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Effect of mucosal H^+ and chemical modification on transcellular K^+ current in frog skin

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A transcellular K^+ current (I_K) was established across the skin of the frog *Rana temporaria*, whose apical K^+ permeability had been previously stimulated by exposure to K^+-rich media. Short-term ($\leq$ 15 s) mucosal pH-titration of I_K indicated two titrated groups (A and B), with apparent pK_A of 6 and pK_B of 3. The height of the titration steps, A and B, varied from skin to skin. Intracellular (i) H^+-sensitive microelectrode studies on *Rana esculenta* skin (which lacks apical P_K) were conducted in order to assess possible changes in pH_i and basolateral K^+ conductance as a consequence of the rise in mucosal $[H^+]$. Cell pH decreased only at mucosal pH lower than 5.4 which caused a drop in basolateral K^+ conductance as estimated from *I-V* records of the serosal membranes. These effects were much too slow to account for the fast mucosal pH effects on I_K (*Rana temporaria*). Thus, we conclude that the two-step titration curves reflect solely the interaction of external H^+ with the mucosal side of apical membrane K^+ channels. Exposure to the SH-reagent PCMB, and to the carboxy-modifying EEDQ markedly reduced total I_K at neutral pH; however, PCMB seemed to preferentially affect titration step B while EEDQ virtually eliminated step A. When the saturating I_K kinetics were studied at different mucosal pH, protons showed a 'mixed' type inhibition of K^+ current in the range of titration step A; at pH values less than 5, protons blocked I_K by competition with K^+ ions. These results are compatible with the presence of two K^+ channel populations in the apical membrane which are discernible by their different interactions with external protons and chemical modifiers.

Introduction

Apical K^+ channels in frog skin seem to be unique to the brown grass frog, *Rana temporaria*. A serosally oriented $[K^+]$-gradient gives rise to an inward current which is blocked by external Cs^+, H^+, Cd^{2+} or Ba^{2+} (for an extensive review, see Refs. 1 and 2). A transcellular K^+ current, I_K, can also be observed in K^+-secreting skins where the serosal Na^+,K^+-ATPase provides the driving force [3]. The secretory task of the epidermis can be challenged by loading the animal in vivo with KCl, or by bathing the isolated skin in a solution of high K^+ concentration [2,4]. Apical K^+ permeability is also dramatically enhanced by treatment with oxytocin [5]. The data reported here show that mucosally applied protons block I_K in a dual way, i.e. in a 'mixed' type at more neutral pH but competitively with K^+ in the more acidic pH range. A 'two-step' titration curve and the differential susceptibility of the titration steps to chemical modification suggest the existence of more than one type of K^+ channel population.

Methods and Materials

I. K^+ permeable Rana temporaria

Since not all skins showed a significant spontaneous K^+ permeability, different procedures were used to enhance I_K. In most cases, apical K^+ permeability (P_K) was stimulated, either by immersing the frogs in vivo in KCl-containing water or by bathing the whole excised skin in KCl-Ringer [4,7]. In a few cases, skins from untreated frogs were exposed in vitro to apical KCl-Ringer (NaCl on the serosal side) which induces a

Abbreviations: EEDQ, *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline; G_b, conductance of basolateral membrane; I_K, K^+ current; I_R, relative K^+ current; I_{sc}, short-circuit current; I_t, transepithelial current; K_m, Michaelis constant; P_K, K^+ permeability; PCMB, *p*-chloromercuribenzoate; pH_i, intracellular pH; pH_{muc}, mucosal pH; V_b, potential difference across basolateral membrane; V_t, transepithelial potential difference.

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long-term stimulation of I_K [6] as a result of recruitment of apical K^+ channels [2]. These methods usually stimulated I_K by a factor of 3 to 8 when compared to 'spontaneously' K^+ permeable skins, and in some tissues the I_K exceeded Na^+ transport rate of skins bathed with NaCl-Ringer on both sides [7].

Abdominal skins of *Rana temporaria* were mounted in a vertical Ussing-type lucite chamber (3 cm^2 skin surface area). The small mucosal chamber compartment allowed a fast (< 1 s) solution exchange by syringe-injection [8]. With a conventional 4-electrode arrangement the short-circuit current, I_{sc}, could be measured with an automatic voltage-clamp device. The solutions used were (in mmol/l):

Na^+ Ringer : 115 NaCl, 2.5 $KHCO_3$, 1 $CaCl_2$
K^+ Ringer : K^+ replacing Na^+
choline Ringer : choline replacing Na^+.

Na^+ Ringer was always used on the serosal side (pH 8.0, air-bubbled). I_K was determined by correcting I_{sc} for the shunt current obtained in the presence of 5 mmol/l mucosal barium acetate. I_K kinetics were recorded with mucosal mixtures of K^+- and choline Ringer, or K^+- and 50 μmol/l amiloride-containing Na^+ Ringer, respectively (Amiloride was a gift of Merck, Sharp and Dohme).

Previous work [6,9] and a series of preliminary experiments (unpublished) revealed that the three mucosal buffers tested by us, namely, Tris, bicarbonate and phosphate, had no influence on the pH-titration curve of I_K. The results presented here were obtained with 2.5 mmol/l bicarbonate, and mucosal pH was adjusted by adding HCl. The solution was gassed with air.

For chemical modification we treated the skins with

(a) 0.1 mmol/l *p*-chloromercuribenzoate (PCMB) in K^+ Ringer for approx. 30 min,

(b) 5 mmol/l *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) in K^+ Ringer for approx. 10 min.

The exposure time was chosen such that I_K dropped to about 50% of its original value. Titration curves were recorded before this irreversible chemical treatment and in the new steady-state after washout of PCMB or EEDQ.

As can be seen from Fig. 1 (cf. Results), continuous acidification leads to a biphasic decrease in I_{sc} with fast initial and secondary slow components. We measured the 'specific' transcellular I_K and its reaction to supposedly extracellular (mucosal) protons in the following way:

When a skin bathed with mucosal K^+ and serosal Na^+-Ringer at neutral pH reached a steady I_{sc} the mucosal K^+ solution was quickly replaced by choline (or Na^+ plus amiloride) solutions of neutral pH, which nearly abolished I_{sc}. We then applied a brief pulse of mucosal K^+ Ringer at the desired acidic pH for periods less than 15 s. The K^+- and pH-dependent I_{sc} deflections were then corrected for shunt effects by repeating

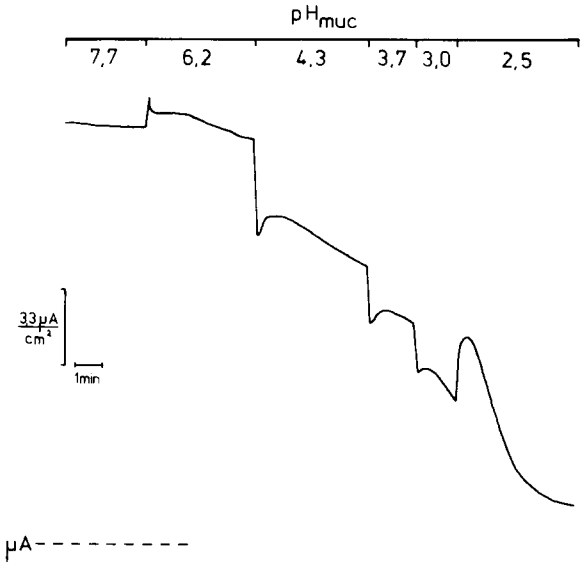

Fig. 1. Time course of short-circuit current during stepwise acidification of the mucosal K^+ Ringer (serosal Na^+ Ringer).

the protocol in the presence of the K^+-channel blocker Ba^{2+} (5 mmol/l). Short-term titration was restricted to the pH range 7.4 to 3.0. The symbol I_R used in various figures is the relative I_K (100% for $[K^+]_{muc} = 117.5$ mmol/l at pH 7.5; no treatment with group reagents). For titration curves, pK values were estimated at the region of the curves' inflection points. (The determination of pK_B may not be exact since titration was not extended below pH 3 (cf. Fig. 1) although the titration curve in this region is very steep which might reduce the error.)

II. Microelectrode studies on Rana esculenta

The rationale for doing the experiments on *Rana esculenta* was to assess the dependence of basolateral K^+ conductance (G_b) on mucosally applied protons, and to determine whether mucosal pH effects on I_K could result in part from a reduction in G_b. For these studies we preferred to use frog skins which did not possess a spontaneous apical K^+ permeability. *Rana esculenta* skin fulfils this condition with the added advantages of non-induction of an apical P_K by K^+ Ringer (unpublished observation) and detailed information available on the pH sensitivity of G_b [10]. Intracellular pH (pH_i) and basolateral membrane K^+ conductance (G_b) were measured as a function of mucosal Ringer pH in the short-circuited epithelium of the ventral skin of *Rana esculenta* isolated by collagenase treatment (exposure to Worthington crude collagenase 0.4 mg/ml at 30 °C for 1.5 h). The tissue was mounted in a horizontal modified Ussing chamber which allowed fast solution changes (flow rate: 60 bath volumes (1.0 ml) per min). The electrophysiological techniques used

for simultaneous measurement of pH_i and cell membrane current-voltage relations are given in detail elsewhere [10] and are described briefly here:

Transepithelial potential difference (V_t) and current (I_t) (mucosal solution as reference) were controlled automatically using a voltage-current clamp amplifier under computer command. Transbasal voltage (V_b) was recorded (with reference to the serosal solution) from microelectrodes impaling either intact skin from the apical, or the collagenase-treated isolated epithelium from the serosal side. In the latter case, intracellular pH was measured with double-barreled (voltage- and H^+-sensitive) microelectrodes the reference side of which was used to record membrane potential and I_t-V_t relations.

Current-voltage curves of the basolateral membrane were determined by recording the response of V_b to a stepwise sweep of V_t over the range ± 200 mV. The high impedance dual electrometer output was stored on computer for later analysis.

Results

I. pH-titration of apical P_K by H^+

Fig. 1 shows a long-duration experiment showing the effects of stepwise decreases in mucosal pH (pH_{muc}): At neutral pH, I_{sc} strongly depends on mucosal K^+ (23 $\mu A/cm^2$) but is close to zero (not shown) with mucosal choline Ringer, so $I_{sc} \approx I_K$. I_{sc} is hardly changed upon slight acidification. At higher acidity, the observed fall in I_{sc} is complex which can be best described by an instantaneous decrease followed by a slow decline. We interpret the very fast effect seen within the first 15 s after a solution change to be the response of apical K^+ channels to *external* H^+ since this happens before significant changes in pH_i can occur (as will be shown in the microelectrode studies). The subsequent slow I_K depression might be due to a rise in intracellular $[H^+]$ after continued mucosal H^+ exposure, which results in a reduction of serosal K^+ permeability (see below). Finally, the positive 'bump' at pH 2.5 might, under these extreme conditions, indicate a fraction of I_{sc} carried by H^+. The inhibitory effect of mucosal pH on I_{sc} is half-maximal at $pH_{muc} \approx 3.0$ (see also Fig. 2) although bearing in mind that, over the whole range of pH_{muc}, I_{sc} may not necessarily be identical with I_K. Titration curves using the rapid deflection of I_{sc} were determined for 19 skins; 18 showed a two-step profile with considerable variation in step height. Three examples are depicted in Fig. 2: For skin 1, the first step in the slightly acidic pH_{muc} range (step A, between pH 7.4 and 4.5) is hardly expressed, but for skins 2 and 3, the rapid inhibition amounts to about 40% and 65% of total I_K, respectively. The inflection point for titration step A (pK_A) is close to pH 6. For titration step B, below pH_{muc} 5, only a rough estimation can be made for the

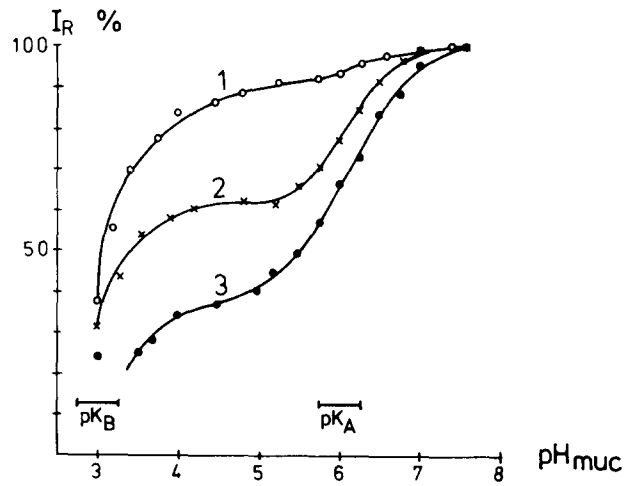

Fig. 2. Three selected titration curves of the apical K^+ conductance of frog skin (*Rana temporaria*). I_R, representing the relative I_K (100% = I_K for $pH_{muc} = 7.5$ and $[K^+]_{muc} = 117.5$ mmol/l) is plotted versus mucosal pH (serosal NaCl Ringer). Approximate points of inflection indicated by pK_A and pK_B. Fit by eye. Skin 1: ○, 100% = 24.5 $\mu A/cm^2$; skin 2: ×, 100% = 35.0 $\mu A/cm^2$; skin 3: ●, 100% = 15.8 $\mu A/cm^2$.

inflection points: $2.5 < pK_B < 3.5$. The estimated range of $pK_B \approx 3.0$ for skins 2 and 3 is close to the ones derived previously from one-step titration curves of spontaneously K^+-permeable skins [6,9]. It appears that the relative height of the titration steps is unrelated to the initial K^+ current at pH_{muc} 7.5 which was ($\mu A/cm^2$) 24.5 for skin 1, 35.0 for skin 2 and 15.8 for skin 3. Thus, we also conclude that the method for I_K stimulation with high-K^+ salines does not determine the height of a given titration step. In fact, our data record shows no correspondence between K^+ current magnitude, and the occurrence or size of either titration steps A or B. Fig. 3 displays the contribution of step B to total I_R for all titrated skins ($n = 19$). It is evident that step B is the most pronounced for the majority of the epithelia. While

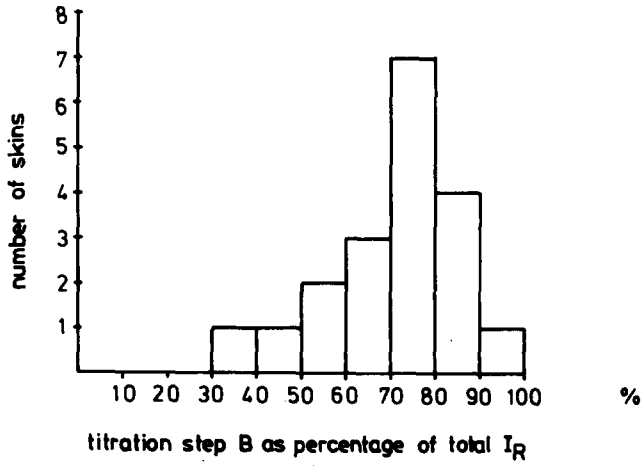

Fig. 3. Histogram of relative magnitude of the second titration step (with $pK_B = 3$) for all titrated skins. There is no correlation with the method of pretreatment for P_K stimulation.

only a single skin showed just the high-acidity titration step B, no skin could be found displaying titration step A alone. The average height of step B is 70% of total I_K.

II. Differential modification of the titration characteristics with group-specific reagents

Group-specific reagents could interfere with the protonation process as characterized by titration curves. Target sites might be identical with the ones for H^+ but allosteric interference is also possible. We now describe such effects obtained by exposure to the mucosally applied SH-reagent PCMB [11], and the carboxy-modifier EEDQ [12]. For testing each modifier, three skins were chosen with titration step B falling between 60% and 75% of the control I_K at pH_{muc} 7.5 (100% I_R): The relative currents at each pH_{muc} value were averaged after normalization to pH_{muc} 7.5. Fig. 4 demonstrates that both agents are blockers of I_K, and I_R measured at pH_{muc} 7.5 was drastically and irreversibly decreased to about 40% of the control value at pH_{muc} 7.5.

Moreover, the appearance of the two-step titration curves was greatly changed. PCMB changed the relative step height ratio, B/A, from 2.3 to 1.0: While step A is hardly affected by PCMB, step B is substantially reduced. In other words, the reduction of I_K by PCMB could simply be due to an attack by the blocker on the same component (B) of I_K which can also be blocked by a more acid pH_{muc}. On the other hand, EEDQ treatment altered the B/A relationship from 1.5 to 4. Unlike the action of PCMB, the carboxy-modifier thus led to a pronounced reduction of both titration steps, with step A being practically eliminated.

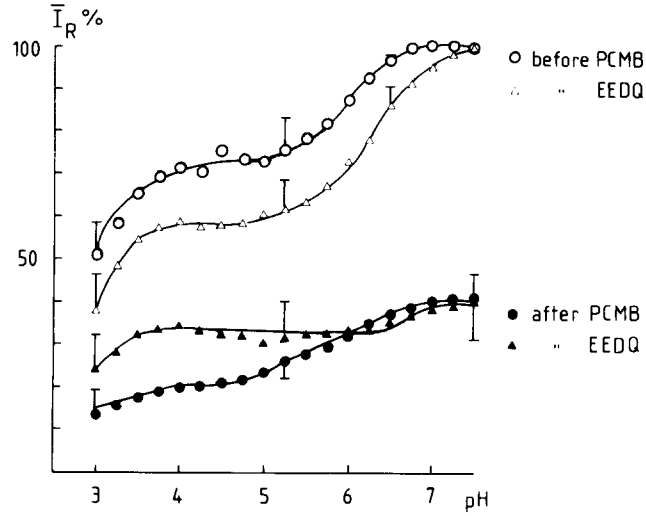

Fig. 4. Averaged ($n = 3$) titration curves of the apical K^+ conductance (expressed as relative K^+ current, I_R) before (open symbols) and after (filled symbols) treatment with the SH-reagent PCMB (circles) or the COOH-reagent EEDQ (triangles). Selected S.E. indicated by bars. Fit by eye.

III. Kinetic type of I_K inhibition by H^+

It was demonstrated previously [6,13] that I_K through the skin of *Rana temporaria* saturates with increasing mucosal $[K^+]$. Interestingly, increasing maximal current as well as Michaelis constant were observed when skins were allowed to develop an enhanced apical P_K during long-term exposure to high mucosal K^+ concentration [6].

Here we examined the I_K kinetics in seven preparations and compared the dependence of I_K on mucosal K^+ concentration at pH_{muc} 7.4 with those obtained at

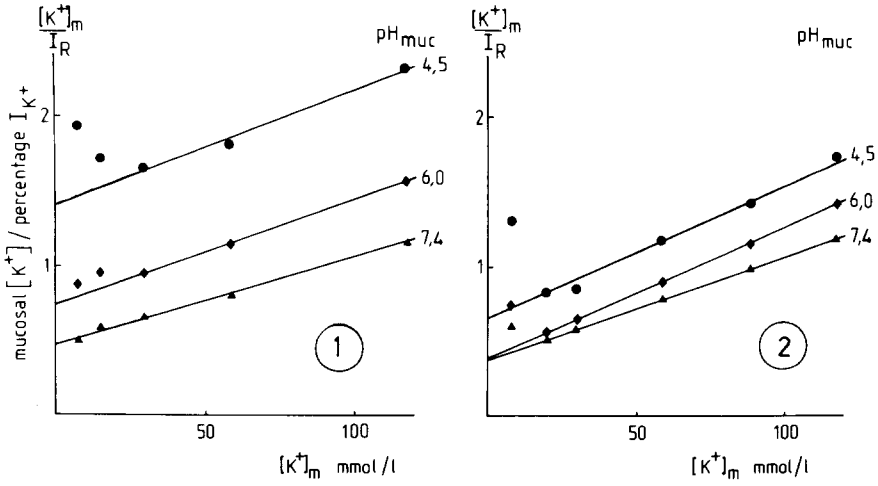

Fig. 5. Hanes plots for the I_K kinetics at pH_{muc} 7.4, 6.0 and 4.5 for two selected skins (1 and 2). The lines result from linear regression according to the linearized form of the Michaelis-Menten equation

$$[K^+]_{muc}/I_R = (1/I_{R,max}) \cdot [K^+]_{muc} + K_m/I_{R,max}$$

for the $[K^+]_{muc}$ range > 20 mmol/l. Initial absolute I_K at pH_{muc} 7.4 (equivalent to 100% I_R) for preparations 1 and 2 are 4.0 and 41.3 $\mu A/cm^2$, respectively. For more details see Methods.

pH_{muc} 6.0 and pH_{muc} 4.5. Although the titration curves indicate that the second titration step starts at pH_{muc} 4.5, we preferred not to acidify further in order to avoid a possible H^+ influx (see below).

The kinetics of I_K inhibition by H^+ were different for the two titration steps and showed considerable variation among individual skins. Two examples (skins denoted 1 and 2) are depicted in Fig. 5, where a Hanes diagram ($[K^+]_{muc}/I_R$ vs. $[K^+]_{muc}$) was chosen for linear representation of Michaelis-Menten kinetics. At mucosal K^+ concentrations above 20 mmol/l the plot is clearly linear which suggests Michaelis-Menten behaviour at all pH_{muc} values. However, at pH_{muc} 7.4, the apparent Michaelis constant (K_M) in these, as in other experiments, was considerably higher than the K_M found for short-term exposure to mucosal K^+, but was comparable to the K_M determined after the long-term effect was fully established, as was the case in all our experiments. At lower $[K^+]_{muc}$, a deviation in the sense of 'overproportional inhibition' by H^+ was observed and became more noticeable with increasing acidity. This latter effect may involve changes in pH_i (see below), and the Hanes plot in this region may not reflect the action of H^+ solely at the mucosal face of the apical membrane.

Closer examination of linear region of the Hanes plot reveals that both epithelia show competitive interaction between external K^+ and H^+ when pH_{muc} 6.0 and 4.5 are compared. For the range of the titration step A (comparing pH_{muc} 7.4 with pH_{muc} 6.0) the upward shift is not quite parallel for skin 1, whereas in skin 2, the lines simply rotate around the ordinate intercept. The latter behavior would be expected if the inhibition of I_K by K^+ was, in this pH_{muc} range, of the uncompetitive type where maximal K^+ current and apparent Michaelis constant are decreased by the same factor. When all seven skins were taken into account, we always found a competitive inhibition in the more acidic pH_{muc} range (step B) but a considerable 'mixed' scatter, i.e. between 'uncompetitive' and nearly 'competitive' type of inhibition for step A. In addition, it is the ratio of the step heights B/A which appears to be consistently related to the type of H^+ blockade. Moreover, the method used for I_K stimulation (thus the magnitude of I_K) shows neither a correlation with the type of H^+-inhibition kinetics nor with the step height ratio B/A.

IV. Localization of the titration effect

Recently, Harvey et al. [10] succeeded in measuring basolateral K^+-conductance in frog skin (*Rana esculenta*) in response to alterations of intracellular pH.

pH_i changes were brought about by either serosal pH variation, by cell acidification via a rise in solution CO_2-pressure, or by stopping the serosal Na^+/H^+ or Cl^-/HCO_3^- exchangers [14]. Serosal K^+ conductances were derived from basolateral current-voltage curves (linear!) and monitored as a function of pH_i, which was recorded simultaneously with ion-sensitive microelectrodes. These investigators found that quite small changes in pH_i from 7.5 to 7.1 reduced basolateral K^+ conductance (G_b) by up to 50%. Thus, the question arises whether our short-term protocol of mucosal pH changes will affect intracellular pH, and therefore, G_b, which might perhaps further decrease I_{sc}, since I_K reflects the state of both apical and basolateral K^+ conductance.

In order to check the possible effects of mucosal pH changes on G_b independently of the effects of mucosal H^+ on apical K^+ conductance, we used the species *Rana esculenta* which does not possess an apical membrane K^+ conductance (or transepithelial I_K).

Cell impalement with microelectrodes was always started when NaCl Ringer was present on both sides of the tissue and the quality of impalement was verified by monitoring fractional apical resistance measurements when Na^+ transport was blocked with mucosal amiloride (10^{-4} mol/l). When the impalement criteria were satisfied, KCl Ringer at pH 8.3 was substituted for NaCl Ringer on the mucosal side and pH_{muc} was decreased in one unit steps to pH_{muc} 3.3 and finally returned to pH_{muc} 8.3. The tissue was maintained at each pH_{muc} value for approx. 3 min (similar to the protocol in Fig. 1). during which pH_i was continuously monitored while G_b was calculated every 60 s from I_b-V_b relations. Fig. 6 shows such an experiment in an isolated epithelium (serosal impalement with double-barreled H^+ sensitive microelectrode) in which it can be seen that pH_i and also G_b began to decrease only when pH_{muc} was lowered to 5.4. Very noticeable decreases in pH_i and G_b occur at pH_{muc} 4.4 and 3.3. The effect of

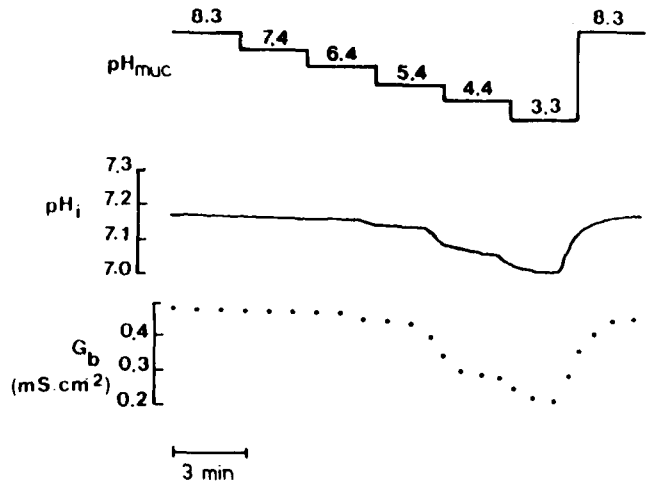

Fig. 6. Recording of pH_i and basolateral membrane K^+ conductance, G_b, following stepwise changes in apical solution pH_{muc} in the isolated epithelium of *Rana esculenta*. Cell impalement was with double-barreled H^+-sensitive microelectrode from the basolateral side. KCl Ringer on the apical side, and NaCl Ringer equilibrated in air and containing 2.5 mmol/l HCO_3^- on the serosal side.

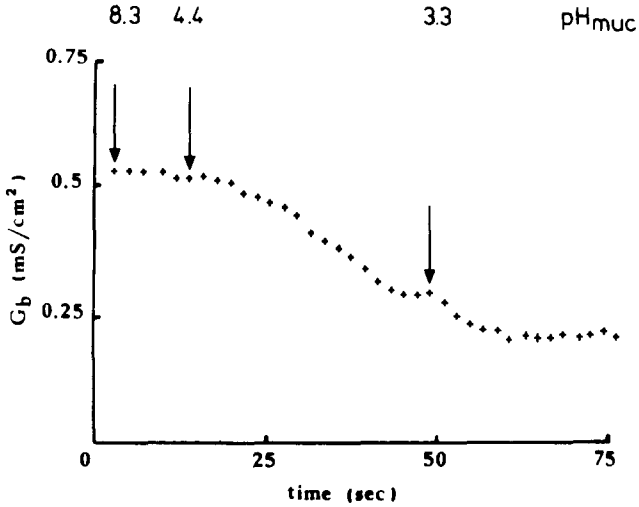

Fig. 7. Time course of the effects of stepwise changes in pH_{muc}, from 8.3 to 4.4 and 3.3, on basolateral membrane K^+ conductance in whole skin from *Rana esculenta*. Cell impalement with 1 mol/l KCl-filled microelectrode from the apical side.

low pH_{muc} on pH_i and G_b was, however, fully reversible on return to KCl Ringer of pH_{muc} 8.3.

In order to examine more closely the time course of pH_{muc} effects on conductance, we measured G_b over shorter intervals (every 2.5 s) in isolated whole skin (apical impalement) while pH_{muc} was suddenly decreased from 8.3 to 4.4 and 3.3 (Fig. 7). Analysis of basolateral *I-V* relations at the three different pH_{muc} values revealed that acidification from 8.3 to 4.4 hardly changed G_b within the 15 s allowed for our short-term titration protocol. Exposure times longer than 30 s were seen to produce a substantial decrease in G_b. This latent decrease in G_b at low pH_{muc} may be responsible for the slow secondary decrease in I_K observed for long-term exposure to mucosal H^+ (cf. Fig. 1), and perhaps also for the deviation from Michaelis-Menten kinetics at a high $[H^+]_{muc}/[K^+]_{muc}$ ratio (cf. Fig. 5).

Discussion

1. Evidence for an extracellular site of mucosal H^+ titrating I_K

Since I_K is a transcellular current it will be influenced by variations in basolateral membrane K^+ conductance, which is itself extremely sensitive to changes in pH_i over the physiological range [10]. Since, so far, basal properties seem to be identical for the two frog species *Rana temporaria* and *Rana esculenta*, the use of *Rana esculenta* for this type of experiment appears justified. We found that pH_i was insensitive to decreasing pH_{muc} to 5.4 whereas further acidification between pH_{muc} 5.4 and 3.3 produced only slow changes in pH_i and G_b (complete after 2 min, see Fig. 6). This latent decrease in basolateral membrane K^+ conductance at very acidic pH_{muc} could account for the secondary slow inhibition of I_K found under these conditions (Fig. 1). The titration curves, however, were computed during the first 15 s following step changes in pH_{muc}, and during this time period there is little significant change in pH_i or G_b (Fig. 7).

Another argument which supports a unique external site of action of H^+ during brief pulses of acidic mucosal solutions is that brief external acidification to pH_{muc} 4.3 *increases* Na^+ current in frog skin [8] and this is opposite to what would be expected if pH_i had also decreased [10].

Since our data suggest H^+/K^+-competition in the range of titration step B, we conclude that this competition should take place at the *outer* face of the external membrane where $[K^+]$ was changed systematically (with the tacit assumption of no change in intracellular $[K^+]$). This interpretation would also be consistent with the idea of chemical attack by PCMB and EEDQ being exclusively from the outside (see below). Externally located carboxylate was recognized as a H^+-titration site for cation channels in excitable membrane [15], or for Na^+ channels in epithelia (for review, see Ref. 1).

K^+ ions will enter the cell via apical K^+ channels and exit via serosal K^+ channels. A block of I_K by internal H^+, acting at the cytosolic mouth of the *serosal* K^+ channel, could, indeed, be the cause for the observed overproportional I_K decrease at high $[H^+]_{muc}/[K^+]_{muc}$ ratios. This conclusion is also supported by our microelectrode studies which showed a pronounced decrease of G_b at external $pH_{muc} < 5.4$. Since the latter findings pertain to H^+ exposure times of clearly more than 30 s, our results favour a purely apical reaction between H^+ and K^+ conductance in the 15 s exposure protocol. Thus, the buffer-independent, fast and quickly reversible effects of external H^+ are interpreted as titration of externally located, negatively charged components of apical K^+ channels.

2. Interpretation of two-step titration curves

Usually with epithelial channels, conductance reduction by a competitive blocking ion could be explained by assuming an all-or-none block of a channel [1,2].

We think that a simple interpretation of the two-step titration curves is possible with the assumption of two K^+ channel populations X and Y as represented by the two titration steps, with acidic groups of $pK_A \approx 6$ and $pK_B \approx 3$. Each channel population might display multisite, single-file permeability characteristics [13], and their gating or single-channel conductance, as observed by means of noise analysis [1] might even be indistinguishable from each other. We assume, a priori, that an all-or-none block of these channels occurs after interaction of external H^+ with the titratable groups, one with pK_A, the other with pK_B. Population X may possess both titratable groups but titration of A would then render the ensuing titration of B undetectable.

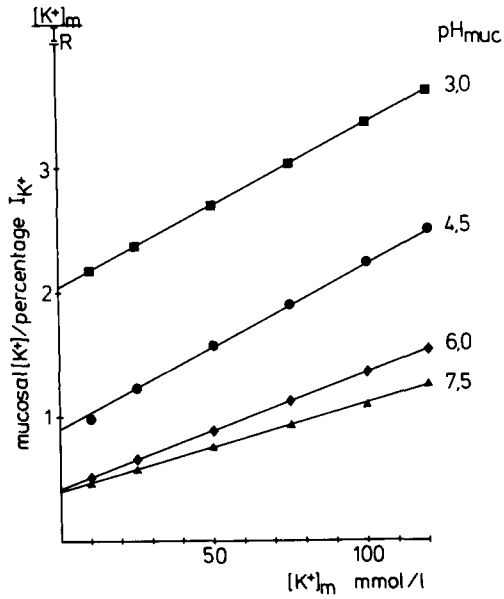

Fig. 8. Hanes plot for the theoretical model following Eqn. 1. For details see Appendix.

Thus, the two-step titration curves would show the contribution of each population, X or Y, to the total I_K as observed at neutral pH_{muc}. In the spontaneously K^+ permeable tissues described previously [6,9], a one-step titration curve with pK 2–3 was obtained which is very close to pK_B derived here from two-step titration curves.

3. Inhibition by H^+ and I_K kinetics

With the assumption of two apical K^+-channel populations the interpretation of the I_K kinetics at different pH_{muc} (cf. Fig. 5) becomes straightforward: Titration from pH_{muc} 7.5 to 5.0 would leave step B (and thus channel population Y) unaffected while population X is slowly eliminated. Thus, the expression of one or the other type of inhibition would depend on the ratio of K^+ channel populations. In Fig. 8 a Hanes plot is shown for a hypothetical case where I_K is considered to be a sum of two titratable K^+ channel populations with Michaelis-Menten behaviour and the parameters I_{max} and K_M characterizing each set. In addition, one population (X) is assumed to show a mixed inhibition by H^+ while population Y is blocked competitively by H^+ (*increasing only* K_M). The current equation for the description of this model and its parameters are given in the Appendix. It is evident that, with model parameters similar to the ones observed in the majority of the epithelia, the experimentally found kinetics (cf. Fig. 5) can be mimicked to a satisfying degree. Consequently we propose that the observed titration curves mirror the competitive H^+ block (at site B) of channel population Y at $pH_{muc} < 5$ while population X is blocked *in addition* by an allosteric mechanism at $pH_{muc} > 5$ (at site A).

4. Chemical modification of apical P_K

The two titration steps were both attenuated by PCMB as well as by EEDQ. While the reduction of step A by PCMB seemed almost negligible as compared to step B, the carboxy-modifier EEDQ had, in contrast, a major effect on both components (although step A seemed much more affected than step B). If we accept the view that PCMB specifically binds to SH-groups [11], whereas EEDQ interacts with carboxylate [12], the striking differences in sensitivity for both titration steps may be due to differences in their accessibility or to other determinants of chemical reaction rates. The observation that EEDQ depressed both titration steps argues in favour of COO^- groups being of importance for both putative K^+-channel populations. Regarding the SH-reagent PCMB, an analogous I_K-depressing effect was reported with a related substance, the membrane-impermeant PCMPS, but also with external, presumably also non-permeant, Cd^{2+} [16,17]. The major effect of PCMB was, in our experiments, on titration step B. Although SH-groups would not be titrated within the range of pK values, a participation of SH groups in protonation effects in the range of pK_A cannot strictly be excluded. At present, we favour a model where the SH-group modified by PCMB is in an allosteric relationship with the principal site responsible for K^+ ion-transfer during channel permeation.

5. Modelling two populations of apical K^+ channels

Zeiske and Van Driessche [13] have advanced a two site/three barrier model for the external part of the frog skin apical K^+ channel in which competing ions such as Cs^+, Ba^{2+} and K^+ interact at the inner position and where the other site represents the access path for a number of permeable ions (K^+, Tl^+, Rb^+, NH_4^+). It is unlikely that either of these sites is identical to the group classified by pK_A in the present paper, since we would expect a direct competition of K^+ and H^+ along that pathway. On the other hand, the group with pK_B (K^+-H^+ competition) may be regarded as one of the single-file sites. With this assumption, one carboxy group (pK_A) as well as the SH-group reacting with PCMB (or PCMPS and Cd^{2+}) being located at different allosteric sites, would not interfere with the open-close kinetics controlling the K^+ channel. This may explain why noise analysis apparently showed a single type of K^+ channel, since gating kinetics and competitive block would be identical for both channel types. So far, however, any systematic relation between the occurrence of the two K^+ channel populations and I_K stimulation (i.e., I_K magnitude) does not seem to exist. Although different types of K^+ channels can be found in the same cell

membrane, e.g., in nerve or epithelia [15,18], their origin or purpose in frog skin seem unclear at present.

Appendix

Treating K^+ channels as ion transport enzymes permits a description of the postulated two channel populations expressed simply as a sum of two K^+ current terms: Population X has two H^+-binding sites, one (pK_A) responsible for allosteric, the other (pK_B) for competitive K^+ channel block. Population Y lacks the pK_A site but not the pK_B site.

$$I_{tot} = \underbrace{\frac{\frac{I^x_{max}}{1+\frac{[H^+]_{muc}}{K_A}}\cdot[K^+]_{muc}}{K^x_M\left(\frac{1+\frac{[H^+]_{muc}}{K_B}}{1+\frac{[H^+]_{muc}}{K_A}}\right)+[K^+]_{muc}}}_{\text{population X}} + \underbrace{\frac{I^y_{max}\cdot[K^+]_{muc}}{K^y_M\left(1+\frac{[H^+]_{muc}}{K_B}\right)+[K^+]_{muc}}}_{\text{population Y}} \quad (1)$$

In Eqn. 1, K_M denotes the $[K^+]$ for 0.5 I_{max} and is the apparent Michaelis constant of the assumed saturable K^+ current of each population (not taking into account deviations from Michaelis Menten kinetics at low $[K^+]_m$ which are occasionally observed and might reflect cytosolic pH- and thus G_b-changes rather than apical P_K sensitivity to mucosal H^+). From the few preparations with (nearly) only one-step titration curves (not shown), we estimate $K^x_M \approx 50$ mmol/l and $K^y_M \approx 80$ mmol/l. If we assume, for instance, that $I^x_{max} = I^y_{max}$ (like in Fig. 2, curve 2), then Eqn. 1 predicts (with $K_A = 10^{-6}$ mol/l and $K_B = 10^{-3}$ mol/l) nearly the same type of shifts in the Hanes plots for pH-dependent I_K kinetics (see Fig. 8) as were found experimentally, e.g., in Fig. 5b. A decrease in K_B for channel type X would lead to a more mixed-type appearance of H^+ block.

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