

CELL PHYSIOLOGY OF CULTURED SQUALUS ACANTHIAS RECTAL GLAND EPITHELIUM

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The rectal gland (SRG) from the dogfish shark Squalus acanthias is an important model for hormone-modulated secondary active chloride secretion. Epithelial transport systems homologous to those in the SRG are found in mammalian trachea, intestine, cornea, salivary gland and kidney thick ascending limb. Understanding SRG transport mechanisms and their hormonal control provides important insights into analogous mechanisms in mammalian tissues. SRG physiology has been studied using a variety of experimental preparations including isolated perfused glands, tissue slices, dispersed tubule and cell suspensions, isolated microperfused tubules and plasma membrane vesicles. However, each has one or more shortcomings for analyzing hormone action in the SRG at the cellular and molecular levels. For example, isolated perfused glands are problematic because the endogenous neural supply and hemodynamic effects complicate analysis of direct hormone action on SRG epithelial cells. Tubule and cell suspensions do not permit study of vectorial polarized membrane functions, an important element in the overall process of trans-epithelial transport. Isolated microperfused tubules have been used in the elegant electrophysiologic analysis of SRG epithelium by Greger and co-workers (NIPS 1:134, 1986). However, as with individually perfused mammalian kidney tubules, this approach is technically difficult, complicated by tubular geometry and small amount of material available for conventional biochemistry, and is inaccessible to epithelial voltage clamp techniques.

Recent studies of mammalian and amphibian transporting epithelia using tissue culture methods illustrate the advantages of cultured cells for exploring numerous problems in epithelial biology. Farmer et al. have previously shown the feasibility of primary SRG culture (Bull. MIDIBL 23:83, 1983; Bull. MDIBL 24:20, 1984). The objectives of this study were to: 1. Develop methods for primary culture and establishing long term cell lines of SRG epithelium and 2. Determine if cultures exhibit the physiological properties defined by other investigators using the isolated perfused gland, dissociated SRG cell suspensions or individual microperfused SRG tubules.

Dissociated SRG tubules were prepared using collagenase (2mg/ml, Worthington CLS II) and inoculated into plastic tissue culture dishes or onto type I collagen gels or EHS matrigels. The later is a matrix comprised of the collagens, glycoproteins and proteoglycans found in the basal lamina, the natural substratum for all epithelia. A variety of culture media and serum supplements were tested. Best results were obtained with equal parts Eagle's Minimal Essential Medium and Ham's F12 medium containing insulin, transferrin, selenium and linoleic acid. Urea, trimethylamine oxide and sodium chloride were added to adjust osmolarity to approximately 950 mOsm/L. This medium was usually supplemented with 5% Nu-Serum (Collaborative Research) but similar cell viability, morphology and growth could be obtained using serum-free medium. This finding will be important in future studies of SRG epithelium where complete hormonal definition of the culture medium is necessary.

Following inoculation, tubules attached to the culture substratum in 24

hrs. Tubule attachment was best on type I collagen gels. Cells migrated as a contiguous sheet from tubules and eventually formed a confluent monolayer of polygonal epithelium (Figure 1). On plastic dishes, cells were flattened and frequently displayed an irregular stellate shape. Cell migration on EHS matrigels was very slow and confluent monolayers were never attained. However, these cells appeared to have a more columnar shape than those maintained on collagen gels or plastic substrata. Ultrastructural studies are in progress to determine if they exhibit enhanced cytodifferentiation.

Giemsa stained sub-confluent cultures showed occasional mitotic figures in tubular epithelial cells. Therefore, although monolayer formation was principally the result of cell migration from attached tubules, some cell proliferation also occurred. The finding of mitosis in tubular epithelium indicates that these cells are capable of multiplying in culture and provides a basis for future studies to define growth promoting activities in shark brain and pituitary extracts. This approach has been highly successful with cultured mammalian epithelial cells.

11-20 day old monolayers grown on nylon mesh supported type I collagen gels could be mounted in Ussing chambers for determination of electrophysiologic parameters. Measurements of transepithelial voltage (V_{TE}), short circuit current (I_{SC}) and resistance (R_C) differentiated cultures into 3 groups. Group 1 cultures were established at low tubule inoculation densities and exhibited no V_{TE} or I_{SC} , either under basal conditions or after hormonal stimulation. Group 2 cultures were established at intermediate tubule density and had a low V_{TE} and I_{SC} . These parameters were moderately stimulated by hormones. Group 3 cultures were established at high tubule densities and displayed moderate spontaneous V_{TE} and I_{SC} which could be stimulated dramatically by hormones. Stimulated I_{SC} 's of over $100\mu A/cm^2$ were observed in these cultures. Their resistance averaged $59\Omega cm^2$. Figure 2 illustrates the electrophysiologic properties of a Group 3 culture.

The polarity of the V_{TE} and I_{SC} were consistent with their origin from transepithelial chloride secretion. Experiments using secretory agonists and inhibitors gave results consistent with this interpretation. $1\mu M$ forskolin, $10\mu M$ 2-chloroadenosine and $1\mu M$ vasoactive intestinal peptide all stimulated I_{SC} . Hormone-activated transport was abolished substantially by basolateral perfusion with 0.1 mM bumetanide or 5 mM barium. In addition, $0.1\mu M$ 2-chloroadenosine partially inhibited forskolin-stimulated V_{TE} and I_{SC} . The findings that high concentrations of 2-chloroadenosine stimulate I_{SC} while low concentrations block forskolin-stimulated I_{SC} demonstrate the presence of both stimulatory (A_2) and inhibitory (A_1) adenosine receptors in these cultures.

To determine if cAMP is a second messenger in hormonally stimulated SRG cells, intracellular cAMP levels were measured by radioimmunoassay in cultures exposed to compounds which activated transport activity. As shown in Figure 3, both forskolin and 2-chloroadenosine caused marked elevation of intracellular cAMP levels. In addition, somatostatin caused a small but significant inhibition in forskolin-stimulated cAMP formation. These data are consistent with cAMP being an intracellular second messenger in SRG epithelium.

Unidirectional ^{86}Rb influx was studied in SRG cultures to define possible plasma membrane effects of elevated intracellular cAMP (See Figure 4). ^{86}Rb influx showed both ouabain, bumetanide and Ba-sensitive components,

indicative of ^{86}Rb entry via the Na/K pump, Na/Cl/K cotransport system and K channels respectively. Pre-incubating cells for 60 min in $1\mu\text{M}$ forskolin enhanced bumetanide-sensitive ^{86}Rb influx without effecting the ouabain or Ba-sensitive fluxes. These results indicate that cAMP-mediated stimulation of Na/K/Cl cotransport is an important component of hormone-activated Cl secretion in cultured SRG cells.

This study demonstrates that proliferating monolayer cultures of SRG epithelium can be routinely established which express the hormone receptors, plasma membrane signal transduction mechanisms and transport proteins found in the intact SRG.

SRG cultures provide a new and powerful tool for defining the neuro-humoral signals and second messengers networks which modulate SRG Cl secretion. In addition, the SRG culture system promises to be an important model for analyzing in a controlled experimental setting the effects of environmental pollutants such as mercury, lead and cadmium on plasma membrane functions in osmoregulatory tissues.

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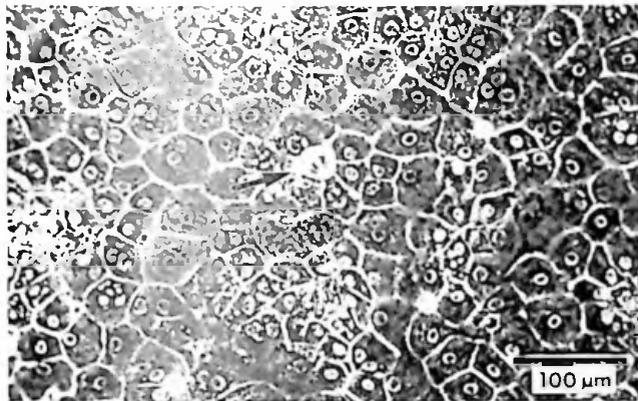


FIGURE 1

14 day old SRG culture grown on a type 1 collagen gel. Arrow indicates a cell in anaphase of the mitotic cycle.

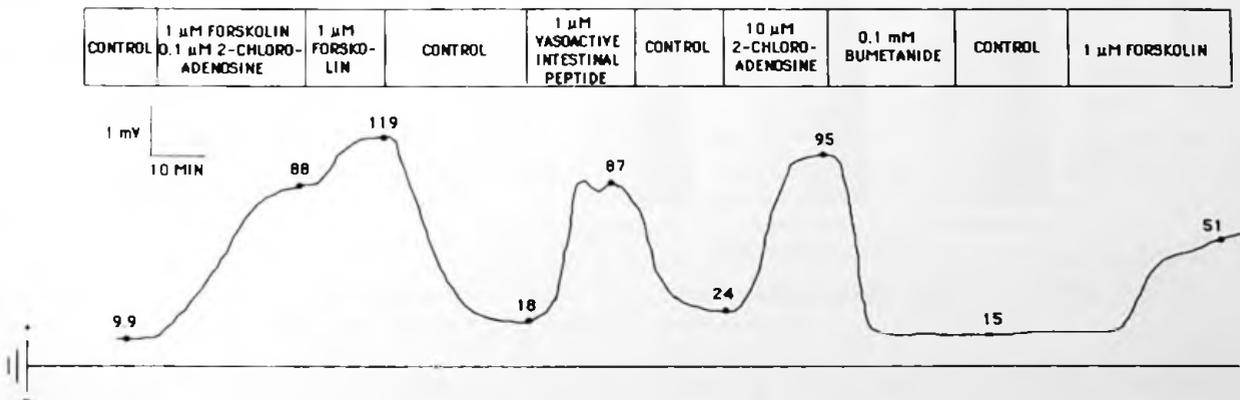


FIGURE 2

Tracing illustrating transepithelial voltage responses to Cl secretory agonists and inhibitors in a 12 day old SRG culture (voltage referenced to apical ground). Values along the curve indicate the short circuit current in $\mu\text{A}/\text{cm}^2$. All test compounds were added to the basolateral perfusate.

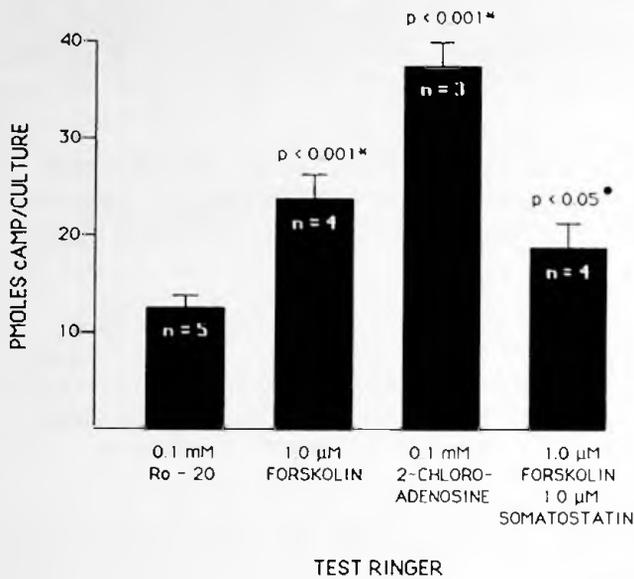


FIGURE 3

Near confluent cultures grown on type 1 collagen gels in 35 mm dishes were incubated with the indicated test compound in shark Ringer for 15 min. Reactions were stopped by addition of TCA and cells extracted for 60 min. The cAMP content of extracts was determined by radioimmunoassay. Asterisk indicates statistical comparison by t-test with control group (Ro-20). Closed circle indicates comparison with forskolin-treated group.

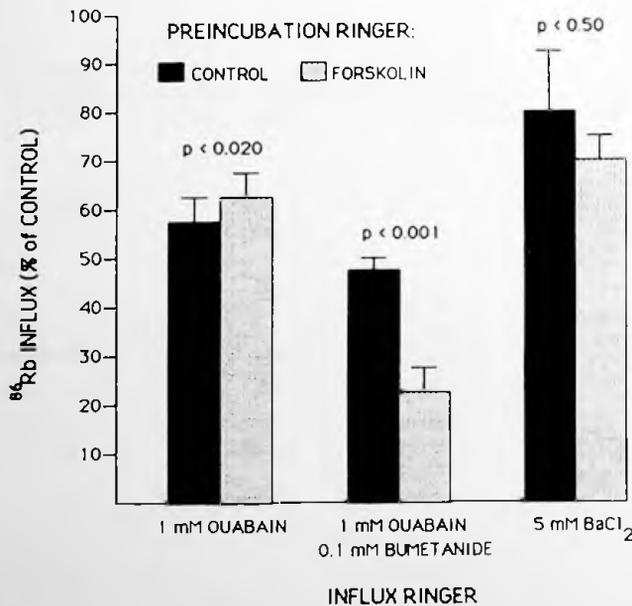


FIGURE 4

Cultures grown in 35 mm tissue culture dishes were rinsed in shark Ringer and preincubated at room temperature for 60 min in Ringer with or without 1 μM forskolin. Cultures were then incubated in the indicated influx Ringer for 2 min, ⁸⁶Rb added and incubation continued for 5 min. Dishes were aspirated, quickly rinsed in 3 changes of ice cold 300 mM MgCl₂ and air dried. ⁸⁶Rb was extracted in 0.1N HCl. Following neutralization, aliquots of the extract were counted by liquid scintillation spectrometry. Control and forskolin-treated cultures were compared statistically by t-test (n=3 for all groups).