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Quantification and distribution of big conductance Ca^{2+}-activated K^+ channels in kidney epithelia

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Abstract

Big conductance Ca^{2+} activated K^+ channels (BK channels) is an abundant channel present in almost all kind of tissue. The accurate quantity and especially the precise distribution of this channel in kidney epithelia are, however, still debated. The aim of the present study has therefore been to examine the presence of BK channels in kidney epithelia and determine the actual number and distribution of these channels. For this purpose, a selective peptidyl ligand for BK channels called iberiotoxin or the radiolabeled double mutant analog ^{125}I-IbTX-D19Y/Y36F has been employed. The presence of BK channels were determined by a isotope flux assay where up to 44% of the total K^+ channel activity could be inhibited by iberiotoxin indicating that BK channels are widely present in kidney epithelia. Consistent with these functional studies, ^{125}I-IbTX-D19Y/Y36F binds to membrane vesicles from outer cortex, outer medulla and inner medulla with B_{max} values (in fmol/mg protein) of 6.8, 2.6 and 21.4, respectively. These studies were performed applying rabbit kidney epithelia tissue. The distinct distribution of BK channels in both rabbit and rat kidney epithelia was confirmed by autoradiography and immunohistochemical studies. In cortical collecting ducts, BK channels were exclusively located in principal cells while no channels could be found in intercalated cells. The abundant and distinct distribution in kidney epithelia talks in favor for BK channels being important contributors in maintaining salt and water homeostasis.

Keywords: Transepithelial transport; Binding studies; Immunohistochemistry; BK channels

1. Introduction

In mammalians, salt concentrations are maintained within a very narrow range despite large variations in daily dietary intake. The kidneys play an essential role in obtaining this homeostasis by paracellular and transcellular re-absorption of NaCl across specialized epithelia cells [1,2]. The transport is mediated by the coordinated function of Na^+/K^+-ATPases, $Na^+/2Cl^-/K^+$ co-transporters and various kind of ion channels [3–5]. Different studies have revealed that K^+ channels are essential in this transport, and the existence of a wide range of channels has been reported so far [6–13] but it is still debated which type of K^+ channels plays the most important role in the trans-epithelial transport. Firm evidence has been reported for the ROMK (Kir1.1 or KCNJ1) channel in both humans and mice. ROMK knock-out mice have an nearly complete lethal phenotype, with only 5% of littermates surviving more than 3 weeks. Adult knock out mice suffer from a severe phenotype partly as a consequence of reduced NaCl absorption [14]. Also in humans, mutations in the ROMK channel display a serious

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phenotype, resulting in Type II Bartter's syndrome manifested by reduced kidney function [15].

Another candidate for an essential role in trans-epithelial transport could be the big conductance Ca^{2+}-activated K^+ channel (BK channel or maxi K channel) which has the highest single channel conductance among the K^+ channels characterized until now, and which is known to be strictly regulated by physiological concentrations of intracellular Ca^{2+}, cAMP, phosphorylation/dephosphorylation and sheer stress [16–22]. There is good evidence that this channel is involved in flow dependent potassium secretion in the kidney [23,24]. A functional role for BK channels in kidney is further emphasized by experiments performed knock out mice where the BK-β1 subunit to the BK channel has been deleted. These animals failed to increase potassium secretion in the distal nephrons as a response to volume expansion and increased flow rate [25]. Despite these indications of a functional role for BK channels in kidney epithelium, there has been some disagreement about the presence, distribution and amount of BK channels in the kidney. Some experiments has reported the existence of BK channels in kidney cortex (proximal tubule and cortical collecting duct), and kidney outer medulla (Thick Ascending Limb) while others have not observed BK channels at all in the kidney [4,13,18,26–31].

Different peptidyl and non-peptidyl agents such as Ba^{2+}, TEA, charybdotoxin (ChTX) and iberiotoxin (IbTX) have been valuable in the characterization of BK channels [32–35]. All of these agents are able to prevent flux through the channel when applied to the extracellular face. This blocking event is obtained by a physical occlusion of the channel pore. IbTX is the only agent considered specific for BK channels.

Attempts to perform IbTX binding were originally hampered by the fact that native IbTX loses its biological activity upon iodination [36]. However, a double mutant of IbTX called IbTX-D19Y/Y36F has been constructed and iodinated with full preservation of the affinity and specificity for BK channels [36]. This recombinant toxin has a low dissociation rate and has turned out to be a useful ligand in quantification of BK channels in both excitatory tissue and colon epithelium [36–39].

In the present study, we have performed flux assays and binding experiments with IbTX and the double mutant IbTX-D19Y/Y36F, respectively, and for the first time, it has been possible to obtain reliable information about the exact number and localization of BK channels in kidney epithelium. The distribution of the channels was confirmed by autoradiography and immunohistochemical studies. Here, it was demonstrated that BK channels were exclusively located in principal cells known to be involved in sodium and water re-absorption. In contrast, no channels could be found in neighboring intercalated cells, which are believed to participate in secretion of protons and bicarbonate.

2. Experimental procedures

2.1. Materials

Trizma (Tris base), MOPS, EGTA, BSA, SUCROSE and polyethylenimine were obtained from Sigma; KCl, NaCl, $BaCl_2$ and $MgCl_2$ were from Merck; Digitonin was from Serva; Tubes from InterMed; Filters were from Advantec; Ouabain and Dowex beads (50 W × 8) were from Fluka. The Dowex beads were converted to the Tris form by 16–24 incubation with Tris base (suspension pH > 10) and subsequent repetitive washing with distilled water until the suspension pH dropped to 7.0–7.4. $^{86}RbCl$ (0.5–12 mci/mg Rb^+) was purchased from Amersham, Purification and iodination of ^{125}I-IbTX-D19Y/Y36F was done as previously described [36].

2.2. Tissue preparation

New Zealand white rabbits (female, approximately 2.5 kg) maintained on a standard commercial diet (0.13% Na^+ and 0.8% K^+) were killed by cervical dislocation and bled. The kidneys were immediately removed and placed on a 150-mm ice cooled Petri dish and outer cortex, inner cortex, outer medulla and inner medulla were separated with a scalpel. For a single kidney, this resulted in approximately 1.25 g of outer cortex, 1.40 g of inner cortex, 0.90 g of outer medulla and 0.50 g of inner medulla. The four different kinds of kidney tissue were placed in liquid nitrogen and subsequent stored at −80 °C.

2.3. Vesicle preparation

Approximately 1 g of kidney outer cortex, inner cortex, outer medulla or kidney inner medulla were homogenized either with 10 ml 250 mM sucrose, 50 mM NaCl, 10 mM MOPS, 1 mM EGTA Tris–HCl pH 7.2 (for binding experiments) or with 250 mM sucrose, 50 mM KCl, 10 mM MOPS, 1 mM EGTA Tris–HCl pH 7.2 (for flux assays) with 5 strokes at 1000 rpm in a glass/teflon homogenizer (Braun-Melsungen) at 0 °C. The homogenate was subjected to low-speed centrifugation (Sorvall SS-34) 6300×*g* for 15 min at 4 °C. The supernatant containing membrane fractions was decanted and saved at 0 °C while the pellet was resuspended in 10 ml 250 mM sucrose, 50 mM NaCl, 10 mM MOPS, 1 mM EGTA Tris–HCl pH 7.2 (for binding experiments) or in 250 mM sucrose, 50 mM KCl, 10 mM MOPS, 1 mM EGTA Tris–HCl pH 7.2 (for flux assays).

The resuspended pellet was subjected to another low-speed centrifugation (Sorvall SS-34) 6300×*g* for 15 min at 4 °C. The two supernatants were mixed and subjected to a high-speed centrifugation (Sorvall SS-34) 41700×*g* for 35 min at 4 °C. The supernatant was discarded and the pellet resuspended in 20 mM Tris pH 7.2 (for binding experiments) or in 250 mM sucrose, 50 mM KCl, 10 mM MOPS, 1 mM EGTA Tris–HCl pH 7.2 (for flux assays) and

homogenized in the glass/teflon homogenizer (10 strokes) at 0 °C. The membrane preparations were stored at −20 °C.

2.4. Flux assay

Flux assays were performed essential as previously described [37]. In short, total K^+ channel activity in plasma membrane vesicles was measured as the potential-driven $^{86}Rb^+$ uptake at 25 °C that could be blocked by 4.4 mM $BaCl_2$ as described earlier [42]. To minimize a possible background flux due to Na^+/K^+-ATPase activity, the vesicles were pre-incubate in 0.5 mM ouabain, 0.1 mM vanadate, 0.5 mM $MgCl_2$, 250 mM Sucrose, 50 mM KCl, 10 mM Mops, 1 mM EGTA for 30 min. Afterwards, 0.9 mM furosemide was added to minimize a possible unspecific flux through the $Na^+/2Cl^-/K^+$ co-transport system present in the distal colon epithelium. Vesicles from kidney cortex, kidney outer medulla and kidney inner medulla were then pre-incubated for 30 min with 100 nM of IbTX to examine the inhibitory effect of this toxin on the Ba^{2+}-sensitive K^+ uptake. The assay was allowed to proceed for 40 min before the vesicles $^{86}Rb^+$ uptake was measured.

2.5. Binding assay

Binding of ^{125}IbTX-D19Y/Y36F was measured essential as previously described (Koschak et al. [36]). The incubation was carried out in 0.5 ml medium consisting of 10 mM NaCl, 20 mM Tris–HCl, 0.1% BSA, pH 7.4. Nonspecific binding was defined in the presence of 10 nM IbTX. Incubation was carried out at 23–26 °C for either 15–24 h (permeabilization experiments) or 60–72 h (saturation experiments). All serial toxin dilutions were performed in 150 mM NaCl, 20 mM Tris–HCl pH 7.4 and always added directly to the incubation medium to avoid adsorption phenomena. Digitonin was allowed to incubate ≥20 min with membrane vesicles before the ^{125}IbTX-D19Y/Y36F was added to the reaction mixture. The protein concentration of outer cortex, inner cortex, outer medulla and inner medulla membrane vesicles were 875 μg/ml, 300 μg/ml and 140 μg/ml for permeabilization experiments and 300 μg/ml, 800 μg/ml, 300 μg/ml and 100 μg/ml for saturation experiments. At the end of incubation, samples were rapidly filtered through Toyo Advantec GC 50 glass fiber filters (presoaked for at least 60 min in 0.3% (w/v) polyethylenimine), followed by two washes with ice cold filtration buffer consisted of 150 mM NaCl, 20 mM Tris–HCl pH 7.4 (3 ml per wash). Samples were filtered individually on a Millipore 1002530 filter apparatus. In each experiment, triplicate assays were routinely performed and the data averaged.

2.6. Immunohistochemistry

Eight Wistar male rats were anesthetized with pentobarbital (i.p.) and perfusion fixed through the left ventricle. As fixative were used phosphate buffered saline (PBS) plus 3000 i.e. heparin per liter PBS followed by (n=4) 0.1 M phosphate buffer with 4% paraformaldehyde for 10 min or (n=4) 0.1 M phosphate buffer containing 4% paraformaldehyde and 0.1% glutaraldehyde. The kidneys were removed, and post fixed for and additional 24 h in 0.1 M phosphate buffer plus 4% paraformaldehyde. Kidney fixed in 4% paraformaldehyde was embedded in paraffin and 5 μm serial sections were cut and mounted on Super Frost Plus object slides. Sections were dehydrated in xylene–ethanol series, endogenous peroxidase activity was blocked in 1% H_2O_2 in 100% methanol, washed three times 5 min in PBS followed by incubation in PBS plus 1% human serum albumin (hSA) for 1 h. Primary antibody rabbit anti-BK-channel [38,39] was diluted 1:3000 in PBS plus 1% hSA and sections were incubated over-night at 4 °C. Then washed three times 5 min in PBS and incubated in a swine anti rabbit biotinylated antibody 1:500 (E0353 Dako A/S, Denmark) in PBS, plus 1% hSA, for 1 h at room temperature. Washed 3 times in PBS followed by incubation with StreptAvidin–Biotin Complex 1:500 (Vector Elite, Vector, Burlingame; USA) for 1 h, washed 3 times in PBS and then incubated with 0.05% Di-aminobenzidine (DAB) in 0.01% H_2O_2 for 10 min and finally washed 3 times in distilled water. One of the two sections on each slide was counterstained with PAS, before mounting in DEPEX.

Kidneys fixed in 4% paraformaldehyde and 0.1% glutaraldehyde were processed for immunohistochemistry followed by Epon embedding. Wibratome sections 40 to 60 μm in thickness were cut, and collected in a 30% w/v sucrose in PBS, after at least 5 h in this solution, the sections were rapidly frozen in liquid nitrogen and thawed 3 times in order to permeabilize the tissue before immunolabeling. All steps in the following protocol were carried out at 4 °C. After permeabilization sections were collected in PBS plus 1%(human serum albumin, Statens Serum Institut, Denmark) hSA for at 8 h followed by incubation in the primary antibody (rabbit anti-BK-channel, [38,39]) diluted 1:1000 or 1:3000 in PBS plus 0.1% hSA for 72 h. Then sections were washed 4 times 2 h in PBS followed by incubation in the secondary antibody (donkey anti rabbit-biotin conjugated (Jackson ImmunoResearch Laboratories, PA, USA) diluted 1:500 in PBS plus 1% hSA for 12 h. Then sections were washed 4 times 2 h in PBS followed by incubation for 12 h in StreptAvidin–Biotin–Horse Radish Peroxidase complex (Vector elite kit, Vector) diluted 1:200 in PBS. Then sections were washed 4 times 2 h in PBS followed by incubation in 0.05% DAB without H_2O_2 for 30 min, and the transferred to 0.05% DAB in 0.01% H_2O_2 in PBS for 15 min at 20 °C. The chromogene reaction was stopped by washing sections 3 times in PBS and then transfer them to PBS containing 1% H_2O_2 for 15 min followed by wash in distilled water. Sections were dehydrated in ethanol and propylene oxide and embedded in Epon and 1 μm serial sections for light microscopy were cut. Each second section was counterstained with toluidin blue.

2.7. Control

Omission of primary antibody or liquid phase pre-absorption of the primary antibody with the immunogene resulted in no staining.

2.8. Autoradiography

Two rabbits and two male Wistar rats were anaesthetized with i.p. pentobarbital and killed by bleeding just before the kidneys were removed. The kidneys were opened and washed twice in ice-cold PBS before frozen in solid CO_2. Tissues were cryosectioned and serial 5 μm sections mounted on Super Frost Plus objective slides and stored at −80 °C until used. Sections for autoradiography were thawed and incubated for 10 min in PBS without potassium plus 0.1% bSA fraction V (cat #A-8022 sigma). Then, incubated in iodinated iberiotoxin ^{125}I-IbTX-D19Y/Y36F (60.000 cpm for either 1.5 or 4.5 h) or cold IbTX. After incubation, sections were washed two times 5 min in ice-cold PBS without potassium followed by 1 min in ice-cold distilled water. Sections were then dried and fixed in paraformaldehyde vapor at 60 °C for 5 h. Sections were dipped in Ilford emulsion LM-1 (Amersham Biosciences, Denmark) and exposed for 2 weeks before development. Each second section was counterstained with hematoxylin before mounted in DEPEX.

2.9. Analysis of data: radioligand binding studies

The results from saturation binding experiments were subject to a Michaelis–Menten analysis were the equilibrium dissociation constant (K_d) and the maximal receptor concentration (B_{max}) was determined using the one-site binding equation:

$$Y = \frac{B_{max} * X}{K_d + X}$$

where Y=receptor concentration and X=radioligand concentration. The correlation coefficient for these plots was >0.97. Data from saturation experiments were transform to a Scatchard analysis. B_{max} and K_d values determined from this linear regression were approximately identical to the values determined from the one-site binding equation.

2.10. Statistics

All data points are given as means. $n \geq 3$ the exact number is indicated in relevant figures. Error bars are given as standard deviations.

2.11. Protein determination

The concentration of membrane protein was determined according to Peterson [40] using BSA as a standard.

2.12. Abbreviations

Tris (Trizma base), hydroxymethyl aminoethan; MOPS, 3-(N-morpholino) propanesulfonic acid; EGTA, ethylene glycol bis-(β-aminoethyl ether) N, N, N', N'-tetraacetic acid; BSA, Bovine serum albumin; IbTX, Iberiotoxin; ChTX, Charybdotoxin; TEA, Tetraethylammonium.

3. Results

3.1. Identification of BK channels by flux assay

For an initial investigation of the abundance of Ca^{2+}-activated maxi K^+ channels in kidney epithelium, we performed a sensitive flux assay which has earlier proven very useful for measurements of ion channels in epithelia plasma membrane vesicles [37,41–43]. In this assay, the Ba^{2+}-sensitive $^{86}Rb^+$ uptake in the presence and absence of IbTX was measured. Influx of $^{86}Rb^+$ through Na^+/K^+-ATPase or $Na^+/2\ Cl^-/K^+$ co-transporters was minimized by including ouabain, vanadate, $MgCl_2$ and furosamide in the buffer medium.

In Fig. 1, shown are some experiments where the $^{86}Rb^+$ uptake into vesicles from kidney cortex, outer medulla and inner medulla have been measured at three different conditions, namely, in control membranes (no addition) and after the addition of either 100 nM IbTX or 4.4 mM BaCl. As can been seen, Ba^{2+} blocks approximately 40% of the $^{86}Rb^+$ uptake, and since Ba^{2+}, used in this concentration, is known to block essential all K^+ channels present, the Ba^{2+} sensitive fraction of the $^{86}Rb^+$ uptake into the vesicles can be considered as a measure of the total K^+ channels activity in the membrane vesicles. The addition of IbTX to the different kinds of membranes resulted in a block of the Ba^{2+}-sensitive $^{86}Rb^+$ uptake of 36%, 29% and 44% for cortex, outer medulla and inner medulla, respectively, indicating that BK channels are abundant in these preparations. In this experiment, no further separation of kidney outer and inner cortex was performed.

Results obtained from this kind of flux assay can merely be considered semiquantitative. This is due to the fact that iberiotoxin blocks BK channels only when applied to the extracellular part of the pore. Since it is not possible to control the orientation of the vesicles used in this experiment, it is inevitable that some vesicles will be oriented inside-out, meaning that iberiotoxin will never be able to get in contact with the extracellular part of the channel. As a consequence, not all channels are subject to block by iberiotoxin, resulting in an underestimation of the number of BK channels present in these vesicles. Another reason why this kind of assay can only be considered semi-quantitative is that vesicles have to be completely sealed to perform reliably. This is a condition that is impossible to control, again disrupting the use of this assay for quantitative purposes.

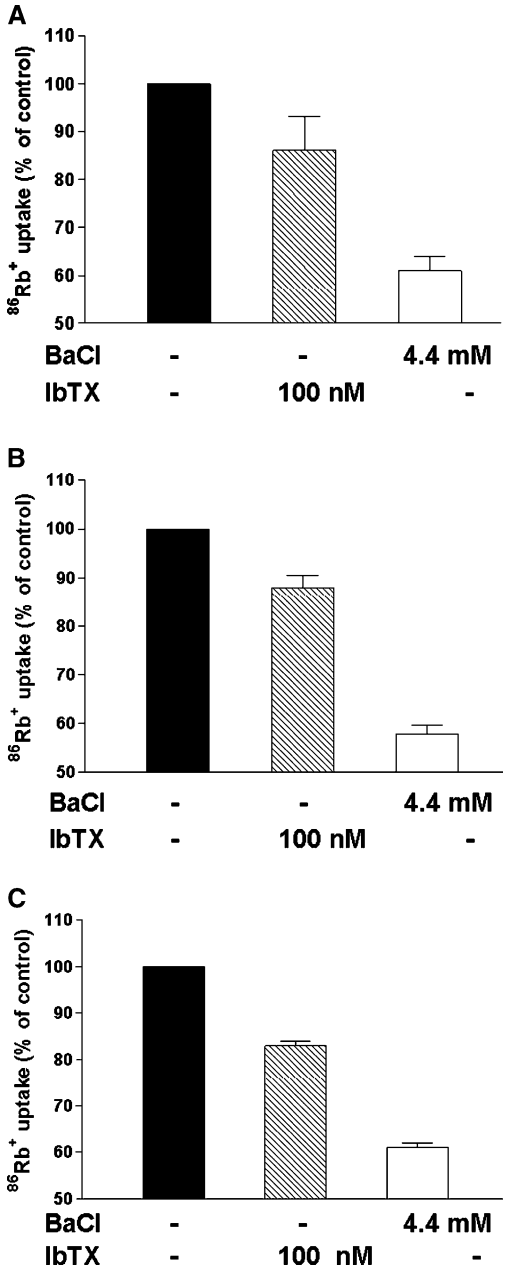

Fig. 1. Ba^{2+} sensitive $^{86}Rb^+$ uptake in kidney epithelia vesicles. Membranes were reconstructed from A cortex, B outer medulla and C inner medulla. Black bars are control situations where no IbTX or Ba^{2+} were presented, hatched bars are $^{86}Rb^+$ uptake in the presence of 100 nM IbTX and white bars are $^{86}Rb^+$ uptake in the presence of 4.4 mM Ba^{2+}. All experiments were performed as quadruplicates and the data averaged. Error bars are S.E.M. values.

3.2. Binding of ^{125}I-IbTX-D19/Y36F to permeabilized epithelial kidney membranes

All binding experiments were performed using the radiolabeled iberiotoxin analog ^{125}I-IbTA-D19Y/Y36F. Unspecific binding was defined in the presence of large excess (>1000 times) of cold iberiotoxin and specific binding was determined as the difference between total and unspecific binding. All data presented in this paper show specific binding.

An essential request for carrying out reliable quantification of BK channels in kidney epithelia is the ability of ^{125}IbTX-D19Y/Y36F to interact with all channels present. Since ^{125}IbTX-D19Y/Y36F is only able to block BK channels by interacting with the extracellular face, channels located in vesicles oriented inside-out can never be subject to ^{125}IbTX-D19Y/Y36F binding. It is therefore essential to make sure that all vesicles in the preparation are fully opened. To obtain a complete permeabilization of the vesicles, we therefore examined the effect of adding increasing amounts of the detergent digitonin to the vesicles.

In Fig. 2, binding of ^{125}I-IbTX-D19Y/Y36F to inner medulla membranes in the presence of different amounts of digitonin is shown as percent binding compared to a control experiment where no digitonin was added. As seen in the figure, the optimal concentration of digitonin for inner medulla was 0.250 mg digitonin/mg membrane protein. For outer cortex and outer medulla, a complete permeabilization was obtained using 0.250 to 0.375 mg digitonin/mg protein (data not shown). All further quantification studies (saturation experiments) were performed using these concentrations of digitonin.

The significant decrease in ^{125}IbTX-D19Y/Y36F binding in the presence of high digitonin concentrations is presumably due to the fact that high detergent concentrations comprise filter retention of solubilized channels protein.

3.3. Quantification of BK channels in kidney epithelium

To obtain information about the number of BK channels in kidney epithelium, we performed saturation binding experiments with 10 different concentrations of ^{125}IbTX-

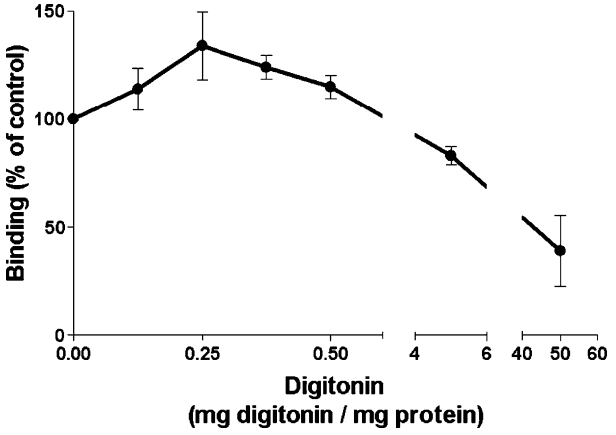

Fig. 2. Permeabilization of membrane vesicles by digitonin. Inner medulla membrane vesicles (70 μg protein/sample) were incubated for ≥20 min with different concentrations of the detergent digitonin before specific binding of ^{125}I-IbTX-D19Y/Y36F (15.000 CPM/sample; 20 h incubation) was determined. Specific binding is shown as a function of the digitonin/membrane protein ratio. Error bars are given as S.E.M. values and $n=3$.

D19Y/Y36F ranging from approximately 10 times below to about 10 times above the expected K_d value. The specific binding, determined as the difference between total binding and non-specific binding, was a saturable function of the ^{125}IbTX-D19Y/Y36F for outer cortex, outer medulla membranes and inner medulla membranes. For inner cortex membranes, no difference between total and non-specific binding was observed, independently of the concentration of ^{125}IbTX-D19Y/Y36F used (Fig. 3B). The data obtained from outer cortex, outer medulla and inner medulla were transformed into Scatchard plots. As can be seen in the insert in Fig. 3, straight lines were obtained indicating that ^{125}IbTX-D19Y/Y36F interacts with a single class of receptor sites in these parts of the kidney. The B_{max} and K_d values from these experiments were calculated by non-linear fitting and are summarized in Table 1.

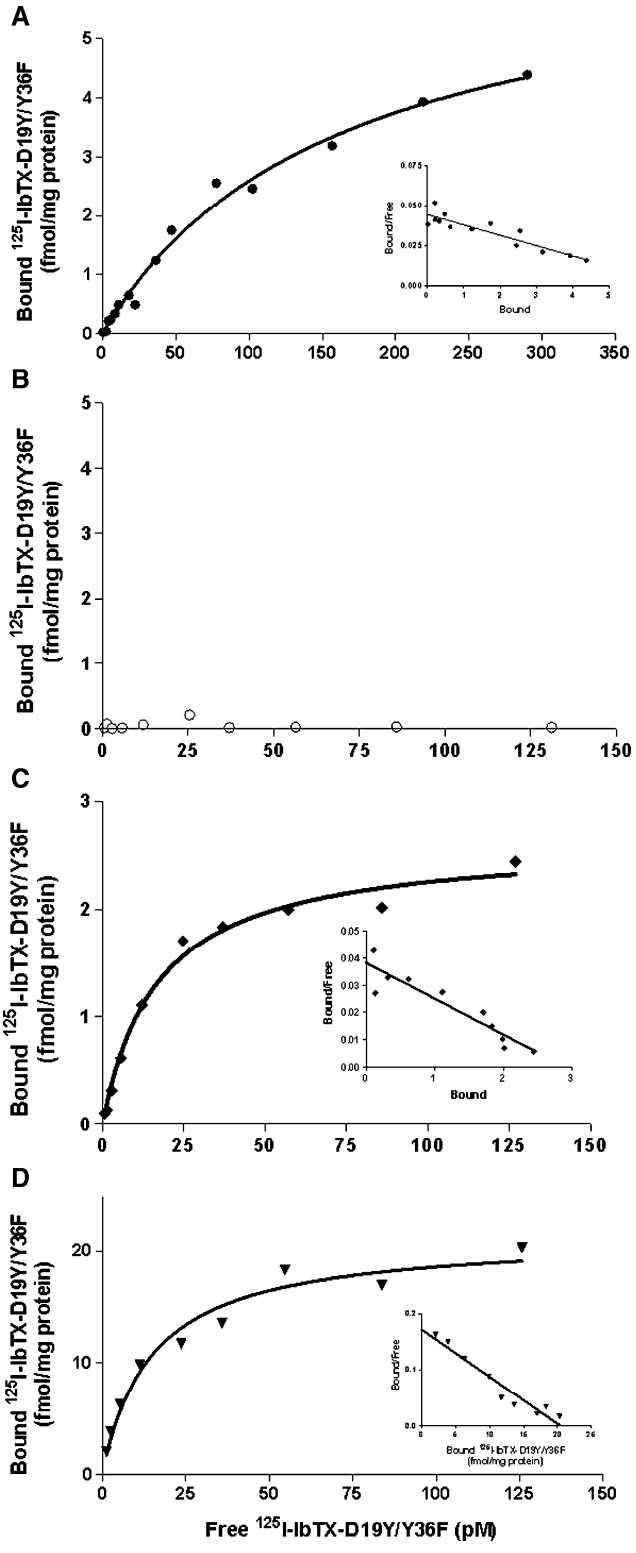

Table 1
Distribution of BK channels in kidney epithelia

Tissue	B_{max} (fmol/mg protein)	K_d (pM)	Source
Kidney outer cortex	6.8	160.2	This study
Kidney outer medulla	2.6	17.0	This study
Kidney inner medulla	21.4	14.8	This study
Colon (surface membranes)	78	9.7	[37]
Colon (crypt membranes)	8	8.5	[37]
Frontal cortex	191	36.7	[39]
Hippocampus	141	23.3	[39]
Hypothalamus	29	19.1	[39]
Smooth muscle	420	5.2	[39]

Maximal receptor concentration (B_{max}) and equilibrium constants (K_d) was determined by ^{125}I-IbTX-D19Y/Y36F binding (this study and Grunnet et al. [37]) or ^{3}H-IbTX-D19Y/Y36F binding (Knaus et al. [39]). B_{max} and K_d values are calculated from 4 independent experiments.

These binding experiments indicate that ^{125}IbTX-D19Y/Y36F is a suitable ligand for quantification of Ca^{2+}-activated maxi K^+ channels in kidney epithelium, and that the assay is sufficiently sensitive to determine the differences between channel density in various parts of the kidney epithelia.

3.4. Immunohistochemical and autoradiographical identification of BK channels

To further confirm the existence of BK channels in the kidney, we performed autoradiography and immunohistochemical studies. Autoradiography was performed with ^{125}I-IbTX-D19Y/Y36F on both rabbit and rat kidneys. As shown in Fig. 4, staining was observed in tubular structures in the outer cortex, and in the smooth muscles of vascular tissue for both rat and rabbit. PAS staining of sections from the

Fig. 3. Quantification of BK channels by ^{125}I-IbTX-D19Y/Y36F binding. Saturation binding experiments with ^{125}I-IbTX-D19Y/Y36F were performed with A, outer cortex (150 μg protein/sample); B, inner cortex (400 μg protein/sample); C, outer medulla (150 μg protein/sample); and D, inner medulla (50 μg protein/sample) membrane vesicles. Samples were incubated for ≥20 min with 0.250–0.375 mg digitonin/mg membrane protein before specific binding in the presence of increasing concentrations of ^{125}I-IbTX-D19Y/Y36F was determined. Binding was allowed to reach equilibrium by 60–72 h incubation. Data points are means of duplicates. The inserts show the Scatchard analysis by linear transformation of the respective data points. Calculated maximum receptor concentrations (B_{max}) and dissociation constant values (K_d) are summarized in Table 1.

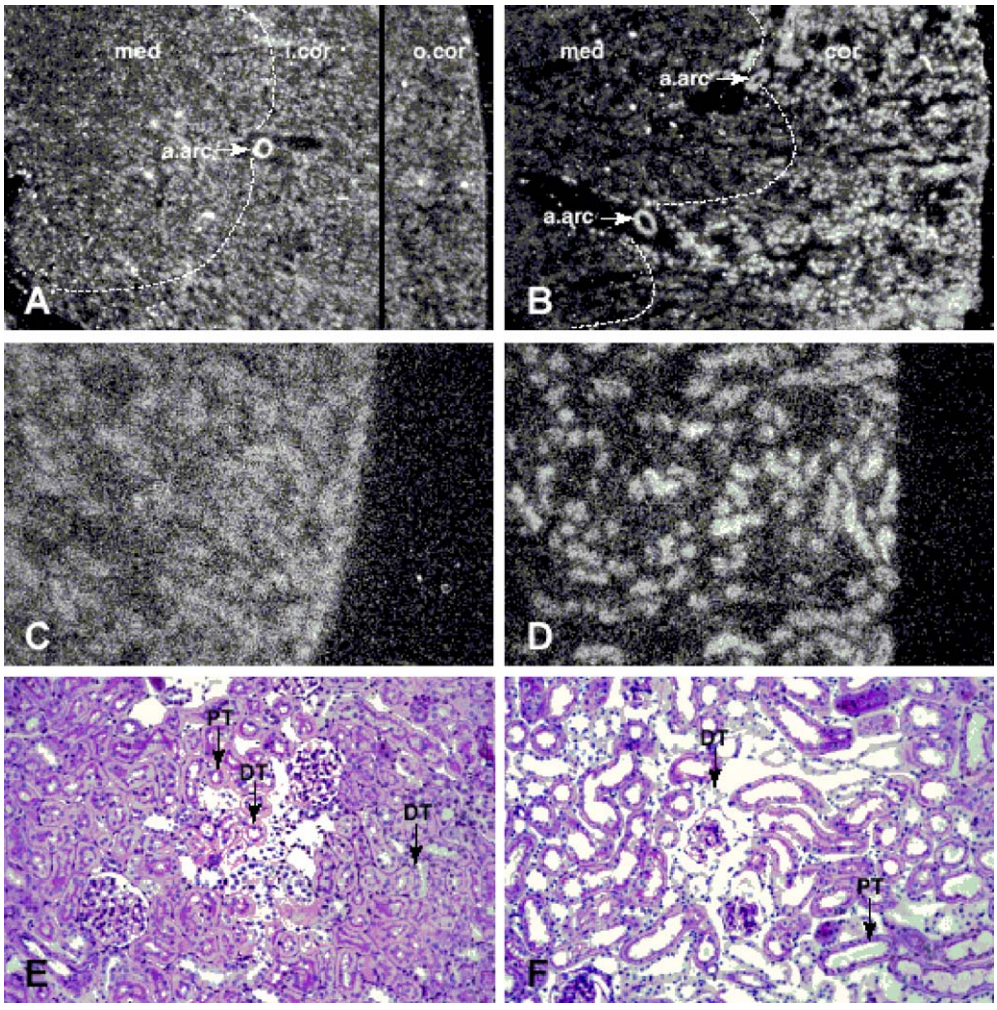

Fig. 4. A–D: Autoradiography performed with ^{125}I-IbTX-D19Y/Y36F. Toxin binding could be demonstrated in rabbit kidney (A, C) and rat kidney (B, D). A and B represent low power micrographs showing outer cortex (o.cor), inner cortex (i.cor), the arcuate arteries (a.arc) defines border between the cortex and medulla (med) and is indicated by a stippled line. C and D show details of cortex. E and F represent PAS staining of perfusion fixed rabbit and rat kidney, respectively. PT, proximal tubulus; DT, distal tubulus, notice the more compact appearance of the tubules in the rabbit kidney compared with the rat kidney.

same area in control kidneys shows that proximal and distal tubules and collecting ducts are harbored here.

Antibodies recognizing the intracellular part of the BK channel have in earlier experiments been valuable tools in characterizing the localization of these channels in excitatory tissue [38,39]. To investigate if these antibodies could also recognize BK channels in non-excitatory tissue, we performed immunohistochemical studies on rat kidneys. As can been seen in Fig. 5, it was possible to demonstrate BK channels in different parts of the kidney. The most intense immunolabeling was found in the parietal and visceral layer of Bowmans capsule, the thin segment of the loop of Henle, distal tubules and in the principal cells of the collecting ducts. In contrast, no staining could be observed in intercalated cell in the collecting duct (Fig. 5D, insert). Staining was also observed in the smooth muscles of the arcuate arteries. Staining was not found in the proximal tubules.

4. Discussion

4.1. Binding of iberiotoxin to kidney epithelial BK channels

The existence of BK channels have been described in almost every nephron segments in the kidney either by patch-clamp studies or by planar lipid bilayers experiments. Using these techniques, the presence of BK channels has been suggested in glomerulus (mesanglial cells) [20], in proximal tubule [27,29,31,22] in medullar and cortical thick ascending limb of Henle's loop [29,44] and in the cortical collecting duct [7,18,23,24]. Furthermore, the existence of mRNA coding for BK channels have been demonstrated by reverse transcriptase PCR in all nephron segments except for proximal straight tubule and proximal convoluted tubule [30].

Even though many of these studies have identified BK channels solely by electrophysiological studies and not by

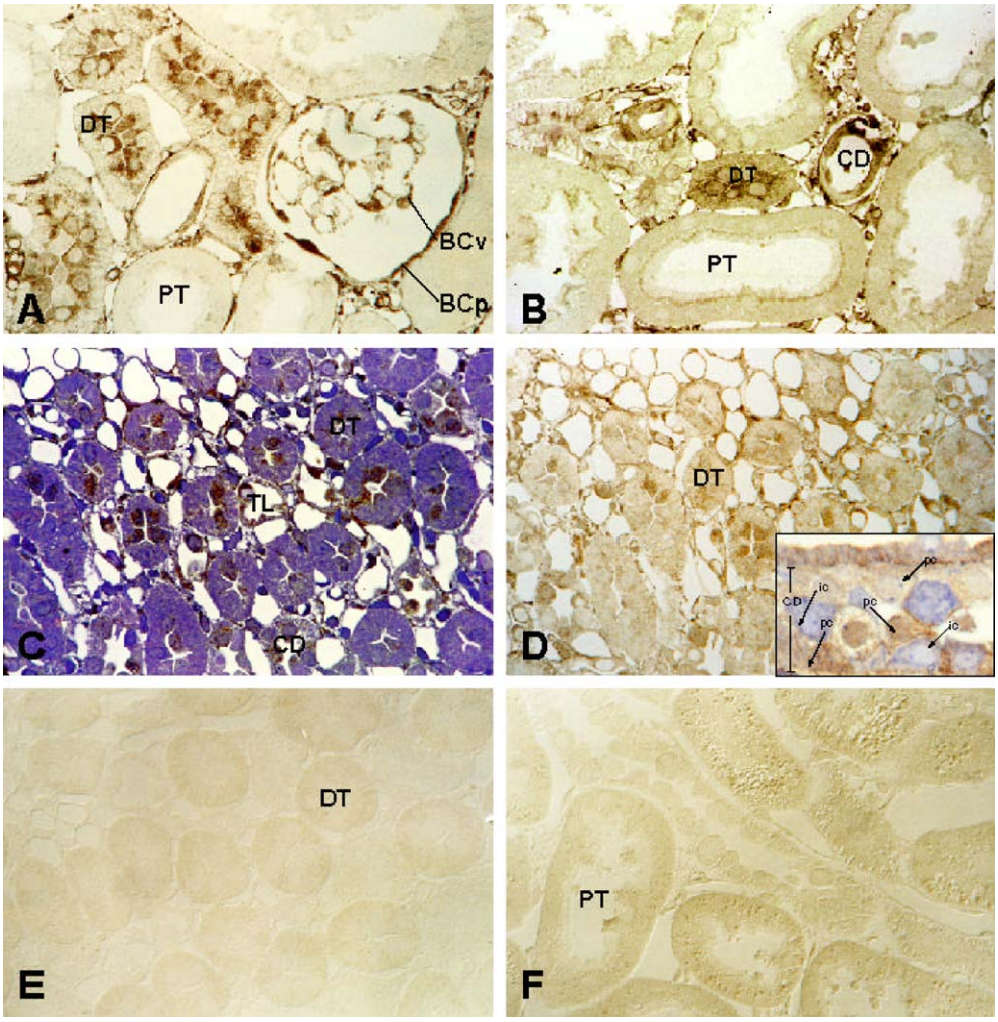

Fig. 5. Immunostaining against BK channels in sagittal sections of rat kidney. (A) Immunoreactive cells were observed in the visceral and parietal layer of Bowmans capsule (BCv resp. BCp) and in the distal tubulus (DT). In contrast, the proximal tubulus (PT) was devoid of staining. (B) Staining could also be observed in the collecting duct (CD). (C) Toluidin stained section from *outer* medulla showing immunoreactivity in the thin limb of Henle's loop (TL). The slide is orientated so that inner medulla will appear in direction of the upper left corner of the picture. (D) As C but without toluidin staining, insert shows toluidin stained section of collecting duct (CD) with immunoreactivity in the parietal cells (pc) but not in the intercalated cells (ic). With this staining the intercalated cells are bluish, in contrast to the more pale principal cells. No immunostaining was observed after omission of the primary antibody in medulla (E) or in cortex (F).

using specific blockers of BK channels, the chance of BK channels to be very abundant throughout the kidney seems likely. This notion supports the idea that this type of Ca^{2+}-activated K^+ channel plays an important role in the kidney.

Although numerous very detailed electrophysiological studies on BK channels in the kidney have been performed, the exact distribution and quantification of these channels have never been determined. For such a purpose, it is necessary to use ligands that bind specifically to the channel with high affinity. Iberiotoxin has these properties and was used either in its native form or as a double mutant called IbTX-D19Y/Y36F which has preserved all its biological properties [36]. We used this toxin to perform binding experiments to four different parts of the kidney namely outer cortex, inner cortex, outer medulla and inner medulla.

As was demonstrated in Fig. 3, ^{125}I-IbTX-D19Y/Y36F binding to outer cortex, outer medulla and inner medulla was a saturable function of the ligand concentration. This indicates that ^{125}I-IbTX-D19Y/Y36F interacts with a single class of receptor sites in the kidney.

Considering the fact that ^{125}I-IbTX-D19Y/Y36F has access to the extracellular parts of all BK channels present due to the permeabilization by digitonin and assuming a 1:1 stoichiometry for ^{125}I-IbTX-D19Y/Y36F binding to BK channels, the density of channels in the different membrane fractions can be determined directly from the calculated B_{max} values. These results are summarized in Table 1, where the results from other BK quantification studies have been included for comparison. As can be seen, the amount of BK channels is comparable to what have been demonstrated in other excitatory and non-excitatory tissues, supporting the idea that this type of K^+ channel might play an important role in epithelia transport in the kidney.

Independent of the amount of ^{125}I-IbTX-D19Y/Y36F used, we were not able to demonstrate any specific binding to the inner part of cortex. The lack of BK channels in this part of the kidney confirms the idea that different kinds of ion channels can have very distinct expression patterns.

An interesting phenomenon observed in the binding studies was that K_d values in cortex and medulla were rather different. The K_d values from outer and inner medulla are in the same range as has been observed for ^{125}I-IbTX-D19Y/Y36F binding in other tissues (see Table 1), while the K_d values for outer cortex is approximately 10 times higher. An explanation for this difference in affinity for ^{125}I-IbTX-D19Y/Y36F binding to BK channels in the different part of the kidney could be the presence or absence of a β-subunit. BK channels are composed of four identical α-subunits and the existence of four additional β-subunits has been demonstrated [45–49]. The presence of β-subunits are known to affect the Ca^{2+} sensitivity of the BK channel, but, to our knowledge, no information is available about any function β-subunits could have on iberiotoxin binding. In contrast, it has been demonstrated that charybdotoxin, a toxin very related to iberiotoxin, binds more efficiently to BK channels when a β-subunit is present [50]. This could indicate that the same phenomenon could also exist for iberiotoxin binding and thereby explain the observed inconsistency between the observed K_d values.

4.2. Physiological role of BK channels in kidney epithelia

BK channels have been suggested to play an important role in Na^+ re-absorption. The thick ascending limb of Henle (pars recta distal tubulus) is thought to play an essential role in the regulated absorption of salt in the kidney, which is in nice agreement with BK channels being present here (outer medulla). The existence of BK channels in the outer part of the kidney indicates that these channels might play an important role in the distal tubule. As was shown by immunohistochemical studies (Fig. 5D, insert), BK channels are in cortical collecting ducts located in principal cells and not in intercalated cells. Since principal cells are believed to be responsible for salt and water re-absorption while intercalated cells are thought to be involved in H^+ and HCO_3^- secretion, this distinct distribution supports the idea that an important physiological role of these channels could be participating in maintenance of water and salt homeostasis. Another key function of distal tubule is that regulation of the K^+ homeostasis and BK channels could also be important in this process. The presence of BK channels in cortical collecting ducts is consistent with functional studies and immunohistochemical localization of BK channels in rabbit and mouse kidney, respectively [23]. Other more detailed studies of the cortical collecting duct point to the fact that BK channels are more abundant in intercalated cells compared to principal cells [18,24]. If these discrepancies are due to the methods applied or the age of the rabbits use are, however, unknown.

BK channels were also found in the inner medulla suggesting that these channels could be present in the inner collecting duct. The physiological relevance of BK channels in this part of the kidney could be involvement in regulatory volume regulation. Epithelia cells in the inner medulla part of the kidney are challenged by great fluctuations during different diuretic states. Experiments have shown that BK channels might be influenced by changes in osmolarity [27] and this fact together with the high abundance of BK channels in inner medulla could suggest that BK channels play an important role for cell volume regulation in epithelia.

Exact quantification of BK channels in epithelia tissue by binding studies can be compromised by vascular contamination. The protocol applied in this study is, however, optimized to isolation of only epithelia tissue. More importantly, the complete lack of ^{125}I-IbTX-D19Y/Y36F binding to the inner part of cortex talks against BK channel from vascular tissue being present. The relative high amount of BK channels in kidney epithelia compared to excitatory tissue where very high amounts of channels are expected (see Table 1) was surprising since the abundance of ion channels in epithelia is normally rather modest. An attempted explanation for this high amount of voltage-gated BK channels in epithelia tissue that normally do not undergo large variations in resting potentials could be that the BK channel actually plays a role in setting the membrane potential in these cells. When Na^+ re-absorption through the luminal membrane in kidney epithelia is increased by aldosterone, a slight depolarization will inevitably happen. To retain the membrane potential at resting level, it is necessary to have a voltage-gated K^+ channel that does not inactivate which is exactly the case for BK channels.

In conclusion, we have demonstrated the functional existence of BK channels in kidney epithelia and by performing saturation-binding experiments with ^{125}I-IbTX-D19Y/Y36F made the first attempt to quantify the amount of these epithelial channels present in the different part of the kidney. BK channels could be observed in outer cortex, outer medulla and inner medulla but were absent in inner cortex. Results were confirmed by autoradiography and immunohistochemistry. In addition, these studies revealed a discrete expression of BK channels in principal cells, responsible for salt and water re-absorption. In contrast, no BK channels are found in neighboring intercalated cells responsible for proton and bicarbonate secretion. This distinct distribution combined with the relative high amounts of channels present could indicate that these kinds of K^+ channels have important physiological relevance in kidney epithelia. However, this assumption still has to be confirmed in future studies.

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