.

METHODS

Shark rectal gland membranes were prepared as described by Hannafin et al., (J. Memb. Biol. 75:73, 1982), put into 100 μl aliquots, and stored frozen in liquid N_2. For individual experiments, membranes were thawed and suspended in 20 mM mannitol 1.2 mM Mg and 20 mM Hepes (free acid)(pH 7.4) to disrupt membrane vesicles by hypoosmotic shock.

Adenylate cyclase activity was determined by the method of Solomon (Advances in Cyclic Nucleotide Research, Vol. 10, ed. G. Brooker et al., Raven Press, New York, 1979) with modifications made by Sulakhe described below. Changes from this protocol in a given experiment will be specified. The assay medium contained 20 20 mM creatine phosphate (disodium salt), 8 units of creatine phosphokinase, 2.5 mM $MgCl_2$, 0.5 mM EGTA, 2 mM DTE, 0.1 μg/μl BSA, 70 mM NaCl, 1 mM cAMP (free acid), 10000 cpm ^{3}H cAMP, 50 μM GTP, 0.33 mM R01724, 5 U/ml adenosine deaminase (ADA), and 50 mM glycylglycine buffer (pH 7.4). The experiment was initiated with the addition of 20-40 μg of protein per tube. After 5 minutes, an ATP reaction mixture with a specific activity of 25-100 cpm/pm cold ATP was added to give a total volume of 150 μl. The final ATP concentration was 0.5 mM. After incubating this mixture at 16°C (sea water temperature) for 10 minutes, the reaction was stopped with the addition of 150 μL of a stopping solution which contained 2% SDS, 1 mM ATP and 120 mM glycylglycine buffer (pH 7.4). The Dowex columns were washed once with 10 ml of 1N NaOH to remove protein, then twice with 10 mls of

distilled water, then once with 10 mls of 1N HCl, followed by at least 4 washes with 10 mls of distilled water. The protein concentration used in the reaction mixture was determined by Lowry method modified by E. Kinne (personal communication).

RESULTS AND DISCUSSION

Assay Variables: Variables to be considered when performing an adenylate cyclase assay on a tissue for the first time include protein concentration, temperature, and time of incubation. Also, ATP hydrolysis to adenosine must be considered if the effects of adenosine are to be determined. Increasing protein concentration from 10 μg to 67 μg per tube and increasing the time of incubation from 15 minutes to 45 minutes resulted in near linear increases in cAMP production (data not shown). The near linearity of these variables suggests that substrate availability for the reaction did not decrease substantially and that the ATP regenerating system was functioning properly. Increasing the temperature from 16°C (sea water temperature) to 30°C increased the rate of formation of cAMP. Both basal and NECA-stimulated activity increased 2.6 times ($p < 0.001$) at the higher temperature. Although the signal was increased at the higher temperature, we chose to incubate at a physiological temperature (16°C) for the shark.

EFFECTS OF ADENOSINE DEAMINASE, ADENOSINE AGONISTS AND FORSKOLIN

Adenosine deaminase is an enzyme that metabolizes adenosine to inosine which is inactive at adenosine receptors. Figure 1 shows the effect of adenosine deaminase (1.5 U/ml) on basal, 2-chloroadenosine (10^{-4}M)(2 Cl ADO)

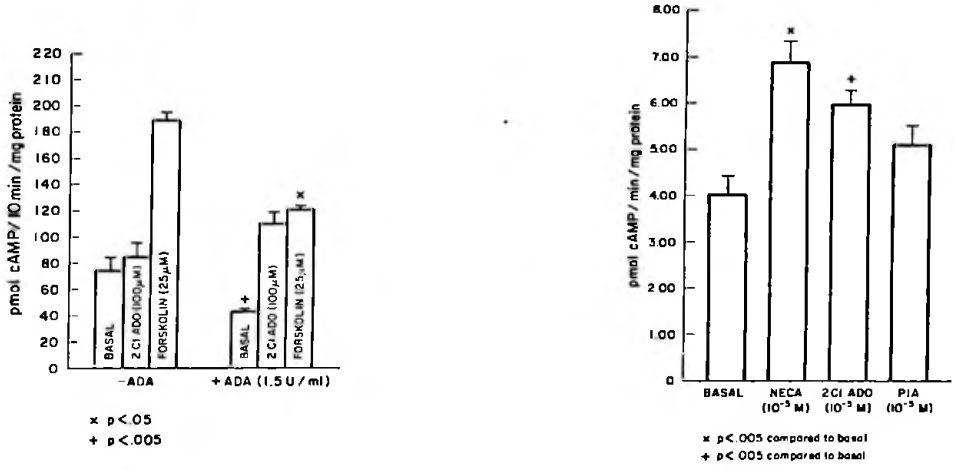

Figure 1.--Effects of forskolin, 2-chloroadenosine and adenosine deaminase on adenylate cyclase activity of basolateral membranes of the shark rectal gland (Squalus acanthias).

Figure 2.--Comparison of the effects of NECA, 2-chloroadenosine and PIA on basal adenylate cyclase activity of isolated basolateral membranes of the shark rectal gland (Squalus acanthias). Assay performed in the presence of adenosine deaminase (% U/ml).

stimulated and forskolin (25 μM) stimulated cyclase activity. 2-Cl ADO is an analogue of adenosine which is not metabolized by adenosine deaminase and forskolin is a direct activator of adenylate cyclase. Adenosine deaminase reduced basal activity by 41% ($p < 0.05$) and forskolin (25 μM) stimulated activity by 35% ($p < 0.005$). This decrease can be explained by the removal of adenosine formed from the hydrolysis of the high concentration of ATP used in the assay. Stimulation from adenosine formed from ATP would be additve to the basal and forskolin stimulation and adenosine deaminase would remove this additional stimulation. ATP stimulation by hydrolysis to adenosine was shown in the perfused rectal gland (this Bulletin, Kelley et al.). Note that the effect of 2 Cl ADO to increase cyclase activity can only be demonstrated in the presence of adenosine deaminase which removes the effect of background adenosine in the reaction mixture.

Three analogues of adenosine, NECA, 2 Cl ADO, and phenylisopropyladenosine (PIA), stimulated adenylate cyclase above basal activity. In one experiment basal activity was 4.02 $\pm$ 0.39 pmoles cAMP/min/mg protein.

NECA (10^{-5}M) stimulated 1.7 times to 6.88 ± 0.44 ($p < 0.005$), 2 Cl ADO (10^{-5}M) stimulated 1.5 times to 5.96±0.30 ($p < 0.005$) and PIA stimulated 1.2 times to 5.01 ± 0.48 (p = ns)(see Figure 2). It is important to note that the order of potency of the various analogues is NECA > 2 Cl ADO > PIA, which is the same order of potency for an R_a receptor (Londos et al., Proc. Natl. Acad. Sci. USA 77:2251-2554, 1980). This order of potency was described previously in the perfused gland (Forrest et al., Bull. MDIBL 20:152-155, 1980 and Kidney Int. 21:253, 1982), and tissue slice experiments (Poeschla et al., Bull. MDIBL 22:S15-S18, 1982).

A dose response curve was also determined for NECA and PIA as shown in Figure 3. It is interesting that low

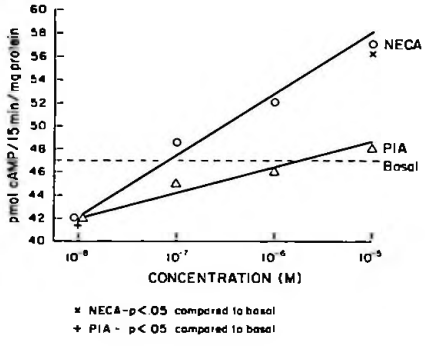

Figure 3.--Dose response to NECA and PIA on adenylate cyclase activity of isolated basolateral membranes of the shark rectal gland (Squalus acanthias).

concentrations of PIA ($p < 0.05$) and possibly NECA (p=ns) seemed to inhibit cyclase activity. PIA was also the more potent inhibitor which is consistent with the order of potency for an R_i receptor. This inhibition supports our previous hypothesis for the presence of an inhibitory (R_i) adenosine receptor (Poeschla et al., Bull. MDIBL 22:S19-S23, 1982). Higher and lower concentration of these analogues need to be examined further to determine the presence of a P site and minimal inhibitory concentrations.

It is interesting to note that NECA (10^{-4}M) only stimulated cyclase activity 1.3 times in this experiment and in all other experiments not more than 2 times; whereas, adenosine analogues stimulate perfused glands maximally as much as 20 times above basal, and increase cAMP measured in tissue slice experiments by RIA as much as 25 times above basal (Poeschla et al., Bull. MDIBL 22:S15-S18, 1982). The reason for this difference is not known. One explanation could be that in the perfused gland and tissue slice experiments adenosine may affect cAMP fluxes from the cell or phosphodiesterase activity as well as adenylate cyclase. In support of these possibilities is our finding that forskolin (200 μM), a drug which stimulates cyclase directly, and VIP (10^{-6}M) stimulate adenylate cyclase more than 6 times above basal activity which is more similar to the increase in cAMP levels measured by RIA (see abstract by Gifford et al, This Bulletin). To further determine the mechanism involved, dose response curves for forskolin and adenosine in tissue slice experiments and adenylate cyclase assays need to be compared under comparable conditions.

In summary, the present work discusses certain assay variables and shows stimulation and inhibition of adenylate cyclase activity. Assay variables tested indicated that the regenerating system was functioning, 16°C was an acceptable temperature at which to perform the assay, and adenosine deaminase was required to prevent stimulation of basal cyclase from the breakdown of ATP in the reaction mixture. Adenosine analogues stimulated cyclase in a dose dependent manner with an order of potency similar to an R_a adenosine receptor: NECA > 2 Cl ADO > PIA. Also, a dose response curve shows inhibition and stimulation of cyclase by PIA and NECA. PIA was a more potent inhibitor than NECA suggesting an Ri type of adenosine receptor. NECA (10^{-4}M) only stimulated at most 2 times above basal. The difference in these maximal stimulations compared to what is seen in tissue slice experiments and perfused glands requires further examination.