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Effect of mercuric chloride on electrical parameters and anion fluxes in the toad skin

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Abstract

The amphibian skin, widely used for studying the transepithelial passage of electrolytes, exhibits anion pathways relatively specific for Cl^-. We studied the effect of $HgCl_2$, 1.0×10^{-4} M on its electrical parameters and unidirectional anion fluxes. In the presence of Cl^-, the transepithelial conductance (G) of the isolated skin of the *Bufo arenarum* toad increased considerably following exposure to $HgCl_2$, whereas short-circuit current (SCC)—reflecting transepithelial Na^+ transport—underwent only slight stimulation. Following the blockade of Na^+ intake by amiloride, 1.0×10^{-4} M, the removal of Cl^- from the solution bathing the epidermal border of the skin brought about a decrease in *G*, and gave rise to a gradient-induced SCC (SCCg) consistent with transepithelial passage of Cl^- along its gradient. Addition of mercaptoethanol, 5.0×10^{-3} M to the bath containing Hg^{2+} fully reversed these effects. The increase in *G* was accompanied by an increase in the unidirectional (epidermal to dermal) fluxes of $^{36}Cl^-$ and $^{131}I^-$, and a decrease in the passage of $^{99m}TcO_4^-$. These results show the effects of $HgCl_2$ to be similar to those of theophylline, although exhibiting a different selectivity. Our data suggest that anion passage following exposure to $HgCl_2$ is, like that stimulated by theophylline, predominantly if not exclusively transcellular, and does not involve a significant opening of the tight junctions.

Keywords: *Bufo arenarum*; Toad skin; Short-circuit current; Transepithelial conductance; Epithelial anion permeability; $^{36}Cl^-$; $^{131}I^-$; $^{99m}TcO_4^-$; Unidirectional fluxes

1. Introduction

Chloride is the most important anion in the extracellular fluid, and its transport across membranes separating body compartments has been the object of intense study since it influences many regulatory functions in the body of animals.

The amphibian skin is a widely used model for studying the passage of Cl^- across membranes; a wide body of work has described in it the existence of pathways for anion passage exhibiting a relatively high specificity for Cl^-.

In that respect, exposure of the isolated toad skin to theophylline, forskolin or cyclic AMP increases considerably the epithelial permeability to Cl^-, a process which is not greatly affected by the presence of amiloride (a specific blocker of sodium transport) or the absence of Na^+ in the epidermal bath (Willumsen and Larsen, 1986; Castillo et al., 1990, 1991; Castillo and Orce, 1995; Katz and Nagel, 1995; Willumsen et al., 2002; Nagel et al., 2002).

Sometime ago we showed that in the skin thus treated, a sodium independent short-circuit current (SCC) can be generated by the application of a Cl^- transepithelial concentration gradient using SO_4^{2-} as a counteranion (gradient-induced SCC, SCCg). The SCCg exhibits a polarity consistent with the flux of Cl^- following its gradient and an intensity that is directly related to the absolute magnitude of the applied gradient (Castillo et al., 1991; Castillo and Orce, 1995).

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We have also shown that under those conditions the unidirectional flux of $^{36}Cl^-$ is greatly increased and the magnitude of the SCCg is not significantly different than the unidirectional flux of $^{36}Cl^-$ (manuscript submitted elsewhere).

The pathway activated by these agents (presumably mediated by an increase in intracellular cAMP) is highly permeable to halides (Cl^-, Br^- and, to a lesser extent, I^-), and selective enough as to exclude other ions present in high concentrations in the solution bathing the skin (SO_4^{2-}, Na^+), thus preventing dissipation of the electrical gradient and explaining the magnitude of the induced SCCg and the large concomitant values of transepithelial potential difference (PD) observed (Castillo et al., 1991; Castillo and Orce, 1995).

During a study of the effect of $HgCl_2$ on aquaporins in the basolateral membrane, we observed that exposure to $HgCl_2$ in the dermal bath caused changes in the electrical behavior of the skin. The present work was performed to characterize the effects of *d*$HgCl_2$ on the electrical parameters of the isolated skin of *Bufo arenarum*. We report here that transepithelial conductance (*G*) increased considerably following exposure of the dermal side of the skin to $HgCl_2$, whereas the SCC—which reflects transepithelial Na^+ transport—underwent only a slight stimulation.

In an attempt to study the selectivity of the pathway, we used different radioactive anions and found that upon exposure to Hg^{2+} on its dermal border which the skin exhibits increased permeability to $^{36}Cl^-$ and $^{131}I^-$, whereas the permeability to $^{99m}TcO_4^-$ decreased concomitantly. $^{99m}TcO_4^-$ was used in order to obtain information on the behavior of the pathway regarding a larger anion.

The combined data described here support the notion that exposure of the dermal side of the isolated toad skin to Hg^{2+} caused the activation of an anion pathway with a relatively high selectivity, distinctly different than both the opening of the tight junctions and the increase in permeability induced by exposure to theophylline.

2. Materials and methods

2.1. Toads

Specimens of *Bufo arenarum*, obtained from local suppliers shortly after capture, were kept on moist sand and soaked overnight in tap water before use. The animals were pithed and the ventral pelvic skin was removed and rinsed in amphibian Ringer's solution containing (in mM): NaCl:105; KCl:2.0; $MgSO_4$:1.0; Tris-HCl buffer (pH 7.5):25; $CaCl_2$:1.0; and glucose:6.0 ("chloride Ringer's").

2.2. Electrical parameters of the isolated amphibian skin

The electrical parameters of the isolated skin were measured by a modification of the short-circuit current (SCC) technique of Ussing (Ussing and Zerahn, 1951). The technique has been described in detail elsewhere (Orce et al., 1980).

The ventral pelvic skin was split in two symmetrical halves, one of which was randomly chosen to be a control. Each half was mounted as a flat sheet between two lucite hemichambers especially designed to reduce edge damage (exposed area: 3.14 cm^{-2}), and each side was bathed in 3 ml of chloride Ringer's solution. At one point, a solution containing no chloride ("sulfate Ringer's", SO4R), made by substituting Na_2SO_4, K_2SO_4 and Ca gluconate for their respective chlorides, and adding mannitol to correct the osmolarity, was substituted for the solution contained in the epidermal hemichamber of both preparations. Osmolality of the solutions was 230–235 mOsm kg H_2O^{-1}; they were stirred and oxygenated with air.

The preparations were kept in the open-circuit condition, and the preparations were short-circuited briefly every 5–10 min to measure the SCC.

The electrical conductance of the tissue (*G*) was estimated by periodically recording the intensity of the current required to briefly (3–5 s) shift the PD by 20–40 mV above or below 0 mV, immediately after measuring the SCC.

2.3. Measurement of anion fluxes

Three isolated skin preparations were made from each toad by tying individual pieces of ventral pelvic skin, epidermal side facing inside, at the end of plastic tubes open at both ends (exposed area: 1.79 cm^2). Two milliliters of Ringer's solution were added to the inside of each tube (epidermal bath), and the preparations were then immersed in 5 ml of the same Ringer's solution (dermal bath).

The solution in contact with the dermal side of the skin was aerated by bubbling atmospheric air through it. The preparations were allowed to stand for 1 h, changing all solutions every 30 min, and all solutions were discarded at the end of this period.

Two milliliters of Ringer's solution containing $^{36}Cl^-$ (4.4 μCi ml^{-1}), $^{131}I^-$ (2.76 μCi ml^{-1}) and $^{99m}TcO_4^-$(9.78 mCi ml^{-1}) ("hot" solution) were then placed inside the tube (epidermal bath), and each preparation was immersed in 5 ml of radionuclide-free Ringer's solution ("cold" solution) (dermal bath). One of the preparations was randomly chosen to be a control; drugs were added to the dermal bath of the other two preparations as required in the protocol.

Duplicate 1-ml samples were withdrawn from the dermal bath every 30 min during 2 h, starting 30 min after addition of the hot solution, either theophylline or $HgCl_2$ was added to the dermal bath, and samples were collected during another 2 h period. An equal volume of "cold" Ringer solution (alone or containing the appropriate concentrations of drugs whenever required) was added to the dermal bath at the end of each sampling period to keep its volume constant. Proper factors were introduced in the processing algorithm in order to compensate for dilution. Duplicate 30-μl samples

were obtained from the hot (epidermal) side of each preparation at the end of the experiment in order to measure the activity of the nuclides used.

^{131}I and ^{99m}Tc activities were measured immediately following collection by means of a CliniGamma automatic well counter (LKB, Finland); readings were corrected for radioactive decay. ^{36}Cl activity was measured in a LS-100C liquid scintillation counter (Beckman, USA), after at least 15 half-lives of ^{131}I had been allowed to elapse.

2.4. Drugs

Theophylline (Sigma Chemical, St. Louis, MO, USA) was made up fresh every day by dissolving the drug at 5.0×10^{-2} M in the Ringer's solution being used for the experiment; final concentration (1.0×10^{-2} M) was achieved by substituting the appropriate aliquot in the inside (dermal) bath. When appropriate, control preparations received an equivalent concentration of glucose. Amiloride, a gift of Merck, Sharp and Dohme (West Point, PA, USA), 1.0×10^{-2} M, was dissolved in distilled water; it was kept at 4 °C, and added directly to the outside (epidermal) bath to a concentration of 1.0×10^{-4} M when required. Mercaptoethanol (Merck, Darmstadt, Germany), 5.0×10^{-3} M, was added directly to the bath. All other drugs were analytical grade and commercially obtained.

When a solution was substituted with another of a different anion composition, the preparation was rinsed twice with 3 ml of the new solution before replacing the final volume in the hemichamber.

2.5. Radionuclides

^{131}I was supplied by the National Atomic Energy Commission of Argentina (CNEA) as $Na^{131}I$. ^{36}Cl was purchased from New England Nuclear as $H^{36}Cl$; it was neutralized with a slight excess of KOH in order to prevent loss of gaseous $H^{36}Cl$. ^{99m}Tc was obtained as $Na^{99m}TcO_4$ from a generator supplied by CNEA and used without further processing.

2.6. Statistics

Experiments were performed at room temperature (20–24 °C). Results are expressed as mean±SEM. Student's *t*-test for paired data was used for statistical analysis, preceded by ANOVA test whenever necessary; a P value of 0.05 or less was considered significant.

3. Results

3.1. Electrical parameters of the isolated amphibian skin

3.1.1. Effect on SCC and G

As shown in Fig. 1, in the presence of 115 mEq l^{-1} Cl^-, exposure to $HgCl_2$ (1.0×10^{-4} M in the dermal bath) (dHgCl$_2$) slightly stimulated SCC, although the observed change was not significantly different than that in the control preparations. SCC increased 34.7±14.1 μA 3.14 cm^{-2}, $P<0.05$ in the $HgCl_2$-treated preparations vs −0.8±3.2, NS, in the controls (P value of the difference, P_{diff}: NS), whereas PD increased 8.8±3.1 mV, $P<0.05$ in the $HgCl_2$-treated preparations vs 6.1±1.3, P<0.01, in the controls (P_{diff}: NS).

In turn, G increased considerably upon exposure to dHgCl$_2$ (by 0.98±0.30 mS.cm^{-2}, $P<0.02$ in the $HgCl_2$-treated preparations vs 0.03±0.05, NS, in the controls; $P_{diff}<0.02$, n=6) (Fig. 2).

Inhibition of transepithelial Na^+ transport by addition of amiloride (AMIL), 1.0×10^{-4} M, to the epidermal bath (eAMIL) significantly inhibited SCC and G in both $HgCl_2$-treated and control preparations, as would be expected following inhibition of an important current carrier. SCC fell

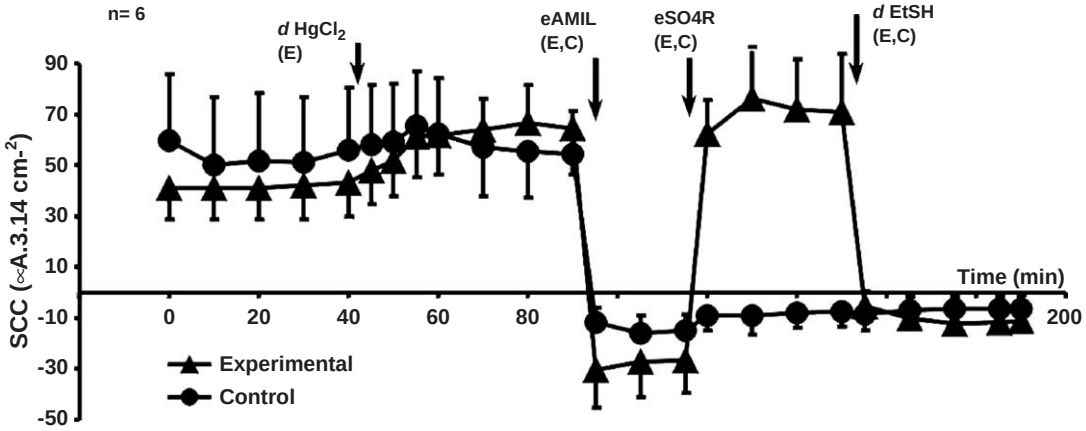

Fig. 1. Effect of mercuric chloride on short-circuit current in the isolated toad skin. After measuring the baseline SCC, $HgCl_2$, 1.0×10^{-4} M, was added to the dermal bath of one preparation of each pair (dHgCl$_2$); controls received the same volume of solvent. Amiloride, 1.0×10^{-4} M, was added to the epidermal bath of all preparations 50 min later (eAMIL), and Cl^- free solution was substituted for the original Ringer's solution in the epidermal hemichamber 25 min later (eSO4R). Finally, mercaptoethanol, 5.0×10^{-3} M, was added to the dermal bath (dEtOH).

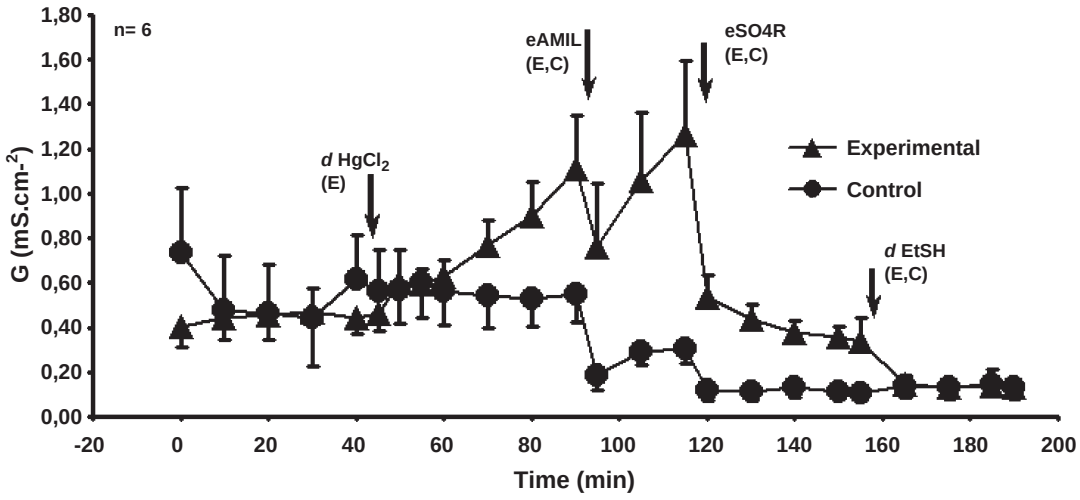

Fig. 2. Effect of mercuric chloride on transepithelial conductance in the isolated toad skin. Legend as in Fig. 1, except the results for *G* are shown.

by −95.8±14.1 μA 3.14 cm^{-2}, $P<0.001$ vs −70.8±16.2 and $P<0.01$, respectively. P_{diff}: NS, and *G* decreased by −0.35±0.10 mS/cm^2, $P<0.02$ vs −0.38±0.11, $P<0.02$, in the controls; P_{diff}: NS.

Further removal of Cl^- from the epidermal bath by substituting SO4R for the ClR originally present (thus creating a dermal–epidermal chloride concentration gradient, ($[Cl^-]grad_{13}$) gave rise to a considerable SCC (gradient-induced SCC, SCCg). SCCg was 107.7±26.3 μA 3.14 cm^{-2}, $P<0.01$ vs 8.0±2.1, $P<0.01$, in the controls; $P_{diff}<0.01$. The polarity of this SCCg was consistent with the passage of Cl^- from the dermal to the epidermal bath. Under these conditions, *G* decreased considerably in both treated and control preparations: −0.92±0.29 mS.cm^{-2}, $P<0.01$ vs −0.21±0.05, $P<0.01$, in the controls; P_{diff}=NS).

Finally, addition of EtSH, 5.0×10^{-3} M (a donor of SH groups), to the $HgCl_2$- containing dermal bath (*d*EtSH) fully reversed the SCCg and the increase in G, bringing both SCC and *G* to values not different from control. SCC in the *d*$HgCl_2$-treated preparations decreased by−83.8±24.4 μA.3.14 cm^{-2}, $P<0.02$ vs −1.5±1.6, NS, in the controls ($P_{diff}<0.02$), whereas *G* fell by −0.23±0.04 mS/cm^2, $P<0.001$, in treated preps vs −0.03±0.02, NS, in the controls; $P_{diff}<0.01$, n=6), bringing *G* in the treated preparations to control values.

3.1.2. Measurement of anion fluxes

In agreement to previous reports, exposure to theophylline (1.0×10^{-2} M in the dermal bath, *d*THEO) increased considerably the epidermal-dermal flux of ^{36}Cl ($J^{36}Cl_{13}$) by more than five-fold (Fig. 3, columns 1 and 2). $J^{36}Cl_{13}$ also increased by more than 3-fold following exposure of skins to *d*$HgCl_2$ (1.0×10^{-4} M) (Fig. 3, columns 3 and 4). The increases were in both cases significantly greater than those recorded in the control, untreated preparations.

Similarly, $J^{131}I_{13}$ also approximately doubled its value following exposure of the dermal border of the skin to theophylline or $HgCl_2$ (Fig. 4, columns 1 and 2).

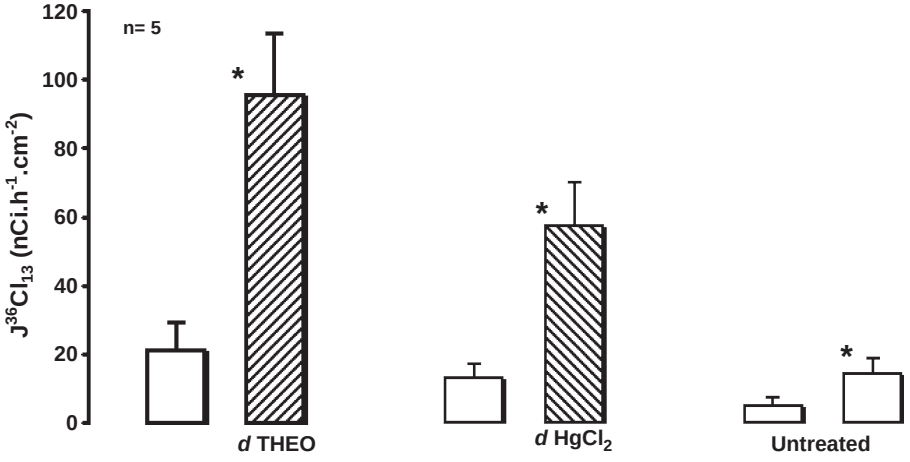

Fig. 3. Effect of theophylline or mercuric chloride on the $^{36}Cl_{13}^-$ flux in the isolated toad skin. The first bar in each pair represents the last reading before the addition of the nuclide mix to the epidermal bath of each preparation; the second bar is the maximum value read for each sample set. *Means different from the value shown in the previous bar with $P<0.05$.

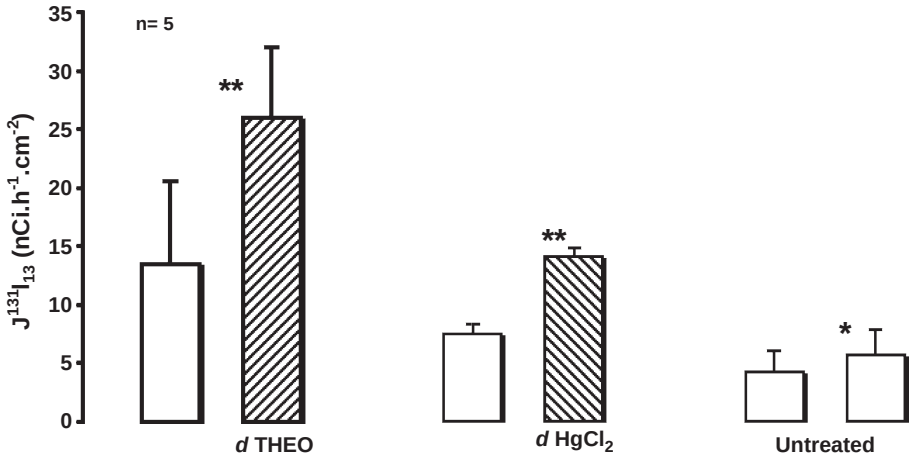

Fig. 4. Effect of theophylline or mercuric chloride on the $^{131}I_{13}^{-}$ flux in the isolated toad skin. Legend as in Fig. 3. *,**Mean different from the value shown in the previous bar with $P<0.05,<0.01$.

In contrast, $J(^{99m}TcO_4^-)_{13}$ increased considerably in the skins exposed to dermal theophylline (Fig. 5, columns 1 and 2), whereas it exhibited a significant decrease in the preparations exposed to dermal $HgCl_2$ (Fig. 5, columns 3 and 4).

4. Discussion

A number of studies have been made describing the effects of Hg^{2+} on transport processes in amphibian membranes, most of them related to blockade of apical water channels, and involving mainly exposure of the epidermal border of the skin to the cation (Ibarra et.al., 1989, 1990; Van Der Goot et al., 1991; Grosso et al., 1993, 1994).

Fewer published reports exist on the effects of Hg compounds on the electrical parameters of the isolated skin of toads and frogs. It has been reported that exposure of epithelia to either Hg^{2+} or Hg compounds brought about variable changes in electrical parameters, spanning from increase in Na^+ transport (Li and Lindemann, 1983) to either inhibition or stimulation, depending on the concentration of the agent used (Bures et al., 1980).

Suwalsky et al. (2000) in a recent study reported that exposure of either side of the skin of the toad *Pleuroderma thaul* to $HgCl_2$ or CH_3HgCl brought about a decline in SCC, which was maximal at around 1.0×10^{-5} M; the effect could be reversed only partially by rinsing. Furthermore, exposure of the isolated skin to the agent brought about irreversible tissue damage, thus precluding further recovery of the skin. They also proposed that the effects of Hg^{2+} could be due to its binding to intracellular structures, suggesting that the cation may easily permeate the cell membrane.

As reported here, exposure of the isolated skin of *Bufo arenarum* to $HgCl_2$ in the dermal solution caused important changes in electrical parameters in the presence of Cl^-, the most prominent of which was a considerable increase in *G*. No inhibition of SCC was observed under those conditions,

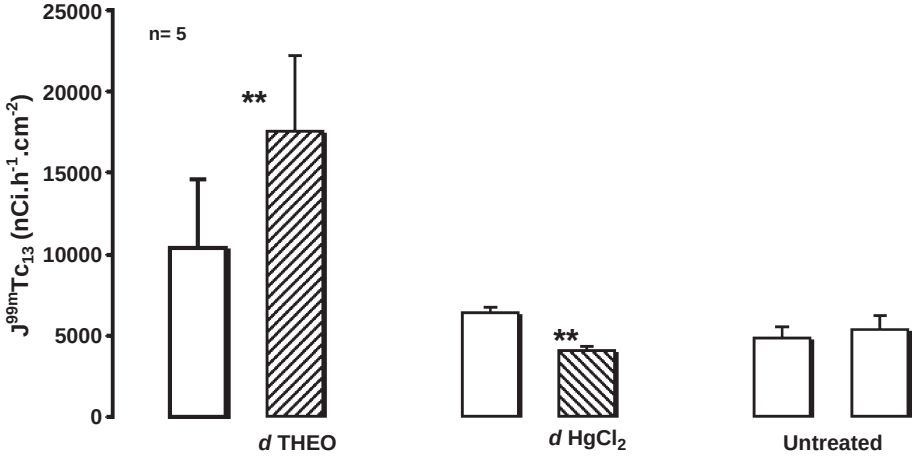

Fig. 5. Effect of theophylline or mercuric chloride on the $(^{99m}TcO_4^-)1_3$ flux in the isolated toad skin. Legend as in Fig. 3. **Means different from the value shown in the previous bar with $P<0.01$.

however, and the decrease in SCC following addition of amiloride to the epidermal bath clearly shows that Na^+ transport continued to take place at the same or slightly increased rate during at least 45 min following addition of the compound, in sharp contrast to the quick decline observed in *P. thaul*. In turn, reversal of the anion permeabilizing effect was complete upon removal of Hg^{2+}, as reflected in the sharp fall in SCCg (Fig. 1) and the full return of *G* to control values (Fig. 2), the speed and completeness of the reversal also arguing against the notion that the effect requires a significant entry of Hg^{2+} into the cell in the *B. arenarum* skin. The return following addition of *d*EtSH of all the electrical parameters of the skin to control values provides further evidence that the effects were not irreversible, despite the fact that the concentration of Hg^{2+} we used was around 10 times higher than in the study by Suwalsky et al. We are unable at this time to provide an explanation for this discrepancy, other than species difference.

Previous work from our laboratory demonstrated that application of a Cl^- concentration gradient across the skin exposed to theophylline elicited an SCCg exhibiting a polarity consistent with the unidirectional flux of the anion (Castillo et al., 1991; Castillo and Orce, 1995).

The results presented here show that in $HgCl_2$-stimulated skins the polarity of the SCCg generated in the presence of a $[Cl^-]grad_{13}$ was also consistent with the passage of Cl^- following its gradient, and confirmed that a considerable concomitant increase in the unidirectional flux of $^{36}Cl^-$ took place in the epidermal-dermal direction.

Our results show that exposure to Hg^{2+} in the dermal bath increases the permeability of the isolated toad skin to $^{36}Cl^-$ and $^{131}I^-$, while decreasing the passage of $^{99m}TcO_4^-$. They also confirm previous reports from our laboratory that theophylline increases the transepithelial permeability to anions in *B. arenarum* skin, although exhibiting a different selectivity, since in this case the unidirectional flux of $^{99m}TcO_4^-$ was increased, as were those of $^{36}Cl^-$ and $^{131}I^-$.

This seemingly general increase in anion permeability following exposure to theophylline raises the possibility that the drug may increase transepithelial permeability by alternate mechanisms, i.e., either by permeabilizing the paracellular pathway, or stimulating dermal gland secretion.

Permeabilization of the tight junctions implies that diffusion of ions in the opposite direction via the poorly selective paracellular pathway masks a large portion of the electrical effects of the transepithelial passage of Cl^-; and is accompanied with a considerable drop in PD, irrespective of the nature of the anion present. Typically, this is observed in the toad skin exposed to hypertonic solution on its epidermal border (Castillo et al., 1991).

In contrast, *G* values in the theophylline-stimulated *B. arenarum* skin bathed in Cl^- free solution (SO_4^{2-} and gluconate substituted for Cl^-) closely correlated with the SCC, and both were rapidly reduced to control values by amiloride at a concentration that abolished the latter; PD values remained elevated in the absence of amiloride (Castillo et al., 1991).

In the skin exposed to Hg^{2+}—as was the case following stimulation with theophylline (Castillo et al., 1991)—the generation of a large SCCg upon removal of Cl^- from the epidermal bath, together with the high PD values registered in the process, are a strong indication that there is no significant increase in paracellular pathway permeability.

In turn, the skin of *B. arenarum* is relatively poor in glands, and its secretory response to norepinephrine is only a small fraction of that seen in the skin of the frog (Castillo and Orce, 1997). In addition, exposure of the isolated amiloride-treated *B. arenarum* skin to high concentrations of the catecholamine failed to elicit the sharp increase in short-circuit current, PD changes and foaming characteristic of glandular secretion that were readily observed in the frog skin undergoing the same treatment (House, 1969; Castillo and Orce, 1997).

In particular, in the *B. arenarum* skin exposed to dermal theophylline, none of these signs are seen, and the increase in SCC following exposure to the drug is fully inhibited in the presence of amiloride, clearly indicating its dependence on Na^+ transport (Castillo et al., 1991).

These results, taken together, strongly suggest that the anion pathway permeabilized following exposure of the *B. arenarum* skin to Hg^{2+} is transcellular, and shares some properties with the anion pathway activated by theophylline. The exact location of the Hg^{2+}-activated channel involved cannot be inferred from our present data, but it can be hypothesized that it may be located to the mitochondria-rich cell, which is known to be the site responsible for the increased passive passage of anions in the toad skin stimulated by agents known to increase intracellular cyclic AMP (β-adrenergic agonists, prostaglandin E_2) (Larsen et al., 2003).

The skin of amphibians is a multiple-layered heterocellular structure whose different cell types exhibit different functions (Willumsen et al., 2002). The existing evidence clearly points to the mitochondria-rich cell as a highly specialized pathway for passive transepithelial transport of chloride (Willumsen et al., 2002; Larsen et al., 2003). In agreement with observations in other amphibian epithelia, high Cl^- passive permeability is activated in the skin of *B. arenarum* by forskolin, theophylline or cAMP (Castillo et al., 1990, 1991; Castillo and Orce, 1995). Furthermore, the known anion selectivity sequence suggests that Cl^- passage may take place via a CFTR-Cl^- channel-like pathway, as demonstrated for *B. bufo* (Willumsen et al., 2002; Larsen et al., 2003).

Our data do not allow us to infer the mechanism involved in the response of the skin of *B. arenarum* to Hg^{2+} in the dermal bath. It has been reported, however, that CFTR-dependent Cl^- currents in *Xenopus laevis* oocytes were sensitive to $HgCl_2$, suggesting that modification of endogenous cysteines involved in channel gating may alter channel activity (Ketchum et al., 2002).

These changes in the electrical parameters of the toad skin represent a modification of anion transport via a mechanism not subjected to control by the organism, and can thus be considered a toxic effect. Such an effect would be deleterious to cell and tissue function, contributing to explain the toxic effects observed following prolonged exposure to Hg^{2+}, a rather ubiquitous environmental contaminant.

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