GROWTH CONDITIONS

Flasks were maintained at either 25°C or 16°C. At the higher temperature both sealed flasks and those open to a 1% CO_2 atmosphere were examined; at 16° all flasks were sealed in room air. Although initial settling of fragments occurred under all conditions, extended growth was observed at the lower temperature. Since flasks were frequently fed, pH was maintained relatively constant at 7.4-7.5. However, it is expected that for longer term culture, 1% CO_2 or HEPES buffering would improve growth.

Attempts to culture cells on collagen coated surfaces were also made using both commercially prepared collagen (Sigma, acid soluble rat tail collagen), and rat tail extracts (Hall et al., Proc. Nat. Acad. Sci. 79:4672-5676, 1982) as prepared by Dr. John Valentich. No growth was observed on collagen, but in three separate batches, when fragments were placed on semi-solid gels, they appeared to reorient and form clusters with central lumina similar to those described in MDCK cells (Hall et al., op. cit., 1982).

GROWTH CHARACTERISTICS OF PRIMARY CULTURES

Within an hour of plating, cells appeared individually and in small clusters scattered over the surface. These cells were flattened epithelioid with winged pseudopods. Frequently, cells appeared to secrete droplets of a low density material. These droplets did coalesce but did not mix with the culture medium, indicating some lipid content.

Growth of epithelioid cells from larger tubule fragments occurred 24-48 hours after initial plating. Patches of outgrowth were characterized by the presence of large, highly vacuolated cells at the leading edge and uniform cells with large oval nuclei and dense cytoplasmic granular inclusions throughout the field (Figure 1). Fibroblastic cells in-

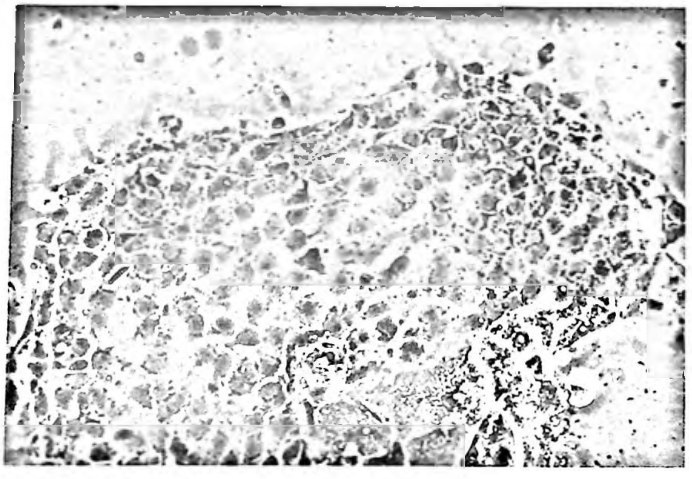

Figure 1.--Primary monolayer culture of shark rectal gland tubule cells (48 hours). Phase contrast, 125x.

frequently appeared 48-72 hours after plating, and followed the epithelioid patch growth. Primary cultures were occasionally contaminated by both bacteria and/or yeast but infection was minimal when prewashes with triple antibiotics were used.

Attempts to pass the cells using either trypsinization or cell scraping were not successful. There was no growth and very poor viability in transferred flasks.

In summary, primary monolayer cultures of tubular cells of the rectal gland were obtained and were viable for 4-5 days. These cells appear sufficient large and numerous for further characterization of transport properties using patch clamping and other electrophysiological techniques.

ATP INDIRECTLY STIMULATES CHLORIDE SECRETION BY HYDROLYSIS TO ADENOSINE IN THE RECTAL GLAND OF SQUALUS ACANTHIAS

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ATP has been shown to affect many tissues either directly through an ATP receptor (Burnstock, G.,

Pharmacological Reviews, 24: 509-581, 1972) or indirectly through hydrolysis to adenosine and subsequent binding to adenosine receptors. Recently, Korman et al., have suggested that ATP stimulates cyclic AMP mediated ion transport in intestinal epithelial cells through an ATP receptor (Biochimica et Biophysica Acta 721: 47-54, 1982). Forrest et al., have suggested the presence of both stimulatory adenosine receptors (R_a) (Bull. MDIBL 20: 152-155, 1980 and Kidney Int. 21: 253, 1982), and inhibitory adenosine receptors (R_i) (Poeschla et al., Bull. MDIBL 22: S19-S23, 1982) in the rectal gland of the dogfish shark Squalus acanthias. Since an ATP receptor has been proposed in other intestinal epithelia, we investigated the rectal gland for its presence. With the use of theophylline, 8-Bromo-ATP (8 BrATP) and adenosine deaminase (ADA), we show that ATP stimulates chloride secretion indirectly by hydrolysis to adenosine with subsequent adenosine receptor interaction.

Rectal glands were perfused as previously described (Forrest et al., Bull. MDIBL 20: 152-155, 1980) with elasmobranch Ringer's solution at 15°C with cannulae in the artery, vein and duct of the gland. Measurements of chloride secretion were made at 10 minute intervals. Glands were perfused for three 10 minute periods (30 minutes total) with each drug. Data presented are the mean ± SEM of these three periods unless otherwise stated. Chloride secretion is expressed in μEq Cl/hr/gww.

ATP stimulated chloride secretion, but this stimulation was readily blocked by theophylline. Figure 1 shows

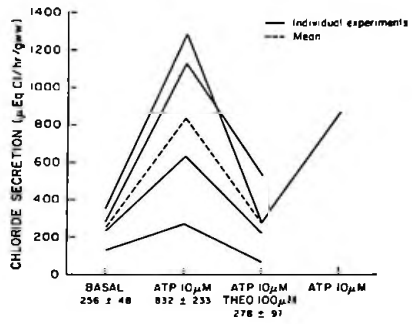

Figure 1.--ATP (10 μM) stimulation of chloride secretion and complete inhibition with theophylline. Solid lines are individual experiments while the dotted line is the mean.

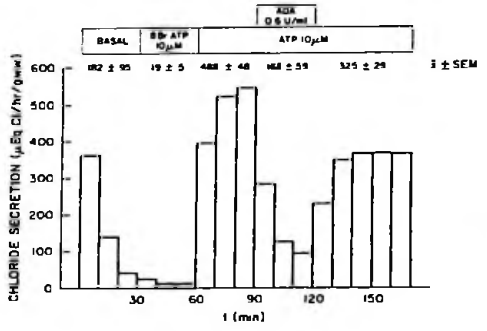

Figure 2.--Effect of 8 BrATP (10 μM), ATP (10 μM), and ADA (0.6 U/ml) on chloride secretion. Individual bars represent chloride secreted in one ten-minute period.

the effect of these drugs in four perfused glands. From a mean basal value of 256 ± 48, ATP (10^{-5}M) stimulated chloride secretion to 832 ± 233 ($p < 0.02$, paired t). In the presence of theophylline (10^{-4}M), however, secretion dropped almost to basal levels at 278 ± 97 ($p < 0.05$, paired t). Since theophylline is a specific adenosine receptor blocker with no effect on ATP receptors (Burnstock, G., In: Cell Membrane Receptors for Drugs and Hormones: A Multidisciplinary Approach. Ed. Straub, R.W. and Bolis, L., p. 107-118, New York: Raven Press, 1978), our results suggest that not ATP, but adenosine formed by the hydrolysis of ATP is stimulating chloride secretion. An adenosine receptor (R_a) that is blocked by theophylline has already been characterized in this tissue (Forrest et al., Bull. MDIBL 20: 152-155, 1980), supporting the idea that adenosine and not ATP is stimulating chloride secretion.

Two additional types of experiments using 8 BrATP and ADA further support this hypothesis. 8 BrATP is an analogue of ATP that is active at ATP receptors, but upon hydrolysis yields an adenosine analogue, 8 BrADO, that is inactive at adenosine receptors (Daly, J. Med. Chem., 25, 1982). Thus, it is a unique tool to distinguish between an adenosine and an ATP response. Similarly, ADA can also distinguish between an adenosine and ATP response. In the presence of ADA any adenosine formed from the hydrolysis of ATP is immediately deaminated to

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