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Ca^{2+} Activation and pH Dependence of a Maxi K^+ Channel from Rabbit Distal Colon Epithelium

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Abstract. To determine if their properties are consistent with a role in regulation of transepithelial transport, Ca^{2+}-activated K^+ channels from the basolateral plasma membrane of the surface cells in the distal colon have been characterized by single channel analysis after fusion of vesicles with planar lipid bilayers. A Ca^{2+}-activated K^+ channel with a single channel conductance of 275 pS was predominant. The sensitivity to Ca^{2+} was strongly dependent on the membrane potential and on the pH. At a neutral pH, the $K_{0.5}$ for Ca^{2+} was raised from 20 nM at a potential of 0 mV to 300 nM at −40 mV. A decrease in pH at the cytoplasmic face of the K^+ channel reduced the Ca^{2+} sensitivity dramatically. A loss of the high sensitivity to Ca^{2+} was also observed after incubation with $MgCl_2$, possibly a result of dephosphorylation of the channels by endogenous phosphatases. Modification of the channel protein may thus explain the variation in Ca^{2+} sensitivity between studies on K^+ channels from the same tissue. High affinity inhibition ($K_{0.5}$ = 10 nM) by charybdotoxin of the Ca^{2+}-activated K^+ channel from the extracellular face could be lifted by an outward flux of K^+ through the channel. However, at the ion gradients and potentials found in the intact epithelium, charybdotoxin should be a useful tool for examination of the role of maxi K^+ channels. The high sensitivity for Ca^{2+} and the properties of the activator site are in agreement with an important regulatory role for the high conductance K^+ channel in the epithelial cells.

Key words: Maxi K^+ channel — Calcium — pH — Charybdotoxin — Rabbit distal colon

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Introduction

Ca^{2+}-activated K^+ channels have been identified in a number of mammalian tissues and can be distinguished by their single channel conductance, calcium sensitivity, voltage dependence and pharmacological properties (*for review, see* 18). From a physiological point of view, the Ca^{2+}-activated K^+ channels are particularly interesting, since they may provide a link between second messenger systems and membrane conductance.

Aldosterone regulates Na^+ reabsorption from the intestinal lumen across the surface epithelial cells in the distal colon as part of the salt and water homeostasis of the organism [35]. In the surface cells, Na^+ is reabsorbed from the intestinal lumen through amiloride-sensitive Na^+ channels [35, 37]. The Na,K-pump in the basolateral membrane creates an electrochemical gradient for Na^+, and the K^+ channels allow K^+ to recycle across this membrane to avoid intracellular accumulation of K^+. Previously, we have identified and characterized Ca^{2+}-activated K^+ channels from the basolateral membrane of the surface cells [41]. Methods were developed for purification of Ca^{2+}-activated K^+ channels and subsequent reconstitution in phospholipid vesicles [15, 16, 20, 41]. Our studies showed that the basolateral K^+ channels are regulated by Ca^{2+} in the physiological range of concentration [41]. Based on the high sensitivity to Ca^{2+}, we suggested that the K^+ channels play an important role in the regulation of the transcellular ion transport. This hypothesis has been challenged in recent studies, where maxi K^+ channels from the basolateral membrane of surface cells of the colon epithelium were incorporated into lipid bilayers and found to require Ca^{2+} concen-

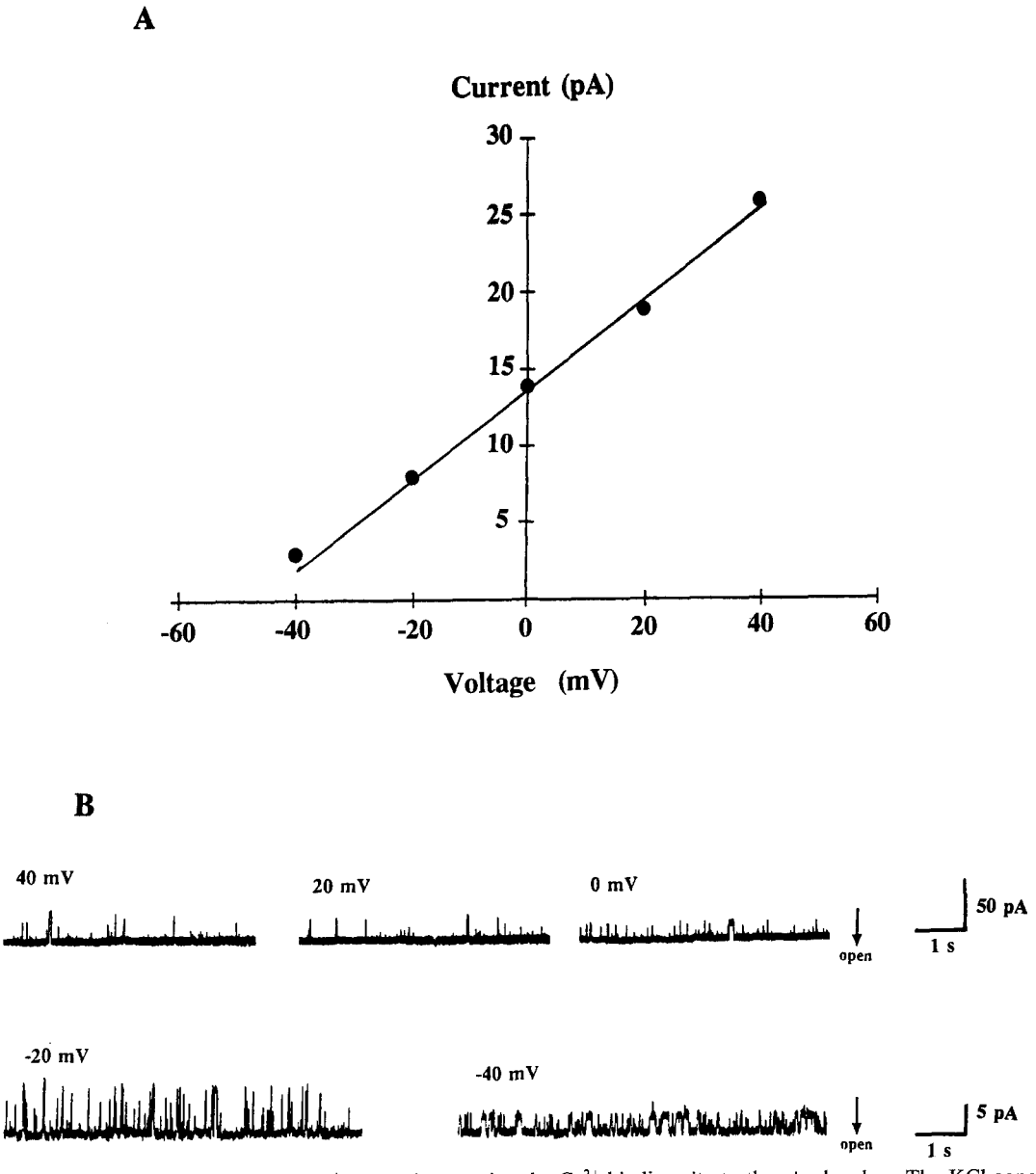

Fig. 1. (*A*) Current-voltage relationship for K^+ channels exposing the Ca^{2+}-binding site to the *cis* chamber. The KCl concentrations were 300 mM in the *cis* and 50 mM in the *trans* chamber. The current-voltage relation was linear at the given circumstances, and the mean single channel conductance was 275 pS. (*B*) Single channel traces for one channel under conditions as described above.

trations much higher than the physiological range to become activated [38].

In the present work, the properties of the maxi K^+ channels from the colon surface cells were therefore reexamined with particular emphasis on the interplay of Ca^{2+}, pH and voltage dependence. The Ca^{2+} activation of the maxi K^+ channel at different potentials, ion gradients and pH values, as well as the sidedness of the effects of Ca^{2+} and H^+ were examined. It was observed that the K^+ channels in some cases lost their high sensitivity to Ca^{2+}; this could be a result of dephosphorylation of the channel protein and may explain the discrepancy in the Ca^{2+} sensitivities found in different studies.

Charybdotoxin from the venom of the scorpion *Leiurus Quinquestriatus Hebraeus*, is a high affinity inhibitor of maxi K^+ channels from a number of tissues [25, 27], while other maxi K^+ channels are insensitive to the toxin [33]. However, questions have been raised concerning the use of the inhibitor in studies of the function and classification of channels in intact tissues, since it has recently been reported that charybdotoxin is ineffective at the membrane potential and ion gradients of intact cells [5].

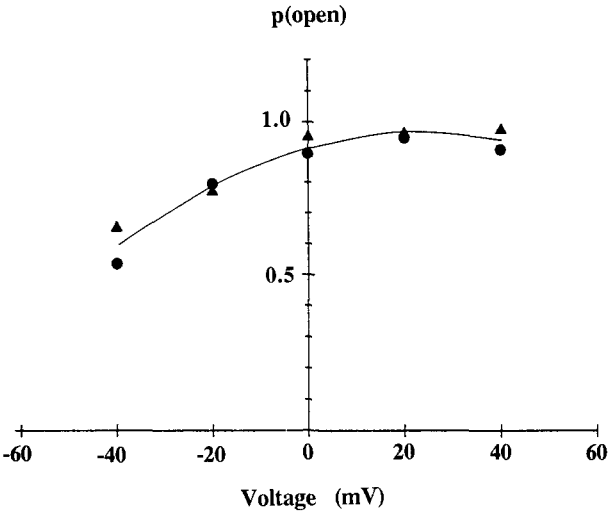

Fig. 2. Relation between open probability and voltage for K^+ channels under conditions as in Fig. 1. The voltage dependence of the open probability is shown for channels exposing their Ca^{2+}-activation site to the *cis* chamber (▲) and to the *trans* chamber (●). The free Ca^{2+} concentration is 200 μM.

We have therefore examined the high affinity inhibitory effect on the maxi K^+ channel at different potentials and ion gradients. It was observed that charybdotoxin could be knocked off the inhibitory site by an outward flux of K^+, but the toxin was indeed effective given the physiological parameters of the epithelial cells.

Materials and Methods

Vesicle Preparation

Basolateral plasma membrane vesicles from rabbit distal colon were isolated from mucosal scrapings as described earlier [42] and resuspended in 250 mM sucrose, 10 mM HEPES-Tris, pH 7.2 to a protein concentration of 10–15 mg/ml [3]. Batches of vesicles were prepared from several animals, frozen in liquid nitrogen, stored at −80°C, and thawed immediately prior to use. For incorporation of ion channels into planar lipid bilayers, the highly enriched surface cell basolateral membrane fraction [42] was used.

Incorporation of Channel Protein into Planar Lipid Bilayers

Planar lipid bilayers consisting of phosphatidylethanolamine (10 mg/ml) and phosphatidylserine (10 mg/ml) were painted over a 0.2 mm drilled aperture in a polystyrene cup (Sarstedt, FRG) placed in a Teflon block [1]. Immediately before use, the lipids were washed twice with pentane, which was evaporated in a stream of N_2, and then redissolved in decane. Initially the bath compartments contained about 2 ml 50 mM KCl, 0.2 mM $CaCl_2$, 10 mM HEPES-Tris, pH 7.2. Formation and thinning of the bilayer was monitored electrically by application of a triangle wave of ±50 mV at a frequency of 10 Hz. To promote fusion of plasma membrane vesicles with the bilayer, the salt concentration in the *cis* compartment was raised to 300 mM KCl before addition of 5 μl aliquots of vesicles containing approx. 15 μg protein. The *cis* compartment was then stirred using a magnetic stirring bar (length: 5 mm; diam.: 1.2 mm) at 50 rotations per minute until gated currents across the bilayer were detected. In some experiments, the salt concentration was raised to 300 mM KCl also in the *trans* chamber after fusion of channels.

The single channel currents were measured using a home-built patch-clamp amplifier connected to the chambers via Ag-AgCl electrodes and 2 M KCl agar bridges. Signals were digitized at 5 kHz using a modified Sony PCM 501 ES digital audio processor and recorded on VHS video tape using a Panasonic AG-6200 recorder. For analysis, recorded data were filtered at 1 kHz by an 8-pole Bessel filter (Krohn-Hite) and sampled at 2 kHz to an IBM AT personal computer using the "Patch and Voltage Clamp Analysis Program" from Cambridge Electronic Design, Cambridge, UK. Alternatively, data were filtered at 200 Hz and recorded on a Gould strip chart recorder. The bilayer chamber was placed on a vibration-free table in a grounded Faraday cage. The cage was noise isolated with 10 mm of noise absorbing material and 4 mm of oil-based noise-deadening material. During the experiments, solutions were changed by additions directly to the chambers. EGTA was added to control the free Ca^{2+}; for each pH value the equilibrium constant for the EGTA-Ca^{2+} buffer system was calculated according to Pershadsingh and McDonald [30]. In experiments using charybdotoxin, bovine serum albumin (50 μg/ml) was added to avoid unspecific binding to the chamber. The voltages were related to the direction of the incorporated channel: a negative voltage was negative with respect to the intracellular face of the channel as it is the case under physiological conditions in the cell. Movement of K^+ from the *cis* to the *trans* chamber was indicated by a negative current and appeared as a downward deflection in the current traces.

Statistics and Curve Fitting

Results are expressed as means. The standard error of the mean (SEM) is given for three or more observations. The Ca^{2+}-activation curves are fitted by the least-squares method (Enzfitter, Elsevier Biosoft) to the Hill equation: $P(o) = (P(o)^{max}\ [Ca^{2+}]^N)/(K + [Ca^{2+}]^N)$, where $P(o)$ is the measured open probability, $P(o)^{max}$ is the maximal open probability determined at a free Ca^{2+}-concentration of 200 μM, $[Ca^{2+}]$ is the free Ca^{2+}-concentration on the cytoplasmic face of the channel, N is the Hill coefficient and K is the apparent equilibrium constant. The apparent free Ca^{2+}-concentration for half-maximal activation can be calculated from the equilibrium constant and the Hill coefficient: $[Ca^{2+}]_{0.5} = K^{1/N}$.

Materials

Phosphatidylethanolamine and phosphatidylserine from bovine brain were from Avanti Polar Lipids, Alabaster, AL; pentane and decane were from Aldrich, Steinheim, Germany. Charybdotoxin was from Latoxan, Rosans, France. All other chemicals were analytical grade.

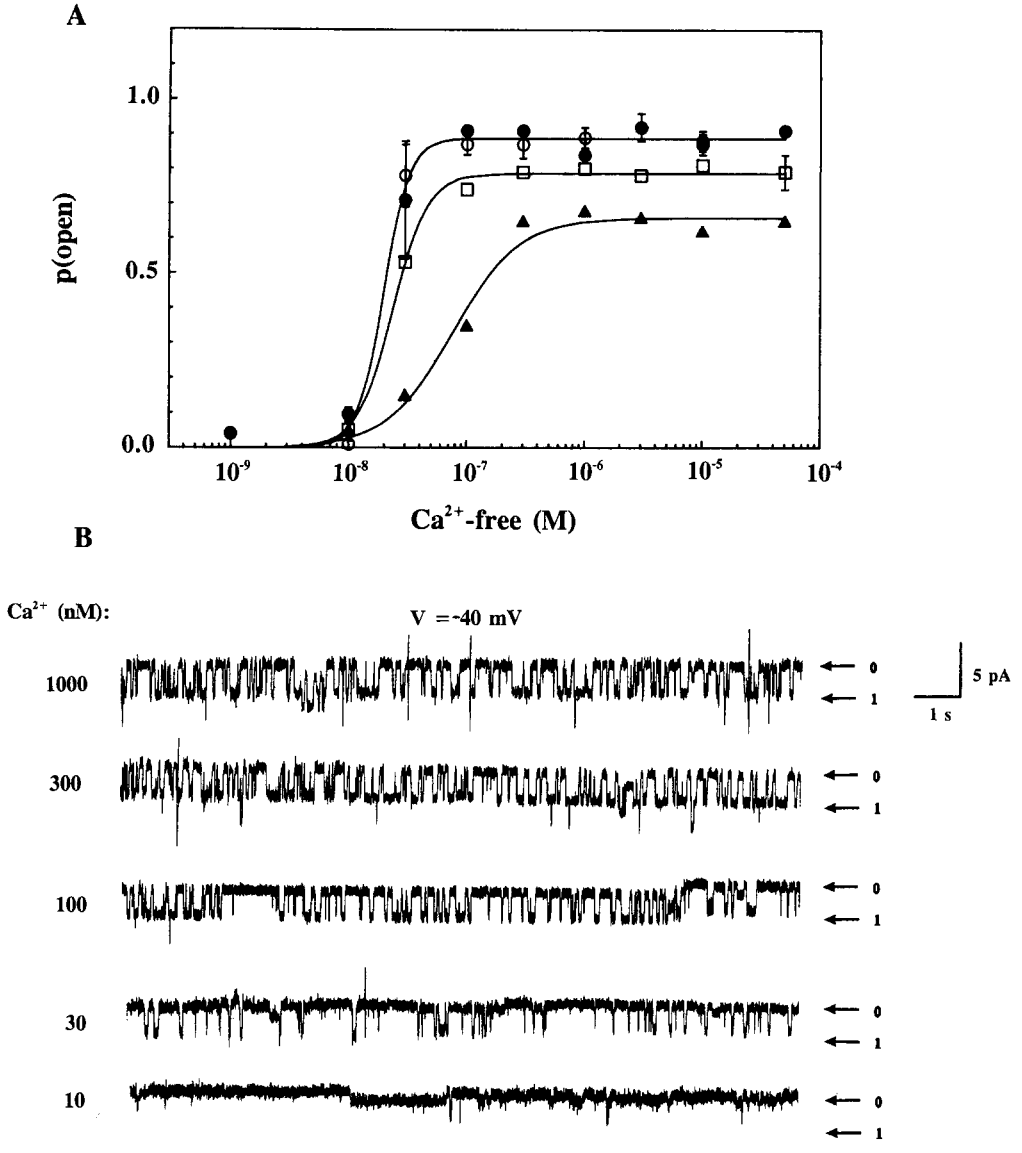

Fig. 3. (*A*) Effect of varying the concentration of free Ca^{2+} on the open probability. In several experiments, K^+ channels were incorporated into bilayers with the Ca^{2+}-activation site facing the *cis* (closed) or the *trans* solution (open) at potentials of 0 mV (●, ○), −20 mV (□) and −40 mV (▲). The *cis* solution contained 300 mM KCl and the *trans* 50 mM KCl. The initial free concentration of Ca^{2+} was 0.2 mM and EGTA was added to obtain the concentrations of free Ca^{2+} shown on the abscissa. Curves were fitted to the Hill equation. (*B*) Single channel traces showing Ca^{2+} dependence for one incorporated channel at a potential of −40 mV.

Results

Incorporation of Ion Channels into the Lipid Bilayer

After addition of membrane vesicles to the stirred *cis* chamber, fusion of vesicles usually occurred within 15 min, and all measurements were done within 30 min after incorporation. For incorporation of single channels only into the bilayer, the following conditions were found to be optimal: a $CaCl_2$ concentration of 200 μM in the chambers, a KCl concentration of 300 mM in the *cis* chamber and of 50 mM in the *trans* chamber and stirring in the *cis* chamber with a speed of approx. 50 turns per minute. In a few cases, low conductance K^+ channels or Cl^- channels were incorporated. These were not examined further.

The orientation in the bilayer of the incorporated channels could easily be determined, since channel activity was abolished by removal of Ca^{2+} from the medium bathing the cytosolic face of the channel. In 61% of the cases (n = 142), the cytoplasmic face

Table. Comparison of Hill parameters from studies on maxi K^+ channels

Tissue	pH	Voltage (mV)	$[Ca^{2+}]_{0.5}$ (μM)	Hill coef.	References
Distal colon	7.2	0	0.020	4.0	Present study
	7.2	−20	0.024	2.9	
	7.2	−40	0.311	1.5	
	6.0	0	4.1	1.3	
Tracheal smooth muscle	7.4	0	0.3	8	*Kume et al., 1990 [17]
	6.6	0	20.0	3	
Cultured renal cells	7.4	0	3	2.7	Cornejo et al., 1990 [9]
	5.8	0	8	2	
Necturus gall bladder	7.4	−30	0.29	3.4	Copello et al., 1991 [8]
	6.9	−30	0.47	2.9	
Skeletal muscle	7.0	30	49	2.11	Laurido et al., 1991 [19]
	6.0	30	54	2.10	

* Parameters are calculated from the given data.

of the channel faced the *trans* chamber, and in 39% the *cis* chamber. In the colon vesicle preparation the distribution ratio of right-side-out vesicles : inside-out vesicles : leaky vesicles is 50 : 25 : 25 [42]. Our results are therefore consistent with the idea that fusion of a right-side-out vesicle leads to incorporation of a channel exposing the extracellular face to the *cis* chamber.

Single Channel Conductance and Voltage Dependence

The *IV* curve of Fig. 1*A* shows that the current voltage relationship for the channel was linear in the examined range and that the conductance for the channel was about 275 pS. Figure 1*B* shows the traces for single channel recordings at different potentials, and it is seen that the channel activity was inhibited at negative potentials corresponding to a hyperpolarization of the membrane in the intact cell.

In Fig. 2 the probability of opening was examined as a function of the potential difference for channels incorporated into the bilayer with different orientations. In both cases, the KCl concentrations were 300 mM in the *cis* chamber and 50 mM in the *trans* chamber. It is seen that the voltage regulation is the same, whether the K^+ channels exposed their Ca^{2+}-activation sites to the *cis* or to the *trans* chamber and that it was therefore independent of the direction of the KCl gradient (300 mM/50 mM). In agreement with these findings, the voltage sensitivity of the maxi K^+ channel from mammalian skeletal muscle plasma membranes is hardly altered by changes in KCl concentration in the range above 50 mM, whereas the P_o *vs.* voltage curve changes significantly when the extracellular KCl is taken below 50 mM [22].

Ca^{2+} Activation

The Ca^{2+} dependence of the incorporated K^+ channels was examined at different membrane potentials (Fig. 3). For each voltage, the Ca^{2+}-activation curves were fitted to the Hill equation (*see* Materials and Methods) and the resulting parameters are shown in the Table. From Fig. 3 and the Table, it is seen that the incorporated K^+ channels were activated by Ca^{2+} in the intracellular range of concentration with a strong dependence on the potential across the channel. The $K_{0.5}$ for Ca^{2+} activation was increased about one order of magnitude, from $2 \cdot 10^{-8}$ to $3 \cdot 10^{-7}$ M, when the potential across the channel was changed from 0 to −40 mV. The Hill coefficient was also dependent on voltage; the Table shows that it was reduced from 4.0 at 0 mV to 1.5 at −40 mV.

The experiments in Fig. 3 show that the sensitivity of the channel to Ca^{2+} was the same whether the Ca^{2+} site was exposed to the *trans* or to the *cis* chamber. Since the KCl concentrations in all cases were 300 mM in the *cis* chamber and 50 mM in the *trans* chamber, this means that the Ca^{2+} sensitivity of the channel was independent of the ion gradient across the channel, as it was the case for the voltage sensitivity. Together, Figs. 2 and 3 show that the maximal activity of the K^+ channels strongly depended on voltage, and that the decrease in $P(o)^{max}$ at negative voltages could not be overcome by high concentrations of Ca^{2+}.

In a few cases, incorporated K^+ channels lost

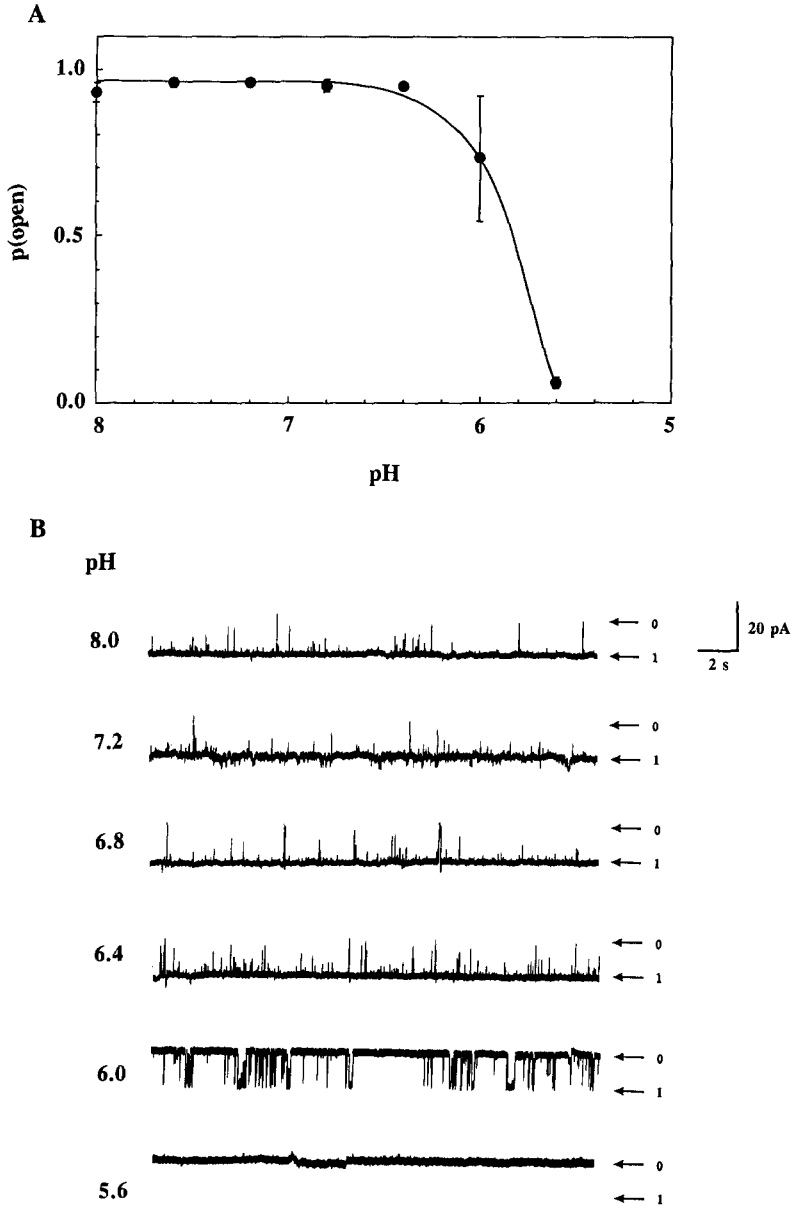

Fig. 4. (*A*) Inhibition of the K^+ channel by protons. K^+ channels were incorporated into the bilayer at pH 8, with 300 mM KCl in the *cis* chamber and 50 mM KCl in the *trans* chamber. The pH was then lowered on both sides of the channel in steps of 0.4 units by addition of HCl, and the open probability at each pH was determined. The potential across the channel was 0 mV and the free Ca^{2+} concentration was 200 μM. (*B*) Effect of pH on one incorporated K^+ channel.

their activity during an experiment; the channels were found to be closed at a free Ca^{2+} concentration of 1 μM. In these cases, the channel activity could be restored by addition of high concentrations of Ca^{2+}, suggesting that the incorporated channels were still in a functional state but with a reduced sensitivity to Ca^{2+}. A low Ca^{2+} sensitivity could also be observed after incubation of plasma membrane vesicles with 1 mM Mg^{2+}, 250 mM sucrose, 10 mM Tris, pH 7.2 at room temperature before fusion with the bilayer. After this treatment, the sensitivity to Ca^{2+} was low in five out of ten cases, i.e. the channels were closed at a free Ca^{2+} concentration of 1 μM. Since Mg^{2+} is known to activate endogenous phosphatases in plasma membrane vesicle preparations [40], it is possible that the decrease in Ca^{2+} sensitivity was due to dephosphorylation of the K^+ channels.

Effect of pH

At a concentration of 200 μM Ca^{2+} and a membrane potential of 0 mV, a reduction of pH from 8.0 to 6.4 did not change the open probability of the channel significantly, whereas it was reduced dramatically by a change in pH from 6.4 to 5.6 (Fig. 4). To avoid the formation of a pH gradient across the bilayer, the pH was changed simultaneously in the *cis* and the *trans* chamber. Since Tris is known to act as a

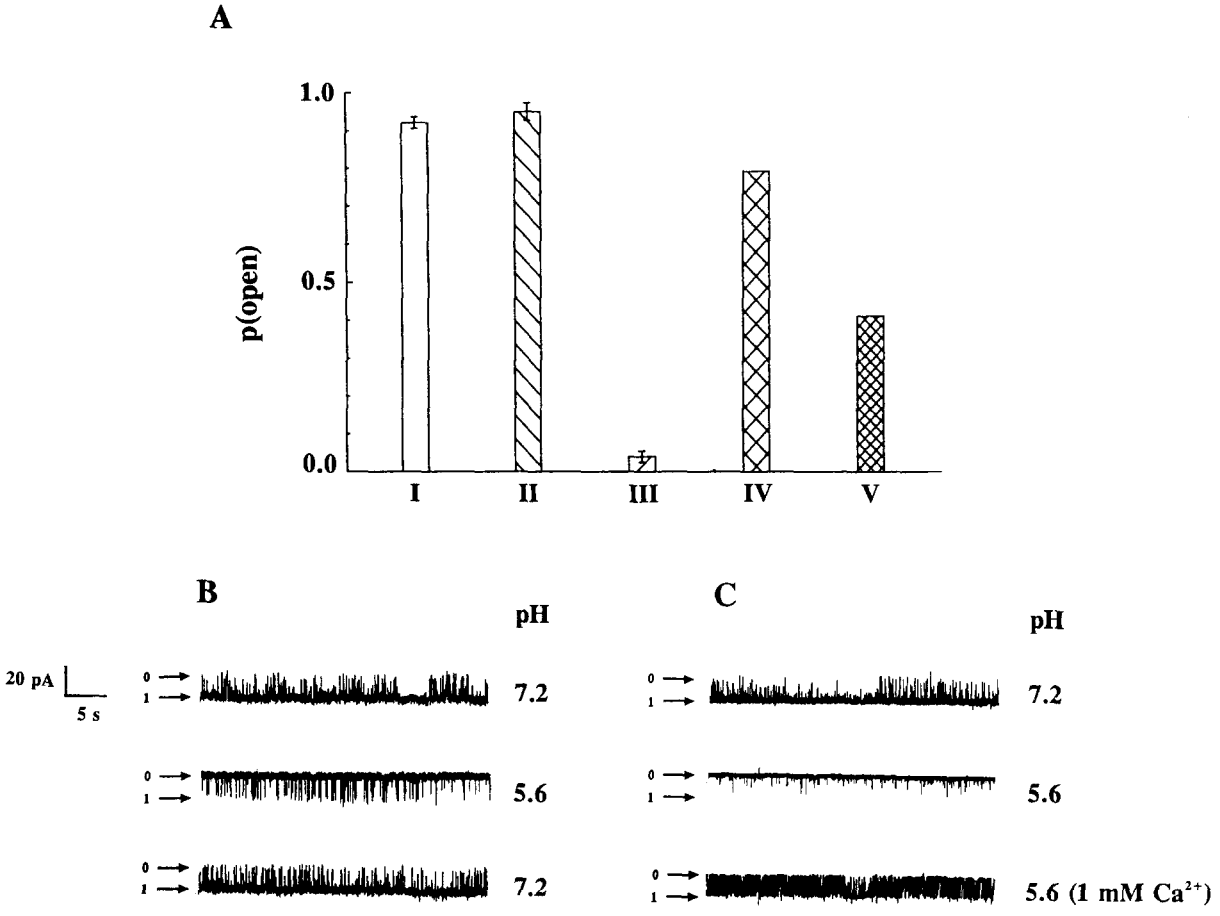

Fig. 5. Sidedness and reversibility of the pH effect on the K^+ channels. Maxi K^+ channels were incorporated at pH 7.2; otherwise, conditions were as in Fig. 4. (*A*) Bar *I* is the control value at pH 7.2. The pH was changed to 5.6 on the extracellular (bar *II*) and the cytoplasmic face (bar *III*) of the channel, respectively. Bar *IV* shows the open probability of channels initially inhibited by pH 5.6, but reactivated by returning pH to 7.2. Bar *V* shows the activity of a channel first inhibited by pH 5.6 and then partially reactivated by addition of 1 mM Ca^{2+}. (*B*) Single channel trace of initial inhibition by pH 5.6 and successive reactivation at pH 7.2. (*C*) Similar experiment, where the reactivation was done by addition of 1 mM Ca^{2+}.

protonophore across lipid membranes, a pH gradient could result in transport of protons from one chamber to the other during prolonged experiments.

To determine the sidedness of the proton inhibition, we made brief changes in pH in either chamber (Fig. 5). It is seen that decreasing the pH on the cytoplasmic face of the channel was responsible for the inhibitory effect shown in Fig. 4, whereas there was no effect on the open probability of changing the pH on the extracellular face of the channel. The H^+ inhibition could be fully reversed by returning the pH to neutral (7.2), demonstrating that the channel had not been denatured by exposure to the acidic pH (Fig. 5*A,IV* and *B*) as it was also shown for the Ca^{2+}-activated K^+ channel from pancreatic beta cells [7]. Figure 5*A,V* and *C* show that even a total block of the K^+ channel by acidification could be partially lifted by addition of high concentrations (1 mM) of Ca^{2+} to the cytoplasmic face of the channel.

In Fig. 6 we examined whether a change in pH affected the single channel conductance. When the pH was changed from 7.2 to 5.6, the channel exhibited a decrease in its probability of being in the open state (*see also* Fig. 4), whereas the amplitude was not affected by the changes in pH. This shows that the H^+ inhibition of the K^+ channel exclusively affected the gating of the channel but not the single channel conductance.

The inhibitory effect of protons could be partially reversed by increasing concentrations of Ca^{2+}, suggesting that protons decreased the sensitivity of the K^+ channel to Ca^{2+}. We have therefore examined the effect of pH on the Ca^{2+} activation of the channel. Figure 7 shows the Ca^{2+} activation of the K^+ channel at pH 6 and a potential of 0 mV. As

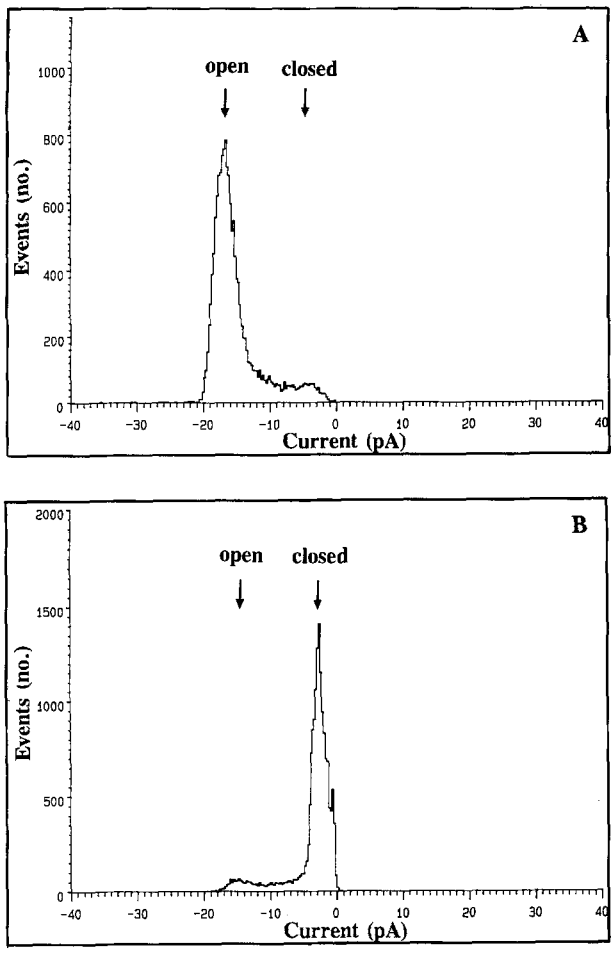

Fig. 6. A frequency histogram of current recordings for one channel incorporated in the bilayer at pH 7.2 (*A*) and at pH 5.6 (*B*). The holding potential was 0 mV, and the KCl concentrations were 300 mM in the *cis* chamber and 50 mM in the *trans* chamber.

would be expected, the Ca^{2+}-activation curve was shifted to the right compared to the activation curve at pH 7.2 (Fig. 3). Fitting the data to the Hill equation showed that the change in pH from 7.2 to 6.0 caused a dramatic change in the $[Ca^{2+}]_{0.5}$ from 0.02 to 4 μM, and a shift in the Hill coefficient from 4.0 to 1.3 (Table). The effect of lowering the pH resembled the effect of rendering the membrane potential more negative; the K^+ channel became less sensitive to Ca^{2+} and the Hill coefficient decreased.

Effect of Charybdotoxin

Figure 8 shows that charybdotoxin blocked the maxi K^+ channel in this study with a high affinity ($K_{0.5}$ = 10 nM) when added to the extracellular face, and the channel therefore belongs to the charybdotoxin-sensitive class of maxi K^+ channels. Furthermore, we examined the inhibition by charybdotoxin in symmetrical KCl (300 mM) solutions. The inhibition by charybdotoxin was strongly dependent on the ion concentrations and the membrane potential. When the K^+ flux was from the outside to the inside, the K^+ channel could be totally blocked by charybdotoxin, but when the flux was reversed with a negative membrane potential even 300 nM charybdotoxin did not block the channel effectively (Fig. 9). Moreover, a block obtained at positive potentials could be lifted by changing the potential to negative values. As charybdotoxin may be an important tool in clarification of the physiological role of maxi K^+ channels in the intact tissue, we have in Fig. 10 examined if charybdotoxin was effective at the membrane potential and ion gradients present in the intact tissue. We found that charybdotoxin was effective at these conditions since the channel was totally blocked by 100 nM charybdotoxin (Fig. 10).

Discussion

Ca^{2+} Activation, pH Dependence and Voltage Sensitivity

Single channel measurements in planar lipid bilayers showed that the high sensitivity to Ca^{2+} of the incorporated maxi K^+ channels depended on the orientation and magnitude of the potential across the membrane and on the pH, while it was independent of the direction of the ion gradient. At a neutral pH, the range of Ca^{2+} concentrations required for activation of the channel was raised from 10–100 nM in the absence of a potential difference to 50 nM–1 μM after application of a potential with an orientation similar to that of the intact cell. A further decrease in the sensitivity to Ca^{2+} was observed when the pH was lowered in the medium bathing the cytoplasmic aspect of the channel.

The effects of lowering pH on the Ca^{2+} sensitivity of maxi K^+ channels from different studies have been listed in the Table. Acidification caused a decrease in the apparent Ca^{2+} sensitivity and in the Hill coefficient (Table). This suggests that H^+ interacts with the Ca^{2+} activation and alters the number of exposed binding sites. The mechanism for the H^+ interaction is not clear. Several authors have suggested that Ca^{2+} and H^+ could compete for binding to the Ca^{2+}-activation site [6, 9, 17]. However, in a recent study [19], the Hill coefficient for Ca^{2+} activation of the maxi K^+ channel from rat skeletal muscle was unaffected by changes in pH, and it was suggested that protons exert their effect allosterically and not by competing with Ca^{2+} for the ion binding sites. The analysis of steady-state data in the

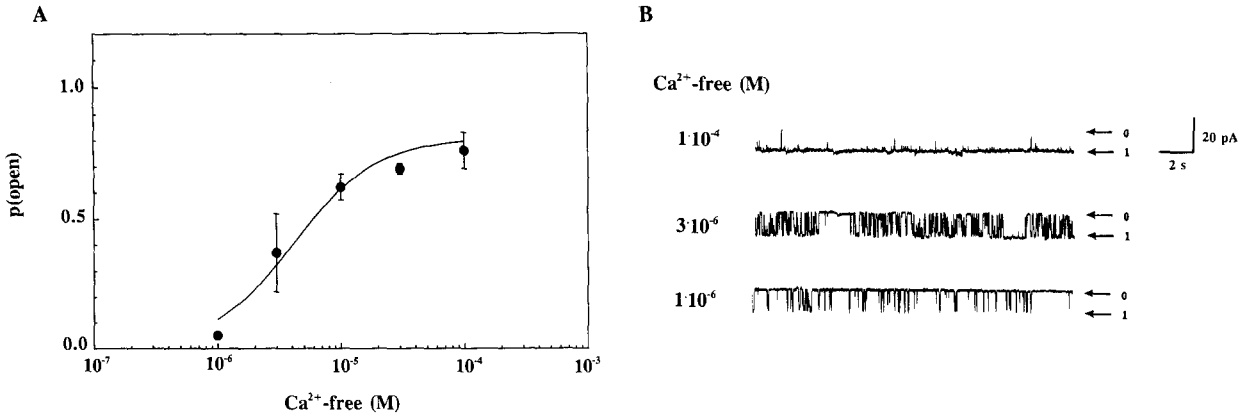

Fig. 7. (*A*) Ca^{2+} activation of K^+ channels at pH 6. K^+ channels were incorporated into the bilayer and pH was lowered to 6.0 by addition of HCl. Subsequently, the free Ca^{2+} concentration was lowered to the shown values on the intracellular side of the channel by addition of EGTA. The membrane potential across the channels was 0 mV and the flux was driven by the electrochemical gradient for K^+ (300 mM in the *cis* chamber and 50 mM in the *trans* chamber). (*B*) Ca^{2+} dependence for one channel at pH 6.0.

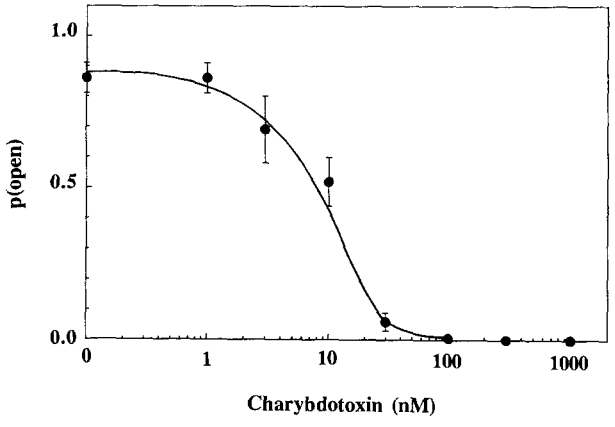

Fig. 8. Charybdotoxin inhibition. The figure shows the effect on the open probability of addition of charybdotoxin to the external face (*trans* chamber) of an incorporated K^+ channel. The potential was 0 mV and the KCl concentrations were 300 mM in the *cis* chamber and 50 mM in the *trans* chamber.

present study does not allow discrimination between these possibilities.

A negative transmembrane voltage reduced both the $K_{0.5}$ for Ca^{2+} activation and the Hill coefficient. A similar tendency is found for the maxi K^+ channel from the rat muscle plasma membrane, where the Hill coefficient ranges from 1.1 at negative voltages to 1.8 at positive voltages [26]. This suggests that voltage may change the number of binding sites accessible to Ca^{2+}. However, it has been difficult to interpret the Ca^{2+}-binding parameters, and the suggested numbers for binding sites involved in the Ca^{2+} activation of the maxi K^+ channels have varied from 2 [26] to 6 [12].

Previous studies have reported high sensitivities to Ca^{2+} for maxi K^+ channels in the range observed in this study. In excised patches of rabbit distal colonocytes, the K^+ channel activity is inhibited at 5 nM and increases markedly at 500 nM free Ca^{2+} [21]. In pancreatic acinar cells [24], acinar cells of the lacrimal gland [11], the basolateral membrane of rat small intestinal enterocytes [28] and *Necturus* enterocytes [36], maxi K^+ channels are activated by Ca^{2+} in concentrations below 1 μM.

In another group of studies, however, the maxi K^+ channels from rat muscle T-tubulus [39], cultured rat muscle [2], cultured renal cells [9], plexus choroideus [4, 6] and pancreas duct cells [13] were found to require Ca^{2+} concentrations as high as 1–10 μM for activation. Also Turnheim et al. [38] reported that very high Ca^{2+} concentrations (1–10 μM) were needed for activation of maxi K^+ channels from the basolateral membrane of surface cells in rabbit distal colon after incorporation into planar lipid bilayers. This is in contrast to the findings in the present study.

Events during the preparation and incorporation into lipid bilayers may modify the K^+ channel protein and thus provide explanations for the differences between the high Ca^{2+} sensitivity observed in this study and the low sensitivities reported earlier [38]. The decrease in Ca^{2+} sensitivity of the K^+ channels after incubation with Mg^{2+} is consistent with a dephosphorylation of the channel, since Mg^{2+} ions are known to activate endogenous phosphatases in plasma membrane preparations [40]. Similar phenomena have been observed in other studies. The inwardly rectifying K^+ channel from opossum kidney cells is inactivated in the presence of Mg^{2+} and reactivated in the presence of ATP. These effects are ascribed to dephosphory-

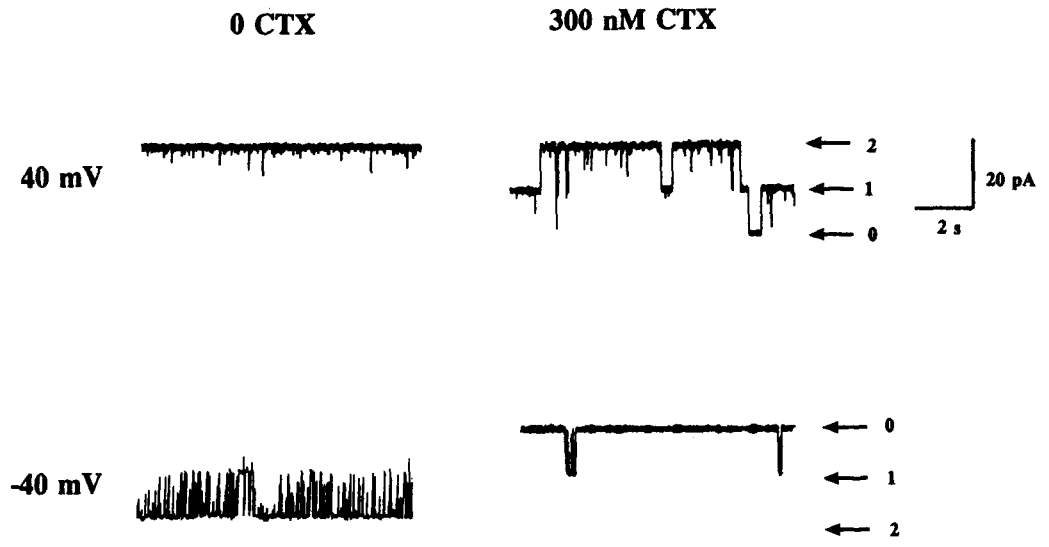

Fig. 9. "Knock off" of charybdotoxin. Two K^+ channels were incorporated into the bilayer with the Ca^{2+}-activation site facing the *cis* chamber. The chambers contained symmetrical KCl solutions (300 mM). The K^+ channel activity was measured at potentials of 40 and −40 mV in the absence and presence of 300 nM charybdotoxin in the *trans* chamber.

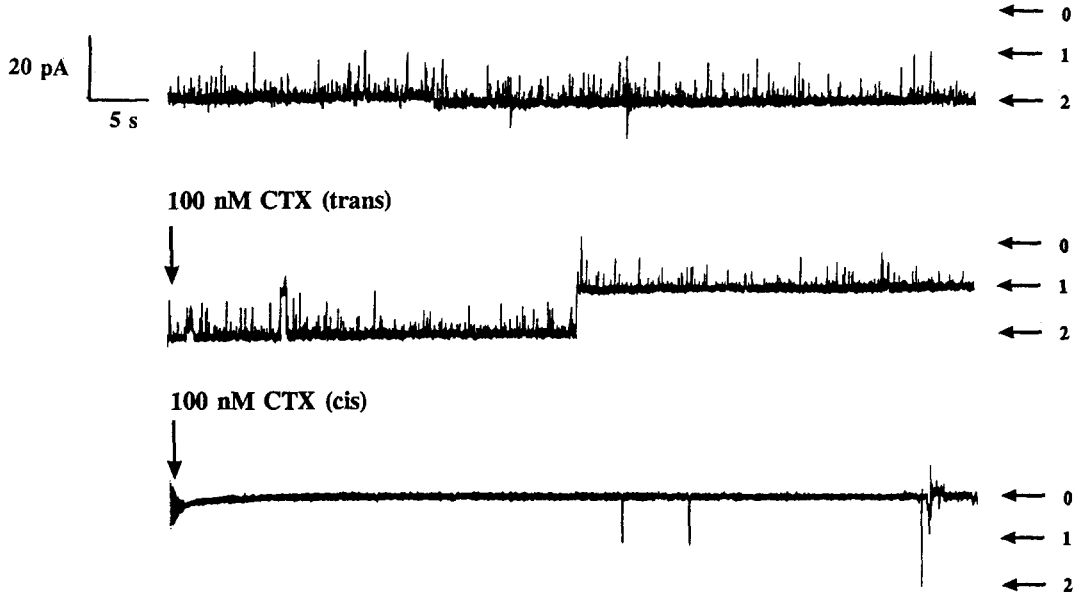

Fig. 10. Effect of charybdotoxin at physiological conditions. Two channels were incorporated into the bilayer with opposite directions at a potential of −40 mV and the following ion gradients: 140 mM KCl, 5 mM NaCl in the *cis* chamber and 140 mM NaCl, 5 mM KCl in the *trans* chamber. Addition of 100 nM charybdotoxin to the *trans* chamber resulted in inhibition of the K^+ channel incorporated with the intracellular aspects facing the *cis* chamber. When 100 nM charybdotoxin was added to the *cis* chamber, the channel incorporated in the opposite direction was inhibited.

lation and phosphorylation of the channel protein [29]. The sensitivity to Ca^{2+} for the maxi K^+ channel from pancreatic duct cells can be increased by phosphorylation from a cAMP-dependent protein kinase [13], and Ca^{2+} as well as cAMP activates the maxi K^+ channel in crypt cells from rabbit colon [21]. In rat brain, charybdotoxin-sensitive maxi K^+ channels are activated by phosphorylation from a cAMP-dependent protein kinase, whereas charybdotoxin-insensitive maxi K^+ channels are not [33]. Since the maxi K^+ channels in the present study are charybdotoxin sensitive, it is probable that high sensitivity to Ca^{2+} requires phosphorylation by a protein kinase, and that de-

phosphorylation of the protein reduces the Ca^{2+} sensitivity by several orders of magnitude.

Other possible explanations for the variety in Ca^{2+} sensitivities from preparation to preparation could be the absence or presence of calmodulin, or partial proteolytic digestion of the channel protein during preparation. We have earlier reported that the Ca^{2+}-activated K^+ channel activity from kidney tubulus is stimulated by calmodulin, and that the proteins can be purified by calmodulin affinity chromatography [16]. These results have been reproduced for the maxi K^+ channel from distal colon [20]. In kidney [15] as well as in distal colon [34], the domain mediating Ca^{2+} activating of the K^+ channel appears to be very sensitive to proteolysis in membrane preparations.

Charybdotoxin Inhibition

Charybdotoxin inhibited the maxi K^+ channel from the basolateral membrane of the surface cells of the distal colon with an affinity comparable to that found in other single channel studies of charybdotoxin-sensitive maxi K^+ channels [18, 27]. We found that the block depended on the direction of the K^+ flux through the channel, and that even a block obtained at 300 nM charybdotoxin could be relieved by an outward flux of K^+. Our findings are in agreement with the notion that charybdotoxin physically plugs the pore of the Ca^{2+}-activated K^+ channel [23], and that the inhibitor can be knocked off by a stream of K^+ through the channel.

In the present study, charybdotoxin was indeed effective at the ion gradients and potentials of the intact epithelium, and it may therefore be used in clarification of the role of the maxi K^+ channels in the intact colon. This is in contrast to a recent study on the maxi K^+ channel from smooth muscle, where charybdotoxin was found to be without effect at physiological ion gradients and membrane potential [5]. Differences in the amino acid sequence in the pore forming parts of the K^+ channels may explain the differences in charybdotoxin block found for channels from different tissues.

Consequences for Physiological Regulation

The observations in this study raise the question of how the Ca^{2+}-activated maxi K^+ channel can be involved in regulation of transcellular Na^+ transport. The Na,K-pump in the basolateral membrane creates an inward electrochemical gradient for Na^+, which drives the influx of Na^+ across the luminal membrane through the amiloride-sensitive Na^+ channels. K^+ ions recycle across the basolateral membrane through K^+ channels to avoid an accumulation of K^+ in the cytoplasm with inhibition of the Na,K-pump [35, 37]. Studies on whole epithelia and cell layers have shown that basolateral K^+ channels are responsible for a significant part of the basolateral K^+ permeability [10], and it is therefore important that the K^+ channels are under strict control, e.g., during hormonal stimulation of the tissue. In agreement with this notion, studies on whole colon have shown that the basolateral K^+ conductance is increased during aldosterone-stimulated Na^+ reabsorption [10].

At resting conditions, the intracellular free Ca^{2+} concentration is 50–100 nM [31] and the membrane potential is −40 mV. As shown in Fig. 3, the open probability of the K^+ channel is 0.2–0.35 under these circumstances. An activation of the K^+ channels via an increase in intracellular Ca^{2+} could be mediated by hormonal stimulation of the tissue. There are no measurements of intracellular Ca^{2+} after hormonal stimulation of the colon, but in other tissues intracellular Ca^{2+} concentrations of 600–2000 nM have been monitored after hormonal stimulation [32].

The reduction of the Ca^{2+} sensitivity of the K^+ channels following hyperpolarization may be part of a physiological feedback mechanism. If hormonal stimulation of the cell increases the intracellular free Ca^{2+} and activates the K^+ channels, the resulting hyperpolarization would lower the sensitivity of the K^+ channels to Ca^{2+} and reduce the open probability.

The pH may also play an important role in regulation of the transport systems involved in the Na^+ reabsorption. In another Na^+ reabsorbing epithelium, the frog skin, it has been shown that stimulation by aldosterone causes an alkalinization of the cytoplasm [14]. Increased pH in the cytoplasm increases the sensitivity of the channels to Ca^{2+}, and furthermore, the Hill coefficient for the Ca^{2+}-activation curve increases with alkalinization. Thus, a given raise in Ca^{2+} concentration will cause a relatively large raise in K^+ channel activity.

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