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THE EXCRETION OF HYDROGEN ION BY THE ISOLATED AMPHIBIAN SKIN: EFFECTS OF ANTIDIURETIC HORMONE AND AMILORIDE

M. G. EMÍLIO and H. P. MENANO

Laboratory of Physiology, Gulbenkian Institute of Science, Oeiras (Portugal)

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SUMMARY

H^+ extrusion by the isolated skins of two amphibia, *Rana ridibunda* and *Bufo bufo*, was studied in order to test for the presence of exchange mechanisms of the type Na^+/H^+ and Cl^-/HCO_3^-, which have been described in several epithelial structures. The preparations were mounted in chambers of the Ussing type, so that the short-circuit current could be used as a function of Na^+ transport and the pH-stat technique was utilized to determine the rates of H^+ extrusion under different experimental conditions. These conditions were either the withdrawal of the ions intervening in the mentioned exchanges (Cl^- or Na^+), or the addition of drugs with well-known effects on Na^+ uptake and transport (antidiuretic hormone and amiloride).

In the frog skin, H^+ excretion was detected in solutions containing either Cl^- or SO_4^{2-}, with identical rates. Again, Na^+ substitution by Mg^{2+} had no effect on H^+ excretion rates, neither did the suppression of Na^+ influx by amiloride or its stimulation by antidiuretic hormone. These experiments were repeated with similar results in gland-free preparations of the epidermis of frog skin separated from the corion by the action of collagenase.

Experiments in toad skin showed that H^+ excretion could not be detected when Cl^- was present in the outer medium, but became apparent if an impermeant anion, SO_4^{2-}, was used. This observation is compatible with the existence of an exchange mechanism of the type Cl^-/HCO_3^-. Secondly, in these preparations H^+ extrusion increased after stimulation with antidiuretic hormone and decreased when amiloride was used or when Na^+ was substituted by Mg^{2+}, suggesting that at least a fraction of the total H^+ efflux is linked to Na^+ influx. In the isolated frog skin this mechanism does not seem to be operative.

INTRODUCTION

H^+ secretion by epithelial structures can be mediated by a cation-exchange mechanism, generally of the type Na^+/NH_4^+ or Na^+/H^+, as in the gills of some fish [1–3] or by an active, pump-mediated, specific process, as in the urinary bladder of the turtle and the toad [4, 5]. Either mechanism might be put forward to explain the phenomenon of H^+ extrusion through the amphibian skin, which has been described in vivo [6, 7] and in vitro [8–10]. According to Garcia Romeu et al. [6], the net Na^+ transport across the skin of the Chilean frog is exactly equivalent to the H^+ efflux; Kirschner et al. [7], working with *Rana pipiens*, found a stoichiometric relationship between the total Na^+ influx and the H^+ efflux, suggesting the presence of an exchange mechanism for the two ions. This type of relationship between Na^+ and H^+ transport was not confirmed in experiments carried out on the isolated frog skin [10]. Namely, no correspondence was found between the short-circuit current and the capacity to acidify the external medium. On the other hand, a Cl^-/HCO_3^- exchange has been shown to occur in the in vivo experiments referred to above in such a way that the H^+ excreted by the skin was buffered by the HCO_3^- and could not be detected when Cl^- was present in the external medium. However, in our former studies no HCO_3^- could be detected in the external bathing medium, although a Ringer solution containing Cl^- was used in the experiments. These differences between results obtained in vitro and in vivo could be due either to the different experimental conditions or to differences between the amphibian species utilized.

The present work was carried out in two different anura, *Rana ridibunda* and *Bufo bufo*. H^+ excretion through the isolated skin was studied under different conditions of Na^+ uptake and transport, namely, in the absence of Na^+ from the external medium, and when the preparations (a) were under the influence of a strong inhibitor of Na^+ uptake, amiloride, or (b) had been treated with antidiuretic hormone as a stimulant of Na^+ transport. The results were always compared with those obtained under "control" conditions, i.e. with the preparations bathed by a Ringer solution with 114 mequiv/l of Na^+ and before the addition of drugs. In order to test the possible existence of a Cl^-/HCO_3^- exchange at the outer border that might conceal the excretion of H^+, the effect of substituting Cl^- by a non-permeant anion, SO_4^{2-}, was also studied. The results suggest that there are some differences between the behaviour of frog skin and toad skin as to the mechanisms of excretion of H^+.

Furthermore, a group of experiments was carried out using the epithelium of frog skin isolated from the corion by the combined action of collagenase and hydrostatic pressure [11, 12]. As these preparations are virtually free of glands, they were used to test whether the secretion of glandular products into the external bathing medium would in some way influence the detection of H^+ excretion. This hypothesis was not confirmed since the results obtained with the isolated sheets of epithelium and with the whole skin were very similar. Unfortunately this method could not be applied to the toad skin, as the very strong adherence between epithelium and corion did not allow the separation of an epithelial sheet with the necessary dimensions for the chamber utilized in the experiments.

METHODS

Frogs of the species *R. ridibunda* were kept in a cold chamber (8–10 °C) in running tap water for 1–7 days before being used. Toads (*B. bufo*) were kept in moist earth at room temperature. Two or three days before the experiments they were transferred to a tank with de-ionized water which was changed daily. After double-pithing the animals, a piece of the abdominal skin was dissected and mounted in a chamber of the Ussing type with an area of 7.1 cm^2, modified to hold a pH electrode. Spontaneous acidification of the external medium was recorded in every experiment

TABLE I

IONIC CONCENTRATIONS IN THE RINGER SOLUTIONS

Data are expressed as mequiv/l.

Ringer solutions	Cl^-	SO_4^{2-}	Na^+	Mg^{2+}	K^+	Ca^{2+}
NaCl	121	–	114	–	2.4	4.8
$MgCl_2$	121	–	–	114	2.4	4.8
Na_2SO_4	–	121	114	–	2.4	4.8
$MgSO_4$	–	121	–	114	2.4	4.8

in a preliminary phase, and then the pH-stat technique was used to measure the rates of H^+ extrusion towards the external half chamber; 2 mM KOH was used as titrant. Short circuiting of the preparations was obtained using an automatic clamping device and the short-circuit current (s.c.c.) was continuously recorded. Further details on methods have been described elsewhere [10].

The solutions used in the experiments were buffered by Tris (0.1 mM) and had the pH value adjusted to 6.9–7.1. The osmolality was compensated to 220 mosm/l with manitol, when necessary. The ionic composition of these solutions is shown in Table I. Ringer substitutions were always done symmetrically in both half chambers, the volume used in each half chamber being 7 ml. The solutions were stirred and aerated by bubbling with air free of CO_2, as it has been shown that this procedure is effective in minimizing the trapping of metabolic CO_2 in the bathing solutions [10].

The isolated epithelium of the frog skin was utilized in a group of experiments. This preparation was obtained by the technique described by Aceves and Erlij [11] and Rajerison et al. [12]. Briefly, this technique consists of submitting a piece of frog skin to the combined action of collagenase (45 munits/ml) and hydrostatic pressure (4–5 cm of water) for 90 min at 35 °C. After this period of incubation a sheet of epithelium can be easily separated from the corion and utilized for the experiments in the same way as the whole skins. Particular details of the different protocols will be given in the next section.

RESULTS

Ion substitution

Cl^-/SO_4^{2-}. In a preliminary phase of this study, NaCl/Ringer was utilized to mount both toad skin and frog skin preparations in order to quantify the H^+ excretion towards the outside solution with a "normal" (114 mM) Na^+ concentration. The toad skin preparations mounted in this Ringer failed to show any capacity to acidify the external medium. The net flux of Na^+, evaluated from the s.c.c. values, was of the same order of magnitude as that obtained in frog skin experiments (Table II, group a). The pH of the outside solution either did not vary or became slightly alkaline (pH 7.2–7.4). On the contrary, when Na_2SO_4 was used instead of NaCl, H^+ excretion became evident as a spontaneous progressive pH decrease of the external medium and it could be quantified by titration (Table II, group b). In view of this fact, SO_4^{2-} was used as the main anion in further experiments with toad skin.

TABLE II

RATES OF H^+ EXCRETION AND Na^+ NET FLUX IN TOAD SKIN AND FROG SKIN PREPARATIONS BATHED IN SOLUTIONS OF DIFFERENT IONIC COMPOSITION

Na^+ net fluxes were calculated from the s.c.c. values. Means and S.E. values are shown. Number of experiments in parentheses. n.d., not detectable. The units of measurement are nequiv · min^{-1} · 7.1 cm^{-2}.

		NaCl	Na_2SO_4	$MgSO_4$	$MgCl_2$	Na_2SO_4
Toad skin						
a (9)	H^+	n.d.	–	–	–	–
	Na^+	52±5	–	–	–	–
b (10)	H^+	–	4.3±0.6	2.8±0.6	–	4.7±0.8
	Na^+	–	72±12	n.d.	–	76±12
Frog skin						
c (10)	H^+	6.8±0.7	6.3±0.8	–	–	–
	Na^+	90±10	65±8	–	–	–
d (9)	H^+	5.8±0.4	–	–	5.4±0.4	–
	Na^+	55±7	–	–	n.d.	–

Previous studies [10] had shown that this problem did not arise with frog skin as preparations of this organ did acidify the external medium when chloride-Ringer was used. Nevertheless, ten preliminary experiments were designed to verify whether the measurable H^+ excretion would be different if SO_4^{2-} were used instead of Cl^- in the bathing solutions. Na_2SO_4/Ringer was used alternately with NaCl/Ringer to fill both sides of the chamber for periods of about 60 min and the H^+ excretion rates were monitored under both conditions. The results of these experiments, included in Table II (group c), show that there is no statistically significant difference between the mean values of the rates obtained under both sets of conditions. In all the following experiments with frog skin, Cl^- was used as the main anion.

Na^+/Mg^{2+}. The substitution of Na^+ by Mg^{2+} in the bathing solutions was assayed in order to test whether H^+ excretion would be modified by the absence of Na^+ in the external medium. In these experiments Na_2SO_4/Ringer (for toads) or NaCl/Ringer (for frogs) was used during an initial period of about 60 min and then the chambers were carefully rinsed and refilled with $MgSO_4$- or $MgCl_2$/Ringer, respectively, for a second period of measurements. The s.c.c. and H^+-excretion rates were monitored continuously. In the experiments with toad skin, Na_2SO_4/Ringer was used again in a third period. The results, also summarized in Table III (group b), show that in the toad skin preparations there was a decrease of H^+ rates and a complete blockage of the s.c.c. when $MgSO_4$/Ringer was used, followed by a full recovery of the control values when Na_2SO_4/Ringer was introduced. In the experiments carried out in frog skin no significant difference was found between the rates of H^+ excretion obtained either with NaCl- or $MgCl_2$/Ringers. The s.c.c. fell to very low values in the absence of Na^+, but an inversion of the current was not observed (Table II, group d).

Effects of antidiuretic hormone and amiloride

The next experiments were designed to study the H^+ excretion of our prepara-

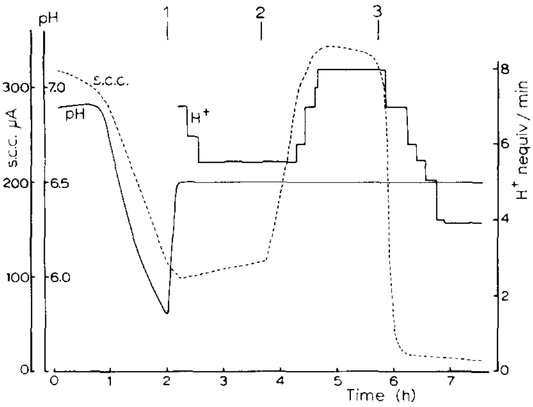

Fig. 1. Effects of antidiuretic hormone and amiloride on H$^+$-titration rates of a toad skin preparation. 1. Beginning of titration at pH 6.5. 2. Antidiuretic hormone added to the internal medium (100 munits/ml). 3. Amiloride added to the external medium (10^{-4} M).

tions under the influence of drugs that modify the uptake of Na$^+$ by epithelial structures, either by stimulation (antidiuretic hormone) or blockage (amiloride). The time course and the results of two such experiments carried out on a toad skin and a frog skin are outlined in Figs 1 and 2, respectively. After a preliminary phase during which the s.c.c. and the spontaneous acidification of the external medium were recorded, control values of H$^+$ excretion were determined by titration at a fixed pH (6.5 for toad skins and 7.0 for frog skins). Antidiuretic hormone (Pitressin, Parke Davis) was then added to the solution bathing the corium in a final concentration of 100 munits/ml; 60–90 min later amiloride in a final concentration of 10^{-4} M was added to the external medium. The overall results are summarized in the first six lines of Table III. Both in toad skins and frog skins, the net Na$^+$ flux, evaluated from the s.c.c. values, showed the usual response to the addition of drugs, i.e. a marked increase with Pitressin and a drop to very low values immediately after the addition of amiloride; the basal H$^+$ excretion rates show a dispersion which is not clearly related to the varia-

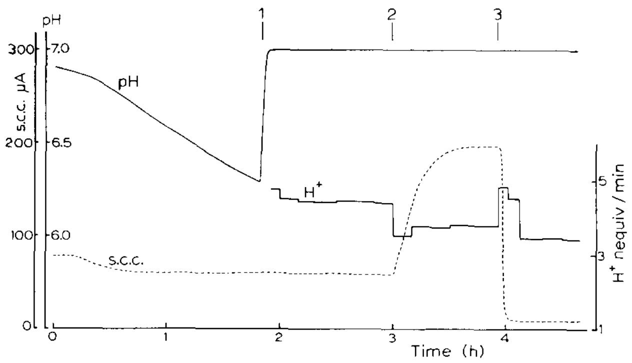

Fig. 2. Effects of antidiuretic hormone and amiloride on H$^+$-titration rates of a frog skin preparation. 1. Beginning of titration at pH 7.0. 2. Antidiuretic hormone added to the internal medium (100 munits/ml). 3. Amiloride added to the external medium (10^{-4} M).

TABLE III

RATES OF H^+ EXCRETION AND Na^+ NET FLUX (nequiv · min^{-1} · 7.1 cm^{-2}) IN TOAD SKIN, WHOLE FROG SKIN AND SEPARATED EPIDERMIS OF FROG SKIN SUBJECTED TO THE ACTION OF ANTIDIURETIC HORMONE AND AMILORIDE

Na^+ net fluxes were calculated from the s.c.c. values. Means and S.E. values are shown. Number of experiments in brackets. *P* values refer to Student's *t* test applied to verify the significance of difference between means. n.s., non significant at 0.05 level.

		Control		Antidiuretic hormone		Amiloride
Toad skin						
a (14)*	H^+	4.6±0.5	$P < 0.01$	8.1±1.0	$P < 0.01$	3.1±0.4
	Na^+	57±10		195±9		15±3
b (8)	H^+	5.3±0.9	—	—	$P < 0.01$	2.5±0.3
	Na^+	47±4		—		0.9±0.2
Whole frog skin						
c (10)	H^+	5.1±0.6	n.s.	4.8±0.7	n.s.	4.6±0.7
	Na^+	57±12		126±15		5±0.6
Separated epidermis						
d (10)	H^+	3.7±0.4	n.s.	3.2±0.4	n.s.	3.1±0.3
	Na^+	50±4		103±13		4±0.6

* Amiloride was used in only six experiments of this group.

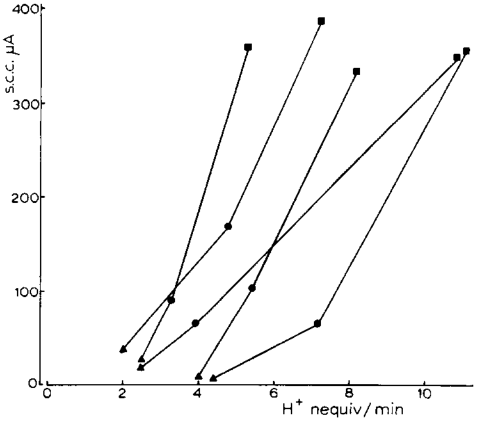

Fig. 3. Plot of H^+ titration rates versus s.c.c. obtained in five toad skin preparations. ●, Control values; ■, values obtained after the addition of antidiuretic hormone; ▲, values obtained after the addition of amiloride.

tions of the s.c.c. in either preparation. However, after the addition of Pitressin toad skins reacted with an increase of H^+ excretion rates and here a certain parallelism was noticed amongst individual skins, i.e., in the plot of H^+ excretion rates versus s.c.c. the slopes are not very different from each other (Fig. 3). On the contrary, no change of H^+ titration rates was verified in the experiments with frog skin. Again, after the use of amiloride the behaviour of the two preparations was different. In the case of toad skin there was a decrease of H^+ excretion rates while in the frog skin experiments these rates do not change significantly. As amiloride inhibited only about 75 % of the s.c.c. of toad skins and the mean value of the H^+ excretion rates after amiloride was not statistically different from the control values, a further group of eight experiments was carried out in toad skins where amiloride alone was used, in order to obtain a blockage of the s.c.c. more complete than in the preparations which had been subjected to the action of antidiuretic hormone. In these experiments a complete inhibition of the s.c.c. was obtained, and the mean rate of H^+ excretion after amiloride was significantly lower than the control value (Table III).

Isolated epidermis of the frog skin

Sheets of frog skin epidermis isolated from the corion by the action of collagenase were used following the same protocol as described before to study the action of antidiuretic hormone and amiloride. Preliminary experiments had shown that the s.c.c. and conductance of these preparations were identical to those of whole skins, suggesting that the Na^+ transport capacity is fully maintained. As the isolated epidermis is virtually free of glands [11, 12] contamination of the external solutions by glandular secretions which could alter the results of H^+ titration [13] was thus avoided. The time course of a typical experiment is shown in Fig. 4. The overall results of this group, shown in the last lines of Table III, are similar to those obtained with whole skins with respect to s.c.c. and H^+ titration rates under the different experimental conditions.

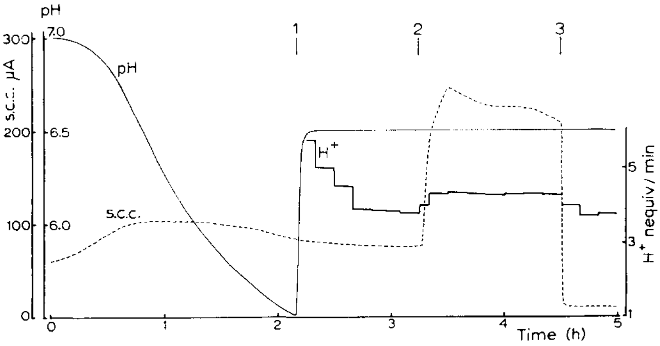

Fig. 4. Effects of antidiuretic hormone and amiloride on H^+ titration rates of a separated epidermis of frog skin. 1. Beginning of titration at pH 6.5. 2. Antidiuretic hormone added to the internal medium (100 munits/ml). 3. Amiloride added to the external medium (10^{-4} M).

DISCUSSION

The results described above confirm previous observations on the capacity of the isolated amphibian skin to extrude H^+. Under our experimental conditions, it has been shown that there is no accumulation of metabolic CO_2 to account for the decrease of pH of the outside solutions, which suggests that the H^+ is excreted as such [10]. Moreover, according to our findings, it seems that there is some important differences in the processes of H^+ excretion by the toad skin or by the frog skin. In the first place, H^+ excretion is not detectable in toad skin preparations when Cl^- is present in the outside solutions but becomes evident when Cl^- is substituted by SO_4^{2-}. A similar situation was found by Garcia Romeu et al. [6] in their experiments in vivo with the frog *Calyptocephalella gayi*, and this was explained by the existence of a Cl^-/HCO_3^- exchange mechanism at the outer border of the epithelium in such a way that HCO_3^- acts as a buffer towards H^+ that might be extruded simultaneously. In the isolated skin of *R. ridibunda*, on the contrary, H^+ extrusion to the outer medium is already apparent in normal chloride-Ringer, where a pH gradient of 3–4 units may develop between both surfaces of the preparation, as was shown in previous studies [10]. In the present experiments, the rates of H^+ titration obtained in chloride-Ringer were not different from the rates obtained in sulphate-Ringer. These facts suggest that a Cl^-/HCO_3^- exchange process may be acting in the toad skin preparation, but could not be detected in the frog skin under our experimental conditions. Moreover, the H^+ excretion of the isolated frog skin did not seem to be influenced by changing conditions of sodium uptake and transport. In fact, rates of H^+ titration did not change significantly either with the withdrawal of Na^+ from the bathing solutions or by the use of drugs with well-known effects on those functions (amiloride and antidiuretic hormone). Our results are in good agreement with the findings of Erlij et al. [14] in the isolated frog skin. On the contrary, when toad skin preparations were used in the same type of experiments there were variations in the H^+-titration rates which seem closely related with the variations of the s.c.c. caused by the experimental conditions mentioned above (absence of Na^+, use of amiloride or antidiuretic hormone). In fact, a marked decrease of H^+ excretion was noticed when Na^+ was substituted by Mg^{2+} in the external medium, or when the uptake of Na^+ was inhibited by amiloride, while the addition of antidiuretic hormone was followed by a rise in H^+ titration rates together with the usual rise of the s.c.c. These observations suggest the possible existence of an exchange mechanism of the type Na^+/H^+, conditioning at least part of the H^+ extrusion. Nevertheless, it is worthwhile noticing that the total H^+-excretion rates are very small when compared with the amount of Na^+ transported by the skin. In fact it is shown in Table III, for example, that the net flux of Na^+ under control conditions is about 57 nequiv/min in these preparations while the H^+ excretion rates are about 5 nequiv/min. The ratio H^+/Na^+ is even smaller under the effect of antidiuretic hormone. On the other hand, some H^+ is excreted even when no Na^+ is present in the external medium or when uptake is inhibited by amiloride. This remaining efflux, which is about 50% of the total amount, may be explained by the presence in the toad skin of a mechanism of the same type as the one present in the frog skin, which has some characteristics of an "active" H^+ pump [10]. Further studies will be necessary for a better characterization of this phenomenon in the toad skin.

In summary, our results are compatible with the existence of an Na^+/H^+ exchange mechanism functioning in the isolated toad skin, and responsible for at least a fraction of the total H^+ flux detected by titration in the external bathing medium. However, this mechanism would only account for 1/10 of the total Na^+ net flux and does not seem essential for the operation of Na^+ uptake in all species. Such an exchange could not be demonstrated in the isolated frog skin, where H^+ extrusion seems totally independent of the simultaneous uptake of Na^+. The fact that similar rates of H^+ titration are obtained either in separated epidermis of the frog skin or in preparations of the complete skin, show that this function is not dependent on the presence of glands and probably pertains to the active strata of the epithelium. It is interesting to notice that a complete inhibition of the s.c.c. by amiloride could not be obtained in toad skin preparations after pre-treatment with antidiuretic hormone, although the same concentration of the drug produced a total inhibition of the s.c.c. when the hormone had not been used previously. This particular behaviour was not seen in frog skin preparations.

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