Journal of Physiology (1991), **434**, *pp.* 577–590
With 7 figures
Printed in Great Britain

CYTOSOLIC pH REGULATES MAXI K^+ CHANNELS IN *NECTURUS* GALL-BLADDER EPITHELIAL CELLS

BY JULIO COPELLO, YOAV SEGAL AND LUIS REUSS

From the Department of Physiology and Biophysics, University of Texas Medical Branch, Galveston, TX 77550, USA

(*Received 13 July 1990*)

SUMMARY

1. The patch clamp technique was used to study the effects of internal and external pH on the Ca^{2+}- and voltage-activated maxi K^+ channel present in the apical membrane of *Necturus* gall-bladder epithelial cells.

2. When the pH of the solution bathing the cytosolic side of inside-out patches (pH_i) was lowered from 7·9 to 6·9, with internal free Ca^{2+} concentration ($[Ca^{2+}]_i$) buffered below saturation levels for the channel gating sites, channel open probability (P_o) decreased. At saturating Ca^{2+} concentrations, P_o was near 1·0, and unaffected by pH_i. The results are consistent with a competitive interaction between Ca^{2+} and H^+ at regulatory binding sites. Kinetic analysis assuming competitive binding yields a Hill coefficient for H^+ of 1·3.

3. At sub-maximal $[Ca^{2+}]_i$, changing the pH of the solution bathing the extracellular surface of the patch (pH_o) between 8 and 7, had no effect on maxi K^+ channel P_o, but lowering pH_o to 6 or 5 significantly reduced P_o. At saturating $[Ca^{2+}]_i$, P_o was independent of pH_o.

4. There were no effects of either pH_i or pH_o on single-channel conductance.

5. Inasmuch as reductions in either pH_o or pH_i decrease maxi K^+ channel P_o, changes in maxi K^+ channel activity account in part for the reduction of apical membrane K^+ conductance elicited by acidification of the bathing medium. The dominant effect of pH on maxi K^+ channels is on the cytosolic surface of the membrane.

6. The change in P_o elicited by small changes in $[H^+]_i$ ($\delta P_o/\delta[H^+]_i$) is $-7{\cdot}6\ \mu M^{-1}$, compared to $\delta P_o/\delta[Ca^{2+}]_i = 2{\cdot}6\ \mu M^{-1}$, both at $V_m = -30$ mV and at physiological intracellular $[H^+]$ and $[Ca^{2+}]$. This implies that $[H^+]_i$ and $[Ca^{2+}]_i$ have opposite effects on channel P_o at physiological levels and underlines the importance of pH_i in channel gating.

INTRODUCTION

Large-conductance Ca^{2+}- and voltage-activated K^+ channels (maxi K^+) are expressed in many cells types (Rudy, 1988; Latorre, Oberhauser, Labarca & Alvarez, 1989). The role of this channel in excitable tissues is to repolarize the membrane after depolarization (Latorre, Vergara & Hidalgo, 1982). In exocrine glands, these channels appear to contribute to the process of ion and fluid secretion (Petersen, 1986). In

renal tubule cells, the channel is involved in the electrical response to agonists, in cell volume regulation, and possibly in K^+ secretion (Guggino, Suárez-Isla, Guggino & Sacktor, 1985; Hunter, Kawahara & Giebisch, 1986; Strange, 1990).

Patch-clamp and intracellular-microelectrode studies have demonstrated the existence of a Ca^{2+}-activated maxi K^+ channel in the apical membrane of *Necturus* gall-bladder epithelium. This channel accounts for roughly 15% of the apical membrane conductance (G_a) at rest, but is the principal pathway responsible for the rise of G_a with depolarization (Segal & Reuss, 1990*a*, *b*). Acidification of the mucosal bathing solution at constant P_{CO_2} causes a decrease in G_a, reduces the magnitude of the depolarization induced by increasing extracellular $[K^+]$ (Reuss, Cheung & Grady, 1981), and blocks the depolarization-activated component of the apical membrane conductance (García-Díaz, Nagel & Essig, 1983; Stoddard & Reuss, 1988). In addition, raising mucosal solution P_{CO_2} elicits marked cell membrane depolarization (Stoddard & Reuss, 1989). These results suggest that the channels are pH sensitive. In principle, pH regulation of apical maxi K^+ channels could contribute to the regulation of fluid absorption (Reuss, Segal & Altenberg, 1991), K^+ secretion (Reuss, 1981) and cell volume (Cotton & Reuss, 1990), as well as other cellular functions. In the intact epithelium it is difficult to establish unambiguously the pathways involved in the pH effects on cell membrane properties: maxi K^+ channels are not the only K^+-conductive pathways across the apical membrane and changes in membrane voltage may result from alterations in intracellular ion activities, in ionic permeabilities at the apical and/or the basolateral membrane domains, or in rate of electrogenic transporters.

Patch-clamp studies have established that cell acidification decreases maxi K^+ channel activity in *Necturus* choroid plexus (Christensen & Zeuthen, 1987), pancreatic β-cells (Cook, Ikeuchi & Fujimoto, 1984), and cultured renal medullary thick ascending limb cells (Cornejo, Guggino & Guggino, 1989). In the latter preparation, the mechanism of the effect of intracellular pH (pH_i) appears to be an interaction between H^+ and Ca^{2+} at the gating site(s). However, in the preparations listed above the $[Ca^{2+}]_i$ necessary to activate maxi K^+ channels was greater (*ca* 20-fold) than in *Necturus* gall-bladder, which suggests differences in the properties of the Ca^{2+}-gating sites and perhaps in the pH_i sensitivity.

In this paper we use the patch-clamp technique to assess the effects of internal and external pH on the apical maxi K^+ channel of *Necturus* gall-bladder epithelium. This channel is interesting because of its gating properties (see above) and because the results of single-channel experiments can be correlated with extensive intracellular microelectrode studies. In addition, the effects of external pH (pH_o) on maxi K^+ channels have not been investigated in detail. We aimed to document the effects of pH and to assess the importance of H^+ as a modulator of channel activity relative to Ca^{2+}, presumed to be the chief channel modulator *in vivo*.

A preliminary report of this work has been published in abstract form (Copello, Segal & Reuss, 1990).

METHODS

Preparation

Patch-clamp experiments were carried out on dissociated epithelial cells from *Necturus* gall-bladder. These cells express a maxi K^+ channel with identical properties to those of the channel

found in the apical membrane of the intact epithelium (see Segal & Reuss, 1990*a*, *b*; Altenberg, Copello, Cotton, Dawson, Segal, Wehner & Reuss, 1990). Higher success rates in obtaining high-resistance seals in dissociated cells favour the use of this preparation.

Mudpuppies (*Necturus maculosus*) were obtained from Nasco Biologicals (Fort Atkinson, WI, USA) or Kon's Scientific (Germantown, WI, USA), kept in tap water at 5 °C, and anaesthetized by immersion in a 1 g/l solution of tricaine methanesulphonate. Gall-bladders were excised, sliced open, drained of bile, pinned mucosal side up on a Sylgard-coated Petri dish, and bathed with NaCl Ringer solution containing (in mM): 97·5 NaCl, 2·5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 sucrose, 10 HEPES–NaOH (pH 7·4). Cell suspensions were prepared by a 7 min incubation of scraped epithelial sheets in 1 mg/ml hyaluronidase, followed by centrifugation and resuspension of the cells in hyaluronidase-free NaCl Ringer (Segal & Reuss, 1990*a*). Aliquots of the suspension were transferred to the experimental chamber, which was positioned on the stage of an inverted microscope (Diaphot; Nikon, Garden City, NY, USA).

Electrophysiology

Patch pipettes were constructed from TW150-6 borosilicate glass (World Precision Instrument Inc., New Haven, CT, USA) using a two-stage vertical puller (Model PP-103; Narishige, Japan), fire-polished under microscopic observation at 400 ×, and coated at the tip with Sylgard 184 (Dow Corning Co., Midland, MI, USA), as described by Corey & Stevens (1984).

Gigaohm seals were obtained by lightly touching the dissociated cells with patch pipettes and applying gentle suction. Patches were excised by tapping the microscope stage. Identification of maxi K^+ channels was easy based on their high conductance. To establish the orientation of the excised patch, we changed the Ca^{2+} concentration in the bath from 10^{-3} to 10^{-7} M; in inside-out patches, this manoeuvre changes the channel activity from near maximal to near zero, whereas in outside-out patches there is no effect. In some cases, we also added 1 mM-tetraethylammonium (TEA^+) or 0·1 mM-Ba^{2+} to the bath solution, taking advantage of the different sensitivities of maxi K^+ channels to these agents when applied to the intra- or extracellular surfaces (Segal & Reuss, 1990*b*).

The effects of pH_i on maxi K^+ channels were studied using inside-out membrane patches, usually exposed to a pipette solution containing (in mM): 100 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 HEPES–KOH (pH 7·4). The cytoplasmic surfaces of these patches were bathed with a solution containing (in mM): 100 KCl, 10 NaCl, 1 $MgCl_2$, 10 HEPES–KOH (variable pH). Ca^{2+} was buffered with 3 mM-1,2-bis-(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA) to a free [Ca^{2+}] varying from 0·1 to 1 μM. Free Ca^{2+} was adjusted to the desired levels (between 0·1 and 1 μM) adding amounts of $CaCl_2$ calculated according to the stability constants of Ca^{2+}–BAPTA and Mg^{2+}–BAPTA (Tsien, 1980) taking into account temperature, pH and ionic strength of the solution (Harrison & Bers, 1987). BAPTA was chosen for these studies because the apparent stability constant of the Ca^{2+}–BAPTA complex varies by only *ca* 20 % in the range of pH values tested in our experiments (Tsien, 1980; Harrison & Bers, 1987). We corroborated the calculation of free Ca^{2+} levels with a Ca^{2+}-sensitive electrode (TIPCA, World Precision Instruments Inc., New Haven, CT, USA). Calibrations were carried out with the solutions according to Tsien & Rink (1980) and also with 5 mM-ethyleneglycol-bis(β-aminoethylether)-*N*,*N*′-tetraacetic acid (EGTA) K^+ Ringer solutions with variable free Ca^{2+} spanning the range between 10^{-6} and 10^{-7} M. The amounts of added Ca^{2+} in the latter solutions were calculated from the stability constants of Ca^{2+}–EGTA and Mg^{2+}–EGTA published by Martell & Smith (1974). There was no change in the voltage output of the electrode when the pH was changed between 6·9 and 7·9 in low-Ca^{2+}, unbuffered solutions. The pH insensitivity of electrodes based on the calcium ionophore ETH 1001 has been established by others (Ammann, 1986). Ca^{2+} electrode measurements showed small differences between calculated and measured free Ca^{2+} levels, likely to arise from batch-to-batch variability in the purity of BAPTA (Harrison & Bers, 1987).

The effects of pH_o were studied in patches in the outside-out configuration. The pipette solution contained (in mM): 95 KCl, 1 $MgCl_2$, 20 HEPES–KOH (pH = 7·4). The [Ca^{2+}] was 1 mM, or buffered to 0·3 μM with 5·0 mM-EGTA. The external surfaces of these patches (bath solution) were superfused with KCl solutions of variable pH (ranging between 5 and 8) and containing 1 mM-Ca^{2+}.

Single-channel currents were measured with a List EPC-7 patch clamp (List Electronic, Darmstadt-Eberstadt, Germany) and stored on videotape using an Indec IR-2 digital instrument recorder (Index Systems, Sunnyvale, CA, USA). Patch recordings of 20–60 s duration were filtered at 2 kHz with an eight-pole Bessel filter (Frequency Devices, Haverhill, MA, USA) and played onto

a strip chart recorder (f_c = 125 Hz) for analysis by hand, or digitized at 2·5 kHz using a Data Translation DT2801-A analog-to-digital conversion system (Data Translation, Inc., Marlborough, MA, USA) for analysis by computer. Current–voltage (I–V) relations were determined by measuring the unitary current amplitude at each holding voltage.

Single-channel open probability (P_o) was estimated from

$$P_o = (1/N_c) \sum_{i=1}^{N_c} i P_i, \quad (1)$$

where i is a summation index, N_c is the number of channels in the patch (determined by maximal activation with 1 mM-Ca^{2+}), and P_i is the fraction of time during which i channels are open. This expression assumes that channels in multiple-channel patches gate independently and identically. In the figures, holding voltages correspond to membrane voltages (V_m), and are reported with respect to the extracellular compartment (pipette solution in inside-out, or bath solution in outside-out patches).

Statistics and curve fitting

Unless otherwise noted, experimental values are expressed as means ± standard error of measurement (S.E.M.). Curve fitting was done by non-linear regression analysis of pooled data. Fitting routines were based on the Marquardt–Levenberg algorithm and are commercially available (Sigmaplot 4·0, Jandel Scientific, Corte Madera, CA, USA; NFIT, Island Products, Galveston, TX, USA).

RESULTS

Effects of pH_i on maxi K^+ channels

The effects of pH_i were assessed initially with $[Ca^{2+}]_i$ buffered to 0·3 μM, a value close to the free Ca^{2+} concentration measured in *Necturus* gall-bladder epithelial cells with Ca^{2+}-sensitive microelectrodes (Palant & Kurtz, 1987). At pH_i = 7·4, which is within the range of normal pH_i values reported in this preparation (Weinman & Reuss, 1982; Stoddard & Reuss, 1989), the single-channel open probability (P_o) rose from 0·13 ± 0·06 at V_m = −65 mV to 0·48 ± 0·15 at V_m = −30 mV (Fig. 1*A* and *C*). These values are in good agreement with those found on cell-attached patches in the apical membrane of intact epithelium, in which P_o ranged between 0·01 and 0·15 at the normal V_m of −60 to −70 mV and rose to 0·79–0·84 with a 30 mV depolarization (Segal & Reuss, 1990*a*).

Single-channel conductance remained essentially unchanged when pH_i was changed in the range of 7·9–6·9 (203 *vs.* 206 pS, Fig. 1*A* and *B*). In contrast, the P_o *vs.* V_m curves showed progressive displacements to the right when the bath solution was acidified from 7·9 to 6·9. The P_o at −65 mV (the resting apical membrane voltage *in situ*) was 0·23 at pH_i 7·9, 0·13 at pH_i 7·4 and 0·01 at pH_i 6·9 (Fig. 1*C*). In two additional experiments (data not shown) we found that elevating pH_i from 7·9 to 8·9 displaced the P_o *vs.* V_m curve to the left, indicating that the channel gating is pH_i sensitive over at least two pH units (see below).

The pooled P_o *vs.* V_m data at each pH_i (Fig. 1*C*) were fitted to a Boltzmann relation as previously done by Segal & Reuss (1990*a*):

$$P_o = \frac{P_o^{max}}{1 + \exp[-N_z F(V_m - V_o)/RT]}, \quad (2)$$

where P_o^{max} is the maximal P_o, N_z is an empiric parameter denoting the effective number of charges which traverse the membrane during transition between open and

closed states, and V_0 is the membrane voltage at which $P_o = P_o^{max}/2$. In our experiments, N_z did not change appreciably with pH_i (1·7 ± 0·4 at pH 7·9, 1·5 ± 0·3 at pH 7·4, and 2·1 ± 0·4 at pH 6·9), indicating that pH_i does not affect the voltage dependence of the gating reaction. In contrast, V_0 became more positive as pH_i was

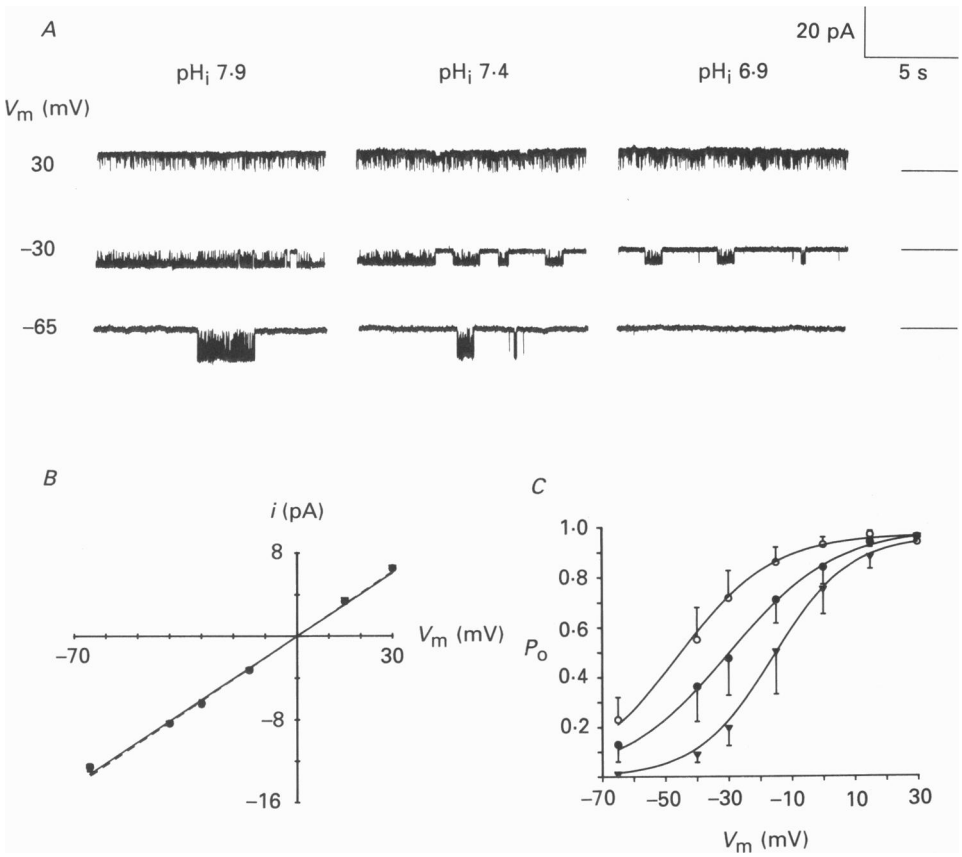

Fig. 1. Effect of pH_i on channels exposed to 0·3 μM $[Ca^{2+}]_i$. Inside-out patches were exposed to 100 mM-KCl on both sides, with 0·3 μM-Ca^{2+} in the cytoplasmic (bath) solution. *A*, example of maxi K^+ channel current as a function of V_m, at pH 6·9, 7·4 and 7·9. *B*, single-channel *I–V* relations at pH 6·9 (▼) and 7·9 (○) ($n = 5$). *C*, single-channel P_o *vs.* V_m at pH_i 6·9 (▼), 7·4 (●) and 7·9 (○) ($n = 6$). P_o was lowered by acidification, whereas single-channel current amplitudes at any voltage between −65 to 30 mV remained unchanged.

lowered, from −46 ± 4 mV at pH 7·9 to −30 ± 4 mV at pH 7·4, and to −16 ± 3 mV at pH 6·9.

The maxi K^+ channel in *Necturus* gall-bladder is activated by cytosolic Ca^{2+} (Segal & Reuss, 1990*a*). At 1 mM $[Ca^{2+}]_i$, the Ca^{2+} gating sites are saturated and the channels are fully activated. Under these conditions, changing pH_i from 8 to 5, in the range of −40 to 40 mV, had no effects on either P_o (Fig. 2*C*), or the *I–V* relations (Fig. 2*A* and *B*).

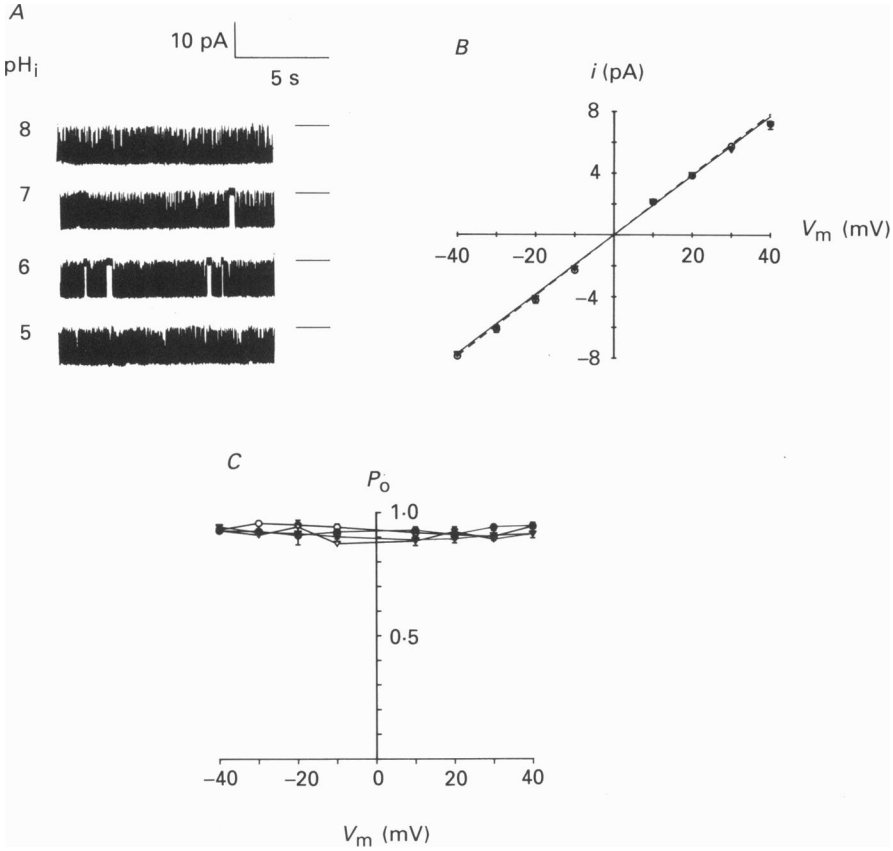

Fig. 2. Effects of pH_i on channels exposed to 1 mM $[Ca^{2+}]_i$. Inside-out patches were exposed to 100 mM-KCl on both sides, with 1 mM-Ca^{2+} on the cytoplasmic (bath) side. *A*, examples of maxi K^+ channel currents at −30 mV, at the pH_i values indicated. *B*, single-channel *I–V* relations at pH_i 5 (▼) and 8 (○) ($n = 4$ experiments). *C*, P_o as a function of V_m at pH_i 5 (▼), 6 (▽), 7 (●) and 8 (○) ($n = 4$). At this $[Ca^{2+}]_i$, P_o is independent of pH_i.

Interactions between H^+ and intracellular Ca^{2+}

The effect of pH_i on Ca^{2+} activation of the channel at $V_m = -30$ mV is shown in Fig. 3. Individual curves were fitted by the Hill equation

$$P_o = \frac{P_o^{max}[Ca^{2+}]_i^{N_{Ca}}}{K + [Ca^{2+}]_i^{N_{Ca}}}, \quad (3)$$

where P_o^{max} is maximal P_o determined at 1 mM $[Ca^{2+}]_i$ (0·95, data not shown), N_{Ca} is the Hill coefficient for Ca^{2+}, and k is an apparent equilibrium constant. K and N_{Ca} give the apparent $[Ca^{2+}]_i$ eliciting channel half-maximal activity according to the relation (see Segel, 1975)

$$([Ca^{2+}]_i)_{0·5} = K^{1/N_{Ca}}. \quad (4)$$

The fitted parameters, shown in Table 1, indicate that lowering pH_i in the

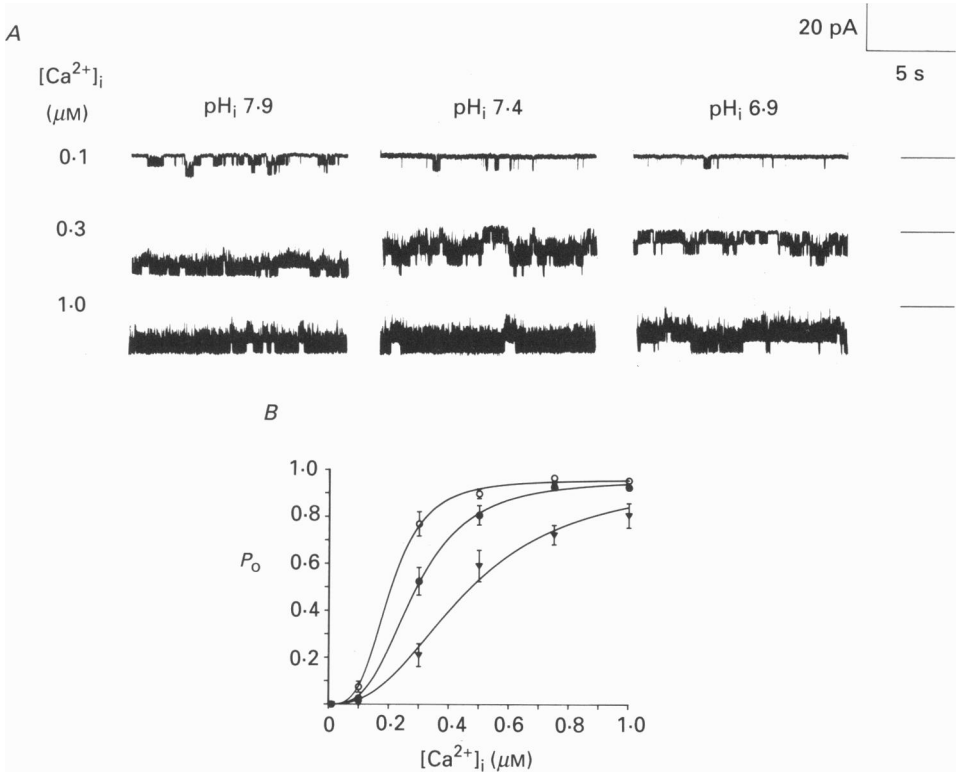

Fig. 3. Effects of pH_i on Ca^{2+} gating of maxi K^+ channels at $V_m = -30$ mV. Inside-out patches were exposed to 100 mM-KCl solution on both sides; $[Ca^{2+}]_i$ and pH_i are indicated. *A*, maxi K^+ channel currents in a patch containing four channels. $[Ca^{2+}]_i$ was buffered to 0·1, 0·3 or 1·0 μM. pH_i was 6·9, 7·4 or 7·9. *B*, P_o at $V_m = -30$ mV and at varying $[Ca^{2+}]_i$ at pH_i 6·9 (▼), 7·4 (●) and 7·9 (○) ($n = 5$). The curves depict fits to the Hill equation (see text). Lowering pH_i decreases the apparent affinity of the gating site for Ca^{2+} and the apparent co-operativity of Ca^{2+} binding (see Table 1).

TABLE 1. H^+ and Ca^{2+} interaction on maxi K^+ channel P_o

pH	N_{Ca}	$K \times 10^3$	$[Ca^{2+}]_{0.5}$ (μM)
7·9	3·7 ± 0·3	2·6 ± 1·1	0·20 ± 0·01
7·4	3·4 ± 0·3	15 ± 5	0·29 ± 0·01*
6·9	2·9 ± 0·3	130 ± 37	0·47 ± 0·02*†

Values shown are means ± S.D. of estimates obtained from the data in Fig. 3 ($n = 5$ experiments). V_m was −30 mV. The P_o *vs.* $[Ca^{2+}]_i$ relations at pH_i 6·9, 7·4 and 7·9 were fitted to eqn (3). P_o^{max} was fixed at 0·95 in all cases (see text).

Since $K = [Ca^{2+}]_{0.5}^{N_{Ca}}$, the values shown have different dimensions and therefore are not statistically comparable.

* $P < 0.01$ with respect to pH 7·9; † $P < 0.01$ with respect to pH 7·4.

physiological range decreases the apparent Ca^{2+} affinity and the apparent number of Ca^{2+} binding sites and/or co-operativity of binding.

The effect of pH_i on P_o at $[Ca^{2+}]_i = 0.3$ μM and $V_m = -30$ mV is summarized in Fig.

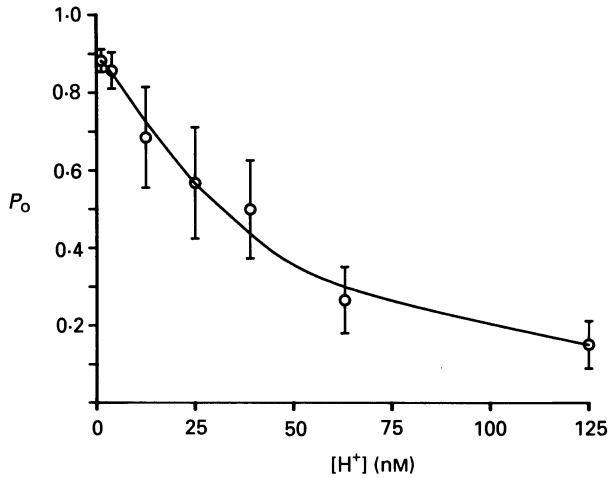

Fig. 4. Effects of pH_i on maxi K^+ channel P_o at $[Ca^{2+}]_i = 0{\cdot}3$ μM and $V_m = -30$ mV. The P_o values at different pH_i were fitted by the Hill equation for multisite competitive inhibition (see text), yielding values of 38 nM for $[H^+]_{0{\cdot}5}$ and 1·3 for N_H; $n = 7$ experiments.

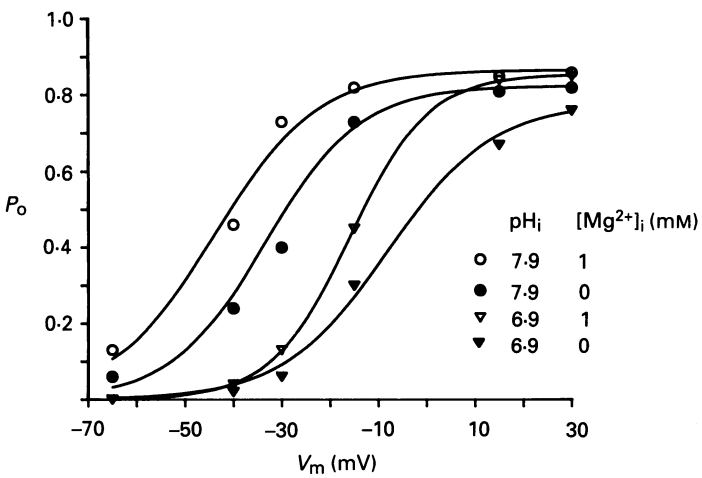

Fig. 5. Effects of pH_i on the activity of a maxi K^+ channel exposed to 0·3 μM-Ca^{2+} in nominally Mg^{2+}-free media. Data represent P_o as a function of V_m at pH 6·9 or 7·9, in the presence of nominally 0 or 1 mM-Mg^{2+} on the cytosolic side. The effect of pH_i on P_o is still present in Mg^{2+}-free medium. However, the channel activity is slightly reduced (see text).

4. Hydrogen ions could interfere with activation of the channel by several possible mechanisms (see Discussion). To estimate the number of inhibitory sites, we assume that H^+ competes with Ca^{2+} for channel regulatory sites. This mechanism has been proposed previously (Christensen & Zeuthen, 1987; Cornejo *et al.* 1989; see also Discussion), and in our case is supported by the observation that high internal Ca^{2+} levels overcome H^+ blocking effects (Figs 2 and 3). The data from Fig. 4 were fitted by the Hill equation for multisite competitive inhibition:

$$P_o = \frac{P_o^0 K'}{K' + [H^+]^{N_H}}, \tag{5}$$

where P_o^0 is P_o measured in the absence of inhibitor, K' is an apparent equilibrium constant, and N_H is the apparent number of H^+ inhibitory sites. $[H^+]_{0.5}$ (half-maximal inhibitory $[H^+]_i$) is given by $(K')^{1/N_H}$. The fit yields values of 38 ± 9 nM for $[H^+_{0.5}]$ (pH_i 7·42) and $1{\cdot}3 \pm 0{\cdot}4$ for N_H. This value of N_H (not significantly different

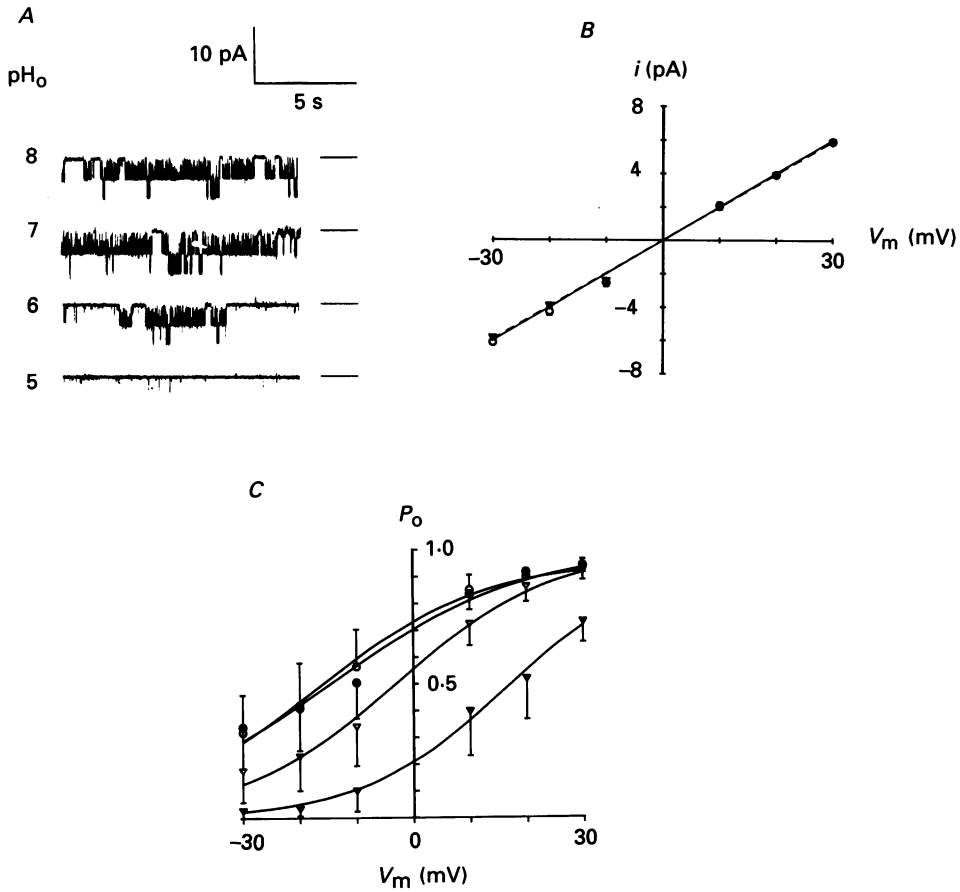

Fig. 6. Effects of pH_o on channels exposed to 0·3 μM $[Ca^{2+}]_i$. Outside-out patches were bathed in symmetrical 100 mM-KCl. $[Ca^{2+}]_i$ was buffered to 0·3 μM with 5 mM-EGTA, at pH_i 7·4. *A*, example of maxi K^+ channel currents at −20 mV, at the pH_o values indicated. *B*, *I–V* relations at pH_o 5 (▼) and 8 (○) (means ± S.E.M. are shown, $n = 4$). *C*, P_o as a function of V_m, at pH_o 5 (▼), 6 (▽), 7 (●) and 8 (○). ($n = 4$). There are no pH effects between 8 and 7. Current amplitudes were not affected by changes in pH_o.

from 1·0) is a minimum estimate of the number of H^+ binding sites if their affinity is high. We cannot exclude the possibility of a larger number of sites with low affinity.

Effects of pH_i in Mg^{2+}-free solutions

Mg^{2+} ions are positive modulators of maxi K^+ channel activity in several preparations, increasing the co-operativity of Ca^{2+} binding (Golowasch, Kirkwood & Miller, 1986; Squire & Petersen, 1987; Trieschmann & Isenberg, 1989). Hence, pH_i

interference with Mg^{2+} modulation is an alternative mechanism for the effect of $[H^+]_i$ on channel activity. To test this possibility, we studied the effects of pH_i on channel activity in the nominal absence of internal Mg^{2+}. Under these conditions, in all experiments, reducing pH_i caused a decrease in P_o. As illustrated in Fig. 5, the shapes

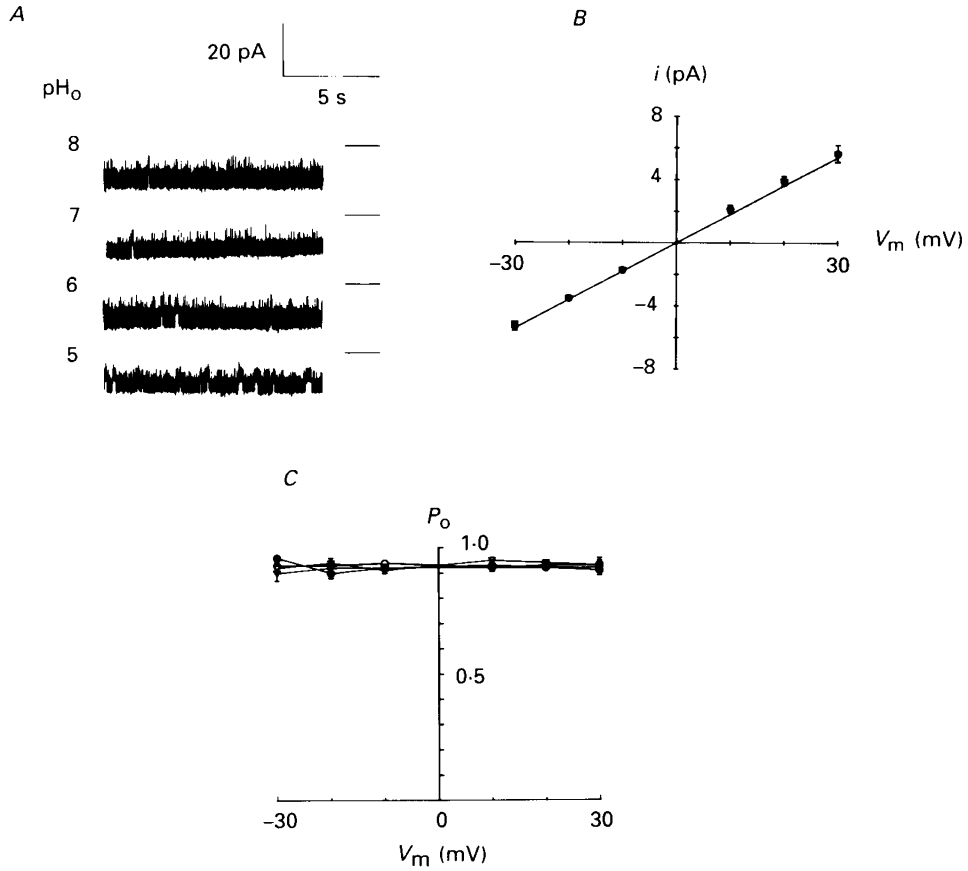

Fig. 7. Effects of pH_o on channels exposed to 1 mM $[Ca^{2+}]_i$. Outside-out patches were bathed in symmetrical 100 mM-KCl with 1 mM-Ca^{2+} in the pipette. At this $[Ca^{2+}]_i$, gating sites are saturated and the channels are fully activated. *A*, example of maxi K^+ channel currents at −30 mV, at the pH_o values indicated. *B*, single-channel *I–V* relations ($n = 4$ experiments) at pH_o 5 (▼) and 8 (○). *C*, P_o as a function of V_m, at pH_o 5 (▼), 6 (▽), 7 (●) and 8 (○) ($n = 4$). At this $[Ca^{2+}]_i$, P_o and single-channel conductance are independent of pH_o.

of the P_o *vs.* V_m curves were unchanged, either at pH_i 6·9 or 7·9, but in the absence of Mg^{2+} the curves were displaced to the right. This indicates that Mg^{2+} directly or indirectly activates the channel. However, under these conditions the Mg^{2+} effects were minor compared with those of intracellular pH or Ca^{2+}. These results suggest that the pH_i effects on maxi K^+ channels are predominantly Mg^{2+} independent.

Effects of pH_o on maxi K^+ channels

The effects of pH_o on maxi K^+ channel properties were assessed by changing the pH in the bath solution superfusing outside-out patches. The $[Ca^{2+}]_i$ in the pipette solution was kept at 0·3 μM (buffered with 5 mM-EGTA). The results, shown in Fig. 6, indicate that changes in pH_o have no effects on channel activity in the range between 8 and 7. However, lowering pH_o further, to 6 or 5, reduced channel activity. It was necessary to limit the range of voltages to -30 to $+30$ mV in most of these experiments in order to obtain successful recordings at all pH_o values, because of instability and short mean life of the outside-out membrane patches.

It is conceivable that the reduction in P_o at low pH_o values is caused by acidification of the pipette solution and hence mediated at an internal site. However, this possibility is unlikely because of the high buffering power of the pipette solution ($\sim$ 12 mM) and because the rise in $[Ca^{2+}]_i$ (expected because H^+ displaces Ca^{2+} from EGTA) should counteract the putative pH_i change, resulting in an elevation in P_o.

When Ca^{2+} in the pipette solution ($[Ca^{2+}]_i$) was raised to 1 mM, in order to saturate the Ca^{2+} gating sites, the pH_o effects were absent (Fig. 7). These observations suggest that extracellular H^+ regulates the Ca^{2+} gating sites, but this effect is sizable only at $pH_o \leqslant 6$.

DISCUSSION

The effects of internal and external pH on *Necturus* gall-bladder maxi K^+ channels were assessed in excised membrane patches using the patch-clamp technique. Neither pH_i nor pH_o significantly affected single-channel currents, implying that pH changes do not affect conduction. In physiologically relevant ranges, P_o was reduced by intracellular but not by extracellular acidification.

Comparison with previous studies

Calcium- and voltage-activated maxi K^+ channels from several cell types are inhibited by cytosolic H^+ (Cook *et al.* 1984; Christensen & Zeuthen, 1987; Cornejo *et al.* 1989; Merot, Bidet, Le Maout, Tauc & Poujeol, 1989; Tabcharani & Misler, 1989).

On the basis of channel open- and closed-time analyses, Christensen & Zeuthen (1987) and Cornejo *et al.* (1989) proposed that acidification decreases the Ca^{2+} affinity of Ca^{2+} binding sites. The disappearance of pH_i blocking effects at saturating $[Ca^{2+}]_i$ (see Results) is consistent with H^+–Ca^{2+} competition at the Ca^{2+} gating sites. The assumption that inhibition is purely competitive leads to an estimate of one H^+ inhibitory site. Recently, Laurido, Wolff & Latorre (1990) have reported that H^+ may reduce the Ca^{2+} affinity of Ca^{2+}-activated K^+ channels from rat skeletal muscle by both competitive and non-competitive mechanisms.

Models of maxi K^+ channel gating have grown increasingly complex, involving numerous open and closed states. Ca^{2+}-binding sites, allosteric regulatory sites, e.g. for internal Mg^{2+}, and effects of surface charge (Golowasch *et al.* 1986; Squire & Petersen, 1987; McManus & Magleby, 1988; MacKinnon, Latorre & Miller, 1989). This is not surprising, in light of the growing appreciation of the multisite nature and complex regulation of calcium binding proteins (Cheung, 1983; Strynadka &

James, 1989). It is likely that H^+ can interact with several channel binding sites. Possible mechanisms of these effects are: (1) inhibition of Ca^{2+} association with Ca^{2+} binding sites, by competitive or non-competitive mechanisms, (2) promotion of Ca^{2+} dissociation, (3) stabilization of channel closed states, and (4) destabilization of channel open states. Our analysis of steady-state P_o data does not allow us to discriminate among these possibilities.

The current results in *Necturus* gall-bladder and previous results in *Necturus* choroid plexus (Christensen & Zeuthen, 1987) show that lowering pH_i displaces to the right the P_o *vs.* V_m curve, both in the presence and in the nominal absence of Mg^{2+}. Hence, the effects of pH_i are not mediated by H^+ interference with Mg^{2+} at the maxi K^+ channel Ca^{2+} modulatory site(s). In their study of the effects of membrane surface charge on maxi K^+ channels, McKinnon *et al.* (1989) found, in addition to changes in P_o, significant effects on channel conductance and on channel block. In contrast, in our studies changes in pH_i from 6·9 to 7·9 had no effect on single-channel conductance (see Results) or on Ba^{2+} block (data not shown). We also ruled out that the effect of pH_i is due to titration of a group that makes the channel activity independent of Ca^{2+}. At $[Ca^{2+}]_i$ below 10^{-8} M, and $V_m \geqslant 20$ mV, channel P_o is greater than 0·05 and remains unaltered during pH changes between 9 and 7 (data not shown). These observations indicate that pH_i has no effects independent of Ca^{2+}.

At $[Ca^{2+}]_i$ below saturation, pH_o also affects single-channel P_o, but appreciable effects occur only at $pH_o \leqslant 6$, and not in the range in which pH_i elicits marked changes in channel activity. The effects of pH_o were only evident when Ca^{2+} on the cytosolic surface of the patch was buffered at levels below saturation. As discussed above, it is unlikely that this effect is secondary to H^+ permeation of the membrane patch, and titration of internal sites. An alternative explanation is an indirect, or 'allosteric', modification of the gating site from the opposite membrane surface. This has been proposed as an explanation for the effects of angiotensin II on smooth muscle maxi K^+ channels (Toro, Amador & Stefani, 1990). External pH was reported to have no effects on the activity of maxi K^+ channel in cultured medullary thick ascending limb cells (Cornejo *et al.* 1989), but the intracellular Ca^{2+} levels were not specified.

pH regulation of maxi K^+ channels in gall-bladder epithelium

Necturus gall-bladder maxi K^+ channels have a P_o of about 0·15 at rest (Segal & Reuss, 1990*a*). Hence, inhibition by H^+ could explain, at least in part, the depolarization of the apical membrane promoted by luminal solution acidification (Reuss *et al.* 1981) or by elevation of luminal CO_2, which acidifies the cell (Stoddard & Reuss, 1989).

Our data permit comparison between the Ca^{2+} and H^+ dependence of channel gating. By using eqns (3) and (5) and the best-fit parameters from the data in Figs 3 and 4, we calculate the partial derivatives $(\delta P_o/\delta[Ca^{2+}]_i)_{pH,V_m} = 2{\cdot}6\ \mu M^{-1}$ and $(\delta P_o/\delta[H^+]_i)_{pCa,V_m} = -7{\cdot}6\ \mu M^{-1}$, at $V_m = -30$ mV, $pH_i = 7{\cdot}4$, and $[Ca^{2+}]_i = 0{\cdot}3\ \mu M$ (pCa *ca* 6·5). Therefore at near-physiological intracellular Ca^{2+} and H^+ concentrations, their effects in gall-bladder maxi K^+ channels are of similar magnitude. In contrast, in maxi K^+ channels with a lower affinity for Ca^{2+} (e.g. pancreatic β-cells), a tenfold increase in $[Ca^{2+}]_i$ is necessary (in the micromolar range) to compensate for a unit change in pH_i (near the physiological range) (Cook *et al.* 1984).

In summary, our observations suggest that small changes in intracellular pH near the physiological level may contribute to the modulation of maxi K^+ channels and hence could play a role in regulating apical membrane conductance and K^+ secretion. Under *in vivo* conditions, changes in pH_i could also affect maxi K^+ channels by indirect pathways involving changes in cytosolic Ca^{2+} activity and perhaps in the levels of other channel-modulating agents.

We are grateful for the comments of G. A. Altenberg, C. U. Cotton, S. A. Lewis and B. J. Simon on a preliminary version of this paper. We also thank K. Dawson and S. B. Puppio for technical help, J. Chilton for computer-related assistance and L. Durant for secretarial help. This research was supported by National Institutes of Health Grants DK-38734 and GM-07200.

REFERENCES

ALTENBERG, G., COPELLO, J., COTTON, C., DAWSON, K., SEGAL, Y., WEHNER, F. & REUSS, L. (1990). Electrophysiological methods for studying ion and water transport in *Necturus* gallbladder epithelium. *Methods in Enzymology* **192**, 650–683.

AMMANN, D. (1986). *Ion-Selective Microelectrodes. Principles, Design and Application*. Springer-Verlag, Berlin.

CHEUNG, W. Y. (1983). *Calcium and Cell Function*, vol. IV. Academic Press, London.

CHRISTENSEN, O. & ZEUTHEN, T. (1987). Maxi K^+ channels in leaky epithelia are regulated by intracellular Ca^{2+}, pH and membrane potential. *Pflügers Archiv* **408**, 249–259.

COOK, D. L., IKEUCHI, M. & FUJIMOTO, W. Y. (1984). Lowering of pH_i inhibits Ca^{2+}-activated K^+ channels in pancreatic B-cells. *Nature* **311**, 269–271.

COPELLO, J., SEGAL, Y. & REUSS, L. (1990). Effects of pH on apical maxi K^+ channels in *Necturus* gallbladder epithelium. *FASEB Journal* **4**, 447.

COREY, D. P. & STEVENS, C. F. (1984). Science and technology of patch-recording electrodes. In *Single-Channel Recording*, ed. SAKMANN, B. & NEHER, E., pp. 53–68. Plenum Press, New York.

CORNEJO, M., GUGGINO, S. E. & GUGGINO, W. B.(1989). Ca^{2+}-activated K^+ channels from cultured renal medullary thick ascending limb cells: effect of pH. *Journal of Membrane Biology* **110**, 49–55.

COTTON, C. & REUSS, L. (1990). Tetraethylammonium (TEA^+) inhibits changes in cell volume induced by alterations of mucosal solution K^+ or Cl^- concentrations in *Necturus* gallbladder epithelium. *Journal of General Physiology* **96**, 39a.

GARCÍA-DÍAZ, J. F., NAGEL, W. & ESSIG, A. (1983). Voltage-dependent K conductance at the apical membrane of *Necturus* gallbladder. *Biophysical Journal* **43**, 269–278.

GOLOWASCH, J., KIRKWOOD, A. & MILLER, C. (1986). Allosteric effects of Mg^{2+} on the gating of Ca^{2+}-activated K^+ channels from mammalian skeletal muscle. *Journal of Experimental Biology* **124**, 5–13.

GUGGINO, S. E., SUÁREZ-ISLA, B. A., GUGGINO, W. B. & SACKTOR, B. (1985). Forskolin and antidiuretic hormone stimulate a Ca^{2+} activated K^+ channel in cultured kidney cells. *American Journal of Physiology* **249**, F448–455.

HARRISON, S. M. & BERS, D. M. (1987). The effect of temperature and ionic strength on the apparent Ca-affinity of EGTA and the analogous Ca-chelators BAPTA and dibromo-BAPTA. *Biochimica et Biophysica Acta* **925**, 133–143.

HUNTER, M., KAWAHARA, K. & GIEBISCH, G. (1986). Potassium channels along the nephron. *Federation Proceedings* **45**, 2723–2726.

LATORRE, R., OBERHAUSER, A., LABARCA, P. & ALVAREZ, O. (1989). Varieties of calcium-activated potassium channels. *Annual Reviews of Physiology* **51**, 385–399.

LATORRE, R., VERGARA, C. & HIDALGO, C. (1982). Reconstitution in planar lipid bilayers of a Ca^{2+}-dependent K^+ channel from transverse tubule membranes isolated from rabbit skeletal muscle. *Proceedings of the National Academy of Sciences of the USA* **79**, 805–809.

LAURIDO, C., WOLFF, D. & LATORRE, R. (1990). Effect of pH in a Ca^{2+}-activated K^+ channel [K(Ca)] from rat skeletal muscle. *Biophysical Journal* **57**, 507a.

MACKINNON, R., LATORRE, R. & MILLER, C. (1989). Role of surface electrostatics in the operation of a high-conductance Ca^{2+}-activated K^+ channel. *Biochemistry* **28**, 8092–8099.

McMANUS, O. B. & MAGLEBY, K. L. (1988). Kinetic states and modes of single large-conductance calcium-activated potassium channels in cultured rat skeletal muscle. *Journal of Physiology* **402**, 79–120.

MARTELL, A. E. & SMITH, R. M. (1974). *Critical Stability Constants*, vol. 1, *Amino Acids*. Plenum Press, New York.

MEROT, J., BIDET, M., LE MAOUT, S., TAUC, M. & POUJEOL, P. (1989). Two types of K^+ channels in the apical membrane of rabbit proximal tubule in primary culture. *Biochimica et Biophysica Acta* **978**, 134–144.

PALANT, C. E. & KURTZ, I. (1987). Measurements of intracellular Ca^{2+} activity in *Necturus* gallbladder. *American Journal of Physiology* **253**, C309–315.

PETERSEN, O. H. (1986). Calcium-activated potassium channels and fluid secretion by exocrine glands. *American Journal of Physiology* **251**, G1–13.

REUSS, L. (1981). Potassium transport mechanisms by amphibian gallbladder. In *Ion Transport by Epithelia*, ed. SCHULTZ, S. G., pp. 109–128. Raven Press, New York.

REUSS, L., CHEUNG, L. Y. & GRADY, T. P. (1981). Mechanisms of cation permeation across apical cell membrane of *Necturus* gallbladder: effects of luminal pH and divalent cations on K^+ and Na^+ permeability. *Journal of Membrane Biology* **59**, 211–224.

REUSS, L., SEGAL, Y. & ALTENBERG, G. A. (1991). Regulation of ion transport across gallbladder epithelium. *Annual Review of Physiology* **53**, 361–373.

RUDY, B. (1988). Diversity and ubiquity of K channels. *Neuroscience* **25**, 729–749.

SEGAL, Y. & REUSS, L. (1990*a*). Maxi K^+ channels and their relationship to the apical membrane conductance in *Necturus* gallbladder epithelium. *Journal of General Physiology* **95**, 791–818.

SEGAL, Y. & REUSS, L. (1990*b*). Ba^{2+}, TEA^+ and quinine effects on apical membrane K^+ conductance and maxi K^+ channels in gallbladder epithelium. *American Journal of Physiology* **259**, C56–68.

SEGEL, I. H. (1975). *Enzyme Kinetics: Behavior and Analysis of Rapid Equilibrium and Steady-state Enzyme Systems*. John Wiley, New York.

SQUIRE, L. G. & PETERSEN, O. H. (1987). Modulation of Ca^{2+}- and voltage-activated K^+ channel by internal Mg^{2+} in salivary acinar cells. *Biochimica et Biophysica Acta* **899**, 171–175.

STODDARD, J. S. & REUSS, L. (1988). Voltage- and time-dependence of apical membrane conductance during current clamp in *Necturus* gallbladder epithelium. *Journal of Membrane Biology* **103**, 191–204.

STODDARD, J. S. & REUSS, L. (1989). Electrophysiological effects of mucosal Cl^- removal in *Necturus* gallbladder epithelium. *American Journal of Physiology* **257**, C568–578.

STRANGE, K. (1990). Volume regulation following Na^+ pump inhibition in CCT principal cells: apical K^+ loss. *American Journal of Physiology* **258**, F732–740.

STRYNADKA, N. C. J. & JAMES, M. N. G. (1989). Crystal structures of the helix-loop-helix calcium-binding proteins. *Annual Review of Biochemistry* **58**, 951–982.

TABCHARANI, J. A. & MISLER, S. (1989). Ca^{2+}-activated K^+ channel in rat pancreatic islet β-cells: permeation, gating and blockade by cations. *Biochimica et Biophysica Acta* **982**, 62–72.

TORO, L., AMADOR, M. & STEFANI, E. (1990). ANG II inhibits calcium-activated potassium channels from coronary smooth muscle in lipid bilayers. *American Journal of Physiology* **27**, H912–915.

TRIESCHMANN, U. & ISENBERG, G. (1989). Ca^{2+}-activated K^+ channels contribute to the resting potential of vascular myocytes. Ca^{2+} sensitivity is increased by intracellular Mg^{2+}-ions. *Pflügers Archiv* **414**, S183–184.

TSIEN, R. Y. (1980). New calcium indicators and buffers with high selectivity against magnesium and protons: design, synthesis, and properties of prototype structures. *Biochemistry* **19**, 2396–2404.

TSIEN, R. Y. & RINK, T. J. (1980). Neutral carrier ion-selective microelectrodes for measurement of intracellular free calcium. *Biochimica et Biophysica Acta* **599**, 623–638.

WEINMAN, S. A. & REUSS, L. (1982). Na^+–H^+ exchange at the apical membrane of *Necturus* gallbladder. Extracellular and intracellular pH studies. *Journal of General Physiology* **80**, 299–342.