Furosemide and Bumetanide Uptake in Dogfish Rectal Gland Slice: Concentration Dependency

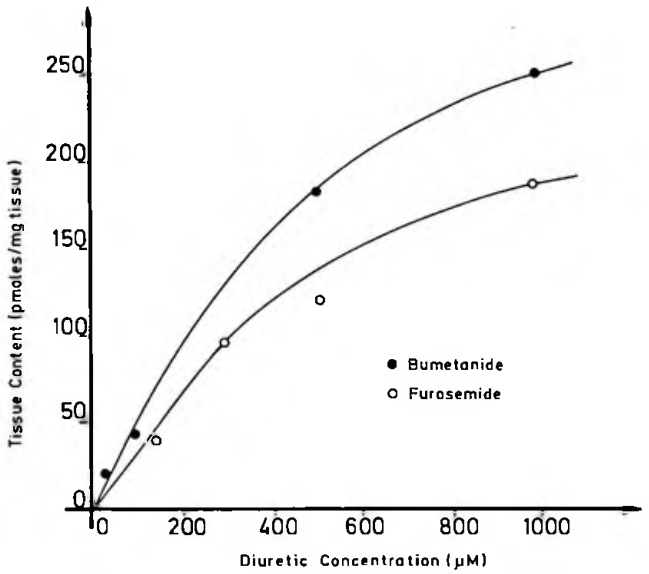

Figure 3. The concentration dependency of furosemide and bumetanide accumulation in dogfish rectal gland slices. Dogfish rectal gland slices were incubated at 15°C in dogfish Ringer's containing increasing concentrations of furosemide (O) or bumetanide (O) for 30 min followed by a 30 min wash period in dogfish Ringer's without the diuretic. Values are the means of 4 experiments and are corrected for the diuretic found in the extracellular fluid spaces as calculated using [^{3}H] inulin spaces.

Thus, it appears that bumetanide and furosemide are taken up into the rectal gland slices in a manner which suggests that some of the diuretic may be specifically bound to the tissue. Future autoradiographs will be attempted to localize the labelled diuretics. Supported in part by USPHS Grant AM 05841 and the Main Affiliate of the American Heart Association.

STRUCTURE OF TIGHT JUNCTIONS IN CHLORIDE-SECRETING EPITHELIA IN THE RECTAL GLAND OF SQUALUS ACANTHIAS

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Tight junctions between epithelial cells play a critical role in recently proposed models of isotonic sodium chloride secretion in the rectal gland of the dogfish shark (Silva et al., Am.J. Physiol. 233:F298, 1977). In these models a neutral sodium chloride carrier in the basolateral membrane is responsible for active movement of chloride into the cell coupled to the inward movement of sodium. A favorable electrochemical gradient for sodium entry is maintained by the activity of basolateral membrane Na-K-ATPase. Whereas chloride then diffuses passively from the cell into the lumen down an electrical gradient, sodium is postulated to move from the extracellular fluid down its electrochemical gradient into the tubular lumen via the paracellular pathway. As part of the primary pathway for transepithelial sodium flux the tight junction must to some extent be permeable to sdoium, but must exclude urea since this solute is practically excluded from the secretion, even though urea is present in high concentration in the extracellular fluid. Although Bulger (Anat. Record 147:95, 1963) stated that tight junctions are present in the rectal gland, Van Lennup concluded that the rectal gland of the elasmobranch did not contain typical

tight junctions between cells, since lanthanum hydroxide filled the intracellular space completely up to the lumen (J. Ultrastr. Res. 25, 94, 1968). The purpose of the present study was to reexamine the properties of the junctional barrier in dogfish rectal gland by determining its fine structure and permeability to ionic lanthanum following in vitro perfusion under basal conditions and during stimulation of chloride secretion.

Rectal glands were obtained from female spiny dogfish (Squalus acanthias) weighing 2 to 6 kg. The rectal gland was removed and cannulae were placed in the rectal gland artery, vein and duct and the gland was perfused by gravity flow from an oxygenated reservoir at a pressure of 3.5-4mm mercury and a flow rate of 4-8 ml/min with a shark-Ringer perfusion solution containing (mM): Na 280: K 5.0; CL 285; Tris 3; Ca 2.5; Mg ;.2; sulfate 0.5; urea 350; glucose 5; pH 7.4-7.5. Bicarbonate and phosphate were omitted from the perfusate to avoid precipitation of lanthanum. Glands were perfused for 40-60 minutes in either the basal state or during stimulation of chloride secretion by the addition of theophylline (0.25mM) and dibutyryl cyclic AMP (0.05mM) to the medium. Following perfusion in either the basal or stimulated state in selected glands, the perfusate was changed to an otherwise identical solution containing ionic lanthanum chloride (3mM) and the perfusion continued for five minutes. All glands were then fixed by perfusion for 15 minutes at 4-8°C with a Karnovsky-type fixative as modified by Doyle (Bull. MDIBL 17:34, 1977 and personal communication) containing 2% glutaraldehyde, 4% paraformaldehyde, 0.1M cacodylate at pH 7.4. Following fixation by perfusion, the gland was sectioned at the level of the rectal gland artery and small pieces (1-2 mm^3) were obtained from both the inner and outer one-third of rectal gland tissue. Sections were incubated for two hours in the fixative mixture and were then washed three times with 0.1M cacodylate buffer and refrigerated. The tissue was processed for electron-microscopy and freeze-fracture replicas were prepared as previously described (Wade et al., Am. J. Physiol., 232:F77, 1977).

Multiple sections from both inner and outer areas of perfused rectal glands from 12 fish were analyzed. Figure 1 is a low power electron micrograph of the luminal membrane as seen by freeze-fracture which demonstrates the

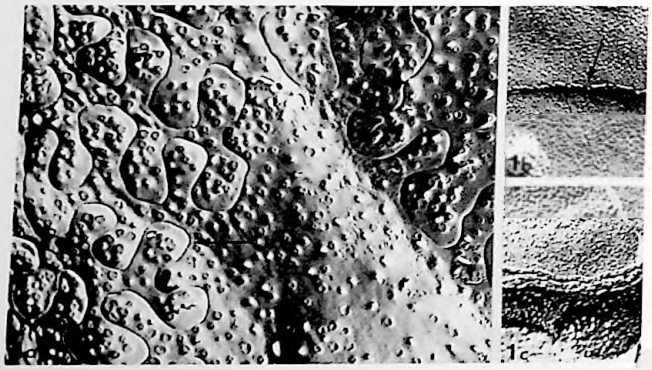

Figure 1. Freeze-fracture electron micrographs of the rectal gland. a) Low power (10,500x) fracture through luminal membrane showing extensive interdigitation of adjacent cells with amplification of the length of tight junction. b) High magnification (80,000x) fracture through the tight junction demonstrating a junctional region of simple structure with discontinuity (arrow). c) High magnification (80,000x) through a tight junction consisting of closely opposed elements.

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Figures 1 and 2 are reduced 50% from the originals. The magnifications given in the legends should be reduced 50%. - Editor

extensive interdigitation of adjacent cells in the gland and the associated amplification in the length of the tight junction (TJ) which is a remarkable feature of this tissue. The mean length of tight junction per area of luminal surface was 86 ± 5.7 meters/cm^2 luminal area as estimated by measurements in 28 micrographs from 8 fish. No difference in junctional length was observed between inner and outer regions of the gland. Examination of junctional structure by freeze-fracture revealed marked heterogenity in structure (Figures 1b and c). Figure 1b demonstrates a junctionof simple structure consisting primarily of a single junctional element which is interrupted (arrow). Junctions with such simple structure are relatively rare. Figure 1c illustrates the most complex junctional structure found, which consists of three closely opposed continuous duples elements. The junctions of the rectal gland are remarkable in that there is usually little or no space between adjacent junctional elements (Figure 1c), a feature which makes

attempts to count the number of junctional elements somewhat arbitrary. The junctional depth (distance between lumen and intercellular space) ranged between 15 and 60 nm. No changes in the length of the tight junction per lumen area or junctional structure were observed in rectal glands from three fish which were adapted to 68% dilute seawater for 4-7 days.

In contrast to the simple junctional structure (1 or 2 elements) previously described in very leaky epithelia, including the renal proximal tubule and another secretory tissue, the avian salt gland (Riddle & Ernst, J. Memb. Biol. 45:21, 1979); the shark rectal gland shows a marked heterogeneity of structure but usually has three or more closely opposed single elements in a narrow band, as recently reported independently in preliminary form by Ernst et al. (J. Cell Biol. 83:83a, 1979).

In glands perfused with lanthanum chloride, lanthanum was seen in the lateral intercellular spaces in both basal and stimulated glands. While the incidence of lanthanum filling the intercellular spaces varied considerably (mean 30.7 ± 10.4% of spaces, range 9-96%, n=603), no consistent difference between glands perfused in the basal state (4 sections) and glands stimulated with theophylline and dibutyryl cyclic AMP (5 sections) was detected in this study. Figure 2 illustrates the presence of ionic lanthanum in intercellular spaces of an unstimulated gland. When viewed at high magnification, (Figure 2 insert), it is clear that lanthanum fills the intercellular space up to the tight junction but does not penetrate to the lumen.

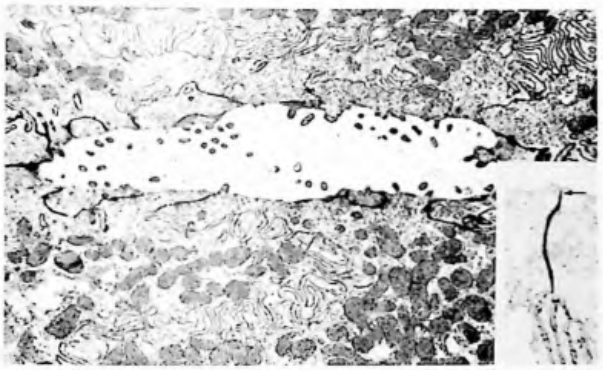

Figure 2. Low power (10,500x) cross-section through a tubule of the rectal gland demonstrating the presence of ionic lanthanum in the lateral intercellular spaces. Insert (40,000x) shows failure of lanthanum to penetrate through the tight junction (arrow) into the lumen.

These experiments indicate that: (1) tight junctions are present in the dogfish rectal gland and their extensive length ($86 m/cm^2$) represents a remarkable feature of this epithelium; (2) heterogeneous morphology of junctional elements is present; and (3) these tight junctions are impermeable to ionic lanthanum introduced from the extracellular space during *in vitro* perfusion in both the basal and stimulated gland. While some studies have emphaszied that the number of junctional elements is proportional to transmural electrical resistance in transporting epithelia (Claude and Goodenough, J. Cell. Biol., 58:390, 1973), DiBona and Mills (Fed. Proc. 38:134, 1979), have suggested recently that junctional length per area may contribute importantly to physiologic differences between "leaky" and "tight" epithelia. For example, frog urinary bladder, a high-resistance epithelium, contains about $6.5 m/cm^2$ of junction while the low resistance proximal tubule of the rabbit contains 50 to 100 meters per cm^2. It is likely that both the junctional length per area and junctional structure area important determinants of the physiologic role of paracellular pathways in transporting epithelia. Although measurements of electrical resistance in single isolated perfused tubules of the rectal gland are not yet available, the present observation of a high value for junctional length per luminal area is consistent with the possibility that the paracellular pathway has an important role in this tissue as required in the present model of sodium chloride secretion.

In contrast to the situation in very leaky epithelia such as the renal tubule where lanthanum has been observed to penetrate the tight junctions, the present study demonstrates that ionic lanthanum added to the perfusate does not penetrate the tight junction of the rectal gland either under basal conditions or during stimulated secretion. These findings differ from those of van Lennup who showed lanthanum within the rectal gland tight junction following fixation of tissue by immersion, a process that may have altered properties of the junction and allowed colloidal lanthanum hydroxide to penetrate this barrier. The findings of the present study are consistent with a permeability barrier capable of restricting the passage of relatively small molecules, specifically urea, throughout the long lengths of tight junction in this tissue. Supported by NIH grant AM 17433 and a grant from Lederle Laboratories.

CALCIUM SELECTIVE MICROELECTRODES

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Polar lobe formation in the fertilized egg of the marine mudsnail, Ilyanassa obsoleta, is a microfilament-dependent process that results in the formation of a prominent constriction slightly below the cell equator. Intracellular calcium (Ca^{++}) has been implicated in conformational changes in the actin-containing microfilaments during this shape change (Conrad and Davis, Develop. Biol. 74:152-172, 1980). The role of Ca^{++} in this process is difficult to assess since cytoplasmic ionic Ca^{++} activities are unknown and greatly influenced by the presence of efficient intracellular sequestering systems. We have completed initial attempts to make ion-selective microelectrodes for intracellular free Ca^{++} activity measurements $(Ca)_c$.

The difficulty in fabricating such an electrode is in achieving the very low Ca^{++} sensitivity ($10^{-7} - 10^{-9}$M) and high selectivities necessary for intracellular measurements. Recently a Ca^{++} selective compound that may satisfy these requirements was synthesized and formulated into a liquid ion exchanger (Simon et al., in Calcium Transport and Cell Function, Annals NYAS, Scarpa and Carafoli (eds), 307:52-70, 1978). The use of this ion exchanger in a microelectrode has been reported (Youmans et al., Fed. Proc. 38:963, 1979).

Open tip microelectrodes (tip diameter < 1 μM) were fabricated from 1 mm OD borosilicate glass using a Kopf pipette puller. The glass tip was siliconized to eliminate Na^+ ion selective sites on the glass interior surface and make the surface hydrophobic. This was done by drawing into the tip ~300 μM of a 5-10% solution of siliconizing compound dissolved in an organic solvent. Five siliconizing compounds were tested (chlorotrimethyl silane, dichlorodimethyl silane, methyltrichloro silane, Prosil [PCR Research Chemicals], and Dow Corning 500) in three different solvents (n-chloronapthalene, 1-octanol and ethyl acetate). Pipettes were then baked for 1 hour at 125°C to evaporate the solvent. Approximately 200 μM of the Ca^{++} ion exchanger (kindly supplied by Dr. W. Simon) was drawn into the pipette tip by applying a vacuum to the open end of the pipette while the tip was immersed in the exchanger.

Several electrodes were successfully fabricated and calibrated in Ca^{++} standard solutions made by adding known weights of $CaCl_2$ to doubly distilled water. These standards were buffered to pH 7.1 with 20 mM TRIS buffer and contained background ion concentrations of 22 mM M_g^{++}, 20 mM Na^+, 200 mM K^+, 254.5 mM Cl^-, 11.5 mM HCO_3^-. The background ions were present to simulate the intracellular marine egg environment for electrode selectivity determinations. All standards below 10^{-5}M Ca^{++} contained 3 mM EGTA to buffer trace Ca^{++} contamination in the distilled water. Ca^{++} concentrations in the EGTA solutions were calculated from published data (Portzehl et al., Biochim. Biophys. Acta 79:581-591, 1964).

A calibration plot of potential vs log Ca^{++} for one electrode is shown in Figure 1. The calibration circuit has been described previously (Duffey, et al., J. Memb. Biol. 42:229-245, 1978). The linear portion of the plot has a slope of +29 mV/decade and detection limit of 3×10^{-7} M Ca^{++}. The selectivity constants (Simon, et al) for Na^+, Mg^{++}