Perfusion of Isolated Tubules of the Shark Rectal Gland

ELECTRICAL CHARACTERISTICS AND RESPONSE TO HORMONES

JOHN N. FORREST, JR., FONG WANG, and KLAUS W. BEYENBACH, *Department of Medicine, Yale University School of Medicine, New Haven, Connecticut 06510; Division of Biological Sciences, Cornell University, Ithaca, New York 14853; The Mount Desert Island Biological Laboratory, Salsbury Cove, Maine 04672*

ABSTRACT Both the mammalian thick ascending limb of Henle's loop and the shark rectal gland actively transport Cl against an electrochemical gradient by mechanisms involving hormone-sensitive NaCl transport. In contrast to mammalian renal tubules, individual tubules of the shark rectal gland previously have not been perfused in vitro. Using a combination of renal slice and microdissection techniques we were able to isolate and perfuse single rectal gland tubules without the use of enzyme treatment. Single tubules consistently generated lumen-negative transepithelial voltages (V_t) of −1.8 mV when perfused and bathed with identical shark Ringer's solution. The addition of cyclic AMP, vasoacative intestinal peptide (VIP), and adenosine to the bath increased V_t to −7.5, −9.0, and −4.3 mV, respectively (all $P < 0.02$ compared with paired controls). Each stimulation could be reversed by addition by furosemide to the bath. The adenosine response was inhibited by theophylline, a specific inhibitor of adenosine receptors. The tubules had a low transepithelial electrical resistance of 12–26 $\Omega \cdot cm^2$ and exhibited a transepithelial permselectivity for small cations. These results indicate that tubules of the rectal gland can be perfused in vitro and have receptors for VIP and adenosine. Cyclic AMP and secretagogues hyperpolarize the membrane consistent with electrogenic chloride transport, and these effects are reversed by furosemide, an inhibitor of coupled sodium-potassium-chloride co-transport. The response of V_t to cyclic AMP and furosemide, the transepithelial electrical resistance, and the cation selective permeability of tubules are remarkably similar to measurements in perfused mammalian thick ascending limbs.

INTRODUCTION

The elasmobranch rectal gland has been considered a useful model for sodium-coupled electrogenic chloride transport in a variety of mammalian and nonmammalian tissues (1–4). The gland is easily perfused in vitro (1–2), and at an average weight of 3 g there is sufficient tissue for biochemical studies and isolation of specific membrane fractions (4). Studies using the whole gland perfused in vitro and isolated plasma membrane vesicles have indicated striking similarities between chloride transport in this gland and the mammalian thick ascending limb (TAL)[1] of Henle's loop (1, 4). However, in spite of the important role of the shark rectal gland in comparative studies of NaCl transport, the driving forces for ion transport and hormonal responses have not been measured in the functional unit of the gland, the rectal gland tubule.

In this study we demonstrate the feasibility of perfusing single rectal gland tubules in vitro and of study-

This work was presented in part at the 15th Annual Meeting of the American Society of Nephrology, December 13, 1982, Chicago, IL and reported in abstract form in 1983, *Kidney Int.* 23:255.

This study was supported by grants from the National Institutes of Health (NIH) (AM-17433) and the American Heart Association (81-920) to Dr. Forrest and by an NIH grant (AM 26633) to Dr. Beyenbach. Address reprint requests to Dr. Forrest at the Yale University School of Medicine.

Received for publication 1 June 1983.

[1] *Abbreviations used in this paper:* 8-chlorophenylthiocyclic AMP, 8-cpt-cAMP; TAL, thick ascending limb; VIP, vasoactive intestinal peptide; V_t, transepithelial voltage.

ing transepithelial NaCl transport directly across this epithelium with the advantages afforded by the methods of Burg and Green (5). The results underscore the presence of similar functional elements in tubules of the rectal gland and the TAL of Henle's loop.

METHODS

Isolation and in vitro microperfusion of tubules. Rectal glands from the dogfish shark (*Squalus acanthias*) were obtained and decapsulated as described previously (6). Tubules were isolated in Ringer's solution by freehand dissection from tissues slices (<0.3-mm/thick) cut perpendicular to the long axis of the gland. Tubules were teased apart proceeding from the subcapsular periphery to the central lumen of the gland until a point of branching was reached. The tubule was teased away from this junction leaving its lumen open at the distal end; the proximal end of the tubule was frayed open using dissecting needles.

Tubules 0.3–1.3 mm in length were transferred to the perfusion bath Ringer's containing: 270 mM NaCl, 4 mM KCl, 3 mM $MgCl_2$, 2.5 mM $CaCl_2$, 1 mM KH_2PO_4, 8 mM $NaHCO_3$, 0.5 mM Na_2SO_4 350 mM urea, and 5 mM glucose. The pH was 7.5–7.6 when gassed with 99% O_2 and 1% CO_2. The temperature of the bath was maintained at 12±2°C. Unless indicated otherwise, identical Ringer's solution was used in the tubular lumen and bath. Lumen were perfused at rates <5 nl/min and the bath was continuously exchanged at flow rates of 3–7 ml/min. Tubules were studied in vitro from 1 to 5 h.

Electrical measurements. The electrical measurements used are described in detail elsewhere (7, 8). Briefly, transepithelial voltage (V_t) was measured via the perfusion pipet with respect to ground in the bath using Ag-AgCl electrodes and agar bridges. Transepithelial resistance was measured by cable analysis (8) in short lengths (<200 μm) of tubules so that adequate voltage deflections could be measured at the distal end when constant current pulses (100–300 nA; 400–600 msec) were injected into the tubule lumen via the perfusion pipet (8). To characterize the transepithelial ion selectivity of unstimulated tubules, transepithelial voltages were examined for bionic diffusion potential when Na in the bath was reduced 10-fold and replaced with choline or Li, or when bath Cl was reduced 10-fold and replaced with isethionate. The voltage data from these experiments were corrected for liquid junction potentials (9). All data presented are mean±SEM, with n indicating the number of tubules studied. Effects of secretagogues and inhibitors were evaluated by a paired t test using each tubule as its own control.

RESULTS

Isolation of tubules. The ease of dissecting single tubules and the length of isolated tubules varied from animal to animal; there was no apparent relationship between the sex or size of the shark and the ease of tubule isolation. After connection to the holding pipets, the average tubule length exposed to the bath was 350±40 μm (n = 24). The outer diameter of tubules ranged from 30 to 80 μm with a mean of 43.5±3.6 μm (n = 13). Most tubules tapered slightly towards the peripheral (subcapsular) end, so that tubule diameters were not always constant. During perfusion, (Fig. 1) the luminal surface of the epithelium usually formed a flat border as in perfused renal tubules.

Basal V_t and response to 8-chlorophenylthio-cyclic AMP (8-cpt-cAMP.) When perfused with identical Ringer's solution in the lumen and bath the mean spontaneous V_t of 37 perfused tubules was −1.8±0.4 mV. In tubules in which V_t was first measured at base line and then after the addition of 8-cpt-cAMP100 μM to the bath, V_t increased from −0.9±0.3 mV to −7.7±1.3 ($P < 0.001$, n = 9, Fig. 2 *A*). This response began within 2 min, was near maximal at 15 min, and remained elevated in the presence of the cyclic AMP analogue. After washout of 8-cpt-cAMP from the bath, V_t gradually fell to base-line values over 20–30 min.

Effects of VIP, adenosine, and furosemide on V_t. Both vasoactive intestinal peptide (VIP) and adenosine increase chloride secretion 10–20-fold in the perfused whole rectal gland (2, 10). However, since VIP and adenosine have vasomotor effects in the perfused whole gland (11) it was of interest to determine the direct effects of these agents on the epithelium. The effects of VIP (Fig. 2 *B*) were studied in tubules perfused in a stationary bath to reduce the quantity of hormone required. Under these conditions basal V_t was −4.1±1.2 mV and rose to −9.0±2.5 mV ($P < 0.02$), n = 8) after addition of 1 μM VIP to the bath.

As shown in Fig. 3 *A* the addition of either 100 μM adenosine (five tubules) or 2-chloroadenosine (four tubules) to the bath increased V_t from basal levels of −0.9±0.3 to −4.6±0.8 mV ($P < 0.005$, n = 9). Theophylline (100 μM), a specific inhibitor of external adenosine receptors in a variety of tissues (12, 13), was added to the bath during the maximal response to adenosine in three tubules (Fig. 3 *A*). In the presence of adenosine and theophylline, V_t was inhibited within 10 min to basal levels (−0.9±0.4 mV, $P < 0.05$).

Because furosemide inhibits sodium chloride transport in a variety of absorptive and secretory epithelia characterized by secondary active chloride transport (3), it was of interest to determine the direct effects of furosemide in perfused tubules in which the V_t had been increased by 8-cpt-cAMP, VIP, or adenosine. Furosemide (100 μM) added to the bath immediately reversed the hyperpolarization due to 8-chlorophenylthio-cyclic AMP, VIP, or adenosine. The onset of this response was immediate (within seconds) and V_t was reduced to near basal values within 5–8 min. The mean V_t of −9.9±1.9 mV measured in the presence of secretagogues was reduced to −2.1±0.6 following addition of furosemide. (Fig. 3 *B*).

Transepithelial resistance and ion permselectivity of the tubule. In eight unstimulated tubules, the transepithelial resistance averaged 1.92±0.5 kΩ per cm tubule length with a length constant of 68±10 μm.

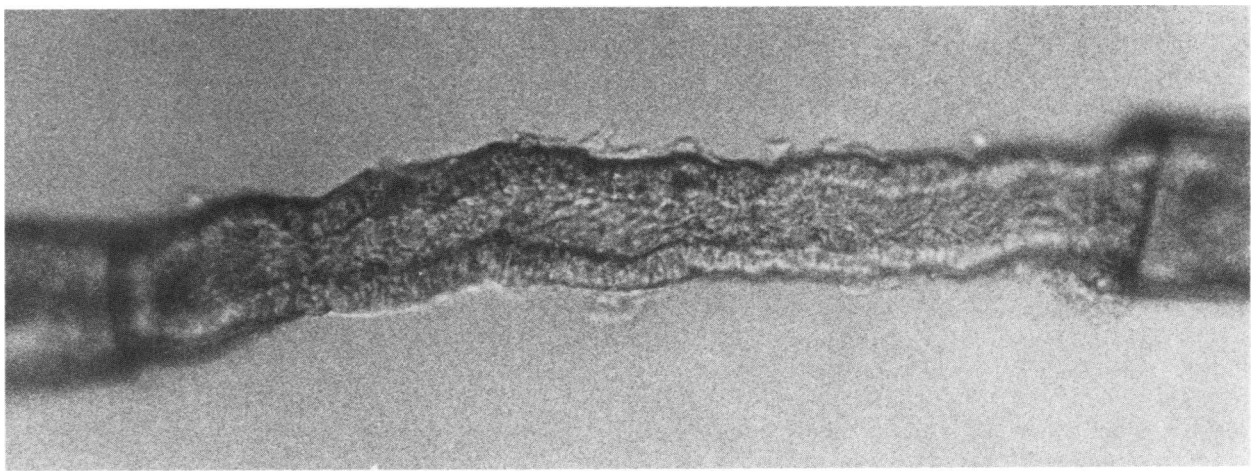

FIGURE 1 In vitro perfusion of a single isolated tubule of the rectal gland of *Squalus acanthias*. × 400.

Hence, the specific tissue resistance was 12.0–26.2 $\Omega \cdot cm^2$ using, respectively, the average inner and outer diameters of the tubules. When sodium concentration in the bath was reduced 10-fold by replacement of bath NaCl with choline chloride, bionic diffusion gradients for sodium and choline resulted in marked hyperpolarization of the lumen by an average of -27.9 ± 0.28 mV (n = 5). This hyperpolarization was immediately reversed on return to the control bath. When bath Na was reduced 10-fold by replacement with lithium, only slight hyperpolarization of the lumen was observed (-4.4 ± 0.1 mV; n = 3). Given the low transepithelial resistance of the tubule these results are consistent with selectivity of the paracellular pathway for small cations such as Na^+ and Li^+. This interpretation is supported by the virtual absence of V_t changes when bath Cl was reduced 10-fold by replacement with isethionate; this substitution depolarized V_t by only 1.9 ± 0.3 mV (n = 3).

DISCUSSION

The shark rectal gland is a Cl-transporting epithelium that secretes an isosmotic fluid containing primarily NaCl (14). In this study the perfusion of single tubules

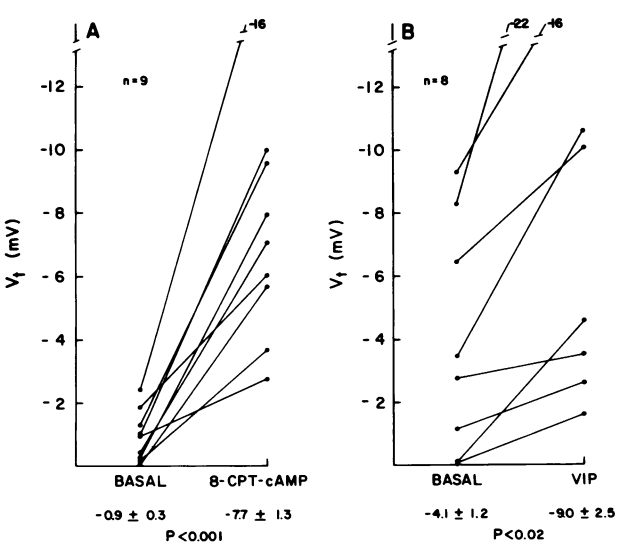

FIGURE 2 V_t in isolated perfused tubules of the rectal gland. (*A*) stimulation of V_t by 8-cpt-cAMP (100 μM); (*B*) stimulation V_t by *VIP* (1 μM). n, number of tubules.

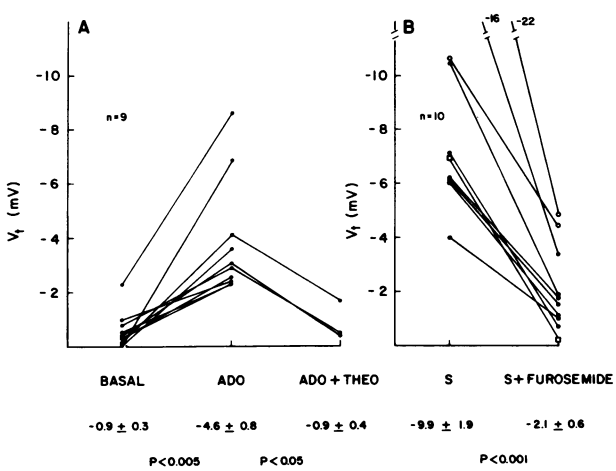

FIGURE 3 V_t in isolated perfused tubules of the rectal gland. (*A*) stimulation of V_t by adenosine (*ADO*) (100 μM) and inhibition of adenosine-stimulated V_t by theophylline (*THEO*). (*B*) inhibition of secretagogue-stimulated V_t by furosemide (100 μM) (●, 8-cpt-cAMP; ○, VIP; □, adenosine). n, number of tubules.

isolated from the gland revealed lumen-negative transepithelial voltages that are consistent with electrogenic Cl transport from bath to tubule lumen. The low electrical resistance measured across the tubule supports the high conductance proposed for this epithelium on the basis of morphologic studies demonstrating an extensive length of tight junctions per area of luminal membrane (86 m/cm^2) (15). It is likely that the tight junctional pathway in shark rectal gland tubules is permselective for Na in view of the high transepithelial diffusion potentials measured for this ion, but not for Cl.

In the perfused whole gland, cyclic AMP, VIP (1, 2), and adenosine (10) stimulate Cl secretion. However, these three agents have been shown to reverse α-adrenergic-induced vasoconstriction in the perfused whole gland (11) leaving unresolved the question of direct effects on the epithelium. The hyperpolarization that we consistently observed with cAMP, VIP, and adenosine indicate that these secretagogues also have direct effects on the tubular epithelial cells. Reversal of adenosine-induced hyperpolarization by theophylline indicates the presence of adenosine receptors on the basolateral membrane of the tubules (12, 13). Similarly, the response to VIP supports the notion of basolateral receptors for this or similar polypeptide hormones. Reversal of secretagogue-induced hyperpolarization by furosemide is consistent with electrogenic Cl transport occurring through a furosemide-sensitive co-transport system (16).

Our initial studies of isolated perfused tubules of the rectal gland underscore the similarities between chloride secretion in this tissue and chloride reabsorption in the mammalian TAL. In both tissues chloride transport occurs against a steep transepithelial electrochemical gradient (1, 5, 17); intracellular chloride activity where measured is above the Nernst equilibrium (18); and chloride transport is dependent on both external sodium and potassium (1, 19–21) and is inhibited by ouabain (1, 5, 17, 20, 22). In both tubules the lumen hyperpolarizes after addition of hormone or cyclic AMP to the bath (22, 23) and this hyperpolarization is reversed by addition of furosemide (5, 24, 25) to that tubular surface (basolateral in the rectal gland and luminal in the TAL) recently shown to possess a sodium-potassium-chloride co-transport system (16, 26). Finally, both tubules are permselective for Na (27) and have low transepithelial resistances: 12–25 $\Omega \cdot cm^2$ in rectal gland tubules in the present study and 11–34 $\Omega \cdot cm^2$ in TAL (5, 27). This remarkable similarity of structural and functional elements, despite differences in the direction of net NaCl transport, indicates the presence of common transport mechanisms in two tubules of widely separate phylogenetic origin.

ACKNOWLEDGMENT

We thank Dr. Mark Knepper, Laboratory of Kidney and Electrolyte Metabolism, National Institutes of Health, for his generous help in initiating these studies.

REFERENCES

1. Silva, P. J., J. Stoff, M. Field, L. Fine, J. N. Forrest, and F. H. Epstein. 1977. Mechanism of active chloride secretion by shark rectal gland: role of Na-K-ATPase in chloride transport. *Am. J. Physiol.* 233:F298–F306.
2. Stoff, J. S., R. Rosa, R. Hallac, P. Silva, and F. H. Epstein. 1979. Hormonal regulation of active chloride transport in the dogfish rectal gland. *Am. J. Physiol.* 237:F138–F144.
3. Frizzell, R. A., M. Field, and S. G. Schultz. 1979. Sodium-coupled chloride transport by epithelial tissues. *Am. J. Physiol.* 236:F1–F8.
4. Eveloff, J., R. Kinne, E. Kinne-Saffran, H. Murer, P. Silva, F. H. Epstein, J. Stoff, and W. B. Kinter. 1978. Coupled sodium and chloride transport into plasma membrane vesicles prepared from dogfish rectal gland. *Pfluegers Arch.* 378:87–92.
5. Burg M., and N. Green. 1973. Function of the thick ascending limb of Henle's loop. *Am. J. Physiol.* 224:659–668.
6. Forrest, J. N., Jr., D. Andrews, and D. C. Davison. 1980. Short-circuit current in flat sheets of the rectal gland of *Squalus acanthias*. *Bull. Mt. Desert Isl. Biol. Lab.* 20:156–157.
7. Beyenbach, K. W., B. M. Koeppen, W. H. Dantzler, and S. I. Helman. 1980. Luminal Na Concentration and the electrical properties of the snake distal tubule. *Am. J. Physiol.* 239:F412–F419.
8. Helman, S. I. 1972. Determination of electrical resistance of the isolated cortical collecting tubule and its possible anatomical location. *Yale J. Biol. Med.* 45:339–345.
9. Barry, P. H., J. M. Diamond, and E. M. Wright. 1971. The mechanism of cation permeation in rabbit gallbladder. *J. Membr. Biol.* 4:358–394.
10. Forrest, J. N., Jr. D. Rieck, and A. Murdaugh. 1980. Evidence for a ribose specific adenosine receptor (R_a) mediating stimulation of chloride secretion in the rectal gland of *Squalus acanthias*. *Bull Mt. Desert Isl. Biol. Lab.* 20:152–155.
11. Shuttleworth, T. J. 1983. Haemodynamic effects of secretory agents on the isolated elasmobranch rectal gland. *J. Exp. Biol.* 103:193–204.
12. Londos, C., D. M. F. Cooper, and J. Wolff. 1980. Subclasses of external adenosine receptors. *Proc. Natl. Acad. Sci. USA.* 77:2551–2554.
13. Bruns, R. F., J. W. Daly, and S. H. Snyder. 1980. Adenosine receptors in brain membranes:binding of N^6-cyclohexyl [^{3}H] adenosine and 1,3-diethyl-8-[^{3}H] phenylxanthine. *Proc. Natl. Acad. Sci. USA.* 77:5547–555.
14. Burger, J. W., and W. N. Hess. 1960. Function of the rectal gland in the spiny dogfish. *Science (Wash. DC).* 131:670–671.
15. Forrest, J. N., Jr., J. L. Boyer, T. A. Arbito, D. Murdaugh, and J. B. Wade. 1982. Structure of tight junctions during Cl secretion in the perfused rectal gland of *Squalus acanthias*. *Am. J. Physiol.* 242:C388–C392.
16. Hannafin, J., E. Kinne-Saffran, D. Friedman, and R.

Kinne. 1983. Presence of sodium-potassium chloride cotransport system in the rectal gland of *Squalus acanthias*. *J. Membr. Biol.* 75:73–83.
17. Rocha, A. S., and J. P. Kokko. 1973. Sodium chloride and water transport in the medullary thick ascending limb of Henle. *J. Clin. Invest.* 52:612–624.
18. Welsh, M. S., P. L. Smith, and R. A. Frizzell. 1980. Intracellular chloride activities in the isolated perfused shark rectal gland. *Bull. Mt. Desert Isl. Biol. Lab.* 20:101–103.
19. Solomon, R. J., P. J. Silva, A. Stevens, J. Epstein, J. S. Stoff, K. Spoles, and F. H. Epstein. 1977. Mechanism of chloride transport in the rectal gland of *squalus acanthias:* ionic selectivity. *Bull. Mt. Desert Isl. Biol. Lab.* 17:59–63.
20. Greger, R. 1981. Chloride reabsorption in the rabbit cortical thick ascending limb of the loop of Henle. *Pflugers Arch.* 390:38–43.
21. Greger, R., and E. Schlatter. 1981. Presence of luminal K^+, a prerequisite for active NaCl transport in cortical thick ascending limb of Henle's loop of rabbit kidney. *Pflugers Arch.* 392:92–94.
22. Hall, D. A., and D. M. Varney. 1980. Effect of vasopressin on electrical potential difference and chloride transport in mouse medullary thick ascending limb of Henle. *J. Clin. Invest.* 66:792–802.
23. Hebert, S. C., R. M. Culpepper, and T. E. Andreoli. 1981. NaCl transport in mouse medullary thick ascending limbs. I. Functional nephron heterogeneity and ADH-stimulated NaCl cotransport. *Am. J. Physiol.* 241:F412–F431.
24. Imai, M. 1977. Effect of bumetanide and furosemide on the thick ascending limb of Henle's loop of rabbits and rats perfused in vitro. *Eur. J. Pharmacol.* 41:409–416.
25. Stoner, L. C., and M. E. Trimble. 1982. Effects of MK-196 and furosemide on in vitro rat medullary thick ascending limb of Henle. *J. Pharmacol. Exp. Ther.* 221:715–720.
26. Koenig, B., and R. Kinne, 1982. The role of potassium in chloride transport of the thick ascending limb of Henle's loop. *Pflugers Arch.* 394:R23.
27. Greger, R. 1981. Cation selectivity of the isolated perfused cortical thick ascending limb of Henle's loop of rabbit kidney. 1981. *Pflugers Arch* 390:30–37.