*Biochimica et Biophysica Acta*, 1104 (1992) 174–178


BBAMEM 75532

# Conductive chloride flux across amphibian skin: inhibition by indacrinone and cobalt ion

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(Received 17 July 1991)

Key words: Chloride flux; Indacrinone; Cobalt; (Amphibian skin)

**When amphibian skin was incubated under conditions in which transepithelial sodium transport was abolished, a conductive transepithelial $Cl^-$ flux arose when $Cl^-$ was removed from one of the compartments. This flux was matched by short-circuit current and it accounted entirely for transepithelial conductance. $Cl^-$ influx was larger than efflux; it was linearly related to the magnitude of transepithelial $Cl^-$ concentration difference. When applied to the epithelial surface of the tissue, divalent metal cations such as $Co^{2+}$, and the ethacrynic acid derivative, indacrinone, reduced rapidly and reversibly both transepithelial $Cl^-$ (in)flux and short-circuit current. Frog skin proved to be more sensitive to these inhibitors than toad skin. Further characterization of transepithelial $Cl^-$ pathway(s) should benefit from the fact that $Cl^-$ across amphibian skin can easily be monitored by the short-circuit current method, and from the availability of agents which inhibit this passive flux rapidly and reversibly.**

## Introduction

Transepithelial ohmic conductance, $G_t$, has been reported to decrease rapidly and reversibly when indacrinone or divalent metal cations were applied to the external surface of frog skin incubated in Ringer's solution, even though sodium transport was stimulated by these agents [1,2]. The decrease in $G_t$ almost equalled in magnitude that which resulted from exposure of the epithelial surface of the tissue to $Cl^-$-free solution. In fact, $G_t$ was not depressed by either indacrinone or divalent cations in the absence of $Cl^-$ [1,2].

In view of these findings, the role assumed by $Cl^-$ in tissue conductance, $G_t$, was examined after inhibition of sodium transport by amphibian skin as $G_t$ is thereby reduced to shunt conductance, $G_{sh}$. The latter, in turn, is known to depend to a large extent on external $Cl^-$ [2]. When $Cl^-$ was removed from one compartment during incubation of skin preparations prevented from transporting sodium, the transepithelial $Cl^-$ flux which resulted, was found to be quantitatively conductive. The agreement between $Cl^-$ flux and electrical measurements in the conditions selected completes observations made initially by Voûte and Meier [3]. Furthermore, divalent metal cations and indacrinone proved capable of interfering readily and reversibly with conductive $Cl^-$ flux. This work has been reported in part in abstract form [4].

## Material and Methods

Studies were conducted on the abdominal skin of the toad, *Bufo marinus* (originating from the Dominican Republic), or the frog, *Rana esculenta*. Toads were maintained on moist peat at room temperature and fed weekly; frogs were stored in running tap water at 4°C. The animals were sacrificed by double pithing whereupon their abdominal skin was dissected free and prepared for incubation according to Ussing and Zerahn [5].

Incubation media were frog Ringer's solution (composition, in mmol/l: NaCl, 115; $KHCO_3$, 2.5; $CaCl_2$, 1.0), or a solution in which $Mg^{2+}$ or $K^+$ were substituted for $Na^+$. Gluconate or sulphate were used to replace $Cl^-$ when required. Osmolality was maintained at 0.225 mosmol/kg $H_2O$ by addition of sucrose when divalent ions were used as substitutes.

The transepithelial movement of $Cl^-$ was measured across short-circuited preparations, using $^{36}Cl$ (Amersham) added to one side, and sampling from both sides at appropriate time intervals after 30–60

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min allowed for equilibration. The overall concentration of $Cl^-$ was determined by a potentiometric method. $^{36}Cl$ was counted by liquid scintillation spectrometry for a sufficient time to keep the statistical counting error at 2% or less.

The drugs used were:
on the chorial side: ouabain, 0.1 mmol/l;
on the epithelial side: indacrinone, 1 mmol/l; amiloride, 0.1 mmol/l; cobalt acetate, 5 mmol/l.

The data were analyzed statistically by standard methods [6].

## Results

### *(1) Permeability of amphibian skin to chloride: contribution of this anion to transepithelial conductance*

When chloride permeability of frog and toad skin was assessed, $Cl^-$ influx and efflux across matched sodium-transporting preparations were of equal magnitude in the short-circuited state (Table I). This is in keeping with the conclusion that transcutaneous $Cl^-$ flux is passive in most Amphibia [7,8]. In these conditions transepithelial chloride movement accounts for a sizeable fraction of transepithelial conductance, $G_t$ [10], as illustrated by Fig. 1. Indeed, after removal of sodium from the epithelial side of toad skin, so that $G_t$ reduces to $G_{sh}$, there was a linear relationship between tissue conductance and unidirectional $Cl^-$ flux. Furthermore, the data suggest that, in absence of sodium transport, no other ion plays a significant role in tissue conductance.

### *(2) Conductive nature of transepithelial chloride flux*

The relationship between $Cl^-$ flux and $G_{sh}$ illustrated above, indicates that this flux is largely conductive. This point was examined further by imposing a transepithelial concentration gradient for $Cl^-$ across

TABLE I

*Transepithelial $Cl^-$ flux across sodium-transporting amphibian skin*

Values are means ± S.E.

| $Cl^-$ flux: direction [a] | $I_{sc}$ ($\mu A\ cm^{-2}$) | $G_t$ ($mS\ cm^{-2}$) | $J_{Cl}$ ($pmol\ cm^{-2}\ s^{-1}$) |
|---|---|---|---|
| Frog ($n = 6$) | | | |
| Efflux | 32 ± 4 | 0.47 ± 0.05 | 49 ± 7 |
| Influx | 33 ± 4 | 0.50 ± 0.05 | 55 ± 12 |
| Difference [b] | 1.0 ± 2.1 | 0.03 ± 0.02 | 6 ± 8 |
| Toad ($n = 3$) | | | |
| Efflux | 10 ± 4 | 0.55 ± 0.07 | 88 ± 12 |
| Influx | 12 ± 5 | 0.45 ± 0.04 | 51 ± 21 |
| Difference [b] | 2.0 ± 1.2 | 0.10 ± 0.04 | 3 ± 31 |

[a] Efflux: from chorial to epithelial surface; influx: from epithelial to chorial surface.

[b] None of the differences (paired experiments) reached statistical significance.

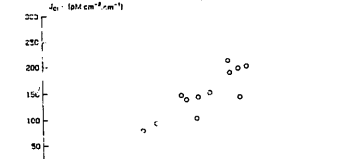


Fig. 1. Chloride flux across toad skin as a function of transepithelial conductance in absence of sodium transport. For this series of experiments, the epithelial surface of the preparation was exposed to Ringer's containing $MgCl_2$, 57.5 mM, instead of NaCl, 115 mM. Residual (shunt) conductance, $G_{sh}$, appears on the abscissa, given that $G_{sh} = G_t - G_{Na^+}$, and that $G_{Na^+} = 0$ in the experimental conditions selected. When $J_{Cl^-}$ is expressed in terms of partial chloride conductance, $G_{Cl^-}$ [9], the following equation obtains: $G_{Cl^-} = -0.03 + 0.78\ G_t$ ($r = 0.87$; $n = 12$; $P < 0.01$).

the tissue when sodium transport was blocked by means of amiloride and ouabain *, or when $Na^+$ was replaced with $K^+$ in Ringer's fluid.

Removal of $Cl^-$ from one side of toad skin incubated as described, generated a conductive flux of this ion, since it was associated with an electrical potential difference across the tissue. This $Cl^-$ flux was matched by the current required to short-circuit the preparation, $I_{sc}$ (Fig. 2). In Ringer's solution, $Cl^-$ flux and $I_{sc}$ were about one order of magnitude larger when $Cl^-$ was made to move inward rather than outward. In fact, efflux was negligible, averaging 19 pmol $cm^{-2}\ s^{-1}$, with $I_{sc} \leqslant 2\ \mu A\ cm^{-2}$. Since this asymmetry has been ascribed to intracellular electronegativity [11], $Cl^-$ flux was measured in $K^+$-Ringer's, so as to depolarize epithelial cells [12]. In these conditions, $Cl^-$ influx still exceeded efflux. On the other hand, both $Cl^-$ influx and (mainly) efflux were of larger magnitude than in Ringer's, as was $G_t$, which is in keeping with an earlier report [13]. It is of relevance to note that $Cl^-$ flux across depolarized tissue remained quantitatively conductive (Fig. 2).

The $K^+$-depolarized preparations did not appear to be irreversibly damaged, even after 4–6 h. Indeed, mean $I_{sc}$ across toad skin examined within 30 min of

* Amiloride was added after at least one hour of exposure of the skin preparations to ouabain. In such conditions the drug usually failed to influence $I_{sc}$ any further, which was considered as evidence for complete inhibition of the sodium pump by the glycoside; furthermore, it failed to interfere significantly with $Cl^-$ flux that averaged 144 pmol $cm^{-2}\ s^{-1}$ across toad skin exposed to ouabain alone, and 168 after subsequent addition of amiloride ($\Delta \pm$ S.E.: 24 pmol $cm^{-2}\ s^{-1} \pm 12$; $n = 7$).

TABLE II

*Conductive chloride influx across toad skin in absence of sodium transport: dependence on chloride concentration gradient*

Values are means ± S.E. These studies were carried out on eight paired toad skin preparations, exposed to Ringer's fluid on the basolateral side (+ouabain), with gluconate replacing $Cl^-$. On the epithelial side, Ringer's (+amiloride) was used as such or diluted 1:1 with a solution containing gluconate as the anion.

| $Cl^-$ concentration on the epithelial side | $J_{Cl^-}$ (pmol $cm^{-2}\ s^{-1}$) | $I_{sc}$ (pmol $cm^{-2}\ s^{-1}$) | $G_t$ (mS $cm^{-2}$) |
|---|---|---|---|
| 115 mol/l | 214 ± 51 | 201 ± 55 | 0.65 ± 0.17 |
| 57.5 mmol/l | 111 ± 31 | 92 ± 27 | 0.33 ± 0.10 |

resuming incubation in standard Ringer's after exposure to $K^+$-Ringer's, was 30 $\mu$A $cm^{-2}$, vs. 25 at the outset ($\Delta$ ± S.E.: 5 ± 6 $\mu$A $cm^{-2}$; $n = 5$; n.s.). $G_t$ was likewise barely affected, averaging 0.8 before, and 1.2 mS $cm^{-2}$ after depolarization ($\Delta$ ± S.E.: 0.4 mS $cm^{-2}$ ± 0.2).

*(3) Transepithelial chloride flux and chloride concentration gradient*

The conductive Cl flux dealt with here appears to be proportional to the $Cl^-$ concentration gradient. Indeed, measurements carried out on paired toad skin preparations incubated in Na-Ringer's fluid indicate that $Cl^-$ influx was reduced by 50% when the concentration of this anion on the epithelial side went from 117 to 58.5 mmol/l; $G_t$ decreased to the same extent (Table II).

*(4) Inhibition of the conductive transepithelial chloride pathway*

Indacrinone and divalent metal cations are effective inhibitors of chloride-related transepithelial conductance [1,2]. These substances rapidly depressed both $I_{sc}$ and $Cl^-$ influx across depolarized preparations, as summarized in Table III. Frog skin proved considerably more sensitive to these inhibitors than toad skin, in agreement with previous observations [1,2]. There was some discrepancy between $I_{sc}$ and $Cl^-$ influx during exposure of frog skin to $Co^{2+}$, for no apparent reason.

The preparations appeared essentially unharmed, at least after short exposure (< 30 min) to these agents. Indeed, in the case of toad skin treated with $Co^{2+}$, $I_{sc}$ and $G_t$ had returned to control levels within half an hour ($\Delta I_{sc}$ ± S.E.: 1.4 $\mu$A $cm^{-2}$ ± 1.4, and 0.05 mS $cm^{-2}$ ± 0.05, respectively; $n = 7$). There was no lasting effect of indacrinone on $I_{sc}$ across frog skin either ($\Delta$ ± S.E.: 3.6 $\mu$A $cm^{-2}$ ± 2.3; $n = 8$); on the other hand, $G_t$ failed to recover completely ($\Delta$ ± S.E.: 0.17 mS $cm^{-2}$ ± 0.08).

*(5) The conductive chloride pathway and transepithelial conductance of non sodium-transporting amphibian skin*

The changes in $G_t$ and in $Cl^-$ flux for frog and toad skin examined as described, were closely correlated.

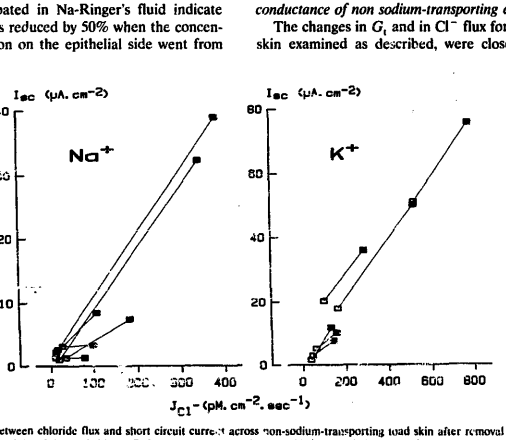


Fig. 2. Relationship between chloride flux and short circuit current across non-sodium-transporting toad skin after removal of chloride on one side of the tissue. Sets of the abdominal skin of *Bufo marinus* were incubated in $Na^+$-Ringer's (left) or $K^+$-Ringer's (right). In the former case, sodium transport had been blocked by ouabain, and subsequent addition of amiloride. Net chloride flux resulted from removal of $Cl^-$ from the external (□) or from the internal (■) bath. The lines connect data points from matched preparations.

TABLE III

*Inhibition by indacrinone and by cobalt of conductive chloride influx across depolarized amphibian skin*

Values are means ± S.E. Short-circuit current ($I_{sc}$) and $Cl^-$ influx ($J_{Cl^-}$) were measured on amphibian skin incubated in $K^+$-Ringer's. Data were collected for the 40 min period prior to addition of inhibitor (control) and again for the 20–60 min period which followed. There were 6–8 preparations for each of the four sets of experiments.

| Experimental conditions | $I_{sc}$ (pmol $cm^{-2}$ $s^{-1}$) | $J_{Cl^-}$ (pmol $cm^{-2}$ $s^{-1}$) | $G_t$ (mS $cm^{-2}$) |
|---|---|---|---|
| Frog skin | | | |
| Control | 686 ± 92 | 664 ± 69 | 1.94 ± 0.04 |
| $+Co^{2+}$ | 78 ± 22 | 166 ± 11 | 0.72 ± 0.04 |
| Inhibition * | 88 ± 4 | 73 ± 1 | 60 ± 5 |
| Control | 966 ± 221 | 951 ± 180 | 2.35 ± 0.40 |
| + Indacrinone | 415 ± 169 | 427 ± 132 | 1.33 ± 0.32 |
| Inhibition *: | 68 ± 10 | 62 ± 8 | 47 ± 5 |
| Toad skin | | | |
| Control | 544 ± 78 | 586 ± 78 | 1.87 ± 0.23 |
| $+Co^{2+}$ | 415 ± 57 | 476 ± 95 | 1.51 ± 0.18 |
| Inhibition *: | 22 ± 5 | 22 ± 13 | 18 ± 4 |
| Control | 406 ± 105 | 425 ± 97 | 1.43 ± 0.34 |
| + Indacrinone | 249 ± 63 | 274 ± 65 | 0.85 ± 0.19 |
| Inhibition *: | 33 ± 11 | 32 ± 7 | 36 ± 6 |

* Inhibition refers to (1 − residual value/control value) × 100, calculated for individual preparations.

Thus the tentative conclusion was drawn that even in $K^+$-depolarized preparations, $K^+$ did not contribute to transepithelial conductance. This was borne out by the lack of effect of $Ba^{2+}$ on $G_t$ in the conditions selected (data not shown).

For toad skin, whether depolarized or not, there was a direct relationship between $I_{sc}$ and $G_t$, irrespective of the direction of $Cl^-$ flux (Fig. 3). This reflects the observation that, for a given set of preparations, transepithelial electrical potential difference appeared to be largely independent from the magnitude and direction of net $Cl^-$ flux. Indeed, values for this parameter were independent from the direction of $Cl^-$ flux in Na-Ringer's solution, ($\Delta \pm$ S.E.: 2.6 mV ± 5.3). Furthermore, the transepithelial electrical potential difference across matched $K^+$-depolarized preparations was also close to the value obtained in Na-Ringer's, again irrespective of the direction of $Cl^-$ flux.

## Discussion

Soon after the demonstration by Ussing and Zerahn [5], that there is a straightforward electrical counterpart to net, active transport of sodium by amphibian skin, attention was drawn to the role of $Cl^-$ in transepithelial conductance, $G_t$ [7,10]. Further work led to the conclusion that $Cl^-$ movement across amphibian skin is at least in part conductive, involving channels [14,15]. Thus Voûte and Meier [3] attempted to define experimental conditions in which $Cl^-$ flux was reflected by simple electrical parameters. As shown here, $Cl^-$ flux can be made to be entirely conductive (see also Ref. 16). Indeed, when a transepithelial $Cl^-$ concentration gradient was imposed across toad and frog skin in the absence of sodium transport, the $Cl^-$ flux

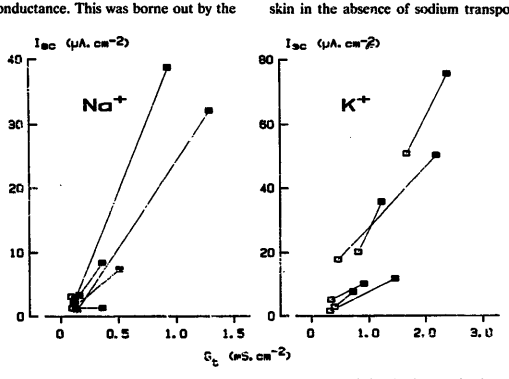


Fig. 3. Relationship between the short-circuit current and tissue conductance across toad skin, in absence of sodium transport. The data correspond to the abdominal skin preparations of *Bufo marinus* dealt with in Fig. 2. The filled symbols represent preparations incubated so that a $Cl^-$ influx was observed, while the open symbols represent matched preparations across which $Cl^-$ efflux took place.

was matched by short-circuit current. Incidently, the relationship between $Cl^-$ flux and $G_{sh}$ on the one hand (Fig. 1), $I_{sc}$ on the other hand (Fig. 2), indicates that artifacts (e.g. edge damage) were negligible in the case of amphibian skin preparations incubated as described.

$Cl^-$ flux across amphibian epithelia is passive in most species. Certainly, this appears to be the case for the skin of *Bufo marinus* and *Rana esculenta* (Table 1). Yet, when preparations were incubated in Ringer's solution in absence of sodium transport, for a given transepithelial $Cl^-$ concentration difference, $Cl^-$ influx was much larger than efflux at short-circuit. Electronegativity of the epithelial cells does not account alone for this asymmetry [11] since $Cl^-$ influx still exceeded efflux across $K^+$-depolarized skin [12]. The reason for the asymmetry observed might lie instead in the apparent requirement for $Cl^-$ on the epithelial side of amphibian skin for activation of apical $Cl^-$ channels [17]. Swelling of mitochondria-rich cells which occurs when $Cl^-$ was present in the epithelial side of the tissue, has also been considered in this respect [3,18].

Both $Cl^-$ influx and efflux were appreciably larger across $K^+$-depolarized preparations. This has been ascribed to activation of adenylate cyclase in $K^+$-Ringer's [19].

All these data imply that transepithelial $Cl^-$ flux involves cells of the skin epithelium. Since the principal cells of amphibian skin lack apical $Cl^-$ channels [20], attention has focussed on the other cell population of this epithelium, namely the mitochondria-rich cells [15]. Admittedly, a paracellular route of passage of $Cl^-$ across the epithelium cannot be ruled out at present [21]. Skin glands do not seem to be critically involved, as indicated by studies conducted on frog skin epithelium isolated from underlying chorion [22].

Even though a cellular pathway for transepithelial $Cl^-$ flux is a distinct possibility, $Cl^-$ influx appeared linearly related to the transepithelial $Cl^-$ concentration difference. This is at variance with an earlier report which emphasized the existence of a saturable component of $Cl^-$ flux across frog skin [23]. The fact that the experiments discussed here were conducted in absence of sodium transport might account for this difference.

In view of the conductive nature of the transepithelial $Cl^-$ flux dealt with here, a relationship between $I_{sc}$ and $G_t$ could be expected. In fact, the data suggest that no other ion contributes to tissue conductance in the experimental conditions adopted (Fig. 3). As a counterpart to the relationship observed between $I_{sc}$ and $G_t$, the electrical potential difference recorded across a given set of skin preparations in the open-circuit state, was remarkably little influenced by the experimental protocol.

The conductive $Cl^-$ flux was significantly reduced by $Co^{2+}$ and indacrinone added to the epithelial side, more so in the case of frog skin, as had been observed previously [1,2]. Furthermore, these substances acted on $Cl^-$ flux in a readily reversible way. The rapidity of the response provides an argument for the apical localization of the $Cl^-$ conductive pathway(s).

**Acknowledgments**

We wish to thank Dr. F.C. Herrera for valuable comments, Mrs. Mollet who tirelessly typed this manuscript, and Dr. K. Veith who corrected its english. The belgian Agencies FNRS and FRSM provided financial support. Amiloride and indacrinone were gifts of Dr. Cragoe, Jr., Merck, Sharp and Dohme.

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