ROLE OF PROTEIN KINASES AND PHOSPHATASES IN CHLORIDE TRANSPORT MODULATION IN *FUNDULUS HETEROCLITUS*

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The isolated operculum epithelium of the killifish, *Fundulus heteroclitus* is extremely rich in chloride cells and is a useful model for Cl^--secretion studies (Zadunaisky, *Fish Physiology,* XB, eds. Hoar & Randall, Academic Press, 1984). In this work we used the operculum epithelium to investigate the role of protein kinases and phosphatases in the signals to activate or deactivate the chloride secretion. An increase/decrease in Cl^- secretion was achieved by increasing/decreasing the osmolarity of the bathing solution or by addition of the β-receptor agonist isoproterenol.

Adult killifish, *Fundulus heteroclitus* were acclimated to circulating seawater (32 ppt) and fed daily with a commercial fish food. Experiments were performed from July through August 1997. The fish were immobilised by pithing and the opercular epithelium dissected out and mounted in a modified Ussing chamber with regular teleost Ringer's solution on both sides as previously described (Zadunaisky et al, *J. Membrane Biol.*143, 207 – 217, 1995). The electrical potential difference, the short circuit current and the tissue conductance of the epithelium were measured with a voltage clamp unit. The short circuit current was used as a measure of the Cl^- current (loc.cit.). Data for separate experiments are expressed as means ± SEM.

The mean electrical results for 91 separate preparations were 15.2 ± 0.6 mV and 149 ± 7.1 μAcm^{-2} for electrical potential differences and short-circuit current respectively. The electrical potential difference was measured under open circuited conditions with the apical

side being negative. Increasing the osmolarity with 100 mOsm adding either mannitol or hypertonic Ringer's to both sides produced an increase in the short circuit current to 204 μAcm^{-2} after mannitol addition and to 275 μAcm^{-2} after addition of hypertonic Ringer's. The Cl^- channel blockers diphenyl amine-2-carboxylic acid (DPC) (500 μM), glibenclamide (800μM) and 5-nitro-2- (3-phenyl-propylamino) benzoic acid NPPB (100 uM) completely blocked the steady state Cl^--current and prevented the response to hypertonicity. The effect of DPC has previously been reported (Zadunaisky et al, *J.Membrane Biol.* 143, 207 – 217, 1995).

The K^+ channel blocker Ba^{2+} (1 mM) added to the basolateral side inhibited the Cl^- current to 40% of the control value and partly prevented the response to hypertonicity. The K^+ channel blocker 293B (2 μM) had no significant effect on the Cl^- current.

The addition of the β-receptor agonist isoproterenol (10^{-5} M) stimulated the short-circuit current to 258 ± 10 μAcm^{-2} and this current was inhibited to 40% by 1 mM Ba^{++}. The isoproterenol stimulation was completely blocked in four separate experiments by the specific PKA inhibitor H-89 (10 μM, Calbiochem). Neither H-89 (10 or 20 μM) nor another specific PKA inhibitor KT 5720 (10 μM, Calbiochem) inhibited the activation of Cl^- current after mannitol addition. It has previously been suggested that the primary event of stimulation of β-receptors is an increase in cAMP and an opening of apical Cl^- channels, whereas the primary event after increase in osmolarity is an activation of the basolateral Na^+, K^+, $2Cl^-$ cotransporter (see e.g. Zadunaisky, *Kidney International*, 49:1563–1567,1996). Thus the present results indicate that PKA is involved in the activation of the apical Cl^- channels via stimulation of β-receptors, whereas activation of the Na^+, K^+, $2Cl^-$ co-transporter after hypertonicity is independent of PKA activation. In contrast, the PKC-inhibitor chelerythrine (40μM) added before the mannitol addition significantly inhibited the effect of hypertonicity. Chelerythrine (20μM and 100μM) added after maximum stimulation with isoproterenol plus mannitol also significantly inhibited the Cl^- current to a value that was signinficantly lower than the Cl^- current after isoproterenol alone. Thus PKC seems to be involved in the hypertonic induced increase in Cl^- current as well as in the increase in Cl^- current induced after stimulation of β-receptors.

Also the steady state chloride current seems to be regulated by phosphorylation and dephosphorylation. Thus addition of the serine/threonine PP-1 and PP-2A phosphatase inhibitor Calyculin A (200nM) increased the steady state current significantly in five separate experiments. Addition of the PKC inhibitor chelerythrine significantly inhibited the steady state Cl^- current whereas the PKA inhibitor H-89 had no effect. The steady state Cl^- current thus seems to be determined by continuous phosphorylation and dephosphorylation involving serine/threonine phosphatases of the PP-1 and PP-2A type and PKC but not PKA. Other kinases are likely to be involved.

Dilution of the Ringer's bathing the operculum tissue from 300 to 240 mOsm on both sides resulted in a drastic - slightly biphasic - reduction of the current from 149 ± 7 μAcm^{-2} to 22 ± 3 μAcm^{-2} increasing again during the next hour to 41 ± 10 μAcm^{-2}. In the presence of the phosphatase inhibitor Calyculin A this decrease was augmented and after 1 hr. in the hypotonic medium plus Calyculin A the current was 0 μAcm^{-2} (see also Zadunaisky et al. *MDIBL-bulletin*, 37,1998). This points to a phosphorylation rather than a dephosphorylation being involved in the drastic reduction in current after a reduction in osmolarity.

Conclusively, stimulation of the Cl^- current by transition from fresh- to seawater or by stimulation of β-receptors involves phosphorylation processes by PKC and by PKC plus PKA respectively. Inhibition of the Cl^- current by transition from sea- to fresh water likewise seems to involve serine/threonine phosphorylation by some not yet identified kinases.

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