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Reconstitution in Phospholipid Vesicles of Calcium-Activated Potassium Channel from Outer Renal Medulla

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Summary. A barium-sensitive Ca-activated K^+ channel in the luminal membrane of the tubule cells in thick ascending limb of Henle's loop is required for maintenance of the lumen positive transepithelial potential and may be important for regulation of NaCl reabsorption. In this paper we examine if the K^+ channel can be solubilized and reconstituted into phospholipid vesicles with preservation of its native properties. The K^+ channel in luminal plasma membrane vesicles can be quantitatively solubilized in CHAPS at a detergent/protein ratio of 3. For reconstitution, detergent is removed by passage over a column of Sephadex G 50 (coarse). K^+-channel activity is assayed by measurement of $^{86}Rb^+$ uptake against a large opposing K^+ gradient. The reconstituted K^+ channel is activated by Ca^{2+} in the physiological range of concentration ($K_{1/2} \sim 2 \times 10^{-7}$ M at pH 7.2) as found for the K^+ channel in native plasma membrane vesicles and shows the same sensitivity to inhibitors (Ba^{2+}, trifluoperazine, calmidazolium, quinidine) and to protons. Reconstitution of the K^+ channel into phospholipid vesicles with full preservation of its native properties is an essential step towards isolation and purification of the K^+-channel protein.

Titration with Ca^{2+} shows that most of the active K^+ channels in reconstituted vesicles have their cytoplasmic aspect facing outward in contrast to the orientation in plasma membrane vesicles, which requires also addition of Ca^{2+} ionophore in order to observe Ca^{2+} stimulation. The reconstituted K^+ channel is highly sensitive to tryptic digestion. Brief digestion leads to activation of the K^+ channel in absence of Ca^{2+}, to the level of activity seen with saturating concentrations of Ca^{2+}. This tryptic split is located in a cytoplasmic aspect of the K^+ channel that appears to be involved in opening and closing the K^+ channel in response to Ca^{2+} binding.

Key Words Ca-activated K^+ channel · solubilization · reconstitution · thick ascending limb of Henle's loop · calmodulin inhibitors · trypsin · pH

Introduction

Active reabsorption of NaCl in the thick ascending limb of Henle's loop of the mammalian kidney involves the parallel working of the loop-diuretic sensitive Na,K,Cl-cotransport system and a Ba^{2+}-sensitive K^+ channel in the luminal membrane and a net Cl^- conductance together with the Na,K pump in the basolateral membrane of the tubular cells [14, 16]. Recycling of K^+ through the K^+ channel is required for the maintenance of the lumen-positive transepithelial potential, which drives Na^+ absorbtion and Cl^- excretion across the tight junctions in the tubular epithelium. This paracellular transport raises the efficiency of the transport so that 5 mol NaCl is reabsorbed per mol ATP split [14]. The NaCl transport has been shown to be hormonally regulated in some species (mouse, rat), and the luminal K^+ channel as well as the basolateral Cl^- conductance may play key roles in this regulation [16, 33].

The K^+ channel has been identified in luminal plasma membrane vesicles from the thick ascending limb of Henle's loop, and it has been shown to be activated by Ca^{2+} at physiological concentrations [3, 4]. In a preliminary study it was shown that the K^+ channel could be reconstituted into phospholipid vesicles by dissolving the luminal plasma membrane vesicles in octylglucoside, mixing with soybean phospholipid, and forming vesicles by passing the mixture down a column of Sephadex G 50 [4].

The purpose of the present study has been to examine if the renal K^+ channel can be reconstituted into phospholipid vesicles with full preservation of its native properties, this being a necessary condition for identification of the K^+ channel in purification procedures. For this purpose we have made use of the detergent CHAPS [18]. In reconstituted vesicles it might be expected that some K^+ channels will insert with their cytoplasmic aspect facing the outside of the vesicles, thus allowing for direct titration of the Ca^{2+}-activation site without the use of ionophores. In this system we can study the Ca^{2+} dependence of the K^+-channel activity and examine the sensitivity of the K^+ channel for the effect of varying pH. The activation patterns and the sensitivity to the inhibitors barium, quinidine,

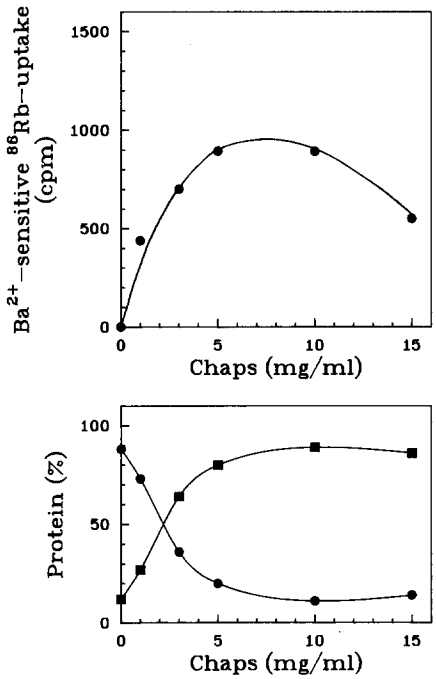

Fig. 1. Solubilization and reconstitution of K^+ channel. Luminal plasma membrane vesicles were prepared on metrizamide gradients and solubilized with CHAPS in 150 mM KCl, 25 mM Tris-HCl, 1 mM EDTA, pH 7.2, at a protein concentration of 1.55 mg/ml as described under Materials and Methods. Soluble protein (■) and nonsolubilized protein (●) were separated by centrifugation at 100,000 rpm for 10 min in a Beckman Airfuge. 50 μl of each supernatant was mixed with 50 μl buffer containing CHAPS 30 mg/ml, 6 μl 2% mercaptoethanol and lipid. For reconstitution, the mixture was passed over dry Sephadex columns and $^{86}Rb^+$ uptake (●) was assayed as described under Materials and Methods

trifluoperazine and calmidazolium are also compared with that in luminal plasma membrane vesicles. The exposure of cytoplasmic aspects of the channel in reconstituted vesicles reveals trypsin-sensitive sites at this membrane surface and therefore permits study of the effect of controlled tryptic digestion on Ca^{2+} activation and channel activity.

Materials and Methods

Membrane Preparation

A crude plasma membrane fraction from pig kidney outer medulla was prepared by differential centrifugation as described earlier [21] using 250 mM sucrose, 50 mM KCl, 1 mM EGTA, 10 mM MOPS-Tris, pH 7.2, for homogenization, resuspension and all subsequent procedures. The membranes were resuspended in the buffer to a concentration of 8–10 mg protein per ml. For isolation of luminal plasma membrane vesicles, 1.5 ml of the resuspended vesicles were placed on linear density gradients of 5–15% (wt/vol) metrizamide (Nyegaard, Oslo, Norway) dissolved in the buffer on top of a 1-ml cushion of 30% metrizamide [4, 7]. After centrifugation for 16 hr at 19,000 rpm in a SW-27 rotor, the gradient was separated into fractions of 1 ml. Luminal plasma membrane vesicles were recovered in fractions Nos. 3–5 from the top at a protein concentration of 1–1.5 mg/ml. Alternatively, the sample consisting of 1.5 ml crude membranes was layered on top of 15% (wt/vol) metrizamide dissolved in the buffer and centrifuged at 35,000 rpm for 1 hr in a Ti-41 rotor or for 3.5 hr at 19,000 rpm in a SW-27 rotor. Two fractions of 1 ml were recovered from the top of the tube. The floating luminal vesicles were found in the second fraction from the top, at a protein concentration of 5–6 mg/ml.

Reconstitution

Prior to solubilization, aliquots of luminal plasma membrane vesicles containing 1 mg protein were collected, following 30 to 100-fold dilution in 10 mM MOPS-Tris, 50 mM KCl, pH 7.2, and then centrifugation for 90 min at 40,000 rpm in a Ti-60 Beckman rotor. The vesicles were resuspended in 200 μl in 250 mM sucrose, 50 mM KCl, 1 mM EGTA, 5 mM DTT, 10 mM MOPS-Tris, pH 7.2, by homogenization in a tightly fitting Teflon glass homogenizer. For solubilization, 20 μl of CHAPS 0.5 M in the buffer was added. 200 μl lipid solution (soy bean phosphatidylcholine, Sigma, Commercial Grade, 50 mg/ml) was also dissolved in the same buffer, sonicated to clarity in a Bransonic 12 bath sonicator, and solubilized by adding 40 μl CHAPS 0.5 M in the buffer. Protein and lipid solutions were mixed in a Carlsberg micropipette and applied to a 1 × 30 cm Sephadex G-50 coarse column that was equilibrated at 20°C with the buffer [15].

Alternatively, reconstitution was carried out by centrifugation on 5-ml columns of Sephadex prepared in disposable plastic syringes in tapered tubes according to the method of Penefsky [28]. Sephadex-G-50 coarse was equilibrated in the buffer and centrifuged for 1 min at 1,000 rpm to remove excess fluid. The mixture of solubilized protein and lipid (100–200 μl) was now added to the columns, and the reconstituted vesicles were harvested by a second centrifugation for 1 min at 1,000 rpm (20°C).

For tryptic digestion 10 μl TPCK-trypsin (MERCK) dissolved in ice-cold 250 mM sucrose, 50 mM KCl, 1 mM EGTA, 10 mM MOPS-Tris, pH 7.2, was added to 300 μl reconstituted vesicles to give the desired final concentration. Incubation with trypsin was at 20°C for 5 min. To stop the digestion 10 μl trypsin inhibitor (Sigma, Type I-S, lyophilized from soybean) was added giving a final concentration three times that of the trypsin.

The esterase activity of trypsin toward TAME (Calbiochem) measured as described earlier [22] was 47 μmol TAME · min^{-1} · mg $trypsin^{-1}$ at 20°C in the present conditions. Earlier the esterase activity was determined to 193–210 μmol TAME · min^{-1} · mg $trypsin^{-1}$ at 37°C [22].

^{86}Rb-Flux Assay

K^+-channel activity is assayed by measuring $^{86}Rb^+$ uptake against a large opposing K^+ gradient as described earlier [4, 10]. Dowex 50 W × 2 (Fluka) was equilibrated with Tris-Cl and poured into Pasteur pipettes to make columns with a void volume of 650 μl. The columns were flushed with 2 × 500 μl ice-cold 350 mM sucrose, 10 mM MOPS-Tris, pH 7.2, and kept on ice until use. To block (Na,K)-ATPase, vesicles were incubated for 10 to 30 min at 20°C with 0.5 mM ouabain, 2.0 mM $MgCl_2$, 100 μM sodiummonovanadate, 250 mM sucrose, 50 mM KCl, 1 mM

EGTA, 10 mM MOPS-Tris, pH 7.2 at 1–2 mg protein per ml. To deplete the vesicles for Ca^{2+}, 3.75 mM EGTA-Tris and 10 μM Ca^{2+}-ionophore A23187 from a 2 mM stock solution in ethanol were added [3].

Prior to assay, external K^+ was exchanged for $Tris^+$ by adding 180 μl of the vesicle medium to ice-cold Dowex columns. The vesicles were eluted with 650 μl 350 mM sucrose, 10 mM MOPS-Tris, pH 7.2, and kept on ice until use. To assay for stimulation by Ca^{2+}, 270 μl vesicles depleted of Ca^{2+} by incubation with 3.75 mM EGTA and A23187, were incubated for 1 min at 20°C in 50 μl 350 mM sucrose, 3 mM EGTA, 10 mM MOPS-Tris, pH 7.2, and 0.1–4.0 mM Ca^{2+} to give the desired concentrations of free Ca^{2+}.

For initiation of the assay 135 μl of the vesicles were mixed with 45 μl reaction medium at 20°C to obtain the following concentrations: 1 mM furosemide, 100 μM $^{86}RbCl$ (5×10^5 cpm/100 μl), 350 mM sucrose, 3 mM EGTA, 10 mM MOPS-Tris, pH 7.2, and $CaCl_2$ to give the desired concentration of free Ca^{2+}. To stop ^{86}Rb uptake, 160 μl of the medium was transferred to an ice-cold Dowex column for removal of external $^{86}Rb^+$. The $^{86}Rb^+$ trapped inside the vesicles was eluted into counting vials with 3 times 500 μl ice-cold 350 mM sucrose, 10 mM MOPS-Tris, pH 7.2, and activity was measured by Cherenkov counting in the tritium channel of a Packard scintillation counter. The assay is usually done in the absence and presence of 5 mM $BaCl_2$ in the reaction medium, and the K^+-channel activity is expressed as Ba-sensitive $^{86}Rb^+$ uptake into the vesicles.

The free Ca^{2+} concentration in the EGTA-Ca buffer systems was calculated according to Pershadsingh and McDonald [30].

Protein was measured by the method of Lowry et al. [25] after precipitation with trichloroacetic acid with bovine serum albumin as standard.

Abbreviations

CHAPS: 3-((3-cholamidopropyl)dimethylammonio)-1-propanesulfonate
DTT: D,L-dithiothreitol
EGTA: ethylene glycol bis-(β-aminoethyl ether) N,N,N',N'-tetraacetic acid
MES: 2-(N-morpholino)ethanesulfonic acid
MOPS: 3-(N-morpholino)propanesulfonic acid
TAME: P-toluenesulfonyl-L-arginine methyl ester
TPCK-trypsin: N-tosyl-L-phenylalanyl-chlormethane treated trypsin
Tris: tris(hydroxymethyl)aminomethane

Results

Solubilization of the K^+ channel and distribution of membrane protein in supernatant and pellet after incubation of the luminal plasma membrane vesicles with increasing concentrations of CHAPS [18] are examined in the experiment in Fig. 1. At CHAPS concentrations above 5 mg/ml, corresponding to detergent/protein weight ratios above 3, we find maximal K^+-channel activity in the supernatant as assayed following reconstitution into phospholipid vesicles. In contrast to the previous experience with the Na,K,Cl-cotransporter [4] a high salt concentration (0.5–1 M KCl) did not affect solubilization of the K^+ channel (*data not shown*). Comparison between detergents showed that CHAPS was superior to octylglucoside for reconstitution of the K^+ channel. Attempts were also made to use the freeze-thaw-sonication method for reconstitution [23] but we found that gel filtration on Sephadex G 50 (coarse) columns was superior.

To determine if the native properties of the K^+ channel are preserved after solubilization and reconstitution, the sensitivity to inhibitors was examined. As shown before, the sensitivity to Ba^{2+} was the same for K^+ channels in reconstituted and in luminal plasma membrane vesicles [4]. The data in Fig. 2 also shows that the sensitivity of the K^+

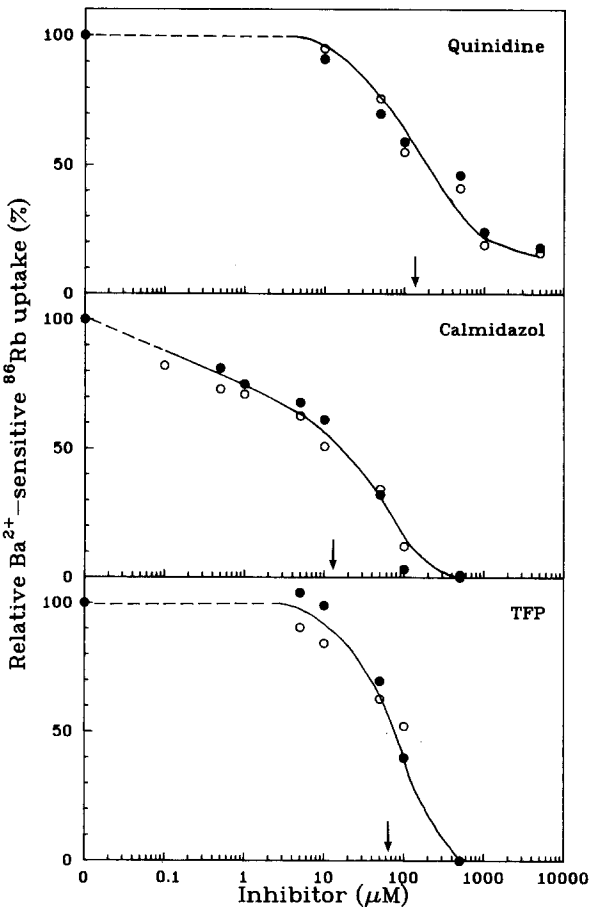

Fig. 2. Effect of quinidine, calmidazolium and trifluoperazine (TFP) on K^+-channel activity in plasma membrane vesicles (○) and reconstituted vesicles (●). $^{86}Rb^+$ uptake into luminal plasma membrane vesicles was measured after incubation for 10 min at 20°C in presence of increasing concentrations of inhibitor. Reconstituted vesicles were prepared from the same batch of plasma membrane vesicles. Inhibitors were added from stock solutions in dimethylsulfoxide. The final concentration of dimethylsulfoxide was 2%, and this did not affect $^{86}Rb^+$ uptake

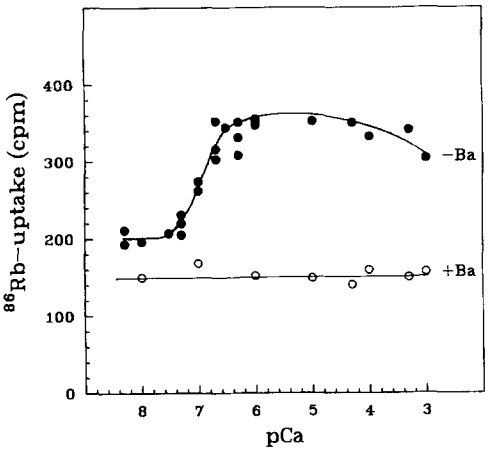

Fig. 3. Ca-activation of Ba-sensitive $^{86}Rb^+$ uptake in luminal plasma membrane vesicles. The vesicles were depleted for Ca^{2+} by incubation with EGTA and ionophore A23187 and Ca^{2+} was added again as described under Materials and Methods. ^{86}Rubidium uptake was measured for 10 min in the absence (●) and presence (○) of 5 mM $BaCl_2$

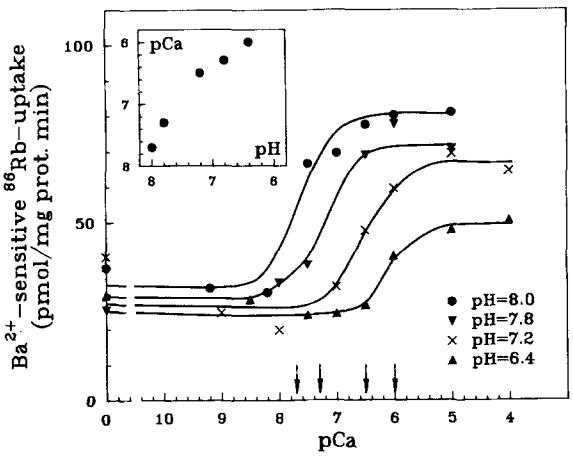

Fig. 4. Effect of changes in pH on Ca^{2+} stimulation of K^+ channel in luminal plasma membrane vesicles. Luminal plasma membrane vesicles were prepared in buffer that was adjusted to the desired values of pH with MOPS-Tris. Free Ca^{2+} in the Ca/EGTA-buffer systems were calculated for each pH value. ^{86}Rubidium uptake was measured after incubation for 10 min in the absence and presence of 5 mM $BaCl_2$ at pH 6.4 (▲), pH 7.2, (×), pH 7.8 (▼) and pH 8.0 (●). The *p*Ca for 50% activation are marked for each pH value with arrows. The inset contains a plot of pH *versus* the *p*Ca at 50% activation

channel to quinidine, calmidazolium and trifluoperazine was the same in plasma membrane and reconstituted vesicles. The antiarrythmic drug, quinidine, blocked Ba^{2+}-sensitive $^{86}Rb^+$ uptake through the channel in both vesicle types with $K_{1/2} \sim 150$ μM. This is in agreement with the sensitivity of K^+ channels in other systems [20, 32]. The calmodulin antagonists trifluoperazine and calmidazolium were used to test for involvement of calmodulin in regulation of K^+-channel activity. The sensitivity to these inhibitors was relatively low, $K_{1/2} \sim$ 15 and 65 μM, respectively. This is close to the $K_{1/2}$ in systems that are known to depend on calmodulin [1, 11, 12, 19].

Ca^{2+} Activation of the K^+ Channel in Plasma Membrane Vesicles

Incubation with a high concentration of EGTA and the Ca^{2+} ionophore A23187 in combination are required for depletion of luminal plasma membrane vesicles for Ca^{2+} and reduction of K^+-channel activity [3]. When added separately, A23187 and EGTA do not alter K^+-channel activity, but together they reduce the Ba^{2+}-sensitive flux to 10–20% of control. This demonstrates that the K^+ channel in the preparation is Ca^{2+} activated when no measures are taken to remove intravesicular Ca^{2+}. The fact that A23187 is required for Ca^{2+} depletion implies that the binding site for Ca^{2+} activation of the K^+ channel is oriented towards the interior of the luminal plasma membrane vesicles. Addition of free Ca^{2+} in physiological concentrations to the Ca^{2+}-depleted luminal plasma membrane vesicles resulted in a fourfold increase in Ba^{2+}-sensitive $^{86}Rb^+$ uptake with an apparent $K_{1/2}$ of 2×10^{-7} M (Fig. 3), confirming the result in ref. 3. The ionophore A23187 is a very hydrophobic structure [34], which is incorporated into the lipid bilayer of the vesicles, and therefore ionophore added at the initial Ca^{2+}-depletion procedure is also supposed to be present in the vesicles after passage of the first Dowex column making the vesicles permeable to Ca^{2+} during the assay. The Ba^{2+}-insensitive $^{86}Rb^+$ uptake is totally unaffected by Ca^{2+}, showing that the Ca^{2+}-activated K^+ channel is totally blocked by Ba^{2+}.

To evaluate the physiological significance of Ca^{2+} activation it is important to know its dependence on cytoplasmic pH. Figure 4 shows the Ca^{2+}-activation curves at pH values covering the range of the cytoplasm. The sensitivity of the K^+ channel to Ca^{2+} is increased from $K_{1/2} = 1 \times 10^{-6}$ M at pH 6.4 to $K_{1/2} = 2 \times 10^{-8}$ M at pH 8.0. The relationship between pH and *p*Ca for this series of titrations is shown in the inset. A straightforward explanation for the data is that H^+ competes with Ca^{2+} [17] for binding to the Ca^{2+}-activation site on the K^+ channel.

Ca Activation in Reconstituted Vesicles

In reconstituted vesicles $^{86}Rb^+$ uptake was inhibited by incubation with 3.75 mM EGTA in the absence of Ca^{2+} ionophore. As shown in Fig. 5, the Ca^{2+}-acti-

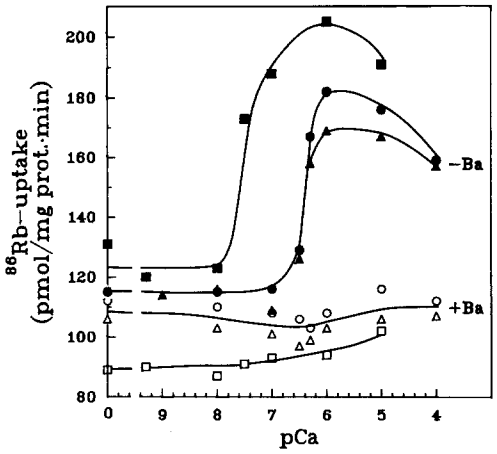

Fig. 5. Ca^{2+} activation of $^{86}Rb^+$ uptake in reconstituted vesicles. The procedure for Ca^{2+} depletion and reactivation by addition of Ca^{2+} was as described for the plasma membrane vesicles. The experiment was done at pH 7.2 in the presence (▲) and absence (●) of ionophore A23187, and at pH 8.0 in the absence of ionophore (■). In all cases the $^{86}Rb^+$ uptake was measured for 10 min at 20°C in the absence (filled symbols) and in the presence (open symbols) of 5 mM $BaCl_2$

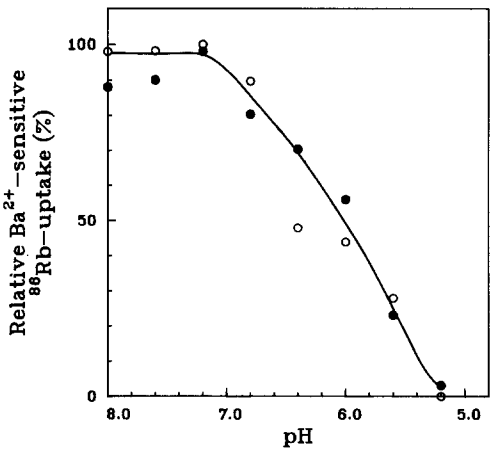

Fig. 6. Inhibition of the K^+ channel by H^+. The figure shows the relative Ba^{2+}-sensitive $^{86}Rb^+$ uptake for plasma membrane vesicles (○) and reconstituted vesicles (●). Data are expressed as per cent of the value at pH 7.2. Plasma membrane vesicles and reconstituted vesicles were prepared at pH 7.2 as described under Materials and Methods. For assay of $^{86}Rb^+$ uptake the media were adjusted to the pH on the abscissa using 10 mM MES-Tris as buffer system instead of MOPS-Tris. The free concentration of Ca^{2+} was 50 μM for all values of pH

vation site on the K^+ channel could be titrated directly with Ca^{2+} ($K_{1/2}$ = 2 × 10^{-7} M at pH 7.2) whether or not ionophore was present. This result shows that most of the active K^+ channels in reconstituted vesicles have their Ca^{2+}-activation sites facing the extra-vesicular medium. This is in contrast to the orientation of the Ca^{2+}-activation sites in luminal plasma membrane vesicles, in which also Ca^{2+} ionophore is required for inhibition. The Ca^{2+} activation of the K^+ channel in the reconstituted vesicles shows the same dependence of pH as in the plasma membrane vesicles. At pH 8.0 the $K_{1/2}$ for Ca^{2+} activation of the K^+ channel in the reconstituted vesicles is found to be 2 × 10^{-8} M as it was the case for the K^+ channel in plasma membrane vesicles (Fig. 5).

The experiment in Fig. 6 shows that the K^+ channel in reconstituted vesicles has the same sensitivity to titration with H^+ as the K^+ channel in luminal plasma membrane vesicles. The K^+ channel was inhibited when H^+ was increased with $K_{1/2}$ = pH 6.2. In this experiment excess Ca^{2+} (50 μM) was present. It is therefore unlikely that the inhibition by H^+ is due to competition with binding of Ca^{2+} for the activation site.

Controlled Proteolysis

As the titrations with Ca^{2+} suggested that the K^+ channel is oriented with its cytoplasmic aspect facing outward in the reconstituted vesicles, we examined if cleavage of trypsin-sensitive bonds would

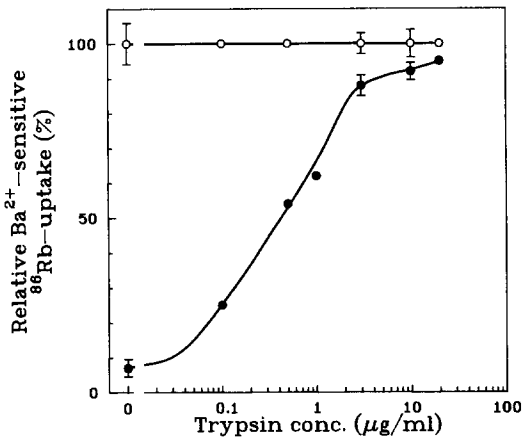

Fig. 7. Effect of tryptic digestion on the K^+ channel in reconstituted vesicles. The figure shows the Ba^{2+}-sensitive $^{86}Rb^+$ uptake at zero Ca^{2+} (●) in per cent of the Ba^{2+}-sensitive $^{86}Rb^+$ uptake and at 10 μM Ca^{2+} (○) after incubation for 5 min at 20°C with increasing trypsin concentrations. For trypsin concentrations 0, 3.0, and 10.0 μg/ml average values ± SEM were calculated for four experiments

affect Ca^{2+}-dependent K^+-channel activity in this system. In luminal plasma membrane vesicles we observed that both Ba^{2+}-sensitive and Ba^{2+}-insensitive fluxes of $^{86}Rb^+$ were insensitive to proteolysis (*not shown*). Data in Fig. 7 show that the reconstituted K^+ channel was highly sensitive to tryptic digestion. A brief incubation (5 min) at 20°C with 3–10 μg/ml TPCK-trypsin leads to activation of K^+-channel activity in absence of Ca^{2+} to the level of

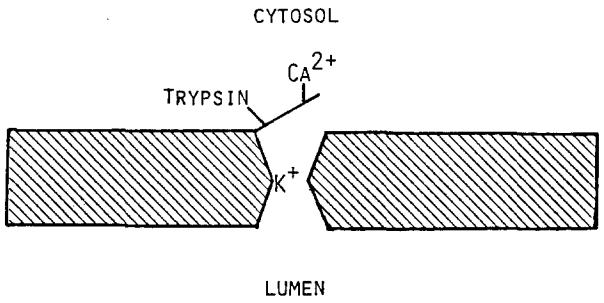

Fig. 8. Schematic model of the Ca^{2+}-activated K^+ channel. In this model two sets of charged groups are suggested. One is the site for Ca^{2+} activation. The other is the group with an apparent pK near 6.2 that may be responsible for channel selectivity or directly involved in K^+ transport through the channel structure. It is suggested that the trypsin cleavage occurs in a part of the protein that is engaged in opening and closing of the K^+ channel in response to Ca^{2+} binding

activity seen with saturating concentration of Ca^{2+}. Both control and trypsin-treated K^+ channels remain sensitive to Ba^{2+} and quinidine. Incubation for longer times (10–20 min) at higher trypsin concentrations (10–20 μg/ml) leads to more unspecific activation of the channel so that $^{86}Rb^+$ uptake becomes insensitive to Ba^{2+}.

This effect of trypsin suggests that a part of the K^+-channel protein which is involved in Ca^{2+} activation has a trypsin-sensitive bond. The structural consequences of this split remain to be examined.

Discussion

This work shows that the K^+ channel in luminal membrane vesicles can be rendered soluble and reconstituted in phospholipid vesicles in a form that is dependent on activation by Ca^{2+} in the physiological range like the K^+ channel in the native plasma membrane vesicles [3]. After reconstitution the K^+ channel shows the same sensitivity to H^+, and it is inhibited by barium, trifluoperazine, calmidazolium and quinidine in the same concentrations as the native K^+ channel in the plasma membrane vesicles. These results show that the K^+ channel can be solubilized in CHAPS and reconstituted in phospholipid vesicles without loss of its native properties. This is an important step towards purification of the K^+-channel protein. Exposure of the cytoplasmic face of the K^+-channel protein to the medium after reconstitution provides the opportunity to examine directly the regulation of the K^+ channel.

The K^+ channel in the reconstituted vesicles is stimulated by Ca^{2+} like the K^+ channel in luminal plasma membrane vesicles. In the reconstituted vesicles, however, we obtain the Ca^{2+} effect in absence of ionophore, and in addition the Ca^{2+} dependence of the K^+ channel in reconstituted vesicles is abolished by tryptic digestion. These findings show that in the reconstituted vesicles the orientation of the K^+ channel must be predominantly cytoplasmic side out. In the plasma membrane vesicles it is necessary to use the Ca^{2+} ionophore to obtain Ca^{2+} effects and there is no effect of tryptic digestion, indicating as discussed in ref. 3 that most of the plasma membrane vesicles are right side out—exposing extracellular aspects of the K^+ channel.

Tryptic digestion of the K^+ channel in the reconstituted vesicles results in cleavage of bonds in the cytoplasmic aspect of the K^+ channel. This cleavage leaves the K^+ channel open even in the absence of Ca^{2+}. Opening of a Ca^{2+}-activated K^+ channel in absence of Ca^{2+} has also been observed after incorporation of trypsin into resealed ghosts of human red cells [37, 38]. This split may occur in a part of the protein engaged in the opening and closing of the K^+ channel in response to Ca^{2+} binding (Fig. 8). It will be interesting to learn if cleavage removes a part of the channel protein, but further examination requires purification of the K^+-channel protein.

At least two sets of charged groups have been titrated with protons in our study. One is the site for Ca^{2+} activation, another is a group with an apparent pK of 6.2. Our results imply that Ca^{2+} and protons bind competitively to the Ca^{2+}-activation site of the K^+ channel; Ca^{2+} opens the K^+ channel whereas protons do not. In the reconstituted vesicles, in the absence of ionophore, we also find competition between H^+ and Ca^{2+} for binding to the K^+ channel (Fig. 5). This confirms that the effect of pH shown in Fig. 4 is indeed an effect on the K^+ channel and is not due to Ca^{2+}/H^+ competition on the ionophore. Ca^{2+}-activated K^+ channels from other tissues have previously been shown to be sensitive to pH in a similar way [5, 6].

Also at a high concentration of free Ca^{2+} (50 μM) the K^+ channel is inhibited at low pH (Fig. 6). From extrapolation of the results in Fig. 5 (inset) the Ca^{2+}-activation site is expected to be saturated with Ca^{2+} even at very low pH at a free Ca^{2+} concentration of 50 μM. The inhibition of the K^+ channel by protons at this concentration of free Ca^{2+} must be due to titration of other groups than the Ca^{2+}-activation site. Previous titrations of Ca^{2+}-sensitive as well as Ca^{2+}-insensitive K^+ channels show a similar dependence of pH with $K_{1/2}$ for inhibition near pH 6.2 [26, 35, 36]. As summarized in the schematical model in Fig. 8, it is a possible explanation that protons compete with K^+ for binding to a cation site that is directly involved in cation translocation through the K^+ channel. We obtain

the inhibition by protons in the plasma membrane vesicles as well as in the reconstituted vesicles. Probably we have no pH gradient in the two situations, as Tris enters the vesicle membrane [13] and hereby facilitates the transport of H^+ across the membrane. In most studies the pH-inhibition is found to take place from the cytoplasmic side of the K^+ channels, but inhibition by protons from the outside has also been reported [2].

The sensitivity of the K^+ channel to the inhibitors tested is independent of the differences in orientation of the K^+ channel in the two vesicle systems. The explanation can be either that the K^+ channel can be inhibited from both membrane surfaces or that the inhibitors are able to penetrate the membrane.

Quinidine and quinine are widely used to block Ca^{2+}-activated K^+ channels. The mechanism seems to be competition with K^+ from the outside of the K^+ channel [32]. Both compounds penetrate lipid membranes, and this can explain that inhibition of the K^+ channel is the same in the reconstituted and plasma membrane vesicles as in other systems [20, 32].

The calmodulin antagonists, trifluoperazine and calmidazolium, are also hydrophobic and freely permeant molecules. This accounts for inhibition of the K^+ channel in the plasma membrane vesicles as well as in the reconstituted vesicles. The concentration of the inhibitors needed to obtain half maximal inhibition is relatively high. Therefore, it is questionable whether these data indicate that calmodulin is involved in the regulation of the K^+ channel. In other systems, Ca^{2+}-activated K^+ channels have been shown to be regulated by calmodulin [27, 29].

Ba^{2+} inhibits most if not all K^+ channels, and different theories for the inhibition mechanism exist. It has been suggested that Ba^{2+}, having approximately the same size as K^+, acts as a blocker of the K^+ channel competing with K^+ [8]. However, recent reports suggest that the action of Ba^{2+} on the K^+ channel is more complicated [20]; Ba^{2+} is found to stimulate the K^+ channel from the inside in micromolar concentrations, while at the same time it inhibits the K^+ channel from the inside as well as from the outside in millimolar concentrations. The stimulating effect of Ba^{2+} observed by Iwatsuki and Petersen [20] probably takes place at the Ca^{2+}-activation site at the cytoplasmic face of the K^+ channel. Ba^{2+} has approximately the same unhydrated crystal diameter as K^+, 0.270 and 0.267 nm, respectively [24]. It is suggested that Ba^{2+} is able to enter the K^+ channel, and that it carries out its blocking effect by binding firmly to negative charges inside the K^+ channel because of its two positive charges compared to the one positive charge of K^+ [24]. This corresponds well with the fact that millimolar concentrations of Ba^{2+} block the K^+ channel from the inside as well as from the outside and that Ba^{2+} blocks the K^+ channel even after removal of the Ca^{2+}-activation site by tryptic digestion.

The sensitivity for Ca^{2+} for the Ca^{2+}-activated K^+ channels differs widely from tissue to tissue [31]. Several K^+ channels are gated by Ca^{2+} and voltage in combination, so that positive membrane potentials facilitates the channel opening by low levels of Ca^{2+} or even in the absence of Ca^{2+}, whereas a negative membrane potential makes the K^+ channel need higher concentrations of Ca^{2+} to open [5, 6, 9].

If the K^+ channel is inside out in the reconstituted vesicles and right side out in the plasma membrane vesicles, we have imposed during the assay a membrane potential with opposite orientation relative to the channel. The similar sensitivity to Ca^{2+} in the two situations therefore implies that this K^+ channel is insensitive to voltage. However, this question can only be unambiguously answered by electrophysiological experiments.

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