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Effects of Anions and/or Cell Volume on the Permeance of an Apical Water Pathway Induced by Hg in Toad Skin Epithelium

A. Grosso[1], P. Meda[2], R.C. de Sousa[1]

[1]Departments of Physiology and Medicine, School of Medicine, University of Geneva, C.M.U., 1 rue Michel-Servet, 1211 Geneva 4, Switzerland

[2]Department of Morphology, School of Medicine, University of Geneva, C.M.U., 1 rue Michel-Servet, 1211 Geneva 4, Switzerland

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Abstract. Hg compounds block membrane transport units behaving as water channels. Here we show that Hg induces an apical water pathway in toad skins pretreated with 10^{-3} M CH_3ClHg or $HgCl_2$, added to the outer bathing medium. Washing with SO_4-Ringer caused a several-fold increase in net water flow (J_w) and osmotic permeability coefficient (P_f) that was reversed by re-exposure to Cl- or NO_3-Ringer and mimicked by gluconate-Ringer. These P_f changes could be elicited repeatedly and were present if, and only if, anion replacements took place in the inner bathing solution. Such inner polarity was related to the anion permeability of the epidermal basolateral membrane: impermeant anions (SO_4, gluconate) increased P_f; permeant anions (Cl, NO_3) did not change basal P_f but reversed the high P_f induced by impermeant anions. Hg induced the appearance of aggregates that persisted despite repeated washings of the skins during 4–5 h, and whether P_f was high (SO_4-Ringer) or low (Cl-Ringer) before skin fixation.

The Hg-induced apical water pathway in toad skin appears to be a unique model for studying the interplay between cell volume, cell ionic composition and water permeability.

Key words: Hg compounds—Water permeability—Toad skin epithelium—Apical aggregates—Anion replacements—Cell volume

Introduction

The mechanisms and pathways for water transport across cell membranes are a central issue in cell biology. On the basis of biophysical evidence, the presence of water channels was postulated in a variety of membrane systems, characterized by high values of osmotic water permeability coefficient (P_f) and low values of the activation energy (E_a) for osmotic flow [11, 28, 46]. These systems include: the plasma membrane of erythrocytes [28, 29]; the plasma and/or the endosomal membranes of some cell types from urinary epithelia of mammalian [48, 49] and anuran [25, 39, 46] origin. It has also been shown that, when such membranes are exposed to Hg compounds, P_f decreases and E_a increases [28, 33, 46]. More recently, similar effects of Hg were found in oocytes of *Xenopus laevis* injected with mRNAs prepared from cells with high P_f membranes [41, 47, 51] and in proteoliposomes where the channel-forming CHIP28 protein has been incorporated [45, 50]. Collectively, these data suggest that Hg blocks the permeance of membrane structures behaving as water channels.

In ventral skins of toads *Bufo marinus*, Hg added to the external bathing medium can either prevent or reverse the increase in P_f induced by hydrosmotic agents such as vasopressin and isoproterenol, depending on whether the exposure to the metal compound preceded or followed the stimulation of water flow, respectively [17]. In the course of experiments designed to investigate the reversibility of the Hg-induced block of the hydrosmotic response in toad skin, we noticed that replacement of SO_4 for Cl in the bathing Ringer solution caused a several-fold increase in P_f that was reversed by re-exposure of the epidermis to standard Cl-Ringer. These anion effects did not depend on the stimulation of the epidermis by a hydrosmotic agent, since similar changes were observed in skins exposed to Hg alone.

The marked sulfate-induced increases in P_f,

Correspondence to: A. Grosso

whose magnitude was as high as that observed in skins challenged with a hydrosmotic agent [8, 9, 12], called for a morphological examination of the epidermis that disclosed the appearance of intramembrane particle (IMP) aggregates in the apical membrane of granular cells. The water pathway associated with these aggregates appeared to be closed in toad skins bathed with Cl-Ringer and open in skins bathed with SO_4-Ringer. Moreover, the anion effects showed sidedness with an epithelial polarity related to the permeability of the basolateral membrane to anions.

The results reported here suggest that the anion-induced changes in water permeability observed in toad skins pretreated with Hg are exerted on the cytoplasmic side of an apical water pathway. The permeance of such a pathway seems to be influenced, under isosmotic conditions, by epithelial cell volume changes and/or by changes in intracellular ionic composition, particularly those concerning chloride.

Materials and Methods

Toads *B. marinus* were purchased from Charles D. Sullivan (Nashville, TN) and kept in large basins, at a room temperature of 25°C. After double-pithing the toads, the abdominal skin was excised and divided in two symmetrical pieces, following the median line of the animal. For most experiments only the pelvic region was taken but, for some experiments, two additional symmetrical pieces of a more orad region were also excised. Each piece was mounted as a diaphragm between glass hemichambers, the area exposed to the bathing media being 4.15 cm^2.

Bathing Media and Chemicals

The composition of the standard Ringer solutions was as follows (in mM): *Standard Cl-Ringer:* NaCl, 112; $MgSO_4$, 1; KH_2PO_4, 1.2; $KHCO_3$, 2; $CaCl_2$, 1. *Standard SO_4-Ringer:* Na_2SO_4, 56; K_2SO_4, 1.25; $CaSO_4$, 1; Tris, 10; mannitol, 65. *Gluconate-* and *nitrate-Ringer:* same as standard Cl-Ringer, except for replacement of Cl by gluconate or nitrate, respectively.

Most of the studies with SO_4-Ringer were carried out with the standard solution indicated above. A few experiments were carried out with a SO_4-Ringer solution having the same composition of the standard Cl-Ringer, except for replacement of Cl by SO_4. The effects on J_w caused by these two SO_4-Ringer solutions were not different.

In some studies, the anion replacements were asymmetrical, i.e., either in the external or in the internal medium, exclusively. Moreover, for a specific protocol (*see* Results), a partial anion replacement was made by mixing equal volumes of standard SO_4-Ringer and standard Cl-Ringer.

The pH of the internal solution was 7.8, that of the external solution 7.6. The chemicals used were: $HgCl_2$ or CH_3ClHg (Fluka); isoproterenol (Sigma).

In this work Hg was added only to the outer bathing medium. For brevity, it is sometimes referred to as apical Hg. Unless specified otherwise, its concentration was 10^{-3} M and the incubation time, 60 min.

Osmotic Gradient, J_w and P_f

The outer surface of the epidermis faced a hemichamber prolonged by a horizontal pipette. Transepithelial net water flow (J_w) was continuously monitored by means of an automatic electronic device that followed *pari passu* the displacement of the liquid meniscus inside the horizontal pipette (for details, *see* [12, 16]). J_w, expressed in $\mu l \cdot min^{-1} \cdot cm^{-2}$, was averaged over periods of 2 min.

The osmotic gradient imposed across the skins was the following: *internal* (dermal) side, standard Cl-Ringer or modified Ringer solutions (220 mosmol Kg^{-1} H_2O); *external* (epidermal) side, a tenfold dilution of the corresponding internal solution (22 mosmol Kg^{-1} H_2O). Where needed, the osmolality of the various Ringer solutions was adjusted to 220 mosmol Kg^{-1} H_2O with mannitol. This standard osmotic gradient was not appreciably modified by the net transfer of water that took place in the different experimental conditions reported in this work. Therefore, changes in J_w were exactly paralleled by changes in the osmotic permeability coefficient P_f, a parameter derived from the coefficient of osmotic flow, L_{PD} [5, 12].

The results are expressed as mean ± SEM. Where applicable, P values were obtained by means of the Student's t-test for paired data.

Freeze Fracture

Sixteen skins across which J_w had been previously determined, were removed from the glass chambers and rapidly immersed in 2.5% glutaraldehyde buffered with 0.1 M phosphate buffer, pH 7.4, for 10–60 min. Following repeated rinsing in phosphate buffer, small skin fragments were infiltrated with 30% phosphate-buffered glycerol and frozen in Freon 22 that had been cooled in liquid nitrogen. Fracture and shadowing were carried out in a Balzers BAF 301 apparatus (Balzers High Vacuum, Balzers, Liechtenstein). The replicas were washed in a sodium hypochlorite solution, rinsed in distilled water, mounted on Formvar- and carbon-coated grids and examined in a Philips EM 301 electron microscope (Philips, Eindhoven, The Netherlands).

Replicas were screened with no prior knowledge of the different experimental conditions, using criteria defined elsewhere [8]. Briefly, (i) the first-reacting cell layer of the epidermis was identified by the presence of tight junction fibrils (on P fracture faces) and furrows (on E fracture faces); (ii) granular cells were distinguished from nearby mitochondria-rich cells which featured characteristically elongated, rod-shaped intramembrane particles; (iii) the apical membrane of granular cells, contacting the cells of the overlying *stratum corneum* layer, was identified by the presence of cross-fractured microvilli and desmosomal stalks. The presence of intramembrane particle aggregates similar to those associated with changes in water permeability [7, 8, 9], was assessed by evaluating the apical membrane of 5–41 different granular cells per skin segment (*see* Table 2).

Results

Hg-Induced, SO_4-Sensitive Changes in Water Permeability

Figure 1 summarizes the original set of observations that led to the work presented here. In a toad skin

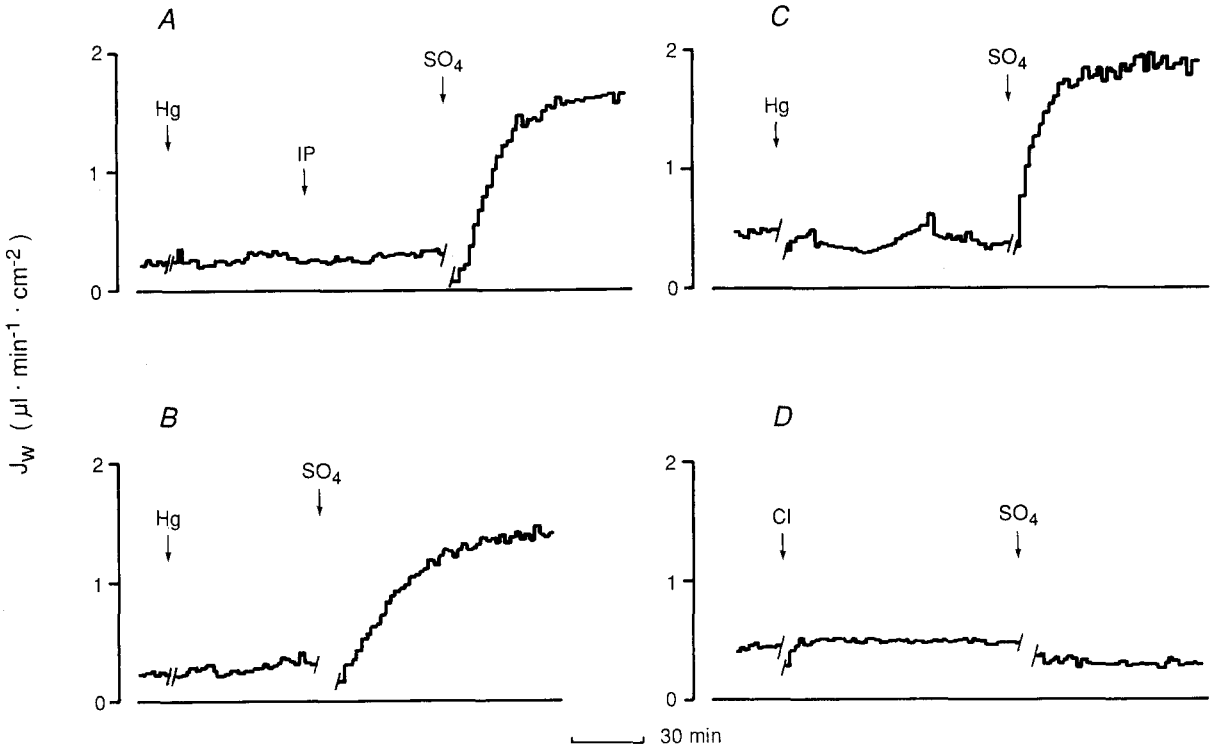


Fig. 1. Typical recordings of the combined effects of Hg and SO_4 on net water flow (J_w). (*A* and *B*) Paired segments of a single skin bathed with Cl-Ringer were pretreated with CH_3ClHg (10^{-3} M) added to the outer, diluted medium. Washing with SO_4-Ringer caused a several-fold increase in J_w. Note (panel *A*) that Hg completely blocked the hydrosmotic response to isoproterenol (IP, 10^{-6} M). (*C* and *D*) Paired segments of another skin showing that SO_4 caused a similar increase in J_w after pretreatment with 10^{-3} M $HgCl_2$ (panel *C*); in the control epithelium, not exposed to Hg, the same anion replacement resulted in a decrease of J_w (panel *D*).

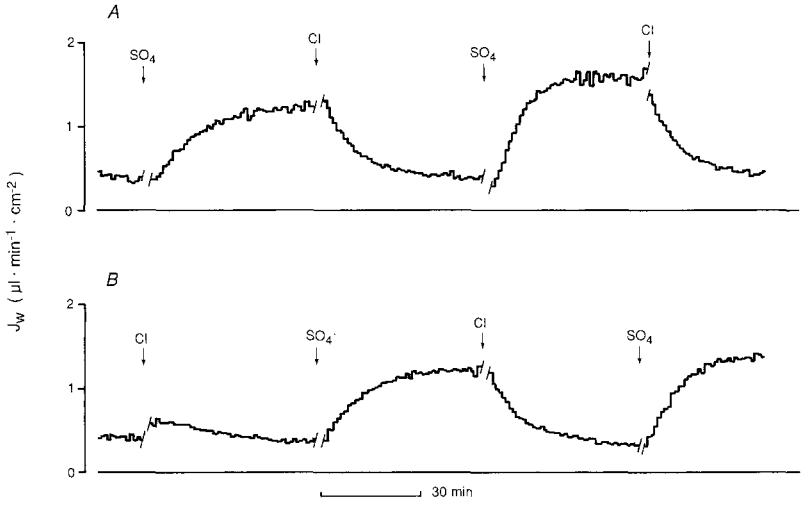


Fig. 2. Cl reverses the increase in J_w induced by SO_4. Two paired skin segments were pretreated with $HgCl_2$, while bathed in Cl-Ringer (panel *A*) or in SO_4-Ringer (panel *B*). In both cases, the SO_4 effect could be elicited twice; Cl-Ringer brought J_w down to its basal value.

segment bathed with standard Cl-Ringer and having its hydrosmotic response to isoproterenol blocked by the presence of Hg in the external medium, a striking, 5.8-fold increase in net water flow (J_w) was seen when both sides of the skin were washed with SO_4-Ringer (Fig. 1*A*). However, the challenge with a hydrosmotic agent was not a prerequisite for the SO_4-induced rise in J_w, since similar increases were found in skin segments pre-exposed to Hg alone, whether the metal compound was organic (Fig. 1*B*) or inorganic (Fig. 1*C*). In contrast, no increase in J_w was seen in control skins not exposed to Hg; the replacement of Cl-Ringer by SO_4-Ringer even caused a slight decrease in water flow (Fig. 1*D*), as previously reported [18].

Iterative Changes in Water Permeability Caused by SO_4 and Cl Ions

Cl-Ringer readily reversed the increase in J_w induced by SO_4-Ringer, down to values close to basal J_w. If this sequence of anion substitutions were repeated, the same type of changes in J_w were observed in a very reproducible manner (Fig. 2*A*). This pattern was found whether the skins, while exposed to Hg, bathed in Cl-Ringer (Fig. 2*A*), or SO_4-Ringer (Fig. 2*B*).

An arbitrary period of 60 min was chosen for the pretreatment of the external surface of toad epidermis with Hg. When this period was reduced to 10 min ($N = 3$), the anion effects remained qualitatively the same (*data not shown*). In general, the repetition of the anion substitutions led to an apparent facilitation of their effects (Fig. 2). The statistical analysis of the magnitude and the kinetics of the anion-induced changes in J_w is summarized in Fig. 3 for three different experimental groups. Table 1 summarizes the results for the dependence of the anion effects on the apical Hg concentration for the range 10^{-4} to 10^{-3} M $HgCl_2$. At 10^{-5} M $HgCl_2$, the results were somewhat variable; SO_4-Ringer did not increase J_w, sometimes there was even a decrease.

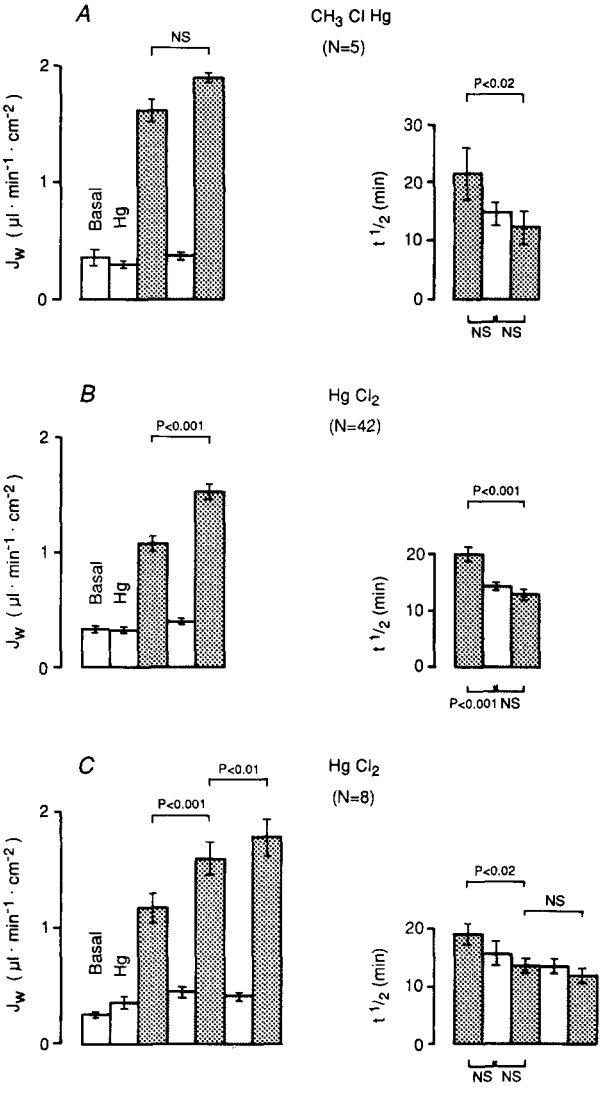


Fig. 3. Statistical analysis of the effects of SO_4 and Cl on transepithelial osmosis across toad skins pretreated with organic (panel *A*) or inorganic (panels *B* and *C*) Hg. On the left are indicated the average values of J_w for the following experimental periods: basal flow, pretreatment with Hg and subsequent washings with SO_4- or Cl-Ringer solutions. On the right is indicated the time ($t_{1/2}$) required to reach 50% of the total increment or decrement in J_w caused by SO_4 or Cl, respectively. Dark stippled bars = SO_4-Ringer. Light stippled bars = Cl-Ringer. NS = not significant.

Search for IMP Aggregates

In view of the large increases in J_w and P_f reported here, the question arose as to whether intramembrane particle (IMP) aggregates were present in epithelia exposed to Hg. Results of skins processed for freeze-fracture examination are illustrated in Figs. 4 and 5 and summarized in Table 2.

In control epidermis, the protoplasmic (P) fracture face of the apical membrane of granular cells from the first-reacting cell layer featured tight junction fibrils, microvilli and desmosomal stalks. Between these structures, intramembrane particles appeared distributed at random and did not form clusters (Fig. 4*A* and Table 2). In contrast, intramembrane particle aggregates were observed in the apical plasma membrane of granular cells in skins fixed in the presence of $HgCl_2$, whether isoproterenol was present (Fig. 4*B*) or not (Fig. 4*C*). Similar aggregates were also seen in skin segments pretreated with Hg alone and subsequently washed twice with SO_4-Ringer and twice with Cl-Ringer

Table 1. Anion-induced effects on J_w and apical Hg concentration

Experimental condition	$HgCl_2$ 10^{-4} M	P ($N = 10$)	$HgCl_2$ 10^{-3} M	P ($N = 10$)
(A) Basal	0.35 ± 0.02		0.39 ± 0.05	
(B) $HgCl_2$	0.36 ± 0.02	NS (B *vs.* A)	0.40 ± 0.03	NS (B *vs.* A)
(C) SO_4-Ringer	0.47 ± 0.04	NS (C *vs.* A)	1.00 ± 0.14	<0.01 (C *vs.* A)
(D) Cl-Ringer	0.67 ± 0.07		0.42 ± 0.04	
(E) SO_4-Ringer	1.03 ± 0.13	<0.001 (E *vs.* C)	1.39 ± 0.14	<0.001 (E *vs.* C)
Experimental condition	$HgCl_2$ $5 \cdot 10^{-4}$ M	P ($N = 8$)	$HgCl_2$ 10^{-3} M	P ($N = 8$)
(A) Basal	0.32 ± 0.04		0.37 ± 0.05	
(B) $HgCl_2$	0.37 ± 0.07	NS (B *vs.* A)	0.29 ± 0.02	NS (B *vs.* A)
(C) SO_4-Ringer	0.71 ± 0.05	<0.01 (C *vs.* A)	0.74 ± 0.08	<0.02 (C *vs.* A)
(D) Cl-Ringer	0.43 ± 0.05		0.34 ± 0.02	
(E) SO_4-Ringer	1.27 ± 0.10	<0.001 (E *vs.* C)	1.18 ± 0.09	<0.001 (E *vs.* C)

For each Hg column, J_w ($\mu l \cdot min^{-1} \cdot cm^{-2}$) corresponds to: (A) basal flow; (B) end of Hg pretreatment period; (C–E) plateau values during skin washings with SO_4- or Cl-Ringer. Top: data from paired skins pretreated with 10^{-4} M and 10^{-3} M $HgCl_2$, respectively. Bottom: data from paired skins pretreated with $5 \cdot 10^{-4}$ M and 10^{-3} M $HgCl_2$, respectively. In SO_4-Ringer J_w rose significantly in all groups, except for the first exposure to SO_4 in the 10^{-4} M group. Plateau J_w during the second exposure to SO_4 was always significantly higher than that of the first exposure. Row by row comparisons showed no significant differences between $5 \cdot 10^{-4}$ and 10^{-3} M groups; the differences were significant ($P < 0.02$) for rows C to E between the 10^{-4} and 10^{-3} M groups.

(Fig. 5 and Table 2). Before fixation, the outer surface of these latter skins had been washed with solutions containing no Hg for more than 5 h. Despite that, aggregates were present whether J_w was high (in SO_4-Ringer) or low (in Cl-Ringer), as shown in Fig. 5*A* and *B* (*see also* Fig. 2 and skins 5*a* and 5*b* of Table 2). The IMP aggregates were irregular in shape, comprised particles of different sizes (Figs. 4 and 5) and were not observed in all granular cells examined.

Sidedness of the SO_4 and Cl Effects

Figure 6*A* shows the effects of asymmetric anion substitutions in the bathing media. After exposure of both sides of the skin to SO_4-Ringer, Cl substituted for SO_4 only in the outer, diluted medium: no decrease in J_w was seen. In a set of seven such experiments, J_w even increased slightly from 1.54 ± 0.17 to 1.71 ± 0.20 $\mu l \cdot min^{-1} \cdot cm^{-2}$, ($P < 0.01$). In contrast, when Cl substituted for SO_4 only in the inner solution, J_w fell as seen before. Conversely, starting at low J_w, exposure of the skins to SO_4-Ringer only on their inner side, produced the full rise in J_w (Fig. 6*A*).

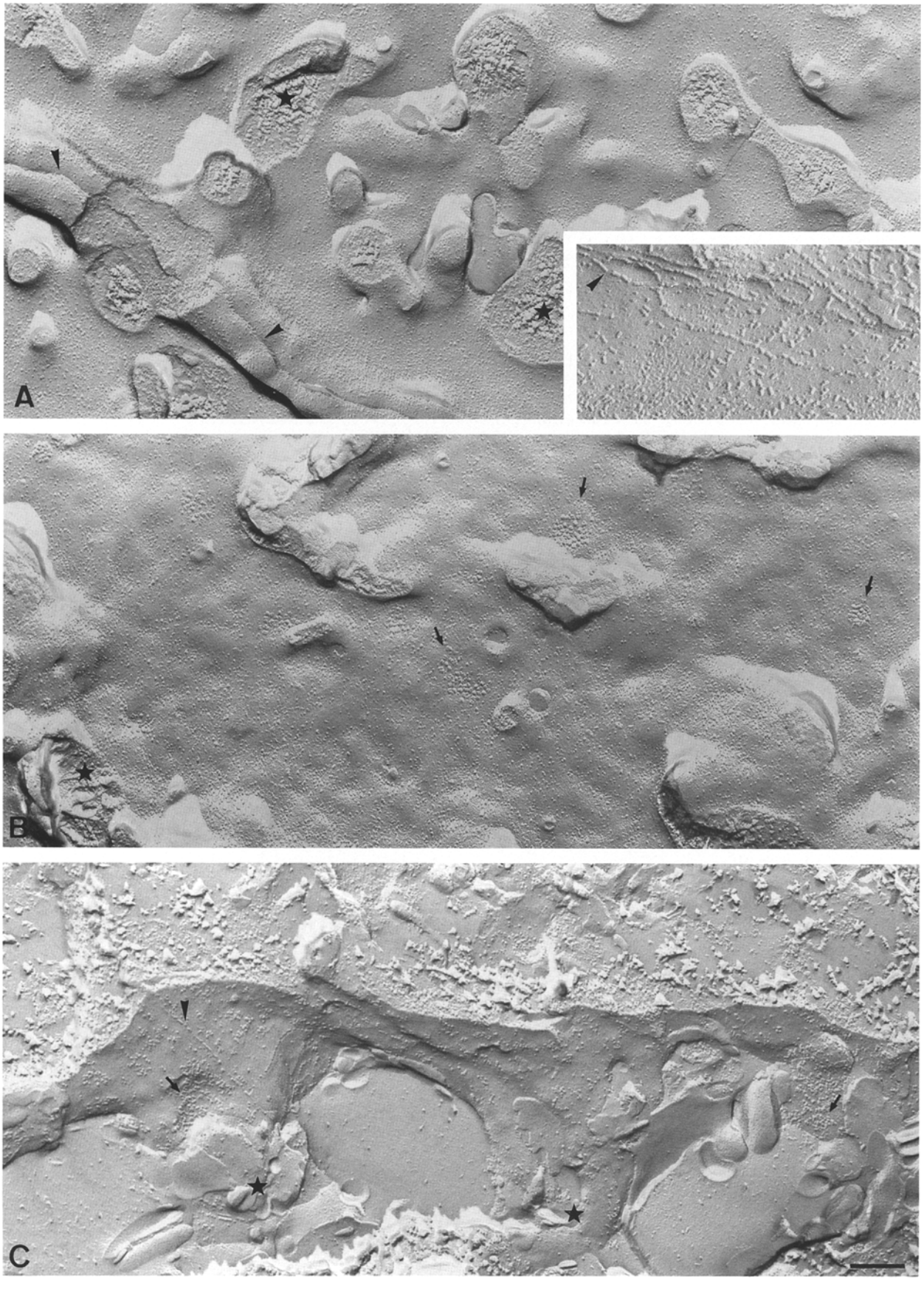
Figure 6*B* is representative of a set of five experiments in which skins that had reached a high plateau value of J_w in SO_4-Ringer were subsequently exposed on their inner surface to a solution constituted by ½ Cl-Ringer and ½ SO_4-Ringer. There was a significant drop in J_w ($N = 5$, $P < 0.001$) that represented 53% of the total drop observed with full Cl-Ringer. Conversely, starting at the low plateau value of J_w in full Cl-Ringer, exposure of the inner surface to ½ SO_4-Ringer led to a ΔJ_w of 0.76 $\mu l \cdot min^{-1} \cdot cm^{-2}$, whereas exposure to full SO_4-Ringer led to a total ΔJ_w of 1.50 $\mu l \cdot min^{-1}$ cm^{-2} (Fig. 6*B*).

Impermeant and Permeant Anions

Anion replacements were carried out with two other chemical species: gluconate, an impermeant anion, like sulfate; and nitrate, a permeant anion, like chloride.

Gluconate-Ringer caused a rise in water permeability similar to that induced by SO_4-Ringer (Fig. 7*A* and Table 3). Analogously, nitrate, like chloride readily reversed the state of high water permeability produced by an impermeant anion (Fig. 7*B* and Table 4).

In other experiments (*data not shown*), several combinations of anion replacements were tested, whose salient features were: (i) J_w rose with sulfate- or gluconate-Ringer in skins previously bathed with nitrate-Ringer; (ii) nitrate-Ringer reversed the effect of gluconate-Ringer; (iii) J_w did not change when nitrate substituted for chloride and vice-versa; (iv) there was a small decrease in J_w when gluconate


A
B
C

substituted for sulfate, and a small rise in J_w when sulfate substituted for gluconate. Even though the magnitude of the water permeabilization effect was higher with sulfate than with gluconate, these two chemically unrelated impermeant anions caused a rise in J_w that was similar in time course, stability of the plateau (at high J_w), reversal by permeant anions and iterative inducibility.

Hg Resistance of the SO_4-Induced High Water Permeability

It remained to be investigated if re-exposure of the outer surface of toad epidermis to Hg would affect the high, SO_4-induced J_w of skins pretreated with this metal. Hg caused only a slight, although significant, decrease in J_w that went from 1.29 ± 0.10 to 1.16 ± 0.08 $\mu l \cdot min^{-1} \cdot cm^{-2}$ ($P < 0.001$; $N = 8$). Starting at low J_w, in Cl-Ringer, Hg did not prevent the SO_4-induced rise in water permeability (*data not shown*).

Discussion

Anion Composition and Water Permeability

Previous work with toad skin showed that Hg prevents the stimulation of water flow by standard hydrosmotic agents, an effect that persists even after thorough and repeated washings with standard Cl-Ringer [17]. The new finding reported here is the marked increase in water permeability when skins thus treated were washed with SO_4-Ringer (Fig. 1*A*). One possible mechanism to explain this finding is that SO_4 re-establishes the permeance of the apical water pathway induced by hydrosmotic agents and blocked by apical Hg. That this was not necessarily the case is shown by the striking SO_4-induced rise in J_w observed in skins only exposed to apical Hg (Figs. 1 and 2).

The salient features of the SO_4 effect in Hg-treated skins are: (i) iterative inducibility; (ii) reversal by Cl or NO_3; (iii) mimicry by gluconate; (iv) inner epithelial polarity; (v) minor (10%) inhibition by Hg upon re-exposure to this metal. The SO_4-induced rise in J_w and P_f was observed in all skins tested. The magnitude of this effect increased, and the time course shortened, with successive exposures to SO_4-Ringer intercalated with exposures to Cl-Ringer (Figs. 2 and 3). In the main experimental group (Fig. 3*B*) basal P_f averaged 15.6 ± 0.86 $\mu m \cdot sec^{-1}$ and rose 3.2- and 4.6-fold during two challenges with SO_4. We have no obvious explanation for these changes in magnitude and time course. Throughout this work, basal P_f values were similar to those reported previously [12], whereas P_f values in SO_4-Ringer were in the range of those induced by vasopressin and high K^+, and only slightly lower than those found with isoproterenol [7–9, 12, 18]. At 60 min, the reversal of the high P_f state due to Cl represented 91% of ΔP_f caused by the previous exposure to SO_4 (Fig. 3*B*). In the two other experimental groups, this reversal ranged from 88 to 103% (Figs. 3*A* and *C*). A strikingly similar reversal was seen in skins exposed to NO_3 (Fig. 7*B* and Table 4), whereas gluconate mimicked the water permeabilization effect of SO_4 (Fig. 7*A* and Table 3).

Since Hg compounds were applied to the outer (epidermal) surface of toad skin, one could envisage the possibility that the effects of both Hg and anions are exerted on the outer side of an apical water pathway. However, our data show that anion replacement only in the inner medium induced the full effect observed when the same replacement was carried out in both inner and outer media (Fig. 6*A*). This inner polarity is apparently related to the anion permeability characteristics of the basolateral membrane of toad epidermis in the following manner: (a) impermeant anions (sulfate, gluconate) increased P_f; (b) permeant anions (chloride, nitrate) did not change basal P_f but reversed the high P_f induced by impermeant anions.

It is noteworthy that Cl/SO_4 substitutions in the outer medium also changed J_w and P_f, but did so slightly and in a direction opposite to that seen with the inner anion effect (*cf.* 2nd arrow in Fig. 6*A*). Qualitatively, these "outer anion" effects are analogous to those found in skins not exposed to Hg, when Cl/SO_4 substitutions took place in both outer

Fig. 4. Apical plasma membrane (P fracture face) of granular cells from the first-reacting cell layer of *Bufo marinus* skin. (*A*) In control epidermis (skin 1 of Table 2), intramembrane particles are distributed individually and homogeneously between microvilli and desmosomal stalks (some of the latter structures are indicated by stars). The arrowheads point to tight junctions, which are a characteristic feature of cells of the first-reacting cell layer. The inset shows a portion of the apical plasma membrane of a mitochondria-rich cell which, even though also present in the first-reacting cell layer, is easily distinguished from granular cells by the presence of rod-shaped intramembrane particles. (*B*) In epidermis exposed to $HgCl_2$ and isoproterenol (skin 3*a* of Table 2), some granular cells featured irregularly shaped aggregates of intramembrane particles (some of these structures are indicated by arrows). (*C*) Aggregates of intramembrane particles (arrows) were also seen in granular cell membranes of epidermis exposed to $HgCl_2$ and Cl-Ringer (skin 3*b* of Table 2). Part of the cytoplasm of a granular cell is seen in the upper part of this photograph. The bar represents 150 nm in *A*, *B* and *C* and 70 nm in the inset.

Table 2. Presence of intramembrane particle (IMP) aggregates in apical plasma membranes of granular cells of toad skin

Skin no.	Experimental condition	Ringer solution	J_w $\mu l \cdot min^{-1} \cdot cm^{-2}$	No. of cells	IMP aggregates
1	Control	SO_4	0.41	17	–
2	Control	Cl	0.30	13	–
3*a*	Hg + IP	Cl	0.50	20	+
3*b*	Hg alone	Cl	0.35	17	+
4*a*	Hg + wash.	Cl	0.30	41	+
4*b*	Hg + wash.	SO_4	1.13	11	+
5*a*	Hg + wash.	Cl	0.40	10	+
5*b*	Hg + wash.	SO_4	1.37	5	+

J_w values correspond to net water flows just before fixation. Skins 3*a* and 3*b* were fixed immediately after Hg exposure, while skins 4 and 5 were fixed several hours after removal of Hg and repeated washings with Cl- and SO_4-Ringer. For skins 3 to 5, paired segments (*a* and *b*) were examined under two different experimental conditions. The anion-induced changes in J_w observed in skin segments 5*a* and 5*b* are shown in Fig. 2*A* and *B*, respectively.

and inner media (*cf.* Fig. 1*D*), as well as to those reported in skins stimulated with high K^+ [18]. By their very rapid onset, they are reminiscent of the so-called hydrosmotic salt effect previously described in toad skin [1, 5].

Hg Effects on Water Pathways

In recent years, a great deal of interest has been focused on the ability of Hg compounds to block membrane pathways behaving as proteic water channels [47], whether these are constitutive membrane components [28, 29, 33, 49] or transport units transiently inserted by regulated exocytosis in granular and principal cells of vasopressin-sensitive epithelia [9, 20, 22, 39, 46]. In toad [20] and frog [21] bladders, exposure to Hg blocks the increase in water flow while still permitting the insertion of apical aggregates similar to those typically found in stimulated epithelia. Our results indicate that the same applies to toad skins pretreated with Hg and challenged with isoproterenol (Fig. 4*B* and Table 2, skin 3*a*).

The conspicuous SO_4-induced increases in P_f observed in skins pretreated with Hg, led to the disclosure that apical Hg induces *per se* the appearance of apical aggregates (Fig. 4*C* and Table 2, skin 3*b*); the latter resemble those described in the hydrosmotic actions of vasopressin, isoproterenol and high potassium in toad skin [7–9, 18]. Two features of Hg-induced aggregates deserve special mention: (a) their presence, whether J_w and P_f were high (in skins bathed in SO_4-Ringer), or low (in skins bathed in Cl-Ringer); (b) their persistence 4–5 h after Hg had been removed from the outer bathing medium. This persistence is in sharp contrast to the rapid retrieval of apical aggregates observed in cAMP-mediated hydrosmotic responses once the biological stimulus is removed [24]. However, there are agents that prevent or delay such a rapid retrieval [14, 19, 34, 35].

Collectively, our data and those of the literature suggest that Hg has at least two effects on water transport: (i) a blocking action on the permeance of constitutive and regulated membrane water pathways; (ii) a polarized exocytic effect characterized by the appearance of apical aggregates in an epithelium (toad epidermis) capable of developing a hydrosmotic response. This exocytic effect is not unique, however, since the SH-reagent *N*-ethylmaleimide (NEM), added to the outer medium, also induces the appearance of long-lasting aggregates in toad bladder [40]. Concerning the NEM effects on J_w and P_f, in both unstimulated and stimulated amphibian bladders, they may be either stimulatory or inhibitory, depending on the concentration of the SH-reagent [2, 36, 40].

Few agents are known to affect water flow when added to the outer surface of vasopressin-sensitive epithelia. In toad skin, the thiol-oxidizing agent diamide prevents the hydrosmotic effect of isoproterenol, but has no effect when the response is fully developed [16]. In toad bladder, phorbol myristate acetate increases P_f and promotes a massive exocytosis of granules from the epithelial granular cells [32]. The mechanism whereby NEM and Hg compounds induced the apical insertion of IMP aggregates very likely implies that these substances cross the apical membrane and have access to some critical site involved in the late, post-cAMP, steps of the hormonal stimulus-hydrosmotic response coupling.

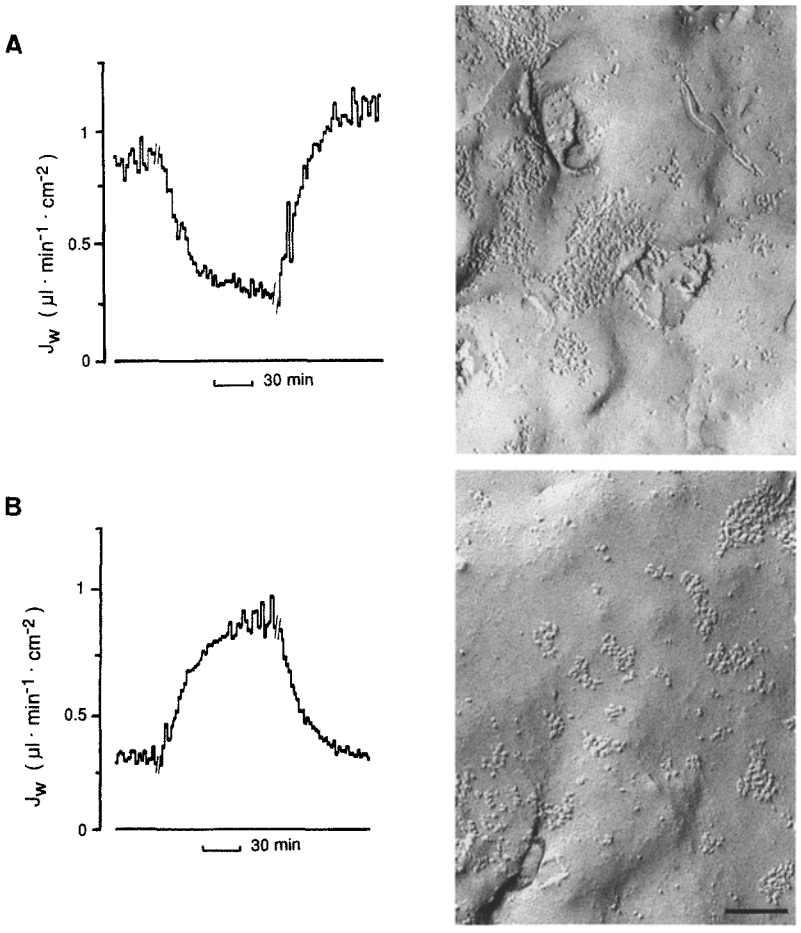


Fig. 5. Irregular aggregates of intramembrane particles were observed in the apical plasma membrane (P fracture face) of granular cells from the first-reacting cell layer, whether *Bufo marinus* skins exposed to $HgCl_2$ were fixed when net water flow was low (panel *A*, skin 4*a* of Table 2) or markedly elevated (panel *B*, skin 4*b* of Table 2), as shown in the corresponding recordings of J_w on the left side of each photograph. The bar represents 100 nm in *A* and *B*.

The NEM-induced rise in P_f can be inhibited by conditions that also inhibit the hydrosmotic response to vasopressin, in particular by exposure to cytoskeleton-disruptive drugs which are known to interfere with exocytosis [40].

Cell Volume, Cell Ionic Composition and Water Permeance

Replacement of Cl by different anions in isosmotic media bathing a variety of cell systems, such as muscle and kidney cortical slices [26, 30, 31], toad bladder [27] and frog skin [13, 15, 44], led to the following observations: (i) in Cl-free media containing an impermeant anion (e.g., SO_4, gluconate), cells lose virtually all Cl, an equivalent amount of cations (K, Na), and decrease in volume; (ii) reexposure to a Cl-containing medium allows reuptake of the ions previously lost and recovery of a normal cell volume. It is also known that, in both isosmotic and anisosmotic conditions, cell volume changes result in alterations of membrane transport systems that tend to minimize, or accelerate the correction of, the volume perturbations; such systems include Cl and K conductance channels, as well as co-transporters and counter-transporters [30, 31, 38, 42–44]. Assuming that analogous volume changes took place in the toad epidermis we studied, the apical water pathway induced by Hg would be closed in epithelia with normal cell volume (Cl- or NO_3-Ringer), and opened in epithelia with decreased cell volume (SO_4- or gluconate-Ringer). A similar correlation could be established, however, with concomitant changes in the cell content of diffusible ions, particularly Cl. To distinguish between these two possibilities, further work is needed with toad skins exposed to a variety of experimental conditions, such as anisosmotic media [42–44], Na-free media containing impermeant cations [31], and media containing weak acid anions like acetate [10, 26, 30]. Recent work in our laboratory, in which anisosmotic conditions were combined with Cl/SO_4 substitutions, indicates that both cell volume and cell Cl appear to influence the permeance of the Hg-induced water pathway; moreover, although a

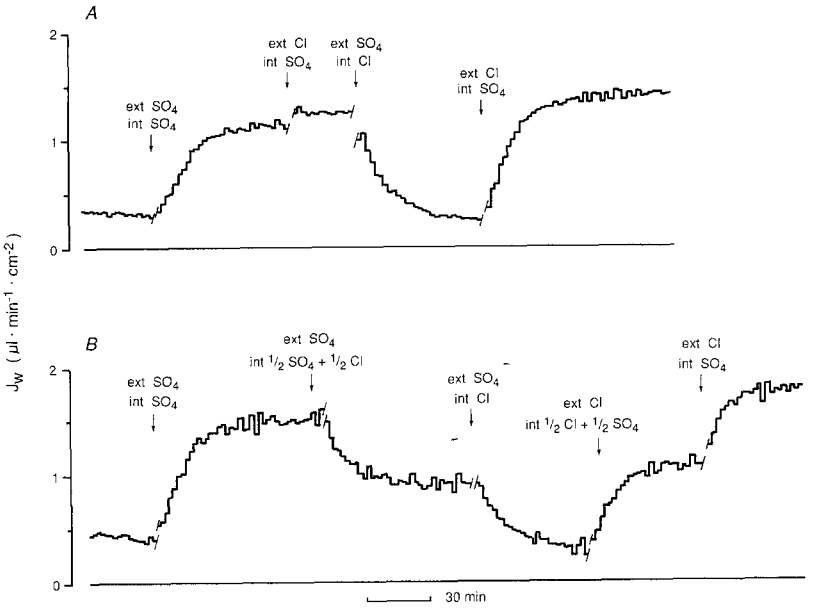


Fig. 6. Sidedness of the anion effects on J_w in toad skins pretreated with $HgCl_2$. (*A*) Asymmetrical anion replacements in the bathing media revealed that the typical effects of either SO_4 or Cl were found only when these anions were present in the inner Ringer solutions. (*B*) Partial (50%) replacement of the anion in the inner solution resulted in a decrement, or in an increment, of J_w that was half of that seen with total anion replacement.

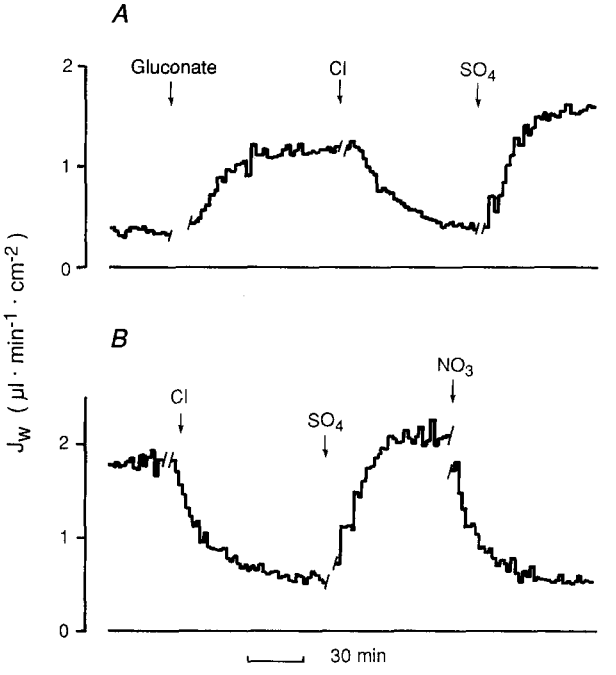


Fig. 7. Effects of impermeant (gluconate) and permeant (nitrate) anions on J_w, in skins pretreated with $HgCl_2$. Gluconate, like SO_4, markedly increased J_w, whereas nitrate, like Cl, reversed the effect of an impermeant anion.

Table 3. Changes in J_w induced by anions in toad skins pretreated with Hg

Experimental condition	J_w $\mu l \cdot min^{-1} \cdot cm^{-2}$	P ($N = 8$)
(A) SO_4-Ringer	1.11 ± 0.18	<0.01 (A *vs.* B)
(B) Cl-Ringer	0.38 ± 0.03	<0.001 (B *vs.* C)
(C) Gluc-Ringer	1.08 ± 0.12	NS (C *vs.* A)

Note the similarity of J_w increments caused by two impermeant anions, SO_4 and gluconate (Gluc).

Table 4. Changes in J_w induced by anions in toad skins pretreated with Hg

Experimental condition	J_w $\mu l \cdot min^{-1} \cdot cm^{-2}$	P ($N = 7$)
(A) Cl-Ringer	0.47 ± 0.04	<0.01 (A *vs.* B)
(B) SO_4-Ringer	1.68 ± 0.24	<0.001 (B *vs.* C)
(C) NO_3-Ringer	0.47 ± 0.06	NS (C *vs.* A)

Note the reversal of the SO_4 effect by a permeant anion, NO_3, down to the values observed in Cl-Ringer.

contribution of the paracellular pathway cannot be completely ruled out, the bulk of the anion-induced changes appears to involve the transcellular pathway associated with the apical aggregates (*submitted for publication*).

Hg compounds are SH-reagents that affect a large number of cell and membrane components, in a manner that might be toxic [4]. Therefore, it can be anticipated that their effects on epithelial water pathways be multiple and complex. Our results do not challenge the well-established blocking action of Hg on water channels [17, 28, 45, 47, 50] but reveal that, after withdrawal of Hg from the bathing

solution, this block persists, or reappears, in conditions where the epidermis is Cl-repleted, while the block vanishes in conditions where the epidermis is Cl-depleted. Whether such anion sensitivity primarily reflects alterations in the apical water pathway and/or in cell volume regulation [4, 37] remains to be established. Note, however, that the water pathway resulting from the exocytic effect of Hg does not appear to differ from that inserted by hormonal stimuli: in fact, preliminary experiments showed that anion-induced P_f changes similar to those reported here were found in toad skins challenged with vasopressin or isoproterenol, fixed with glutaraldehyde and then exposed to Hg (*unpublished observations*).

The mechanism underlying the blockage of the water channel by Hg is still largely unknown. There is evidence that this metal might act on the cytoplasmic side of this structure [41], a view consistent with recent reports on the Hg-induced inhibition of Na-K-ATPase [3, 23]. As a working hypothesis to explain our data, one could postulate that both Hg and Cl affect sites located on the cytoplasmic side of the apical water channel, the occupancy of both sites leading to channel closure, whereas the desorption of Cl results in channel opening. A modulatory effect of intracellular anions on the permeance of epithelial water channels has already been considered by other authors [6]. It is of crucial importance, however, to clearly establish if the characteristics of such a modulation differ in Hg-exposed cells and in cells not exposed to this metal. Until these experimental data become available, further speculation seems unwarranted. It is all too evident that our simple working hypothesis does not account for all the results presented here, but it may be useful to explore new aspects of the water channel behavior. In any event, toad skins pretreated with Hg compounds appear to be a unique in vitro model for studying the interplay between cell volume, cell ionic composition and membrane water permeability.

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