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Purification of Ca^{2+}-activated K^+ channel protein on calmodulin affinity columns after detergent solubilization of luminal membranes from outer renal medulla

Dan A. Klærke, Janne Petersen and Peter L. Jørgensen

Institute of Physiology, Aarhus University, 8000 Aarhus C, Denmark

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A method is developed for purification of the protein of the Ca^{2+}-activated K^+ channel from outer renal medulla of pig kidney. The response of this K^+ channel to physiological concentrations of Ca^{2+} is important for regulation of transtubular NaCl transport. In reconstituted vesicles direct addition of calmodulin doubles Ca^{2+} activation with sufficient affinity ($K_{1/2}$ 0.1 nM) for chromatographic purification of the protein. For purification luminal plasma membrane vesicles are isolated on metrizamide density gradients and solubilized in CHAPS. The fraction of soluble protein retained on calmodulin-Sepharose 4B columns in the presence of Ca^{2+} and eluted by EGTA is 0.7%. The purified protein has high Ca^{2+}-activated K^+ channel activity after reconstitution into phospholipid vesicles. It distributes on two bands of 51 and 36 kDa after gel electrophoresis in SDS. The 36 kDa band is rapidly cleaved by trypsin and may be involved in Ca^{2+} stimulation of the channel. Phosphorylation from cAMP-dependent protein kinase strongly stimulates Ca^{2+}-activated K^+ channel activity and labels the 51 kDa band suggesting that this protein is involved in regulation of K^+ channel opening.

Ca^{2+} activation; K^+ channel; Calmodulin inhibitor; Calmodulin; Affinity chromatography; Phosphorylation; cyclic AMP dependence; Protein kinase

1. INTRODUCTION

The reabsorption of NaCl in the thick ascending limb of Henle's loop involves the integrated function of the loop-diuretic sensitive Na^+,K^+,Cl^- cotransport system and a K^+ channel in the luminal membrane together with the Na^+,K^+ pump and a net Cl^- conductance in the basolateral membrane [1,2]. The K^+ channel is stimulated by Ca^{2+} in physiological concentrations [3,4] and may thus be involved in regulation of transepithelial NaCl transport. As estimated on the basis of ion fluxes and a channel conductance of 135 pS [5], each cell in the TAL contains only 200–400 K^+ channels. This is a very low number relative to 1 million Na^+,K^+,Cl^- cotransport systems and 40 million Na^+,K^+ pumps per cell [6]. This very sparse distribution of the K^+ channel explains the problems encountered in attempts at purification and characterization of its protein.

The Ca^{2+}-activated K^+ channel can be solubilized by CHAPS and reconstituted into phospholipid vesicles with full preservation of its native properties [7]. In the reconstituted vesicles the K^+

Correspondence address: P.L. Jørgensen, Institute of Physiology, Aarhus University, 8000 Aarhus C, Denmark

Abbreviations: ATP, adenosine 5′-triphosphate; CHAPS, 3-((3-cholamidopropyl)dimethylammonio)-1-propanesulfonate; DTT, DL-dithiothreitol; metrizamide, 2-(3-acetamido-5-*N*-methylacetamido-2,4,6-triiodobenzamido)-2-deoxy-D-glucose; Mops, 3-(*N*-morpholino)-propanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; TAL, thick ascending limb of Henle's loop; TFP, trifluoperazine

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channel exposes cytoplasmic aspects to the medium thus allowing for examination of the effect of compounds normally present in the cell cytoplasm. Here we further examine the effects of calmodulin and phosphorylation from cAMP-dependent protein kinase [8,10]. After demonstrating a high-affinity effect of calmodulin on the K^+ channel, a method is developed for purification of the K^+ channel protein on a calmodulin affinity column. Phosphorylation and controlled tryptic digestion are used as tools for identifying the K^+ channel protein after gel electrophoresis.

2. MATERIALS AND METHODS

2.1. *Preparation of plasma membrane vesicles*

Luminal plasma membrane vesicles from pig kidney outer medulla were prepared by centrifugation on 12% (w/v) metrizamide as described [7].

2.2. *Affinity chromatography*

Luminal plasma membranes were resuspended to 8 mg protein per ml in 3.0 ml 250 mM sucrose, 50 mM KCl, 0.1 mM $CaCl_2$, 20 μM PMSF (Sigma), 20 mM Mops-Tris, pH 7.2, and solubilized by adding CHAPS in 400 μl buffer to a detergent:protein ratio of 5:1 (w/w). After centrifugation for 5 min at 100000 rpm in a Beckman airfuge, the supernatant was applied slowly (0.1 ml/min at 20°C) to an 8 ml calmodulin-Sepharose 4B (Pharmacia) column (1 × 12 cm). UV absorption was recorded at 279 nm. After passage of non-absorbed protein, the column was washed with the buffer above with 10 mM CHAPS at 0.2 ml/min for 60 min. To release specifically bound protein, the buffer was changed to 250 mM sucrose, 10 mM CHAPS, 50 mM KCl, 5 mM EGTA, 20 μM PMSF, 20 mM Mops-Tris, pH 7.2.

2.3. *Reconstitution and $^{86}Rb^+$ flux assay*

To assay for K^+ channel activity in the eluate from the affinity column, 200 μl of the fractions were mixed with 240 μl phospholipid solution in CHAPS and reconstituted on 1 × 30 cm Sephadex G-50 coarse column as in [7].

K^+ channel activity at controlled concentrations of free Ca^{2+} was assayed by measuring $^{86}Rb^+$ uptake against a large opposing K^+ gradient as before [7,9] except for minor modifications of the volumes. Inhibitors ($BaCl_2$, pimozide, Janssen Pharmaceutica; TFP, Sigma) and activators (calmodulin, Pharmacia) were added directly to the reaction medium in small volumes.

2.4. *Phosphorylation*

For phosphorylation [10] reconstituted vesicles were incubated for 2 min at 30°C in 10 mM Mops-Tris, pH 7.2, 3 mM $MgCl_2$, 0.5 mM Na_2ATP (Boehringer), 5 μmol cAMP (Merck) and 50 μg/ml cAMP-dependent protein kinase (Sigma). The $^{86}Rb^+$ flux assay was carried out immediately after phosphorylation. For autoradiography, ATP was replaced by 100 μM [γ-^{32}P]ATP (5 μCi/ml).

2.5. *SDS gel electrophoresis and protein determination*

Fractions containing the purified K^+ channel protein were concentrated by pressure filtration (Amicon YM 10) and resuspended in 10 mM CHAPS, 10 mM Mops-Tris, pH 7.2. For gel electrophoresis SDS and mercaptoethanol were added to 9 and 1% (w/v). Glycerol (10%) and bromphenol blue (0.01%) were added after boiling for 3 min and samples were run on a 5–15% linear gradient polyacrylamide gels according to Laemmli [11] and stained with Coomassie blue.

Protein was determined by the method of Lowry et al. [12] after precipitation using trichloroacetic acid and deoxycholate [13] with bovine serum albumin as standard.

3. RESULTS AND DISCUSSION

3.1. *Effect of calmodulin and calmodulin antagonists*

In the reconstituted vesicles the Ca^{2+}-activated K^+ channels are oriented with the cytoplasmic aspects facing outward [7]. Addition of calmodulin to the reconstituted vesicles doubled the Ca^{2+} stimulation of the K^+ channel with a $K_{0.5}$ of 0.1 nM (fig.1), whereas there was no effect of addition of calmodulin to the reconstituted vesicles in the absence of Ca^{2+} (not shown). Addition of calmodulin (1 μM) to the plasma membrane vesicles had no effect either (not shown), because the K^+ channel here exposes its extracellular aspects to the medium [7]. Calmodulin thus binds to the cytoplasmic surface of the Ca^{2+}-activated K^+ channel in the presence of Ca^{2+} with a very high affinity similar to that found for cAMP

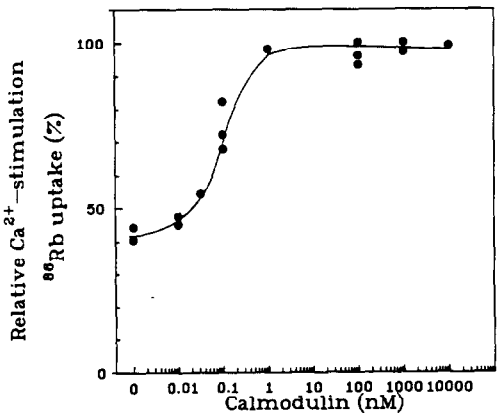

Fig.1. Effect of direct calmodulin addition on the Ca^{2+}-activated K^+ channel. Protein from luminal plasma membrane vesicles was solubilized and reconstituted into phospholipid vesicles as described in section 2. $^{86}Rb^+$ uptake into the vesicles was measured in the presence and absence of 1 μM free Ca^{2+} at increasing concentrations of calmodulin. The Ca^{2+}-dependent $^{86}Rb^+$ uptake was calculated for each concentration of calmodulin in % maximum $^{86}Rb^+$ uptake.

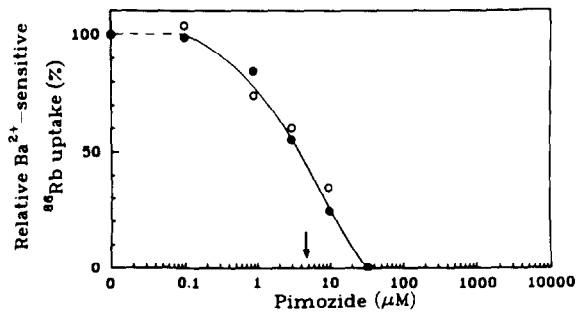

Fig.2. Effect of pimozide on the K^+ channel activity in plasma membrane vesicles (○) and reconstituted vesicles (●). The Ba^{2+}-sensitive $^{86}Rb^+$ uptake into the vesicles was measured after 10 min as before [7] with increasing concentrations of pimozide. The $K_{0.5}$ for the inhibition is indicated by an arrow. Pimozide was added from a stock solution in DMSO. The final concentration of DMSO was 2% and did not affect the $^{86}Rb^+$ uptake. 100% activity corresponds to 110 pmol ^{86}Rb/mg protein per min.

phosphodiesterase. Other enzymes bind calmodulin with 10–1000-times lower affinities [14].

In agreement with these results the potent calmodulin antagonist pimozide inhibited the K^+ channel with $K_{0.5}$ 5 μM in the reconstituted vesicles as well as in the plasma membrane vesicles (fig.2). The inhibition in the plasma membrane vesicles is explained by the high permeability of the hydrophobic pimozide. Earlier experiments have shown that the K^+ channel is also inhibited by the calmodulin antagonists calmidazolium and TFP with $K_{0.5}$ of 15 and 65 μM, respectively [7]. These values are in the range previously observed for other systems involving calmodulin in the regulation [15–18].

In the reconstituted vesicles calmodulin acts directly on the Ca^{2+}-activated K^+ channel, since it is unlikely that the calmodulin effect is mediated via phosphorylation by a calmodulin-dependent protein kinase [19]. Direct calmodulin activation of Ca^{2+}-activated K^+ channels was previously shown in plasma membrane vesicles from adipocytes [20], erythrocytes [21], and in plasma membrane Ca^{2+}-ATPase, where Ca^{2+} and calmodulin are thought to bind to a regulatory site of the protein [23].

3.2. *Affinity chromatography*

High affinity for calmodulin in the presence of Ca^{2+} suggested that the K^+ channel protein could be purified on a calmodulin affinity column as has been done for the plasma membrane Ca^{2+}-ATPase [22].

Fig.3 shows a representative fractionation from a series of about 20 experiments using a calmodulin affinity column. In the presence of Ca^{2+} 99.3% of the soluble protein passed through the column in peak 1 and only 0.7% was retained and subsequently eluted in peak 2 after addition of EGTA. The K^+ channel activity in peak 2 was several fold higher than in peak 1, and estimation of the specific K^+ channel activity (pmol $^{86}Rb^+$/min per mg protein) showed that the K^+ channel protein was highly concentrated in peak 2.

The Ca^{2+}-sensitive $^{86}Rb^+$ uptake into the phospholipid vesicles reconstituted from peak 2 was inhibited by 0.5 mM TFP. At variance with previous data of $^{86}Rb^+$ fluxes in vesicles reconstituted with luminal membrane protein [7], 5 mM $BaCl_2$ did not inhibit the $^{86}Rb^+$ uptake in vesicles containing the purified protein. This suggests that the channel protein is altered or partially

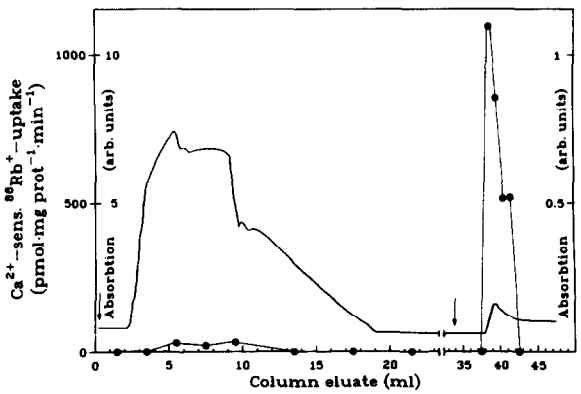

Fig.3. Fractionation of soluble luminal membrane protein by affinity chromatography. Addition of sample and change from buffer containing 100 μM $CaCl_2$ to buffer containing 5 mM EGTA are indicated by arrows. Protein from the fractions was reconstituted into phospholipid vesicles and the Ca^{2+}-sensitive $^{86}Rb^+$ uptake was measured as the difference in $^{86}Rb^+$ uptake in the presence and absence of 1 μM free Ca^{2+} (●). The unbroken line shows the UV absorption with different scales for peak 1 (left) and peak 2 (right).

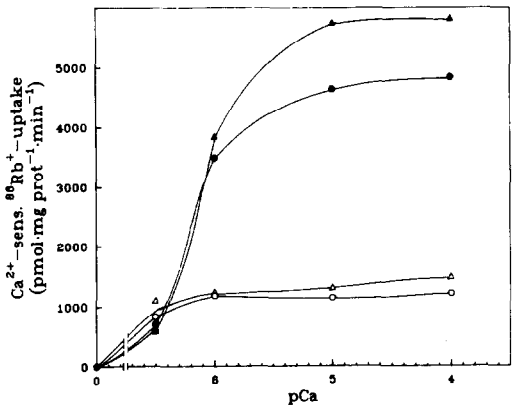

Fig.4. $^{86}Rb^+$ uptake after phosphorylation of purified K^+ channel protein in reconstituted vesicles (▲,●) compared to uptake without phosphorylation (△,○). Protein from peak 2 was reconstituted into phospholipid vesicles and the $^{86}Rb^+$ uptake into the vesicles was measured at different concentrations of free Ca^{2+}. The K^+ channel activity was measured in the presence (▲, △) and absence (●,○) of 0.1 μM calmodulin.

denatured during chromatography. Earlier reports on Ba^{2+} effects on Ca^{2+}-activated K^+ channels have been contradictory [24]. For the present purpose activation of the reconstituted K^+ channel by Ca^{2+} with $K_{0.5} \sim 3 \times 10^{-7}$ M (cf. fig.4 and [7]) is considered an adequate criterion for identification of the K^+ channel protein.

3.3. *Phosphorylation of purified K^+ channel protein*

The experiment in fig.4 examines the effect of phosphorylation by cAMP-dependent protein kinase of the purified protein from peak 2 after reconstitution into phospholipid vesicles. It is seen that phosphorylation stimulated the maximal Ca^{2+}-activated K^+ channel activity 3–4-fold. This is similar to the effect of phosphorylation on the Ca^{2+}-activated K^+ channel activity in vesicles reconstituted with luminal membrane protein (not shown). The calmodulin stimulation of the Ca^{2+}-sensitive $^{86}Rb^+$ uptake in vesicles reconstituted with purified protein was only 10–20% (fig.4), and less than in vesicles reconstituted with luminal membrane protein (138%, fig.1). The K^+ channel has obviously lost some of its sensitivity to calmodulin during affinity chromatography as has been observed for Ca^{2+}-ATPase from the plasma membrane [22]. In contrast, the K^+ channel has been purified in a functional state regarding the response to Ca^{2+} and to phosphorylation by cAMP-dependent protein kinase.

Earlier phosphorylation experiments showed different effects on Ca^{2+}-activated K^+ channels. Ca^{2+} activation of the K^+ channel from heart sarcolemma could not take place unless the K^+ channel was in a phosphorylated state [10]. Phosphorylation of the K^+ channel from snail neurones [25] and snail helix ganglia [8] made the K^+ channel more sensitive to Ca^{2+} but did not change the maximal K^+ channel activity.

3.4. *SDS gel electrophoresis*

Gel electrophoresis in SDS showed a multitude of bands in the sample applied to the affinity column (fig.5, lane A) and in peak 1 (fig.5, lane B); while protein from peak 2 had only two major bands with molecular masses of 51 and 36 kDa (fig.5, lanes C,D). The migration of these two bands was independent of whether mercaptoethanol was added to the sample. Autoradiography after cAMP-dependent protein kinase phosphorylation of the purified soluble protein

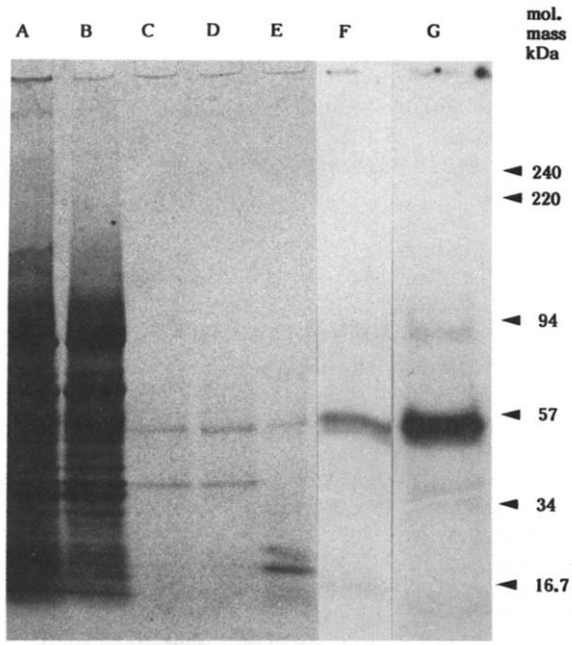

Fig.5. Separation of purified K^+ channel protein and luminal plasma membrane vesicles by gel electrophoresis in SDS. The gel was loaded with 220 μg soluble protein from luminal plasma membrane vesicles (A), 195 μg protein from peak 1 (B) and 20 μg protein from peak 2 (C + D). 20 μg purified protein was incubated for 5 min at 20°C with trypsin (10 μg/ml) as in [7] and then applied to the gel (E). For autoradiography (F + G) 20 μg protein was phosphorylated as described in section 2 before gel electrophoresis. The gel was dried and exposed to a Kodak X-ray film for 7 (F) or 15 (G) days at −80°C.

from [γ-^{32}P]ATP (fig.5) showed ^{32}P labelling of the 51 kDa band. The staining from ^{32}P was distributed on a broad band (fig.5, lane G), which appeared to be a doublet (fig.5, lane F). This suggests contribution from ^{32}P bound to proteins that were invisible after Coomassie blue staining. Since the K^+ channel activity was stimulated 3–4-fold by this phosphorylation (fig.4), our data suggest that the 51 kDa protein forms a part of the channel and that it is involved in control of K^+ channel opening.

Gel electrophoresis after tryptic digestion of the purified channel protein showed that the 36 kDa protein was cleaved much faster than the 51 kDa protein (fig.5, lane E). Previous data showed that the reconstituted K^+ channel is highly sensitive to tryptic digestion, and brief digestion activated the K^+ channel in the absence of Ca^{2+} to the level of activity with saturating concentrations of Ca^{2+} [7]. The result therefore provides indirect evidence for involvement of the 36 kDa protein in opening and closing the K^+ channel in response to Ca^{2+} binding.

Two other laboratories have suggested that a series of proteins with molecular masses in the same range could be involved in formation of K^+ channels. Wen et al. [10] proposed from phosphorylation experiments that one or more proteins of 55, 45, 31, 28 and 14 kDa could be part of the Ca^{2+}-activated K^+ channel from heart sarcolemma. Covalent labelling with apamin identified proteins of 86, 59, 30 and 23 kDa as presumptive components of the apamin sensitive Ca^{2+}-activated K^+ channel in rat neurones [26]. In contrast to this, a complex of 280 kDa isolated by affinity chromatography on quinine and bumethanide columns has been proposed to form both a K^+ channel and a Cl^- channel [27].

In conclusion, we have isolated a small fraction of the protein in luminal plasma membranes in TAL with high Ca^{2+}-activated K^+ channel activity and two predominant protein bands of 51 and 36 kDa. The yield corresponds reasonably well to the amounts of K^+ channel protein that can be estimated to be present in TAL relative to the amount of Na^+,K^+-ATPase (6). The phosphorylation and tryptic digestion experiments provide evidence for involvement of the 51 and 36 kDa proteins in regulation of Ca^{2+}-activated K^+ channel activity. It should however be kept in mind that K^+ channels are very fast transporters. Minute amounts of protein, that are not visible in acrylamide gels, could therefore give rise to significant K^+ channel activities in reconstituted vesicles.

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