CHLORIDE UPTAKE PATHWAYS INTO PRIMARY CULTURES OF SQUALUS ACANTHIAS RECTAL GLAND EPITHELIAL CELLS

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Squalus acanthias rectal gland epithelial cells were isolated and seeded onto fetal calf serum-coated 35 mm culture dishes and maintained at 20°C in a 5% CO_2 atmosphere in shark medium (alphaMEM supplemented with 140 mM NaCl, 3 mM $MgCl_2$, 3 mM $CaCl_2$, 300 mM urea, 150 mM trimethylamine oxide, ITS+ (6.25 mg/l insulin, 6.25 mg/l transferrin, 6.25 ug/l selenous acid, 1.25 g/l bovine serum albumin, 5.35 mg/l linoleic acid; Collaborative Research) and 2% fetal calf serum). The osmolarity of this solution was approximately 1030 milliosmolar, which is within the range of values for tissue fluid osmolarities reported in the shark. Cultures were maintained for between 6 and 10 days, medium being replenished every 2-3 days. Uptake of 2 mM $^{36}Cl^-$ from a Cl^--free shark Ringers' solution (280 mM Na-gluconate, 5 mM K-gluconate, 5 mM Ca-gluconate, 5 mM Mg-gluconate, 300 mM urea, 10 mM HEPES-Tris, pH 7.4) was measured as a function of time at room temperature (20°C - 26°C). Under all conditions uptake was linear for at least 3 minutes. Each time point was determined from triplicate independent samples and uptake rates were calculated as the slope of the line connecting the 1 minute and 3 minute points. Standard deviation of the triplicate samples was routinely less than 10% of the mean.

Total chloride uptake rate was 1.91 nmoles chloride/mg protein-minute. Addition of 100 uM bumetanide reduced uptake to 0.68 nmoles chloride/mg protein-minute (64% inhibition). Removal of Na^+ and K^+ ions from the solution reduced uptake to 0.70 nmoles chloride/mg protein-minute. Under this condition bumetanide no longer inhibited chloride uptake (0.72 nmoles chloride/mg protein-minute), which demonstrates that bumetanide specifically inhibits that portion of chloride influx which requires the simultaneous presence of Na^+ and K^+ at the same membrane surface (Na^+-K^+-Cl^- symporter). This component of chloride uptake was also inhibited by two putative chloride channel blockers (1 mM anthracene-9-carboxylic acid (9-AC) and 1 mM N-phenylanthranilic acid (PAA, also known as DPC)) (Wangemann, P., H. Wittner, A. Di-Stefano, H.C. Englert, H.J. Lang, E. Schlatter, and R. Greger. (1986) Pfluegers Arch. 407 (Suppl. 2): S128-S141; Landry, D.W., M. Reitman, E.J. Cragoe, Jr., and Q. Al-Awqati. (1987) J. Gen. Physiol. 90: 779-798). PAA and 9-AC reduced total chloride uptake by 92% and 75% respectively. To verify that PAA inhibited Na^+-K^+-Cl^- symport activity the effect of PAA on $^{86}Rb^+$ uptake (a marker for K^+ transport) was compared to that of bumetanide (which inhibits specifically K^+ uptake mediated by the Na^+-K^+-Cl^- symporter). PAA (1 mM) inhibited ouabain-insensitive $^{86}Rb^+$ uptake (uptake not mediated by the Na^+-K^+-ATPase) by 19%. Bumetanide (100 uM) inhibited uptake by 24%, and the combination of the two compounds inhibited uptake by 24%. Since the combination of the two compounds inhibited $^{86}Rb^+$ uptake to the same extent as bumetanide alone, this demonstrates that PAA inhibits the Na^+-K^+-Cl^- symporter.

The final set of experiments examined the residual chloride influx pathway (i.e., that uptake not mediated by the Na^+-K^+-Cl^- symporter). Most of the remaining uptake was inhibited about equally by 1 mM PAA, 0.5 mM SITS or PAA plus SITS (81%, 70%, and 81%, respectively). SITS inhibits anion

antiporters, suggesting that this portion of chloride uptake is mediated by an anion antiporter.

During the course of these experiments several interesting points were noticed which are worth mentioning. With regard to chloride transport, under normal conditions pre-treatment of cells with 10^{-5}M forskolin plus 1 mM 1-methyl-3-isobutyl xanthine (FM) for 15 minutes (a procedure which should elevate intracellular cAMP and thereby activate latent conductive chloride channels in the plasma membrane) reduced uptake by greater than 50% in three separate experiments. This is surprising since it would be expected that opening another chloride pathway would increase uptake. When bumetanide was included during the pre-treatment and uptake periods, no change in chloride influx was observed compared to control cell populations, suggesting a role for the Na^+-K^+-Cl^- symporter in this effect. FM pre-treatment of cell populations increased $^{86}Rb^+$ uptake via the Na^+-K^+-Cl^- symporter by approximately 8-fold. An 8-fold increase in the bumetanide-inhibitable portion of chloride uptake would be readily detectable, and was clearly not observed since uptake actually decreased upon FM pre-treatment. The two uptake protocols utilize solutions with differing ionic compositions (2 mM chloride (chloride transport) versus 300 mM chloride (rubidium transport)) and thus cannot be directly compared, but it is surprising that one procedure demonstrates a substantial increase in symport activity whereas the other detects a probable decline in activity.

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