Biochimica et Biophysica Acta, 1067 (1991) 38–42

ADONIS 0005273691002932

BBAMEM 75304

Reconstitution and partial purification of an amiloride-sensitive, cation channel from rabbit kidney

Gary V. Desir

Yale University School of Medicine, Department of Medicine, New Haven, CT (U.S.A.)

(Received 8 March 1991)

Key words: Potassium ion channel; Cation channel; Reconstitution; Amiloride; Protein purification; Proteolipid; (Rabbit kidney)

The aim of the present study was to reconstitute and purify an epithelial potassium channel from rabbit kidney. Renal brush border membrane vesicles (BBMV) were found to contain a potassium conductance which was inhibited by amiloride, 5-(*N*-methyl-*N*-isobutyl)amiloride (MIA) and by barium. Membrane vesicle proteins were solubilized and reconstituted in proteoliposomes. Channel activity was assayed using Acridine orange and the voltage sensitive dye, 3,3'-diethylthiadicarbocyanine iodide ($DiSC_2(5)$). Both methods yielded similar results which indicated the presence of an amiloride-sensitive, cation channel in the proteoliposomes. This channel was more permeable to K than to Na and its activity was increased in reconstituted proteoliposomes as compared to native brush border membranes. We conclude that rabbit BBMV possess an amiloride sensitive cation channel. Channel activity was successfully reconstituted in proteoliposomes and the protein was partially purified during reconstitution.

Introduction

Several types of potassium (K) channels have been detected in the mammalian kidney. The basolateral membrane of the proximal tubule has a barium-sensitive, voltage-gated K channel which recycles K (taken up by the sodium-potassium ATPase pump) across the membrane [1]. Two types of K channels are found in the luminal membrane of the proximal tubule. The first is a small conductance (42 pS) channel which is blocked by barium and apamin [2]. The other is a large conductance calcium and voltage-activated channel. It has been described in cultured cells [3,4] and in luminal membrane vesicles [5]. Calcium and voltage-gated K channels have also been detected in the medullary thick ascending limb [6], and in the cortical collecting tubule [7]. A 32 pS calcium and serotonin-activated *inward rectifier* is found in renal epitheliod cells (MDCK) [8].

Although the kinetic and pharmacologic properties of several of these channels are under intense investigation, little is known about their structural properties. There is evidence suggesting that a calcium-activated K channel has been reconstituted and partially purified on calmodulin affinity columns from pig kidney [9]. Recent data indicate that some renal K channels may belong to the *Shaker* gene family [10]. In these studies, the polymerase chain reaction was used to amplify five clones from rabbit kidney cDNA, which had strong sequence similarities with *Shaker*. Takumi et al., have used the method of expression cloning to identify a 17 kDa membrane protein which may represent a K-selective channel [11]. In the rat kidney, this protein is exclusively located on the luminal membrane of the proximal tubule [12]. In this report we describe the reconstitution and partial purification of an amiloride-sensitive cation channel from luminal membranes of rabbit kidney.

A preliminary summary of this work has been previously published in abstract form [24].

Abbreviations: MIA, 5-(*N*-methyl-*N*-isobutyl)amiloride; AO, Acridine orange; $DiSC_2(5)$, 3,3-diethylthiadicarbocyanine iodide; BBMV, microvillus membrane vesicles; TMACl, tetramethylammonium chloride; KCl, potassium chloride; NaCl, sodium chloride; CHAPS, 3-[(3-cholamidopropy)dimethylammonio]-1-propanesulfonate.

Correspondence: G.V. Desir, Yale University School of Medicine, Department of Medicine, Section of Nephrology, 2073 LMP, 333 Cedar street, New Haven, CT 06510, U.S.A.

Experimental procedures

Membrane preparation

Male New Zealand white rabbits (2–3 kg) were killed by intravenous pentobarbital. Microvillus membrane vesicles (BBMV) were prepared from the renal cortices by magnesium aggregation and differential centrifugation as described previously [13]. The homogenization media contained 200 mM mannitol, 41 mM K^+, 80 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes) at pH 7.5. The final membrane pellets were suspended to a protein concentration of 30–50 mg/ml as determined by the Peterson modification [14] of the Lowry assay [15] using bovine serum albumin as the standard. Membrane vesicles were 10–15-fold enriched in luminal membrane markers (γGTP). Membranes were stored at −70 °C and used within one month of preparation.

Methods for detecting channel activity in vesicles

Fluorescence quenching of Acridine orange. To detect cation conductances in intact vesicles, we used a strategy similar to that described by Reenstra et al. [16]. It relied on the ability of Acridine orange, a fluorescent, pH-sensitive dye, to detect small changes in intravesicular pH. In brief, vesicles were loaded with 2 mM Hepes, pH 7.5 and either 200 mM KCl or 200 mM TMACl. A 200-fold outward cation gradient was imposed by diluting 10 μl of vesicles in 2 ml of buffer containing 200 mM choline chloride, 2 mM Hepes (pH 7.5), 6 μM Acridine orange at 25 °C. If the appropriate conductive pathway is present in the vesicle, the cation of interest, driven its chemical gradient (200-fold in > out), will diffuse out of the vesicle and generate an inside-negative membrane potential. This membrane potential will, in turn, drive protons into the vesicles and result in intravesicular acidification and acridine orange fluorescence quenching. Changes in fluorescence were measured using a Perkin-Elmer MPF-44A spectrofluorometer (excitation 493 nm, emission 520 nm, slit widths were both set at 5 nm). Fluorescence was recorded in arbitrary units on a Perkin-Elmer strip chart recorder. Ionophores were added from ethanol stock to the buffer and the ethanol concentration never exceeded 0.15%.

Fluorescence quenching a voltage-sensitive dye. In some experiments a voltage-sensitive dye, 3,3'-diethylthiadicarbocyanine iodide ($DiSC_2(5)$), was used to confirm that cation efflux was occurring via a conductive pathway. Vesicles were loaded with 2 mM Hepes (pH 7.5) and the chloride salts of various cations. They were diluted in 2 ml of buffer containing 6 μM $DiSC_2(5)$ at 25 °C. If a conductive pathway were present in the vesicle for the cation of interest, it would diffuse out of the vesicle down its chemical gradient (200-fold in > out), and would generate an inside-negative membrane potential. This membrane potential would in turn drive the intravesicular accumulation of the cationic dye $DiSC_2(5)$ with a resulting decrease in its fluorescence. Changes in fluorescence were measured (excitation 643 nm, emission 666 nm, slit widths were both set at 10 nm) and recorded as described above. Ionophores were added from ethanol stock to the buffer and the ethanol concentration never exceeded 0.15%.

Solubilization and reconstitution protocols

BBMV (30 mg/protein/ml) were extracted with a mixture of chloroform/methanol at 22 °C with constant stirring for 1 h. The final ratio of water/methanol/chloroform was 1:3.3:6.3 (v/v). The mixture was transferred to a test tube and left undisturbed for 3 h. The aqueous phase collected to the top. It was carefully removed with a Pasteur pipette and discarded. The organic phase was filtered using a 22 μm filter. To form proteoliposomes, the chloroform was evaporated under nitrogen and the mixture was dialyzed against 4 liters of buffer for 24 h at 4 °C. The addition of exogenous lipid was not necessary since the mixture already contained brush border membrane lipids. Proteoliposomes were loaded with the cation of interest by changing the ionic composition of the dialysis buffer. Following dialysis, liposomes were collected by centrifugation ($100\,000 \times g$ for 2 h), their protein content was measured and was found to represent 0.14% of the starting BBMV proteins. Channel activity was assayed as described above. The reconstituted proteins were separated by gel electrophoresis on a 10% SDS-polyacrylamide gel according to Laemmli [17] and visualized by silver staining.

Materials

Acridine orange was obtained form Eastman Kodak. $DiSC_2(5)$ was obtained for Molecular Probes Inc. Valinomycin was obtained from Sigma. Amiloride and MIA were gifts of Merck Sharp and Dome. Hepes was obtained from Sigma or United States Biochemical Corp. and other compounds were of at least reagent-grade quality from Sigma, Fisher, or Aldrich. Synthetic phosphatidylcholine and phosphatidylethanolamine were obtained from Avanti Polar Lipids Inc.

Results

Detection of an amiloride-sensitive K channel in intact BBMV

Acridine orange (AO) fluorescence quenching was used to test whether BBMV contained a K channel (Fig. 1). In the absence of a K gradient, AO fluorescence was rapidly quenched (1 florescence unit) by the addition of BBMV and then remained unchanged. This initial rapid quench was attributed to mixing and dye

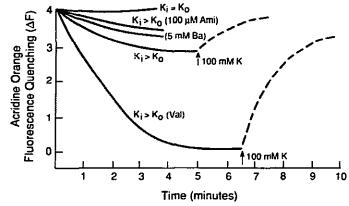

Fig. 1. Detection of an amiloride-sensitive cation channel in BBMV. The protocol for measuring conductive K efflux in intact BBMV using acridine orange is described in Experimental procedures. Vesicles were loaded with 2 mM Hepes (pH 7.5) and 200 mM KCl. A 200-fold outward K gradient was imposed by diluting 10 μl of vesicles in 2 ml of buffer containing 200 mM choline chloride, 2 mM Hepes (pH 7.5). 6 μM Acridine orange at 25° C. 500 μg of BBMV proteins were used to generate each curve. Valinomycin was added where indicated to a concentration of 10 μM. A representative experiment is shown in this figure. Experiments were repeated thrice.

binding artifacts and was subtracted from all subsequent measurements. The imposition of a 200-fold outward K gradient in the presence of the K ionophore, valinomycin, resulted in large decrease in AO fluorescence which reached a maximum value at 4 min (Fig. 1). AO quenching could be reversed by the addition of 100 mM K indicating that it represented intravesicular acidification driven by the inside negative membrane potential and not merely non-specific binding of the dye. In the absence of valinomycin, a 200-fold outward K gradient caused a smaller decrease in AO fluorescence (25% of maximum). K-efflux driven AO quenching was maximum at 4 min and could be reversed by 100 mM external K. Efflux was inhibited by 100 μM amiloride, and by 5 mM barium. 10 μM MIA inhibited K efflux as well as 100 μM amiloride (Data not shown).

Reconstitution and partial purification of the cation channel

We then attempted to reconstitute channel activity in liposomes. Various reconstitution protocols were tested. Several detergents, including Triton X-100, octyl glucoside, Nonidet P-40, 3-[(3-cholamidopropy)dimethylammonio]-1-propanesulfonate (CHAPS) and cholate, were used to solubilize membrane proteins. The protocol which gave the best and most reproducible results was described above and involved the extraction membrane proteins with a mixture of chloroform/methanol and the formation of proteoliposomes by dialysis. Reconstitution of channel activity in proteoliposomes was monitored using acridine orange. A 200-fold outward K gradient significantly quenched Acridine orange fluorescence (Fig. 2A). A similar Na gradient produced lesser quenching of Acridine orange fluorescence (20% of K values). Fluorescence quenching was not observed in the absence of a K or Na gradient or in the presence of a 200-fold outward TMA gradient indicating that the reconstituted proteoliposomes possess a large conductance for K and a lesser one for Na and are impermeable to TMA. To further characterize these conductances, their sensitivity to amiloride and barium was tested. K efflux was inhibited by both amiloride and barium (Fig. 2A). 10 μM MIA inhibited K efflux as well as 100 μM amiloride. Na efflux was similarly inhibited (Data not shown).

To confirm that cation efflux from the proteoliposomes was conductive, efflux driven changes in membrane potential were measured using the cationic, voltage-sensitive dye, $DiSC_2(5)$. As shown in Fig. 2B, a 200-fold outward K gradient quenched dye fluorescence. Amiloride (100 μM) and barium (5 mM) prevented dye quenching. To control for non-specific effects of amiloride and barium on dye behavior, valinomycin was added in the presence of a K gradient. The addition of valinomycin (200 outward K gradient) generated an inside negative-membrane potential in the presence of either amiloride or barium, indicating that the inhibitory effects of these drugs was not the result of their non-specific interaction with the dye. Finally, dye quenching was not observed in the presence of a

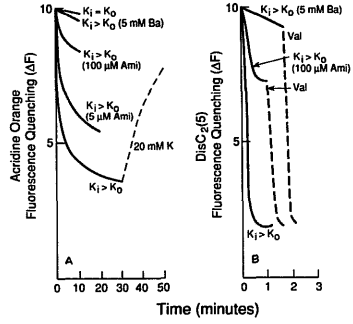

Fig. 2. Reconstitution of an amiloride-sensitive cation channel from BBMV. The reconstitution protocol is described in Experimental procedures. 10 μg of reconstituted protein were used to generate each curve. Vesicles were loaded with 2 mM Hepes at pH 7.5 and 200 mM KCl. A 200-fold outward cation gradient was imposed by diluting 10 μl of vesicles in 2 ml of buffer containing 200 mM choline chloride, 2 mM Hepes (pH 7.5) at 25° C and either 6 μM Acridine orange (panel A) or 6 μM $DiSC_2(5)$ (panel B). Valinomycin was added where indicated to a concentration of 10 μM. A representative experiment is shown in this figure. Experiments were repeated thrice.

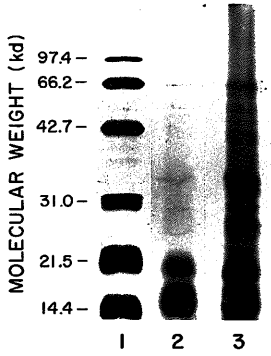

Fig. 3. Gel electrophoresis of reconstituted brush border membrane vesicle proteins. Proteins were separated on a sodium dodecyl sulfate containing 10% polyacrylamide gel using a discontinuous buffer system according to Laemmli. Proteins were visualized by silver staining. Lane 1: Molecular weight standards; Lane 2: 1 μg of reconstituted brush border membrane protein; Lane 3: 5 μg of reconstituted protein.

200-fold outward K gradient when liposomes were made of synthetic, commercially available lipids (phosphatidylcholine and phosphatidylethanolamine, 2 : 1).

The specific acitivity of the K conductance in intact BBMV was 1 FU/mg protein per min (Fig. 1). In contrast, since the reconstituted proteoliposomes only contained 0.14% of the starting membrane protein, their specific activity was 300 FU/mg protein per min (Fig. 2A). It is therefore possible that a 300-fold purification was achieved during reconstitution of the cation channel in proteoliposomes. Partially purified reconstituted proteins were separated by gel electrophoresis and visualized by silver staining (Fig. 3). Only two major protein bands (molecular mass of 15 and 20 kDa) are visualized in the partially purified preparation (Lane 2, 1 μg of protein). Several minor protein bands can also be detected if 5 μg of purified proteins are loaded on the gel (Lane 3).

Discussion

In the preceding studies, we have shown that rabbit luminal membrane vesicles prepared from rabbit kidney cortex (BBMV) contain a K conductance. It is inhibited by amiloride, the amiloride analog MIA and by barium. We could not evaluate whether this channel is also permeable to Na since BBMV contain an active amiloride-sensitive, electroneutral Na-H exchanger. It is unlikely that we are measuring K efflux through sodium channels since the epithelial sodium channel is sensitive to neither MIA nor barium. Our results cannot be explained by postulating the existence of a potassium/hydrogen ion exchanger in these vesicles since such an exchanger, described in the ileum, was found to be amiloride insensitive [18]. K channels have not previously been reported to be amiloride sensitive. However, recent studies suggest that the Maxi K channel present in rabbit proximal tubule is inhibited by the amiloride analog MIA (see Ref. 5).

Reconstituted proteoliposomes, possess electrogenic K and Na conductances which are amiloride and barium sensitive. It is possible that these fluxes are mediated by different pathways. It is more likely that one is dealing with a single pathaway since both fluxes are inhibited by the same drugs, namely amiloride, MIA and barium. It appears that this channel is more permeable to K than to Na. However, the methods used in these studies are not quantitative and do not allow us to accurately estimate the permeability ratio of K versus Na. A more precise characterization of the kinetic properties of the purified channels could be carried out using the planar lipid bilaryer technique. This conductance does not represent nonspecific flux through the lipid bilayer since it could not be detected in liposomes consisting only of synthetic lipids. The specific activity of the reconstituted vesicles appeared to be significantly higher than that of native vesicles (300 FU vs. 1 FU). However, the methods used to estimate channel activity are not quantitative and may underestimate the specific activity of native vesicles.

Several reconstitution protocols were tested and the best results were obtained by solubilizing membrane proteins with organic solvents (chloroform/methanol). This method only solubilizes a few proteins which tend to be hydrophobic, integral membrane proteins. In our studies, the organic phase contained less than 1% of the starting membrane protein. Although, several minor components could be detected, only a few major protein bands were visualized in partially purified protein preparations. We do not yet know if reconstitution of channel activity can be achieved using one of the 2 major protein bands (15 or 20 kDa). It is noteworthy that these two proteins are similar in size to the rat kidney protein which was cloned by Takumi et al. (1988) [11]. This protein is located in the luminal membrane of the proximal and is thought to represent a K-selective channel.

The reconstituted protein can be classified as a proteolipid by virtue of its solubility in organic solvents. In several tissues, proteolipids have been reported to participate in membrane transport. For instance, proteolipids obtained from bovine kidney have been shown to form cation-selective channels in planar lipid bilayers [19]. These channels are at least 3 times more permeable to potassium than to sodium. Their conduc-

tances range from 10 to 100 pS depending on the concentration of added protein to the planar lipid bilayer. The inhibitor sensitivity of these channels was not tested. More recently, proteolipids from renal BBMV have been shown to bind phosphate and are postulated to play a role in the reabsorption of phosphate in the proximal tubule [20]. Finally, the proteolipid phospholamban (molecular mass 5–6 kDa), a major membrane protein of the heart [21,22], is phosphorylated in response to β-adrenergic stimulation and has been shown to form Ca^{2+}-selective channel in planar lipid bilayers [23].

In summary, an amiloride-sensitive, barium-sensitive, cation channel was detected in brush border membrane vesicles prepared from rabbit kidney. Channel activity was reconstituted in proteoliposomes. The reconstituted channel was functionally similar to that present in native membranes in terms of inhibitor sensitivity. It was relatively more permeable to K than to Na. Furthermore, the channel protein was partially purified during reconstitution and can be classified as a proteolipid since it is soluble in organic solvents. It is hoped that the reconstitution protocol described in this report will provide a useful tool for further study of the this channel.

Acknowledgements

I am grateful to Peter S. Aronson for his valuable suggestions. This work was supported by a grant from the Robert Wood Johnson Foundation.

References

1 Gogelein, H. and Greger, R. (1984) Pflügers Arch. 401, 424–426.
2 Merot, J., Bidet, M., Le Maout, S., Tauc, M. and Poujeol, P. (1989) Biochim. Biophys. Acta 978, 134–144.
3 Merot, J., Bidet, M., Le Maout, S., Tauc, M. and Poujeol, P. (1989) Biochim. Biophys. Acta 978, 134–144.
4 Kolb, H.A., Brown, C.D.A. and Murer, H.J. (1986) J. Membr. Biol. 92, 207–215.
5 Zweifach, A, Aronson P.S., Giebisch, G. and Desir, G.V. (1988) FASEB J. 2, A750, in Press. Am. J. Physiol.
6 Guggino, S.E., Suarez-Isla, B.A., Gugino, W.B., Green, N. and Sacktor, B. (1985) Biophys. J. 47, 387a.
7 Hunter, M., Lopes, A.G., Boulpaep, E.L. and Giebisch, G. (1984) Proc. Natl. Acad. Sci. USA 81, 4237–4239.
8 Fridrich, F. Paulmichl, H.A. and Lang, F. (1988) J. Membr. Biol. 106, 149–155.
9 Klaerke, D.A., Karlish, S.J.D. and Jørgensen, P.L. (1987) J. Membr. Biol. 95, 105–112.
10 Desir, G.V., Hamlin, A.H., Reilley, R.F. Hildebrandt, F. and Igarashi, P. (1991) J. Am. Soc. Nephrol. 1, 739A.
11 Takumi, T., Ohhkubo, H. and Nakanishi, S. (1988) Science 243, 1042–1045.
12 Sugimoto, T., Tanabe, Y., Shigemoto, R., Iwai, M., Takumi, T., Ohkubo, H. and Nakanishi, S. (1990) J. Membr. Biol. 113, 39–47.
13 Aronson, P.S. (1978) J. Membr. Biol. 42, 81–98.
14 Peterson, G.L. (1983) Methods Enzymol. 91, 95–119.
15 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
16 Reenstra, W.W., Warnock, D.G., Yee, V.Y. and Forte, J.G. (1981) J. Biol. Chem. 256, 11663–11666.
17 Laemmli, U.K. (1970) Nature 227, 680–685.
18 Binder, H.J. and Murer, H. (1986) J. Membr. Biol. 91, 77–84.
19 Tosteson, M.T., and Sapirstein, V.S. (1981) J. Membr. Biol. 63, 77–84.
20 Vachon, V., Delisle, M.C., Gicquaud, C. and Beliveau, R. (1990) Kidney Int. 37, 462A.
21 Chiesi, M., Gasser, J. and Carafoli, E. (1983) FEBS, Lett. 160, 61–66.
22 Boyot, P., Luu. B., Jones, L.R. and Trifilieff, E. (1989) Arch. Biochim. Biophys. 269, 639–645.
23 Kovacs, R.J., Nelson, M.T., Simmerman, H.K.B. and Jones, L. (1989) J. Biol. Chem. 263, 18364–18368.
24 Desir, G.V. (1989) Clin. Res. 37, 488A.