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Purification and characterization of epithelial Ca^{2+}-activated K^+ channels

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Purification and characterization of epithelial Ca^{2+}-activated K^+ channels. Reabsorption of NaCl in the thick ascending limb of Henle's loop in the kidney and in the surface cells in the distal colon involves the integrated function of several membrane transport systems including ion channels, the Na,K,Cl-cotransport system and the Na,K-pump. To determine if their properties are consistent with a role in regulation of transepithelial transport, Ca^{2+}-activated K^+ channels from the luminal membrane of the TAL cells and from the basolateral membrane of the distal colon cells have been characterized by flux studies in plasma membrane vesicle preparations and by single channel measurements in lipid bilayers. The channels are found to be activated by Ca^{2+} in the physiological range of concentration with a strong dependence on intracellular pH and the membrane potential. The Ca^{2+}-sensitivity of the K^+ channels is modulated by phosphorylation and dephosphorylation and the K^+ channel protein must be in a phosphorylated state to respond to intracellular concentrations of Ca^{2+}. As a step towards purification of the K^+ channel proteins, procedures for solubilization and reconstitution of the K^+ channels have been developed. The observation that the epithelial Ca^{2+}-activated K^+ channels bind calmodulin in the presence of Ca^{2+} have allowed for partial purification of the K^+ channel proteins by calmodulin affinity chromatography. In the sequences for the two cloned Ca^{2+}-activated K^+ channels, the *mSlo* channel and the *slowpoke* channel, putative calmodulin binding regions can be identified.

Water and salt homeostasis of the organism is largely a result of transport in specialized epithelial cells of the mammalian nephron and the distal colon, and the excretion of NaCl in these organs is regulated by hormones, such as ADH and aldosterone. The present paper deals with two Na^+-absorbing epithelia, the thick ascending limb of Henle's loop (TAL) and the distal colon surface cells, and focuses on the role of K^+ channels in the regulation of the transepithelial transport [1–4].

The cells in TAL are characterized by having their K^+ channels in the luminal membrane, while the distal colon surface cells have the K^+ channels in the basolateral membrane (Fig. 1). The localization in TAL allows K^+ to recycle across the luminal membrane, and in the colon the K^+ channels ensure the intact pump-leak system in the basolateral membrane. In both instances there is no major net transport of K^+ across the epithelium. The K^+ channels constitute together with other ion channels, the Na,K-ATPase and the Na,K,Cl-cotransport system the pathways which allow transepithelial NaCl transport to take place [2, 5]. Regulation of the NaCl transport can therefore in principle take place at each of these membrane proteins, and can be influenced by physiological factors such as intracellular Ca^{2+}, phosphorylation-dephosphorylation, pH, cAMP or by drugs, such as the loop diuretics which inhibit the Na,K,Cl-cotransport system, or amiloride which inhibits the Na^+ channels [2, 4, 5].

Considering the physiological function of Ca^{2+}-activated K^+ channels, it remains an open question whether they are important for regulation of transepithelial transport, and in particular if they form links between second messenger systems and membrane conductances. This would require that the Ca^{2+}-activated K^+ channels are activated by Ca^{2+} in physiological concentrations in the conditions found the epithelial cells with respect to membrane potential, pH and ion gradients. Other requirements would be that second messenger systems are coupled to protein kinases and phosphatases catalyzing the transition between active and inactive forms of the channel proteins.

A major purpose of the present review is to examine the properties of the epithelial maxi K^+ channels, with particular emphasis on modulation of the channel activity by intracellular factors such as Ca^{2+}, pH, phosphorylation/dephosphorylation and ATP. It is proposed that these channels may play an important role in the regulation of epithelial ion transport, and that the differences in channel characteristics observed in different studies could be due to modulation of the channel protein by second messenger systems.

Detailed characterization of the Ca^{2+}-activated K^+ channels requires purification of the channel proteins. The sparse distribution of K^+ channels in epithelia explains the problems encountered in attempts at purification and characterization of their proteins. As estimated on the basis of ion fluxes each cell in TAL contains only 200 to 400 K^+ channels, which is a very low number relative to 1 million Na,K,Cl-cotransporters and 40 million Na,K-pumps [8]. In spite of this, the development of procedures for solubilization and reconstitution has allowed purification of proteins with a high specific K^+ channel activity by calmodulin affinity chromatography [9, 10]. The channel proteins can be reconstituted into phospholipid vesicles for comparison of their properties with those of the channels in native plasma membrane vesicles. After incorporation of the purified proteins into planar lipid bilayers, a Ca^{2+}-activated maxi K^+ channel can be identified [11]. Putative calmodulin binding sites can be identified in the two cloned Ca^{2+}-activated K^+ channels, the *slowpoke* channel [12] and the *mSlo* channel [13], suggesting that calmodulin affinity chromatography may also be useful in purification of the cloned K^+ channels.

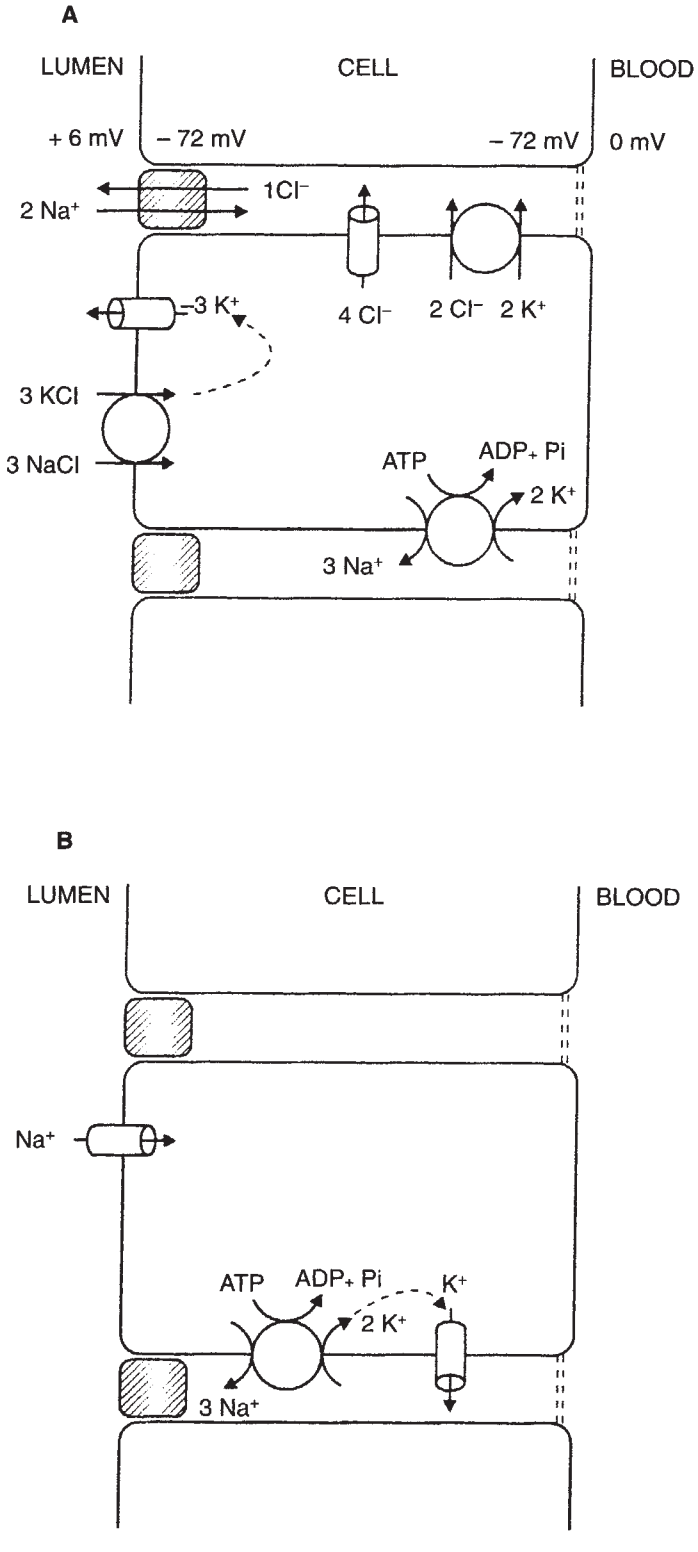

Fig. 1. *Organization of membrane transport systems in two Na^+ absorbing epithelia, the thick ascending limb of Henle's loop (A) and the distal colon surface cells (B).* In both epithelia the Na,K-pump in the basolateral membrane delivers the primary driving force by creating an electrochemical gradient for Na^+. In TAL, Na^+ is transported through the luminal cotransport system and K^+ recycles through the luminal K^+ channels so that there is no net uptake of K^+ in TAL. The luminal K^+ channels creates together with the basolateral Cl^- channels a lumen positive potential that drives the paracellular transport. In the distal colon surface cells, Na^+ is reabsorbed through luminal Na^+ channels and K^+ is allowed to recycle across the basolateral membrane through K^+ channels. Modified from Greger and Schlatter [6] and Wiener, Klaerke and Jørgensen [7].

Procedures for purification of Ca^{2+}-activated K^+ channels

Isolation of plasma membrane vesicles containing Ca^{2+}-activated K^+ channels

Since the number of Ca^{2+}-activated K^+ channels in epithelia is very low, an important first step in the procedures for purification and characterization of the K^+ channels is preparation of plasma membrane fractions with an enriched channel content. In the distal colon surface cells, the Ca^{2+}-activated K^+ channels are located in the basolateral membrane (Fig. 1), and a basolateral plasma membrane vesicle fraction can be prepared from mucosal scrapings using sucrose gradients and Ficoll barriers [14]. In TAL the K^+ channels are found in the luminal membrane (Fig. 1), which has a low area per cell compared to the area of the basolateral membrane. By differential centrifugation a crude plasma membrane vesicle fraction can be prepared from the inner stripe of outer medullar of the pig kidney, where the TAL is a predominant structure [15]. A subsequent centrifugation on a linear metrizamide density gradient allows separation of luminal plasma membrane vesicles containing Na,K,Cl-cotransport systems and K^+ channels from basolateral plasma membrane vesicles, which are characterized by their high content of Na,K-ATPase [16].

Even in the plasma membrane fractions enriched in luminal or basolateral membranes, it must be expected that only a small portion of the plasma membrane vesicles contain a K^+ channel. This means that the K^+ channel activity in the membranes cannot be estimated by conventional isotope influx or efflux methods. In contrast, a very sensitive voltage driven isotope assay is required to obtain reliable measurements of K^+ channel activity [17]. In this assay, $^{86}Rb^+$ uptake into the vesicles against an opposing K^+ gradient is measured. Plasma membrane vesicles prepared in high KCl medium (50 mM) are transferred to a low KCl medium (0.1 mM) and in vesicles with a high K^+ permeability, that is, vesicles containing open K^+ channels, an outside-positive diffusion potential will arise driving the uptake of tracer amounts of $^{86}Rb^+$ into the vesicles. This results in a linear uptake of $^{86}Rb^+$ for at least 20 minutes and a high signal-to-background ratio. To determine the total K^+ channel activity in the vesicles, the $^{86}Rb^+$ uptake is measured in the absence and in the presence of 5 mM $BaCl_2$, and the K^+ channel activity can be expressed as the Ba^{2+}-sensitive $^{86}Rb^+$ uptake into the vesicles.

With this assay it is possible to analyze the properties of Ca^{2+}-activated K^+ channels in the plasma membrane vesicles from TAL and colon after initial depletion of the vesicles for Ca^{2+} with EGTA [7, 18, 19]. The $K_{0.5}$ for the Ca^{2+}-stimulation of the K^+ channels is 200 to 500 nM (Fig. 2). A full stimulation by Ca^{2+} is dependent on the presence of the Ca^{2+} ionophore A23187, implying that the Ca^{2+} binding site of the K^+ channels in the major part of the plasma membrane vesicles is located to the interior of the vesicles. In vesicles from TAL the K^+ channel activity can be stimulated fourfold by Ca^{2+}, and in the colon vesicles the K^+ channel activity is increased by 40% when the free

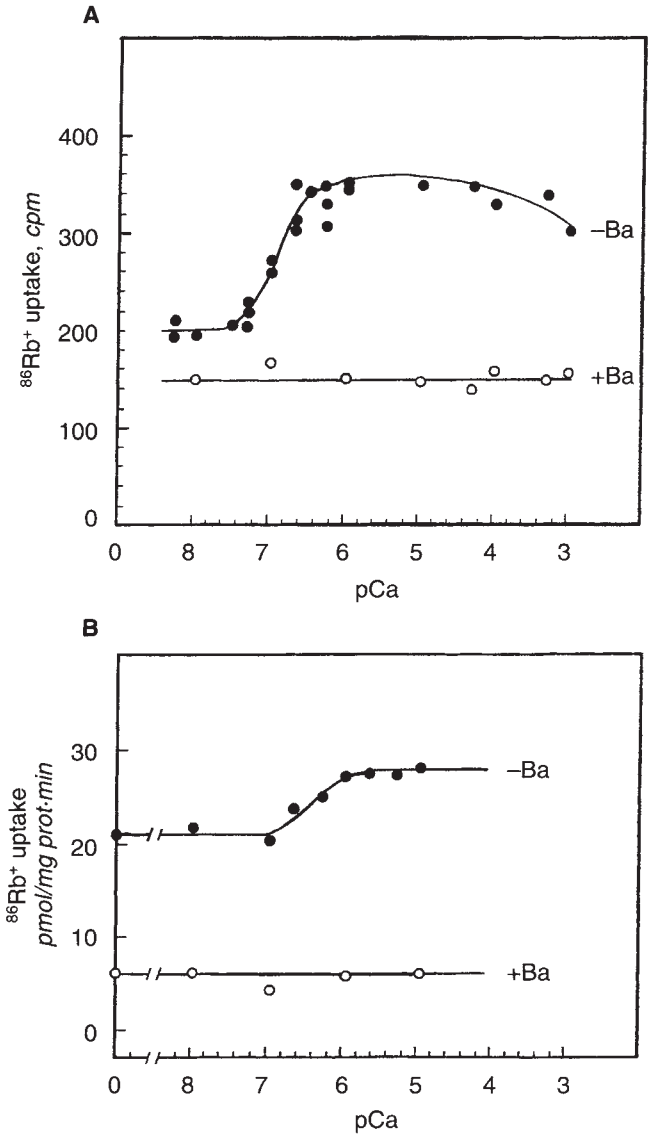

Fig. 2. *Ca^{2+}-activation of K^+ channels in plasma membrane vesicles.* Luminal plasma membrane vesicles from TAL (**A**) or basolateral plasma membrane vesicles from distal colon surface cells (**B**) are depleted for Ca^{2+} by incubation with EGTA and the ionophore A23187, and Ca^{2+} is added again to obtain the shown final free concentrations. The $^{86}Rb^+$ uptake is measured for 10 minutes in the absence (●) or presence (○) of 5 mM $BaCl_2$. From Wiener et al [7] and Klærke et al [9].

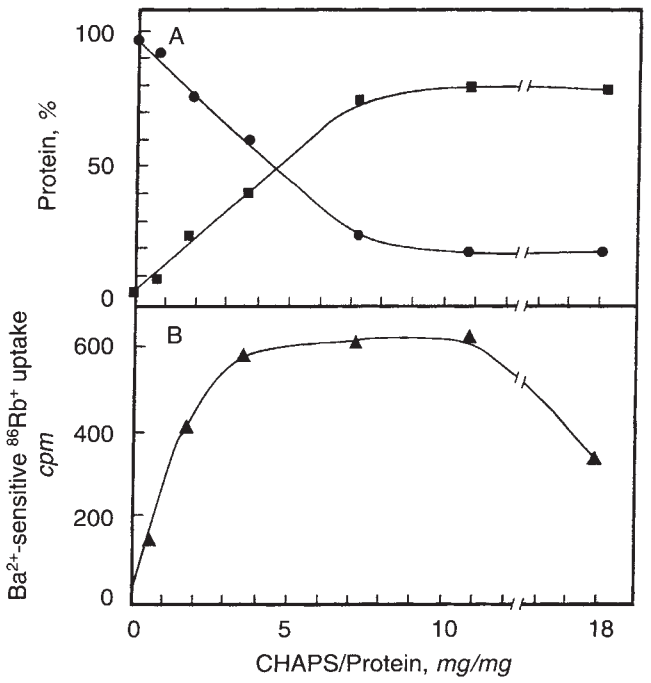

Fig. 3. *Solubilization and reconstitution of Ca^{2+}-activated K^+ channels.* (**A**) Luminal plasma membrane vesicles from TAL are solulubilized with CHAPS and solubilized protein (■) and non-solubilized protein (●) is then separated by centrifugation. (**B**) For reconstitution of solubilized K^+ channel protein aliquots (200 μl) of the supernatant from the experiment in (A) are mixed with solubilized lipid, reconstituted vesicles are formed by removal of the detergent on a Sephadex G-50 column and K^+ channel activity is measured as described in the text. From Klærke and Jørgensen [10].

Ca^{2+} concentration is increased (Fig. 2). These results show that Ca^{2+}-sensitive as well as Ca^{2+}-insensitive K^+ channels are present in the vesicle preparations. In agreement with this, electrophysiological studies have identified Ca^{2+}-activated [20–23] as well as ATP sensitive [24, 25] K^+ channels in these epithelia.

The plasma membrane fractions from TAL and colon with a relatively high content of Ca^{2+}-activated K^+ channels serve as starting material for further studies on purification and characterization of the channels.

Solubilization and reconstitution

Further purification procedures requires solubilization of the K^+ channel protein with detergent. Since solubilization implies removal of the membrane proteins from their natural lipid environment, it is important that the solubilization procedures are optimized and that it is assured that the membrane proteins of interest are not damaged. To examine the properties of ion channels after solubilization, it is necessary to reconstitute the ion channel proteins into phospholipid vesicles.

To find the optimum procedure for solubilization and reconstitution for epithelial K^+ channels a number of different detergents, including CHAPS, $C_{12}E_8$, octylglucoside and cholate, and reconstitution procedures, including freeze-thaw sonication, gel-filtration and dialysis have been tested. The detergent of choice, CHAPS, is a zwitterionic molecule with a high CMC of 4 to 6 mM, which makes it easily removable by gel filtration during the reconstitution procedure [19]. Measurement of the K^+ channel activity in the solubilized plasma membrane protein fraction after reconstitution into phospholipid vesicles shows that the K^+ channel protein is solubilized at a CHAPS/protein weight ratio of 4:1 to 10:1 (Fig. 3). Note that the K^+ channel protein is solubilized at a lower detergent concentration that the bulk of the membrane protein, suggesting that some purification of the channel protein can already take place at the solubilization step (Fig. 3). At high concentrations of detergent the channel activity decreases, probably because the channel protein is denatured if it is totally delipidated (Fig. 3).

Using the channels in plasma membrane vesicles as reference, the sensitivity of the K^+ channels to Ca^{2+}, pH and various

inhibitors is preserved after solubilization and reconstitution [7, 8, 19], indicating that the channels have not been denatured by these procedures.

In the reconstituted veiscles the K^+ channels expose their cytoplasmic aspects to the medium, and this system therefore allows direct examination of compounds normally present in the cytoplasm. Calmodulin is a protein of 148 amino acids known to modulate the binding of Ca^{2+} to proteins. Addition of calmodulin to the reconstituted vesicles doubles the Ca^{2+}-activation of the K^+ channels, whereas there is no effect of addition of calmodulin in the absence of Ca^{2+}. The $K_{0.5}$ for the calmodulin effect is 0.1 nM [9]. A similar effect of calmodulin has also been found for other channels, such as the Ca^{2+}-activated K^+ channels from adipocytes [26]. Regulation by calmodulin is often mediated through phosphorylation [27], but since the calmodulin stimulation of the Ca^{2+}-activated K^+ channels described here takes place under conditions where phosphorylation is unlikely, calmodulin is expected to bind directly to the cytoplasmic aspects of the Ca^{2+}-activated K^+ channels as it is the case for, such as the plasma membrane Ca^{2+}-ATPase. Calmodulin activates the Ca^{2+}-ATPase by preventing interaction between the calmodulin binding region of the protein and the Ca^{2+}-binding sites [28]. Whether a similar mechanism is responsible for the calmodulin activation of Ca^{2+}-activated K^+ channels is not known.

Purification of K^+ channel proteins

Very few attempts at purification or labeling of ion channel proteins exist, probably because of the low amount of channel protein in the cells. However, the observation that calmodulin binds to the Ca^{2+}-activated K^+ channels in the presence of Ca^{2+} provides the basis for a purification procedure of the K^+ channel proteins by calmodulin affinity chromatography as it has earlier been done for the plasma membrane Ca^{2+}-ATPase [29]. The use of affinity chromatography is very convenient, when the proteins of interest only form a very small part of the starting material.

For partial purification of K^+ channel proteins, solubilized plasma membrane protein is added to a calmodulin affinity column. In the presence of Ca^{2+} more than 99% of the solubilized protein does not bind to the column and is eluted in peak 1 (Fig. 4). The bound protein, which can be eluted by addition of EGTA (peak 2), show a very high Ca^{2+}-activated K^+ channel activity after reconstitution into phospholipid vesicles (Fig. 4). The "specific K^+ channel activity" as measured by the voltage driven isotope assay is increased from 8 to 10 pmol $^{86}Rb^+$/mg protein · min in crude plasma membrane vesicles up to 1100 pmol $^{86}Rb^+$/mg protein · min for purified protein reconstituted into phospholipid vesicles indicating the the channel proteins are purified more than 100-fold [10]. The K^+ channels purified from TAL are activated by Ca^{2+} in the same range of concentration as in the native plasma membrane vesicles, but the K^+ channel protein must be phosphorylated from a cAMP-dependent protein kinase to show full activity [9]. Single channel measurements in lipid bilayers after incorporation of protein purified from rabbit distal colon identifies a Ca^{2+}-activated maxi K^+ channel with a single channel conductance of approximately 250 pS (Fig. 4). The Ca^{2+}-sensitivity in these single channel studies [11] seem to be somewhat lower than the Ca^{2+}-sensitivity found in the plasma membrane vesicles from this tissue. This could be due to modification of the proteins during the purification procedure.

Identification of the proteins responsible for formation of the

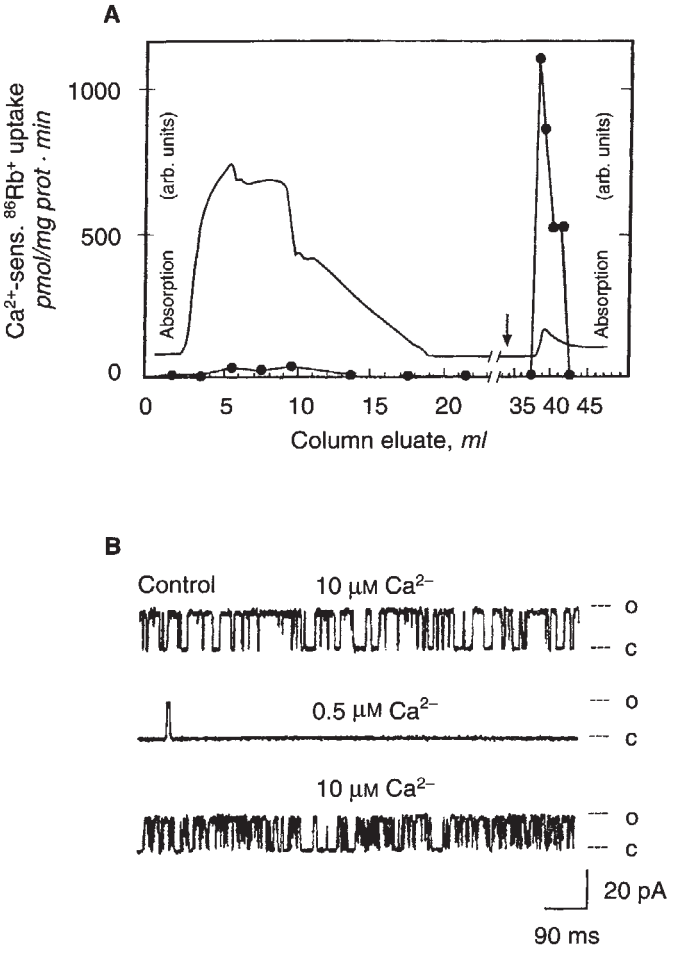

Fig. 4. *Purification of Ca^{2+}-activated K^+ channels by calmodulin affinity chromatography.* **A.** Solubilized plasma membrane protein is added at zero and change from buffer containing 100 mM $CaCl_2$ to buffer containing 5 mM EGTA is indicated with an arrow. Protein from the fractions was reconstituted into phospholipid vesicles and the Ca^{2+}-sensitive $^{86}Rb^+$ uptake was measured as the difference in presence and absence of 1 μM free Ca^{2+} (●). The unbroken line shows the UV absorption at 270 nm with different scales for peak 1 (fractions 2 to 19) and peak 2 (fractions 37 to 42). From Klærke and Jørgensen [10]. **B.** Single channel measurements in planar lipid bilayer of Ca^{2+}-activated maxi K^+ channels purified from rabbit distal colon by calmodulin affinity chromatography. From Lui et al [11], with permission.

Ca^{2+}-activated K^+ channels in the purified material is complicated by the fact that ion channels are very fast transporters. Therefore, it should be kept in mind that minute amounts of protein, that may not be identified in polyacrylamide gel electrophoresis, binding studies, etc., could give rise to significant K^+ channel activity in reconstituted vesicles and in lipid bilayers, where the activity of just one channel molecule is measured. The identification of possible channel forming proteins may therefore be rather uncertain.

SDS gel electrophoresis of the proteins purified by calmodulin affinity chromatography from TAL reveal two major bands of 51 kD and 36 kD, a minor band of 43 kD and minor bands at several other positions [9, 10]. N-terminal amino-sequencing has identified the 43 kD band as actin and the band of 51 kD contains the protein beta-2-glycoprotein I [10]. The 51 kD band is rather broad

Table 1. Purified or labelled Ca^{2+}-activated K^+ channel proteins

Tissue	Proteins (kDa)	Method	Ref.
Kidney (TALH)	51, 36	Calmodulin affinity chromatography	[9, 10]
Distal colon surface cells	35, 60, 120	Calmodulin affinity chromatography	[11]
Tracheal smooth muscle	62, 31	Charybdotoxin binding	[31]
Neurons	23, 30, 59, 80	Apamin binding	[34]
Muscle	14, 28, 31, 45, 55	Phosphorylation	[35]

Table 2. Putative calmodulin binding domains in the sequences for the *slowpoke* and *mSLO* channels

Channel	Sequence	$<\mu_H>$	<Hb>	Net charge
mSlo	475-KNYHPKIRIITQML-488	0.48	−0.10	4
mSlo	482-RIITQMLQYHNKAH-495	0.55	−0.14	4
mSlo	772-RASNFHYHELKHIV-785	0.44	−0.10	4
Slowpoke	815-RASNFHYHELKHVV-828	0.42	−0.12	4

The channel sequences are screened using a computer algorithm to find segments of 14 amino acids having at least 4 positive charges, a mean hydrophobic moment ($<u_H>$) greater than 0.4 and an average hydrophobicity (<Hb>) between −0.25 and 0.6 consistent with formation of positively charged, amphiphilic alpha-helices

and may contain other proteins, but this has not been clarified yet. Repeated attempts at sequencing the 36 kD band have been unsuccessful so far. Two-dimensional gel electrophoresis of the proteins purified from rabbit distal colon have revealed three proteins of 120 kD, 60 kD and 35 kD [11]. These proteins have not been identified by N-terminal sequencing, and it is not clear which are involved in formation of the K-channel.

In one other study a Ca^{2+}-activated maxi K^+ channel has been purified, using charybdotoxin binding to identify the channel proteins. Charybdotoxin is a polypeptide of 37 amino acids known to block different types of K^+ channels, including a certain class of Ca^{2+}-activated maxi K^+ channels [30]. The purified receptor consists of two subunits of 62 kD (α) and 31 kD (β), and after incorporation into lipid bilayers these proteins give rise to formation of a Ca^{2+}-activated maxi K^+ channel with a single channel conductance of 235 pS [31].

It has not been possible to measure charybdotoxin-binding to K^+ channel proteins purified by calmodulin affinity chromatography (Klaerke et al, unpublished observations), even if charybdotoxin blocks the channels in the native tissue [7, 23]. This could be ascribed to the fact that even if iodinated charybdotoxin has been shown to bind to channels from certain tissues such as tracheal smooth muscle [31] and red blood cells [32], the iodinated charybdotoxin in many cases is far less potent that the non-iodinated peptide, because the iodination takes place in the part of the toxin interacting with the channel mouth [33]. The variability in binding of iodinated charybdotoxin could reflect differences in the pore forming region for channels from different tissues.

In a few other studies a number of proteins based on different approaches have been suggested to be involved in formation of Ca^{2+}-activated K^+ channels (Table 1). The apparent molecular weights ranges from 14 kDa to 120 kD, and in all cases more than one protein is suggested. This supports the idea that more subunits of different sizes are involved in the formation of K^+ channels.

Comparison with cloned Ca^{2+}-activated K^+ channels

So far only two Ca^{2+}-activated K^+ channels have been cloned and characterized after heterologous expression, the *slowpoke* channel from Drosophila [13, 36] and the *mSlo* channel from mouse brain and muscle [12]. Both consist of ~1200 aminoacids predicting a molecular mass of approximately 135 kD. The channels are expected to consist of four subunits each having at least six transmembrane segments (S1 to S6) with the pore forming region between S5 and S6, and a long cytoplasmic C-terminus, which may be important for regulation of the channels [12, 13]. Expression of the genes in *Xenopus* oocytes give rise to Ca^{2+}-activated maxi K^+ channels; the *slowpoke* channel is insensitive to charybdotoxin [36], whereas the *mSlo* channel is inhibited by charybdotoxin [12].

Sequence information is only available for one of the putative Ca^{2+}-activated K^+ channel proteins mentioned in Table 1, namely the α-subunit of the charybdotoxin-receptor. Seven peptides from the α-subunit in size from 6 to 29 residues have been sequenced, and all show a high homology with the sequences for *slowpoke* and *mSlo* [37]. The reason for the considerable difference in molecular mass between the α subunit (62 kD) and the deduced mass of the cloned channels (~ 135 kD) is not clear, but possible explanations could be post-translatoric modification of *mSlo* or proteolytic degradation of the α subunit during purification procedures [37].

Since Figure 4 suggests that the calmodulin affinity procedure purifies a Ca^{2+}-activated maxi K^+ channels, it is obvious to search the sequences of the *slowpoke* channel and the *mSlo* channel for possible calmodulin binding domains. Unfortunately, calmodulin binding domains show extreme variability in sequence, however, in most cases they are protein regions of approximately 14 amino acids, which form positively charged, amphiphilic helices. In addition, the hydrophobic residues frequently repeat in the sequences with a 3 to 4 residue period matching that of the α-helix [38, 39]. In the sequences for both cloned Ca^{2+}-activated K^+ channels possible calmodulin binding regions can be identified, and one of these is highly conserved in both channel sequences (Table 2). These findings support the notion that calmodulin affinity chromatography purifies Ca^{2+}-activated maxi K^+ channels, and suggest that the method may be useful for future purification of cloned channels after expression at high levels. However, it remains to be shown experimentally that the cloned channel proteins bind calmodulin.

Single channel measurements of epithelial Ca^{2+}-activated K^+ channels

Regulation by voltage, Ca^{2+} and pH

A detailed study of the regulation of ion channels requires characterization of the currents through single channel molecules. To obtain such measurements the epithelial Ca^{2+}-activated K^+ channels are incorporated into planar lipid bilayers. This method has several advantages. It allows characterization of the Ca^{2+}-activated K^+ channels from membranes not accessible to the

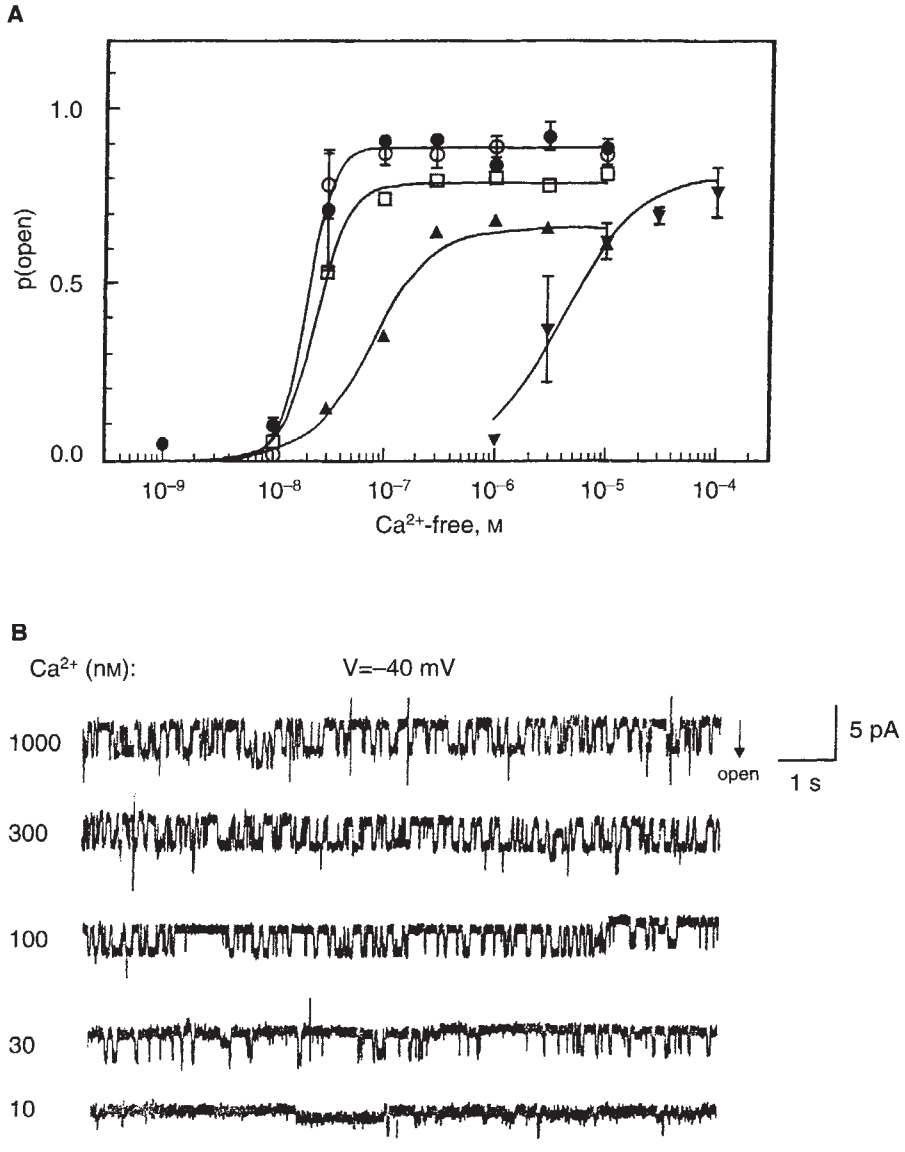

Fig. 5. *Ca^{2+}-activation and pH dependence.* **A.** Epithelial Ca^{2+} activated K^+ channels are incorporated into planar lipid bilayers with a salt gradient of 300 mM KCl (*cis* chamber) to 50 mM KCl (*trans*-chamber) and the Ca^{2+} activation site facing the cis chamber (open symbols) or the trans chamber (closed symbols). The initial free Ca^{2+} concentration is 200 μM and EGTA is added to obtain the concentrations of free Ca^{2+} shown on the abcissa. The Ca^{2+}-activation is examined at pH 7.2 at potentials of 0 mV (○,●), −20 mV (□) and −40 mV (▲) and at pH 6.0 at a potential of 0 mV (▼). At pH 7.2 the Hill coefficient ranges from 4.0 (0 mV) to 1.5 (−40 mV) and at pH 6.0 the Hill coefficient is 1.3 (0 mV). **B.** Single channel traces showing Ca^{2+}-dependence for one incorporated channel at a potential of −40 mV. From Klærke et al [23].

patch clamp technique, such as the basolateral membrane of the rabbit distal colon, and the bilayer technique gives free access to the cytoplasmic as well as the extracelluar face of an incorporated channel providing optimal conditions for an extensive characterization of the asymmetric properties of the channel.

Fusion of basolateral plasma membrane vesicles from rabbit distal colon with the planar lipid bilayer leads to incorporation of channels present in this membrane. The most abundant channel is the Ca^{2+}-activated maxi K^+ channel with a single channel conductance of about 275 pS, whereas low conductance K^+ channels and Cl channels only occasionally are observed [22, 23].

To characterize the Ca^{2+}-regulation of the epithelial maxi K^+ channels, it is necessary to study the Ca^{2+}-activation at different membrane potentials and pH values. Figure 5 shows that the maxi K^+ channels are activated by Ca^{2+} in the intracellular range of concentration as also found by flux studies in plasma membrane vesicles or reconstituted vesicles [7, 19], suggesting that the channels have not been denatured or modified by incorporation into the bilayer. The Ca^{2+}-activation is strongly influenced by the voltage across the channel and the pH on the cytoplasmic face of the channel, whereas even large changes in pH on the extracellular face of the channel has no effect [23]. Lowering the pH on the cytoplasmic face of the channel and rendering the membrane potential more negative have similar effects: the K^+ channels becomes less sensitive to Ca^{2+}, and the Hill coefficient decreases (Fig. 5). The exact mechanisms for the Ca^{2+}-activation and the modulation by voltage and pH are not known.

The decrease in the apparent Ca^{2+}-sensitivity and in the Hill coefficient caused by acidification, 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 [20, 40, 41]. However, in a recent study [42], the Hill coefficient for Ca^{2+}-activation of the

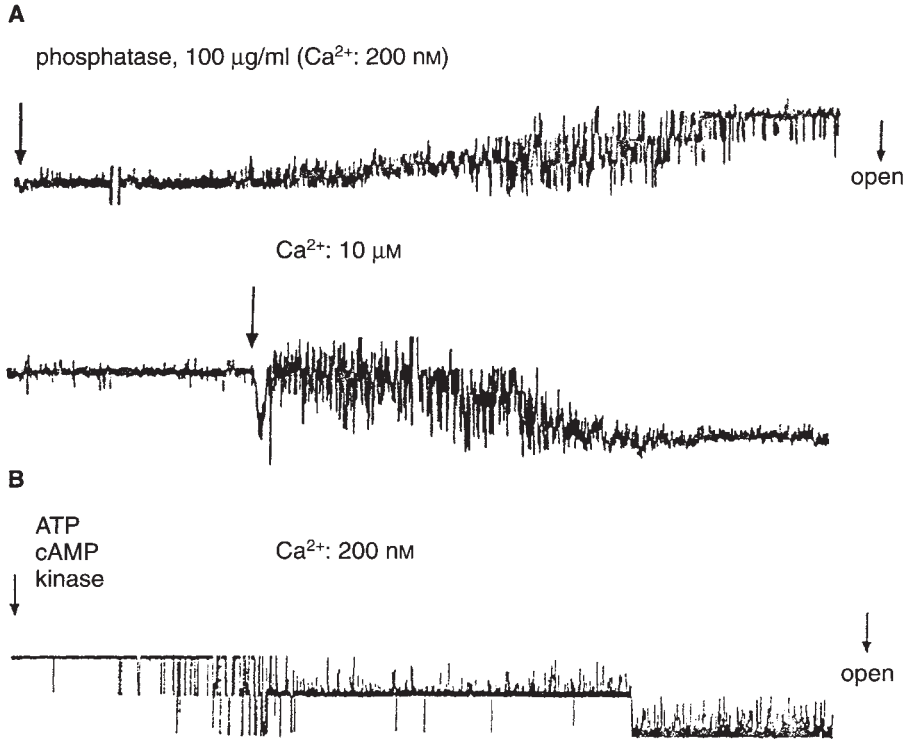

Fig. 6. *Phosphorylation and dephosphorylation of Ca^{2+}-activated K^+ channels.* Several maxi K^+ channels are incorporated into the planar lipid bilayer at a low Ca^{2+} concentration (200 nM). (**A**) Addition of phoshatase (100 μg/ml) inactivated the channels, but the channels can still be reactivated by addition of high concentrations of Ca^{2+} (10 μM). (**B**) Two dephosphorylated maxi K^+ channels are incorporated into the bilayer. They are inactive at the low Ca^{2+}-concentration (200 nM), but can be activated by phosphorylation after addition of ATP, cAMP and protein kinase. Note that the activation by phosphorylation takes place in two ways, either instantly or after a short flicker period. There is no change in single channel conductance after phosphorylation or dephosphorylation.

maxi K^+ channel from rat skeletal muscle is unaffected by changes in pH and it is suggested that protons exert their effect allosterically and not by competition with Ca^{2+}. The analysis of steady-state data in Figure 5 does not permit discrimination between these possibilities.

A negative transmembrane voltage reduces both the $K_{0.5}$ for Ca^{2+}-activation and the Hill coefficient for the epithelial maxi K^+ channels. 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 [43]. 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 [43] to 6 [44].

Regulation by phosphorylation/dephosphorylation and ATP

Phosphorylation is an important modulator of the Ca^{2+} sensitivity of epithelial maxi K^+ channels. Measurements of K^+ channels from TAL and distal colon epithelia in plasma membrane vesicles and after incorporation into lipid bilayers show that the epithelial K^+ channels are activated by Ca^{2+} with a very high sensitivity (Figs. 2 and 5). This high sensitivity to Ca^{2+}, however, is dependent on the channel protein being in a phosphorylated state. If the channels are dephosphorylated by the addition of a phosphatase (Fig. 6) or by activation of endogenous phosphatases in the membrane preparation [23], they lose their high sensitivity to Ca^{2+}. Dephosphorylated channels can regain their high sensitivity to Ca^{2+} by phosphorylation catalyzed by a cAMP dependent protein kinase (Fig. 6), whereas there is no effect of protein kinase C. The shift in Ca^{2+}-sensitivity induced by phosphorylation/dephosphorylation is ~ 1 order of magnitude [45]. This implies that at certain conditions (such as 200 nM free Ca^{2+} and a voltage of 0 mV) phosphorylation changes the channel from being closed to being almost totally open, and that the channels must be in a phosphorylated state to respond to Ca^{2+} in the intracellular range of concentration.

K^+ currents in different types of cells have been shown to be affected by agents that change the activity for protein kinase A or protein kinase C [46]. In the case of Ca^{2+}-activated K^+ channels the protein kinase mediated changes in the channel activity have been suggested to result from direct phosphorylation of the channel proteins and in a number of tissues, including kidney tubule cells [9, 47], pancreatic duct cells [48], heart sarcolemma vesicles [35] and smooth muscle [49], the Ca^{2+}-activated K^+ channels are up-regulated by phosphorylation catalyzed by protein kinase A. Typically, a two- to fourfold change in open probability is observed for these channels after phosphorylation. This is a relatively small response compared to the effect of phosphorylation found for the epithelial K^+ channel from rabbit distal colon (Fig. 6), and it will be interesting to learn if the different responses to phosphorylation for channels from different tissues reflect structural differences in the amino acid sequences, such as resulting from alternative splicing of RNA.

Ca^{2+}-activated maxi K^+ channels may be divided into subclasses based on their response to phosphorylation. In the central nervous system it has been shown that the channels can be either up-regulated [50, 51] or down-regulated by phosphorylation catalyzed by protein kinase A [52]. Reinhart et al [52] have recently identified two populations of maxi K^+ channels in plasma membrane vesicles from rat brain; one population (type 1) is up-regulated by protein kinase A whereas the other (type 2) is down-regulated by protein kinase A, but may be up-regulated by an endogenous protein kinase [53, 54]. Type 1 has a fast opening

kinetics and is sensitive to charybdotoxin, while type 2 has a slow opening kinetics and is insensitive to charybdotoxin. The Ca^{2+}-activated K^+ channels from the rabbit distal colon are up-regulated by phosphorylation catalyzed by protein kinase A, and it has earlier been shown that they are sensitive to charybdotoxin [23]. Using the terminology suggested by Reinhart et al [52], the colon Ca^{2+}-activated K^+ channels must therefore be characterized as type 1 maxi K^+ channels.

The traditional classification of K^+ channels as either Ca^{2+}-activated or ATP-sensitive has been challenged by the observation that ATP can inhibit the epithelial Ca^{2+}-activated maxi K^+ channels from, such as Amphiuma distal tubule [55] and rabbit distal colon [56]. In the distal colon the Ca^{2+}-activated K^+ channels are inhibited by ATP from the intracellular face with a $K_{0.5}$ of ~ 2.5 mM [56]. Since this ATP-inhibition take place in the absence of protein kinase it is probably a direct effect of ATP. Studying ATP-inhibition of Ca^{2+}-activated K^+ channels, it is important to control the free Ca^{2+} concentration in the media carefully, since ATP chelates Ca^{2+} and therefore could give rise to a "false" inhibition by decreasing the free concentration of Ca^{2+}. The ATP inhibition cannot be reversed by ADP as it is the case for "traditional" ATP-sensitive K^+ channels from, such as β cells [57] or kidney tubulus [24, 25]. Also, high concentrations of Ca^{2+} cannot reactivate the channel once it is inhibited by ATP [56], indicating that ATP does not interfere with the Ca^{2+}-activation of the channel. The effect of ATP opens the possibility that Ca^{2+}-activated K^+ channels may be coupled to the metabolic state of the cells.

Analysis of the sequence of the cloned Ca^{2+}-activated K^+ channels, the *slowpoke* channel and the *mSlo* channel, show that the proteins contain several possible phosphorylation sites for protein kinase C and one for protein kinase A in the C-terminal part of the proteins [12, 13]. This part of the channel proteins is expected to be involved in channel regulation, although mutagenis experiments in a putative Ca^{2+}-binding region identified in C-terminal part of the *slowpoke* channel failed to show any change in the Ca^{2+}-regulation of the channel [58]. Consistent with a possible direct effect of ATP on channel activity, a putative ATP binding site is present in the C-terminal part of the *slowpoke* channel [13].

A recent report has shown that the *slowpoke* channel is down-regulated by phosphorylation by protein kinase A [58] and, keeping in mind that this channel is insensitive to charybdotoxin, the *slowpoke* channel must be a type 2 maxi K^+ channel. The *mSlo* channel, in contrast, is sensitive to charybdotoxin, and could therefore be a type 1 channel. However, the effect of protein kinase A catalyzed phosphorylation on the *mSlo* channel remains to be examined, and it is therefore unknown whether this channel resembles the maxi K^+ channels from epithelia.

Physiological role of epithelial Ca^{2+}-activated K^+ channels

The extensive regulation of the epithelial Ca^{2+}-activated K^+ channels opens the question of how these channels can be involved in regulation of transepithelial Na^+ transport. In the TAL several studies have indicated that the luminal K^+ conductance is elevated after stimulation with ADH [1] and studies on the whole colon have shown that the basolateral K^+ conductance is increased during aldosterone-stimulated Na^+ reabsorption [4]. The role of the Ca^{2+}-activated K^+ channels in the hormone induced changes in K^+ conductance has been questioned, since different studies have shown that the Ca^{2+}-activated K^+ channel in cultured TAL cells [20] and in vesicles from distal colon [22] needed Ca^{2+}-concentrations far above the physiological range to become activated. However, the data presented in the present paper suggest that if the K^+ channels are in a phosphorylated state they are indeed sensitive to Ca^{2+} in intracellular concentrations.

In the TAL two types of K^+ channels are found in the luminal membrane, an ATP-sensitive K^+ channel and Ca^{2+}-activated maxi K^+ channels [1]. Hormonal stimulation of this tissue with ADH in many species lead to a rise in intracellular cAMP [59] and in rabbit kidney it has been shown that ADH-stimulation increases the intracellular free Ca^{2+} from 155 nM to 429 nM [60]. It is obvious to suggest that the rise in cAMP will activate cAMP dependent protein kinases, which in turn phosphorylate the luminal maxi K^+ channels rendering the channels so sensitive to Ca^{2+} that they are activated by the relatively modest rise in intracellular Ca^{2+}. If this is the case the luminal Ca^{2+}-activated K^+ channels could be responsible for the raise in luminal K^+ conductance seen after hormonal stimulation, whereas the luminal ATP-sensitive K^+ channels, whose activity is coupled to the metabolic state of the cell through ATP, could be considered as the housekeeping channels responsible for the luminal K^+ conductance at non-stimulated circumstances. This idea is supported by the observation that treatment of cultured TAL cells with ADH and forskolin, an activator of the adenylate cyclase, leads to stimulation of Ca^{2+}-activated K^+ channels [61].

The low Ca^{2+}-sensitivity found for dephosphorylated maxi K^+ channels implies that these channels are not active in unstimulated tissues. This could be the explanation that these channels have only been identified very rarely in patch clamp studies on intact tubuli [24, 25]. In the distal colon surface cells there are no measurements of intracellular cAMP or Ca^{2+} after stimulation of the tissue, but it is suggested that phosphorylation may be an important regulatory mechanism also in this tissue, since Ca^{2+}-activated maxi K^+ channels in distal colon crypt colonocytes are stimulated by cAMP [62].

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.

Also the pH may 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 [63]. Increased pH in the cytoplasm will increase 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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