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Control of Cl^- transport in the operculum epithelium of *Fundulus heteroclitus*: long- and short-term salinity adaptation

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Abstract

The eurohaline fish, *Fundulus heteroclitus*, adapts rapidly to enhanced salinity by increasing the ion secretion by gill chloride cells. An increase of ~ 70 mOsm in plasma osmolarity was previously found during the transition. To mimic this in vitro, isolated opercular epithelia of seawater-adapted *Fundulus* mounted in a modified Ussing chamber were exposed to an increase in NaCl and/or osmolarity on the basolateral side, which immediately increased I_{SC}. Various Cl^- channel blockers as well as the K^+ channel blocker Ba^{2+} added to the basolateral side all inhibited the steady-state as well as the hypertonic stimulation of I_{SC}. The ∃-agonist isoproterenol stimulates I_{SC} in standard Ringer solutions. In contrast, when cell volume was kept at the larger value by simultaneous addition of water, the stimulation with isoproterenol was abolished, suggesting that the key process for activation of the Na^+, K^+, $2Cl^-$ cotransporter is cell shrinkage. The protein kinase C (PKC) inhibitor chelerythrine and the myosin light chain kinase (MLCK) inhibitor ML-7 had strong inhibitory effects on the mannitol activation of I_{SC}, thus both MLCK and PKC are involved. The two specific protein kinase A (PKA) inhibitors H-89 and KT 5720 had no effect after mannitol addition whereas isoproterenol stimulation was completely blocked by H-89. This indicates that PKA is involved in the activation of the apical Cl^- channel via c-AMP whereas the shrinkage activation of the Na^+, K^+, $2Cl^-$ cotransporter is independent of PKA activation. The steady-state Cl^- secretion was stimulated by an inhibitor of serine/threonine phosphatases of the PP-1 and PP-2A type and inhibited by a PKC inhibitor but not by a PKA inhibitor. Thus, it seems to be determined by continuous phosphorylation and dephosphorylation involving PKC but not PKA. The steady-state Cl^- secretion and the maximal obtainable Cl^- secretion were measured in freshwater-adapted fish and in fish retransferred to saltwater. No I_{SC} could be measured in freshwater-adapted fish or in the fish within the first 18 h after transfer to saltwater. As evidenced from Western blot analysis using antiserine-antibodies, a heavily serine phosphorylated protein of about 190 kDa was consistently observed in the saltwater-acclimated fish, but was only weakly present in freshwater-acclimated fish. This observation indicates that acclimatization to saltwater stimulates the expression of this 190-kDa protein and/or a serine/threonine kinase, which subsequently phosphorylates the protein.
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Keywords: Killifish; Ussing chamber; Cell volume; Chloride cell; Cl^- channel; Cl^- secretion; Na^+, K^+, $2Cl^-$ cotransporter

1. Introduction

In 1951 Ussing and Zerahn [1] had constructed what was later named *the Ussing chamber* and conducted the first *short-circuit experiments* to show that only Na^+ was actively transported across the frog skin, while Cl^- followed passively along, and that the short-circuit current equals the Na^+ transport. This short-circuit technique was a breakthrough in the study of membrane transport. In 1952 it was found that in other tissues it was the Cl^- ion that produced the current across the epithelium, building up the potential, which was also the case in the glands of frog skin when stimulated with adrenaline [2].

In 1976 Cl^- transport across the isolated operculum epithelium of the little eurohaline fish, the killifish (*Fundulus heteroclitus*) was described. The opercular epithelia were mounted in small Ussing chambers constructed by Zadunaisky and the experiments were standard short-circuit

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current experiments [3]. The killifish opercular epithelium has proved to be an excellent general model for Cl^- secreting epithelia (see Ref. [4]). It contains typical mitochondria-rich Cl^- secreting cells at the high density of 4×10^5 cells/cm^2 and when isolated, mounted in an Ussing chamber and short circuited, the Cl^- transport from the blood side (basolateral) to the seawater side (apical) equals the short circuit current (see Refs. [3,5]).

The killifish has a remarkable capacity to rapidly adapt to changes in salinity. The acclimation from low to high salinities involves a rapid phase during which the secretion of Cl^- via chloride cells located both in the opercular epithelia and in the gill is rapidly increased. In the intact fish, plasma osmolarity increases by about 70 mOsm when freshwater-adapted fish are transferred to seawater. The recovery to normal plasma osmolarity takes a few days [6]. To mimic the early increase in blood osmolarity during adaptation to higher salinities, mannitol was added to the basolateral side of the isolated operculum epithelium. The authors (loc. cit.) concluded that the signal for the rapid adaptation to higher salinities is actually the increased tonicity of the plasma resulting in cell shrinkage and activation of the Na^+, K^+, $2Cl^-$ cotransporter and the Na^+/H^+ exchanger [6]. Interestingly, cell shrinkage is also the signal that leads to activation of the Na^+, K^+, $2Cl^-$ cotransporter and the Na^+/H^+ exchanger during volume regulatory increase (see, e.g. Ref. [7]). Killifish transferred from seawater to freshwater show a large reduction in plasma osmolarity at 6 h, which is corrected within 24 h [8]. When the operculum epithelium is subjected to hypotonic solutions on the basolateral side, the short circuit current is dramatically decreased [8–10]. There is evidence that this might in part be a result of swelling-activated Cl^- channels on the basolateral membrane [10]. The search for the mechanisms involved in stimulation of Cl^- secretion by basolateral hypertonicity and inhibition by basolateral hypotonicity should therefore start by looking at cell volume regulation in other cells.

The "pump-leak steady-state concept" for cell volume control was originally developed by Leaf [11] and Ussing [12] as well as by Tosteson and Hoffman [13]. This description stands today but the focus during the following years has been on the dynamic and controlled changes that occur in the various leak transport paths as a compensating response to volume changes. The first studies of volume regulation were performed by MacRobbie and Ussing [14] but during the following years most detailed studies on volume regulation have been carried out on symmetrical cells; for a recent review see Ref. [15].

Swelling-activated K^+ conductance was reported in the early 1970s for Ehrlich ascites tumour cells (EATC) [16] and lymphocytes [17]. The Cl^- conductance was expected to be very large so that Cl^- could just follow K^+. The reason for that assumption was that the ^{36}Cl tracer flux was very fast and it was only later realized that this was caused by anionic exchange. The first description of an 1:1 *exchange diffusion* had already been introduced by Ussing in 1947 [18] and an important experimental consequence pointed out by Ussing was that steady-state isotope fluxes should not be used to measure passive leak permeability since a large portion of the flux could be exchange diffusion. Despite this, it was still assumed up until the early 1970s that the rapid unidirectional tracer Cl^- flux that could be measured over the cell membrane was due to huge conductive Cl^- permeability in most cells. Inspired by Ussing, this assumption was tested in EATC and it was found that unidirectional Cl^- transport was completely (>95%) dominated by an exchange diffusion process [19,20], as had previously been found in red blood cells (see Ref. [21]). Thus, the Cl^- conductance was much lower than previously estimated from measurements of unidirectional tracer fluxes [19,20]. Also the bumetanide sensitive anion, cation cotransport system was almost quiescent at steady state in EATC [22]. However, the rate constant for steady-state Cl^- flux was found to rise with both expanding and decreasing volume [23] reflecting an increase in the conductive Cl^- flux after swelling and an increase in the co-transport of anion and cations after shrinkage [22,23]. Since the idea of activation of a conductive pathway by cell swelling in the basolateral membrane of frog skin principal cells was proposed [14] and demonstrated in EATC [14,23,24], a volume-sensitive Cl^- channel has been found in a large number of cell types [25–27].

In 1981 it was shown that the primary process during a regulatory volume increase (RVI) response in EATC was the activation of an otherwise quiescent bumetanide-sensitive Na^+, Cl^- or Na^+, K^+, $2Cl^-$ cotransport system, with subsequent replacement of Na^+ with K^+ via the Na/K pump [22,28,29]. It was not possible to determine whether it was a Na^+, Cl^- cotransport system or a Na^+, K^+, $2Cl^-$ cotransport system with fast recycling of K^+ that was involved. In 1982 Ussing [30] proposed shrinkage activation of a Na^+, Cl^- cotransport system in the basolateral membrane of frog skin. Both in frog skin [31,32] and in EATC [33], however, it was later found that cell shrinkage does activate a Na^+, K^+, $2Cl^-$ cotransporter. This was demonstrated in EATC by a non-steady-state flux ratio analysis [34] in the presence of both, ouabain and Ba^{2+} [33].

Cells that secrete Cl^-, such as the operculum epithelium of the killifish, typically have the NKCC1 isoform of the Na^+, K^+, $2Cl^-$ cotransporter [35], which for instance has been cloned from shark rectal gland [36] and from mouse inner medullary collecting duct [37,38]. The NKCC1 involved in RVI in EATC has more than 90% homology with the basolateral epithelial NKCC1 isoform from mouse kidney (B.S Jensen and E.K. Hoffmann, unpublished results). NKCC1 is thus important for both the secretion of Cl^- and the regulation of cell volume. This begs the question of whether the volume sensitivity of NKCC1 is important for secretion.

As in secretory epithelia, NKCC1 in EATC can be activated after addition of agonists, which induce inositol

phosphate-mediated Ca^{2+} signalling, resulting in KCl loss and cell shrinkage [33,39]. In this case, cotransport activation could be due to an increase in cellular Ca^{2+} (direct or indirect), the drop in cellular Cl^- activity or cell shrinkage. When cell shrinkage was prevented in a K^+ equilibrium salt solution (KCl substituted for NaCl), activation of the cotransporter essentially disappeared [40]. This indicates that either cell shrinkage or a decrease in cellular Cl^- activity is a prerequisite for a pronounced and persistent activation of the NKCC1. However, the experiment cannot distinguish between the drop in cellular Cl^- activity and cell shrinkage. The same problem is encountered in secretory epithelia. The idea that both a drop in intracellular Cl^- activity and cell shrinkage influenced activation of the cotransport system was first proposed by Ussing [30]. That a reduced intracellular Cl^- concentration could play a permissive role for the activation of the cotransporter was subsequently discussed for EATC [29] and for thymocytes by Grinstein et al. [41]. The importance of cell shrinkage versus the drop in cytosolic Cl^- activity in secretory epithelia has remained unclear, but it has been proposed that the key event is the fall in cytosolic Cl^- activity, not the shrinkage [42]. In the present study, we examine the Cl^- current activation in response to isoproterenol, a β-agonist under conditions where the Cl^- activity drops with or without concomitant cell shrinkage.

NKCC1 in EATC is activated after cell shrinkage via activation of protein kinase C (PKC) and myosin light chain kinase (MLCK) resulting in phosphorylation of the myosin light chain (MLC) and translocation of myosin to the surface membrane cortical area [43]. The shrinkage-induced NKCC activation is strongly inhibited by the MLCK inhibitor ML-7 in EATC [44] and in vascular smooth muscle cells [45]. In the present paper we examine whether PKC and MLCK are similarly involved in shrinkage activation of NKCC1 in the operculum epithelium. Our work is thus a further study of the mechanisms behind the short-term activation of the Na^+, K^+, $2Cl^-$ cotransporter (NKCC1), and in addition we shall investigate the long-term acclimation to higher salinities.

2. Material and methods

2.1. Tissue preparation

Adult killifish (*Fundulus heteroclitus*) >8 cm were obtained from estuaries and rivers around Mount Desert Island, Maine. The fish were kept in circulating seawater (32 ppt) in aquariums and fed daily with commercial fish food. Experiments were performed from the beginning of July to September. Adaptation to freshwater was done gradually for 24 h and the fish were kept in freshwater for at least 14 days before being used in experiments. The fish were immobilized by pithing and the opercular epithelium dissected out essentially as described in detail previously [46]. The epithelium was gently teased out from the operculum using micro dissection forceps and placed over the 0.07-cm^2 opening of a teflon disc. The new mounting procedure described by Zadunaisky et al. [6] was used. The technique permits the support of the opercula epithelium with a flat ring that keeps the tissue in place.

2.2. Electrical measurements

The electrical potential difference, the short circuit current (I_{SC}) and the tissue conductance of the epithelium were measured with a voltage clamp unit (University of Iowa 710 C-1). The short circuit current (I_{SC}) was used as a measure of the Cl^- current [6]. After stabilization of the resting potential, the tissue was short-circuited and the current recorded continuously with a chart recorder. Connections to Ussing chambers were made with bridges filled with 3% agar–agar in Ringer solution.

2.3. Solutions and chemicals

2.3.1. Teleost ringer

NaCl 135 mM; KCl 2.5 mM; $MgCl_2$1.0 mM; $NaHCO_3$ 16.0 mM; $CaCl_2$ 1.5 mM; dextrose 1.0 g/l; adjusted to pH 7.4 with 95% O_2 and 5% CO_2.

2.3.2. Stock solutions

Calyculin A (Sigma) 20 μM in DMSO; H-89 dihydrochloride (Calbiochem) 1 mM in DMSO; KT-5720 1 mM in DMSO; chelerythrine chloride (Calbiochem) 2.6 mM in ethanol; $BaCl_2$ 10 mM in HCO_3 free Ringer; diphenylamine-2-carboxylic acid (DPC) (Aldrich Chemical Company, Inc.) 20 mM in ethanol; glibenclamide 20 mM in DMSO; isoproterenol (Sigma) 1 mM in water; mannitol 500 mM in teleost Ringer; 5-nitro-2-(3-phenyl-propylamino) benzoic acid (NPPB) (a gift from Dr. R.Greger) 100 mM in DMSO.

2.4. Western blotting

From the fresh- and saltwater-acclimated fish, respectively, opercular epithelia were equilibrated in teleost Ringer solution and subsequently lysed in ice-cold lysis buffer (62.5 mM Tris, 4% SDS, 18% glycerol) for SDS-PAGE electrophoresis and Western blotting. Samples (45-μg protein) were diluted 1:1 in double Laemmli buffer, boiled for 5 min, resolved by SDS-PAGE gel electrophoresis, and electro transferred onto nitrocellulose membranes. Membranes were blocked for 1 h at room temperature (150 mM NaCl, 13 mM Tris, 5% nonfat dry milk) and incubated with primary antibodies (1:1000 in blocking buffer) overnight at 4 °C and 1 h, at room temperature. The primary antibody was directed against phosphoserin (Biomol). The membranes were washed (150 mM NaCl, 13 mM Tris, 0.02% Triton X-100), incubated for 2 h with horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody

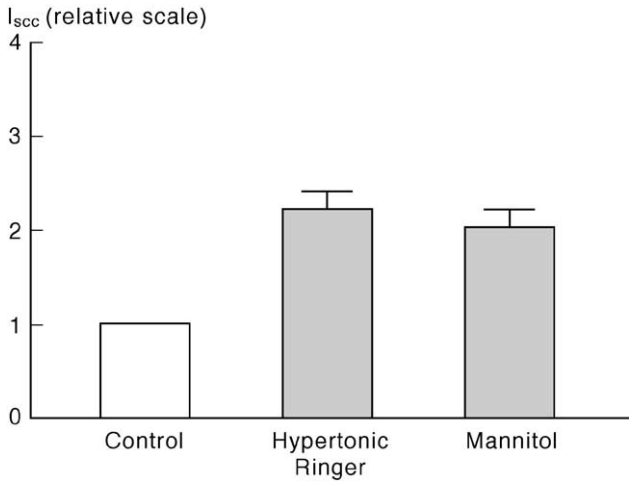

Fig. 1. Increase in short-.circuit current (I_{SC}) in an isolated operculum epithelium from *F. heteroclitus* induced by addition of 100 mM mannitol or hypertonic Ringer (NaCl added) to a similar osmolarity. I_{SC} after addition of mannitol or hypertonic Ringer is given relative to the basal spontaneous I_{SC} of the epithelium reached after approximately 10 min (control). Number of independent experiments: Mannitol ($n=13$), Hypotonic Ringer ($n=5$).

(1:3000), washed, and bound secondary antibody was detected by enhanced chemiluminescence (ECL) (Amersham, Buckinhamshire, England).

3. Results

3.1. Response of isolated opercular epithelia to osmolarity increases induced by hypertonic Ringer solution or mannitol on the basolateral side

Operculum tissues from seawater-adapted fish were mounted in standard Teleost Ringer solution on both sides. The electrical potential difference and the short circuit current were 16.1 ∀ 0.54 mV and 163 ∀ 5.8 A/cm^2, respectively ($n=149$). As previously demonstrated [6], the addition of mannitol (100 mM) produced a rapid, nearly twofold increase in the short circuit current when added to both sides of the preparation or to the basolateral side,

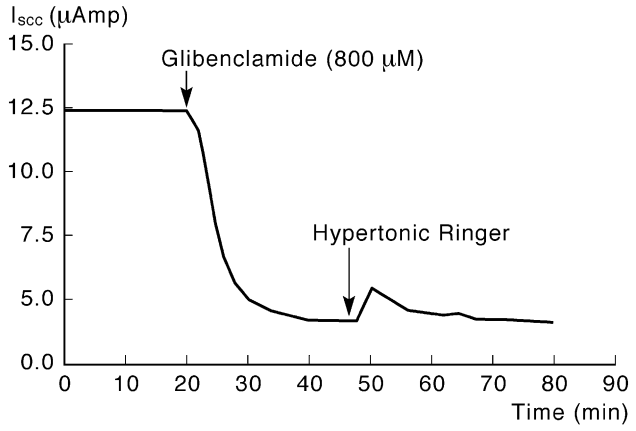

Fig. 3. Lack of response to basolateral hypertonicity on I_{SC} in the presence of the Cl^- channel blocker glibenclamide (800 μM) at the basolateral side.

whereas no response was observed when added to the apical side. Fig. 1 demonstrates that a similar stimulation is achieved if the osmolarity is raised by increasing the concentration of NaCl in the medium on both sides or to the basolateral side, to a final osmolarity of 400 mOsm. (Hypertonic Ringer). Thus it seems to be the osmolarity increase on the basolateral side, and not the increase in the ion concentration, which induces the increase in current.

3.2. Effect of Cl^- channels blockers

Zadunaisky et al. [6] showed that the addition of the Cl^- channel blocker diphenylamine-2-carbolic acid (DPC) to the basolateral side of the operculum epithelium inhibited the Cl^- current and prevented the response to basolateral hypertonicity with 50 mOsm mannitol. The middle panel of Fig. 2 confirms the effect of DPC on the steady state Cl^- current when added on the basolateral side. Addition of DPC to the apical side showed a similar, although significantly slower, inhibitory effect (data not shown). We have moreover used two other well-known Cl^- channel blockers on the basolateral side of the operculum epithelium: the 5-nitro-2-(3-phenyl-propylamino) benzoic acid (NPPB) (100 μM) and glibenclamide (800 μM), and have found a

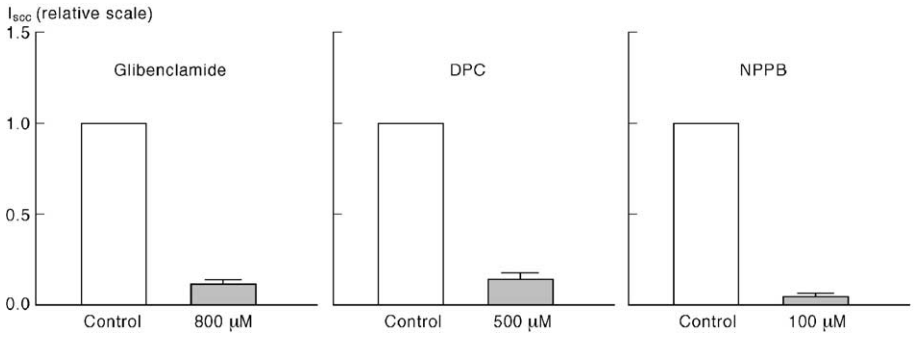

Fig. 2. Significant inhibition ($p<0.001$) of I_{SC} by three different Cl^- channel blockers added to the basolateral side. Number of independent experiments were: glibenclamide ($n=5$), DPC ($n=4$) and NPPB ($n=4$).

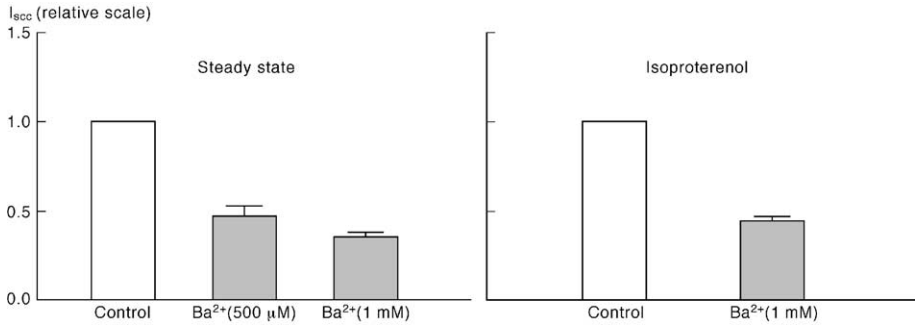

Fig. 4. Left panel: Significant inhibition ($P \leq 0.01$) of I_{SC} by the K^+ channel blocker Ba^{2+} added to the basolateral side. Number of independent experiments: 500 μM ($n=4$) and 1 mM ($n=6$). Right panel: Significant inhibition ($P<0.001$) of the response to isoproterenol (2×10^{-5} M) by addition of 1 mM Ba^{2+} to the basolateral side. Number of independent experiments ($n=6$).

significant inhibition of the steady state Cl^- current (Fig. 2, left and right panels). Moreover, in two experiments with glibenclamide (800 μM) and one with NPPB (100 μM), it is shown that the Cl^- channel inhibitor completely blocks the activation of the Cl^- current by hypertonic Ringer (illustrated for glibenclamide in Fig. 3).

3.3. Effect of potassium channel blockers

To see whether the Cl^- excretion was dependent on a basolateral K^+ channel, we investigated the effect of the K^+ channel blocker Ba^{2+} in two different concentrations. The results are shown in Fig. 4, left panel. The effect of Ba^{2+} was also investigated after stimulation with isoproterenol (Fig. 4, right panel) and after a hypertonic stimulation (data not shown). In all experiments the Ba^{2+} inhibited the Cl^- current significantly (about 50%). The activity of K^+ channels in the basolateral membrane is therefore crucial for maintaining Cl^- secretion. A series of experiments showed that the K^+ channel blocker 293B (a gift from Prof. R. Greger) had no significant effect on the current (data not shown).

3.4. Effects of protein kinase A (PKA) and PKC on agonist- and/or hypertonic stimulated short circuit current

The addition of the ∃-receptor agonist isoproterenol (2×10^{-5} M) stimulated the short-circuit current [see 4] from about 100 A/cm^2 to 259 ∀ 28.5 A/cm^2. The isoproterenol stimulation was completely blocked in four separate experiments (Fig. 5) by the specific PKA inhibitor H-89 (10 ^{S_B}M). Neither H-89 (10 or 20 ^{S_B}M) nor another specific PKA inhibitor KT 5720 (10 ^{S_B}M) inhibited the activation of Cl^- current after mannitol addition (data not shown). The combined effect of isoproterenol (2×10^{-5} M) and mannitol is significantly inhibited by H-89 (10 ^{S_B}M) (data not shown). Thus, PKA is involved in the activation of the apical Cl^- channel via β-receptors, whereas activation of the co-transporter after hypertonicity is independent of PKA activation. In contrast, the PKC-inhibitor chelerythrine (40 ^{S_B}M) added

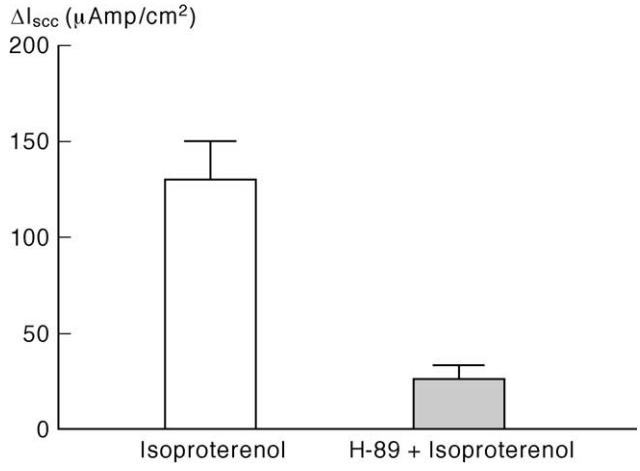

Fig. 5. Significant ($P=0.01$) inhibition of the response to isoproterenol (2×10^{-5} M) by the protein kinase inhibitor H-89 (10 μM). Number of independent experiments: isoproterenol ($n=4$), mannitol ($n=3$).

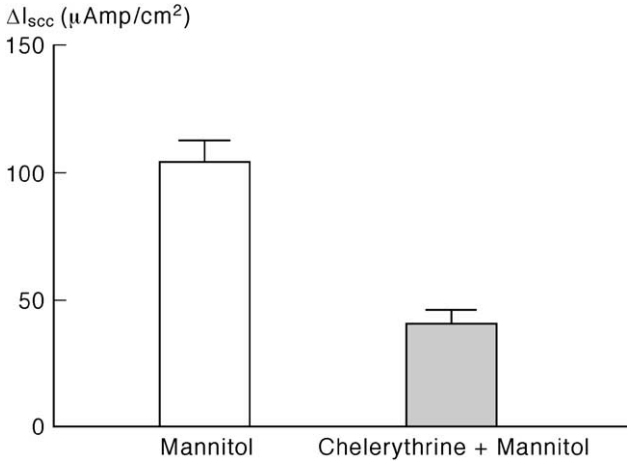

Fig. 6. Significant inhibition ($P=0.014$) of the response to mannitol (100 mOsm) by the protein kinase C inhibitor chelerythrine (40 μM) added to the basolateral side before addition of mannitol. Number of independent experiments: Mannitol ($n=13$), chelerythrine plus mannitol ($n=3$).

before mannitol significantly inhibited the effect of hypertonicity (Fig. 6). Chelerythrine (20 ^{S_B}M and 100 ^{S_B}M) added after maximum stimulation obtained by addition of isoproterenol plus mannitol also significantly inhibits the Cl^- current (see Fig. 7a and b). Thus, PKC is involved in the hypertonically induced increase in Cl^- current as well as in the increase induced by stimulation of β-receptors.

3.5. Effect of MLCK inhibitor on the shrinkage activated I_{SC}

The MLCK inhibitor ML-7 added before mannitol inhibited the stimulatory effect of hypertonicity (Fig. 8). Thus, both PKC and MLCK seem to be involved in activation of Cl^- secretion induced by basolateral hypertonicity.

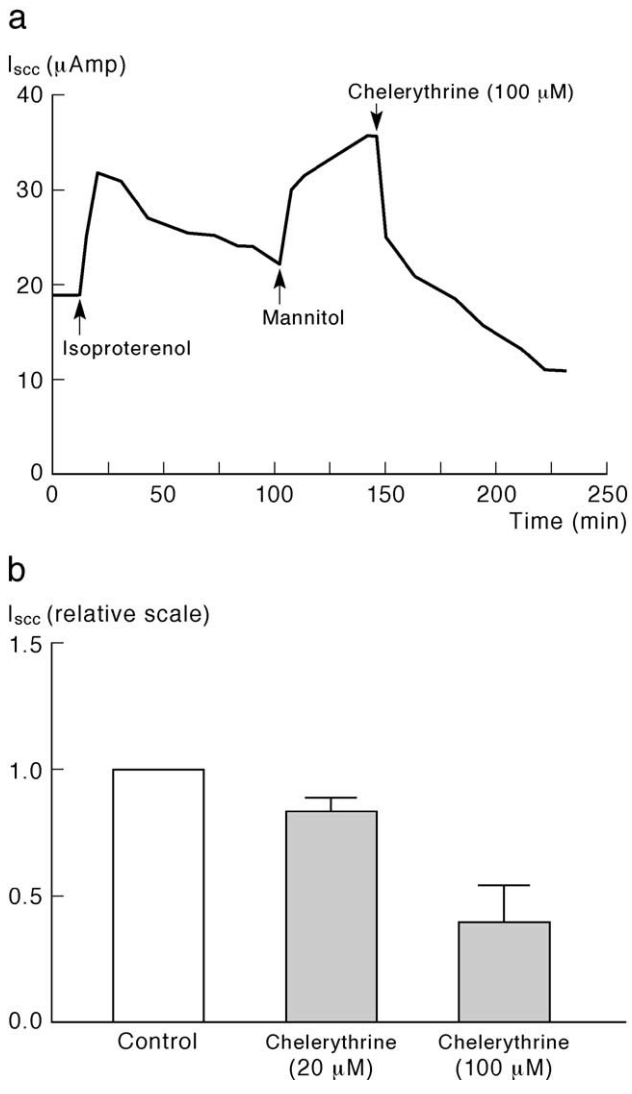

Fig. 7. (a) Inhibition by chelerythrine (100 μM) of I_{SC} stimulated by addition of isoproterenol (2×10^{-5} M) plus mannitol (100 mOsm). (b) Mean value of I_{SC} obtained at the end of experiments like the one shown in (a) given relative to I_{SC} before stimulation with isoproterenol and mannitol. Chelerythrine inhibits the stimulated I_{SC} to a final value lower than the initial steady state value.

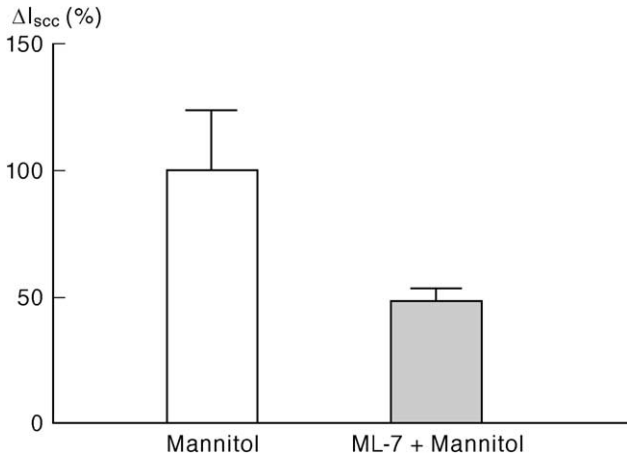

Fig. 8. Response to mannitol (100 mOsm) in the presence and absence of the MLCK inhibitor ML-7 (5 μM) added to the basolateral side before addition of mannitol. Number of independent experiments: mannitol ($n=13$), ML-7 plus mannitol ($n=7$). ($P=0.07$).

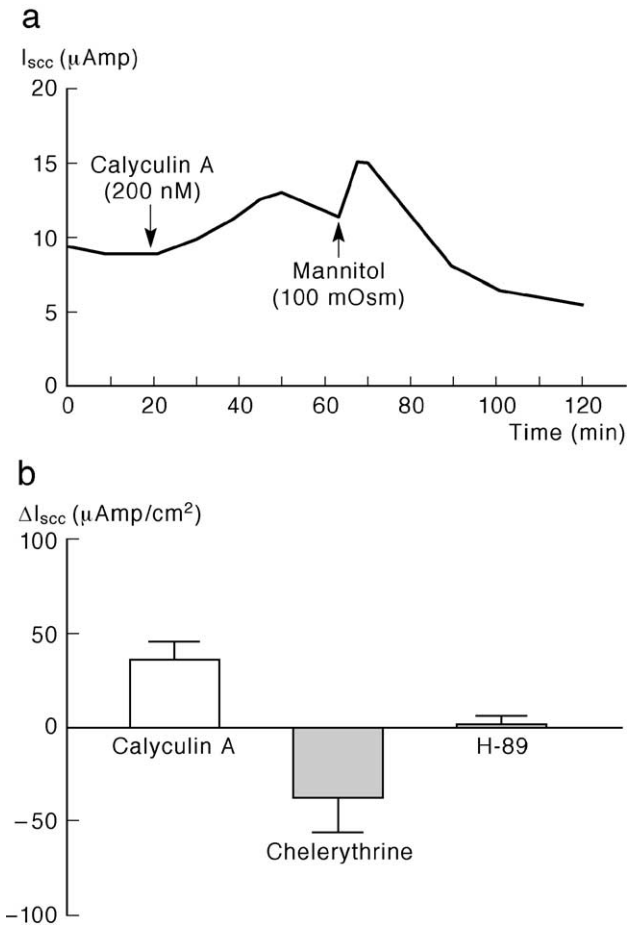

Fig. 9. (a) Stimulating effect of the PP-1 and PP-2A phosphatase inhibitor Cal-A (200 nM) on I_{SC}, and the effect of addition of mannitol (100 mOsm) 40–50 min after Cal-A. (b) Effect of the PP-1 and PP-2A phosphatase inhibitor Cal-A (200 nM), the PKC inhibitor chelerythrine (100 μM) and the PKA inhibitor H-89 (10 μM) on I_{SC}. Cal-A increases the current significantly ($P=0.019$); chelerythrine decreases the current ($P=0.0594$), whereas H-49 has no effect on the steady-state I_{SC}. The number of independent experiments: Cal-A (=5), chelerythrine (=5) and H-89 ($n=3$).

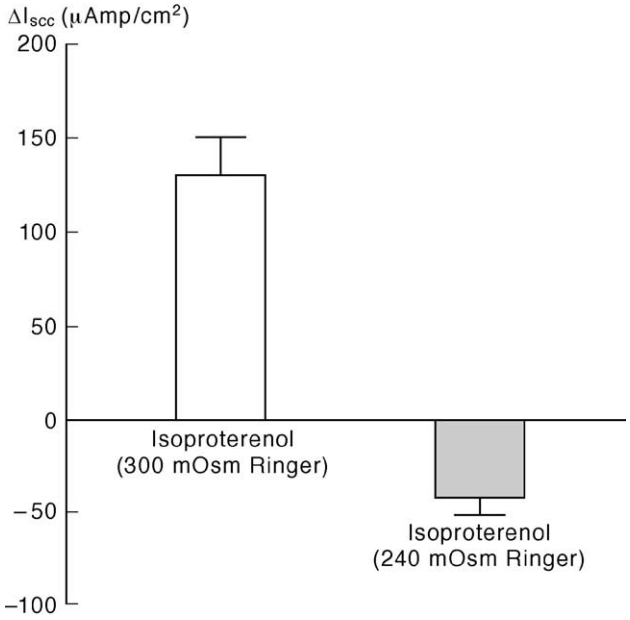

Fig. 10. Change in I_{SC} (as a measure of the Cl^- current) after addition of isoproterenol (2×10^{-5} M) with or without simultaneous addition of 20% distilled water. The change in I_{SC} is calculated as the difference between I_{SC} before addition of the agonist and I_{SC} after a new steady state is attained. The addition of isoproterenol alone in 300 mOsm Ringer stimulated I_{SC} to 258 ∀ 10 A/cm^2, the addition of isoproterenol and water to 240 mOsm resulted in significantly smaller increase in current ($P < 0.01$). In fact, the current was even lover than the steady state current before stimulation. The number of independent experiments: isoproterenol in 300 mOsm Ringer ($n = 12$), isoproterenol in 240 mOsm Ringer ($n = 4$).

3.6. Steady-state Cl^- current is regulated by phosphorylation and dephosphorylation

Addition of the PP-1 and PP-2A phosphatase inhibitor calyculin A (Cal-A) (200 nM) increased I_{SC} as shown in Fig. 9a and b. In three of these experiments, mannitol was added 40–50 min after Cal-A (see Fig. 9a). Cal-A did not increase the effect of mannitol significantly but about 10 min after, the initial increase caused by mannitol the current started to decrease rather dramatically in all three experiments. This effect is unexplained. Fig. 9B also demonstrates that 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 phosphatase of the PP-1 and PP-2A type and protein-kinase C, but not protein-kinase A. Probably other kinases are also involved.

3.7. Is the key process in the activation of the Cl^- current with isoproterenol cell shrinkage or a fall in cytosolic Cl^- activity?

If we stimulated Cl^- transport with a ∃-agonist such as isoproterenol (10^{-5} M) in standard Ringer (300 mOsm) a dramatic increase is seen [4] as also demonstrated in Fig. 5. If, however, the medium is weakly hypotonic (240 mOsm) then all stimulation with isoproterenol disappeared (see Fig. 10). Under both conditions, the cytosolic Cl^- activity drops, resulting in concomitant cell shrinkage when isoproterenol is added in standard Ringer but without concomitant cell shrinkage when isoproterenol is added in hypotonic Ringer. We interpreted this to mean that a certain amount of cell shrinkage is necessary to activate the basolateral Na^+, K^+, $2Cl^-$ cotransporter.

3.8. Long-term acclimation

Fig. 11 shows the steady-state Cl^- current and the maximal Cl^- current after addition of mannitol plus isoproterenol in freshwater-adapted fish and in fish from the same batch retransferred to saltwater and measured at 5, 18, 48, 70, 95.5 and >300 h after the transfer. No current could be measured in freshwater-adapted fish nor in the fish within the first 18 h after transfer to saltwater. Stimulation with hypertonic solution (100 mOsm mannitol) plus the β-agonist isoproterenol, which in saltwater-acclimated fish

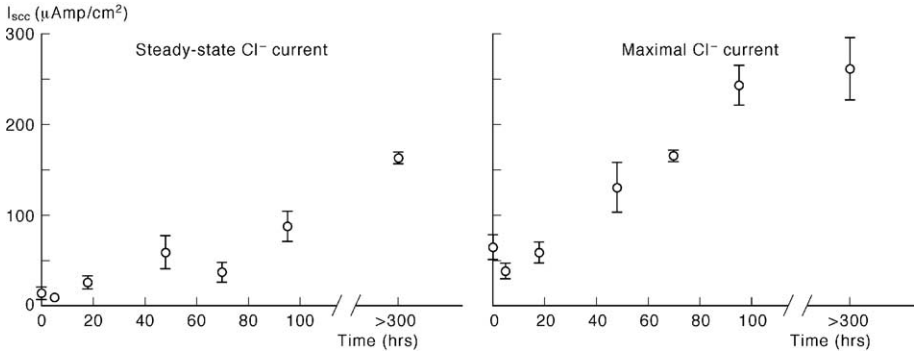

Fig. 11. The steady state I_{SC} (left frame) and the maximal I_{SC} (as a measure of the maximal Cl^- current) after addition of mannitol plus isoproterenol (right frame) in freshwater adapted fish and in fish from the same batch retransferred to salt water and measured at 5, 18, 48, 70, 95.5 and >300 h after the transfer. No significant current was measured in freshwater-adapted fish or in the fish within the first 18 h after transfer to saltwater. Stimulation with hypertonic solution (100 mOsm mannitol) plus the β-agonist isoproterenol, which in salt-water acclimated fish causes a dramatic stimulation of I_{SC} to 260 ± 32 μAcm^{-2} ($n = 14$), did not result in any significant stimulation within the first 18 h after transfer.

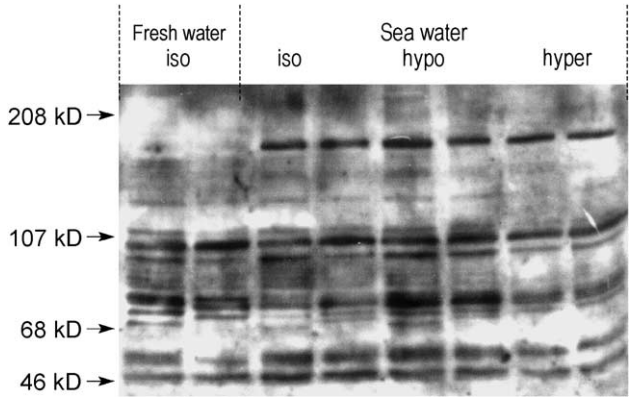

Fig. 12. Western blot analysis of the proteins in the operculum epithelium using anti-phosphoserin-antibodies. Three pairs of opercula epithelia were placed in oxygenated standard Ringer for 30 min. After this pre-incubation period the epitheliums were transferred to an isotonic standard Ringer (*iso*), a hypotonic Ringer (240 mOsm, *hypo*) or a hypertonic Ringer (400 mOsm, *hyper*) for 15 min. Ringers were removed and lysis buffer added as described in Material and methods. Number of independent experiments ($n=4$).

causes a dramatic stimulation of the short circuit current to 260 ± 32 μA/cm^2 ($n=14$), did not result in any significant stimulation within the first 18 h after transfer of the freshwater-adapted fish to saltwater. Not until more than 90 h after transfer to saltwater were the fish fully acclimated, as evaluated from steady-state short circuit current and the maximum current after hypertonic stimulus plus stimulus with isoproterenol. The maximal Cl^- current is taken to represent the number of active cotransporters in the basolateral membrane. A possible long-term adaptation to saltwater could be an increase in the number of Na^+, K^+, $2Cl^-$ cotransport molecules expressed in the basolateral membrane or alternatively an up-regulation of a kinase involved in the activation of the co-transporter. As evidenced from Western blot analysis using antiserine-antibodies (Fig. 12), a heavily serine phosphorylated protein of about 190 kDa is consistently observed in the saltwater-acclimated fish, but is only very weakly present in freshwater-acclimated fish. This observation indicates that acclimatization to saltwater stimulates the expression of this 190 kDa protein and/or a serine/threonine kinase, which subsequently phosphorylates the protein. No difference was observed in the intensity of the 190-kDa band between the operculum epithelium from the saltwater-adapted fish subjected to 15 min in iso-, hypo- or hypertonic Ringer.

4. Discussion

4.1. Cl^- channels, K^+ channels and NKCC1

The basic principle of Cl^- secretion in the operculum epithelium of *F. heteroclitus* is relatively well established [6,47] and similar to that suggested for exocrine glands (see, e.g. Ref. [48]) and other secretory epithelia [35]. It includes a CFTR Cl^- channel on the apical side and a Na^+, K^+, $2Cl^-$ cotransporter (NKCC1), a Na^+/K^+ pump and a K^+ channel on the basolateral side [6,47]. When the Cl^- secretion is stimulated by media that are made hypertonic either by the addition of NaCl or mannitol or after addition of the β-agonist isoproterenol, all transporters must increase their activity either secondarily by boosting the driving force or directly by stimulating the transporter. In agreement with this, we find that the Cl^- channel blockers glibenclamide, DPC and NPPB as well as the K^+ channel blocker Ba^{2+} all inhibit the Cl^- current as well as the stimulation of the Cl^- current with mannitol, hypertonic Ringer or isoproterenol. Inhibition of the Na^+, K^+, $2Cl^-$ cotransporter with bumetanide was previously shown to have a similar inhibitory effect [6]. In the colon, a 293B-sensitive K^+ channel has been identified on the basolateral membrane as the K_VLQT1 channel (see Ref. [35]). We have tested 293B on the operculum epithelium but did not see any effect; thus, it is likely that a different K^+ channel is important on the basolateral membrane of the operculum epithelium.

The driving force for the NKCC1 is definitely not enhanced after stimulation of the Cl^- current with mannitol, thus we must be dealing with a direct activation of the transport system. Since I_{SC} is stimulated to the same degree by media that are made hypertonic either by the addition of NaCl or by the addition of mannitol, we conclude, in concordance with previous suggestions [6], that the rapid signal for adaptation to higher salinities is an increase in plasma tonicity resulting in a cell shrinkage, which activates NKCC1. Cell shrinkage after addition of mannitol was demonstrated in Ref. [6].

4.2. Is the key process in the activation of NKCC1 with an agonist the cell shrinkage or the fall in cytosolic Cl^- activity?

The first step in the stimulation of Cl^- secretion after addition of isoproterenol is a c-AMP-induced activation of the luminal Cl^- channel followed by an enhanced uptake of Cl^- via the basolateral NKCC1. In this case it could be either the cell shrinkage or the fall in cytosolic Cl^- activity [42], which activated the NKCC1. In the present report we find that if we stimulate Cl^- transport with a ∃-agonist such as isoproterenol (10^{-5} M), whilst keeping the medium weakly hypotonic, then the effect of the stimulation is gone (see Fig. 10). Under these conditions, the cytosolic Cl^- activity must still fall caused by opening of luminal Cl^- channels, but the cells do not shrink because of the hypotonic medium which clamps the cell volume to an increased value. We interpret this to mean that a certain amount of cell shrinkage is necessary to activate the basolateral NKCC1, a result which fits to Ussing's studies on the activation of the Na^+, K^+, $2Cl^-$ cotransporter system after cell shrinkage in frog skin epithelium (see, for example, Ref. [31]). We have not actually measured if the cellular Cl^- activity does fall also when cells are treated with isoproterenol under hypo-

tonic conditions, but it is very unlikely that the hypotonic medium should prevent Cl^- loss. In agreement with the present results, it was recently shown in isolated perfused rectal gland tubules [49] that the primary and key process that triggers activation of the basolateral NKCC1 is a transient cell shrinkage. To this end, the rectal gland tubules were stimulated in a weakly hypotonic medium and it was shown that this led to a fall in cytosolic Cl^- activity without any shrinkage. Like in the killifish experiments presented in the present paper, no activation of the Na, K, 2Cl cotransporter was seen under these conditions in the rectal gland tubules [49]. It should be mentioned, as discussed by Zadunaisky et al. [10], that a decrease in current also could result from an eventual swelling activation of a basolateral Cl^- channel.

4.3. Mechanisms involved in stimulation of Cl^- secretion after cell shrinkage: protein kinases

Several known kinases have been shown to be directly or indirectly involved in co-transport activation: PKA, PKC and MLCK (see Ref. [50] for a recent review). PKC activity is stimulated in EATC by hypertonic shock [51] and MLC phosphorylation appears to be important for the shrinkage-induced NKCC1 activation which is strongly inhibited by the MLCK inhibitor ML-7 in EATC cells as well as in the vascular smooth muscle cells (see Refs. [44,45,52]). In agreement with this, both chelerythrine and ML-7 had strong inhibitory effects on the mannitol activation of the Cl^- current in the operculum epithelium. Thus, both MLCK and PKC are involved in the activation. On the other hand, neither of the two specific PKA inhibitors, H-89 and KT 5720, had an effect on the current after mannitol addition, whereas isoproterenol stimulation was completely blocked by H-89 (see Fig. 5). This observation indicates that PKA is involved in the activation of the apical Cl^- channel via c-AMP whereas the shrinkage activation of the Na^+, K^+, $2Cl^-$ cotransporter is independent of PKA activation. The PKC inhibitor chelerythrine inhibited the isoproterenol induced Cl^- current stimulation in agreement with the above discussed hypothesis that this stimulation also involves a shrinkage activation of the Na^+, K^+, $2Cl^-$ cotransporter. Finally, we found that the relatively high steady-state Cl^- secretion seems to be determined by continuous phosphorylation and dephosphorylation involving serine/threonine phosphatases of the PP-1 and PP-2A type and the kinase PKC but not PKA.

4.4. Long-term adaptation

It was found that the possible long-term adaptation to saltwater could be an increase in the number of Na^+, K^+, $2Cl^-$ cotransport molecules in the membrane or, alternatively, an up-regulation of a kinase involved in the activation of the cotransporter. As evidenced from Western blot analysis using antiserine-antibodies, a heavily serine phosphorylated protein of about 190 kDa is consistently observed in the saltwater-acclimated fish but is only weakly present in freshwater-acclimated fish. This observation indicates that acclimatization to saltwater stimulates the expression of this 190 kDa protein and/or a serine/threonine kinase, which subsequently phosphorylates the protein. A preliminary report of these results was published by Hoffmann et al. [53] and similar results are given in preliminary form, indicating that increase in both the amount of NKCC1 and phosphorylation takes place [54].

A serine/threonine kinase (sgk) has been identified in the shark *Squalus acanthias* and it is found to be transcriptionally regulated by cell volume [55]. Using degenerated primers against highly conserved sequences of the sgk kinase, we identified a similar band in the gill epithelium from saltwater-acclimated *Fundulus*, indicating the existence of the sgk transcript in the gill [53]. It is tempting to speculate that this kinase is up-regulated during salt acclimatization and subsequently activates the Na^+, K^+, $2Cl^-$ cotransport by phosphorylation. As suggested in preliminary reports [53,54], it is, however, likely that hypertonicity also enhances the expression of functional NKCC1 as shown in EATC [56]. Transcriptional regulation of a kinase and/or of the Na^+, K^+, $2Cl^-$ cotransporter will be the subject of future investigations. In addition to the increased amount and phosphorylation state of NKCC1, the killifish CFTR gene expression also increases during saltwater adaptation [57].

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