Single cell RT-PCR analysis of CIC-2 mRNA expression in ureteric bud tip

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Huber, Stephan, Bernd Schröpfl, Matthias Kretzler, Detlef Schlöndorff, and Michael Horster. Single cell RT-PCR analysis of CIC-2 mRNA expression in ureteric bud tip. Am. J. Physiol. 274 (Renal Physiol. 43): F951–F957, 1998.—Embryonic epithelia at the tip of the ureteric bud (UB) face the interspace between epithelial and mesenchymal cells and are fundamentally involved in reciprocal signaling during early nephrogenesis. To characterize their membrane conductive proteins, patch-clamp and single cell RT-PCR techniques were applied to embryonic rat UBs [embryonic day 17 (day E17)] microdissected from the outer cortex. Cells at the UB tip had a high whole cell conductance (14 ± 2 nS/10 P, n = 8). The main fractional conductance resembled that of Ca-activated Cl channels in nonepithelial cells, with its time-dependent activation at depolarizing and inactivation at hyperpolarizing voltages. A second Cl-selective current fraction, by contrast, activated slowly during strong hyperpolarization, suggestive of a CIC-2-mediated conductance. To determine the origin of this current, cytoplasm was harvested into the patch pipette, RNA was reverse transcribed, and cDNA amplified by polymerase chain reaction (PCR). GAPDH and CIC-2 PCR products were identified in 23 and 8 (out of a total of 57) single cell cDNA samples, respectively. CIC-2 PCR products were obtained by two alternatively spliced ClC-2 mRNA isoforms. This first and combined approach by patch-clamp and single cell RT-PCR techniques to embryonic epithelium indicates that 1) cells at the UB tip express a phenotype remarkably different from that of postembryonic collecting duct principal cells and that 2) CIC-2 is likely to have a key role in early nephrogenesis.

Embryonic kidney and lung have branching morphogenesis and bud formation in common, and embryonic lung epithelia express the chloride channel CIC-2 (30). Specifically, CIC-2 mRNA shows maximum levels in fetal lung cells and is downregulated after birth, suggesting a particular role of CIC-2 in embryonic epithelogenesis (22). CIC-2 is activated by strong hyperpolarization or by cell swelling (10). The hyperpolarization-evoked whole cell Cl currents generated by CIC-2 typically activate slowly (30), they are not directly dependent on cytoplasmic Ca or ATP concentration, and they have an anion permselectivity of Cl > I (15). By these properties and its voltage dependence, CIC-2 differs greatly from the volume-regulatory Cl channel (24), from Ca-activated channels, and from secretory cystic fibrosis transmembrane conductance regulator channels, respectively (1). CIC-2 regulates cell volume, presumably in concert with volume-sensitive organic osmolyte and anion channel (29), and it probably contributes to Cl secretion in some epithelial cells (2, 7). To evaluate CIC-2 mRNA expression in tUB cells, the technique of single cell reverse transcription-polymerase chain reaction (RT-PCR) technique (20) was adapted for epithelial cells. The data indicate that CIC-2 mRNA is expressed in cells at the tUB.

METHODS

Microdissection of embryonic ureteric buds. As shown in Fig. 1, branching ureteric buds and the attached mesenchyme were microdissected from the outermost cortex of embryonic rat kidney in Ca2+- and Mg2+-free phosphate-buffered solution at 4°C, explanted on coverslips coated with newborn rat tail collagen, and attached to the matrix at their ureteric trunk end. Coverslips had been glued to culture dishes (Nunc, 30 mm) with a central hole. The mesenchymal caps were removed in one or two of the bud tips by additional dissection to obtain direct access to the basolateral membrane (Fig. 4A). The freshly dissected tissue was kept in nephron culture medium (13) at 37°C for 30 min before analysis. All cells investigated by patch-clamp whole cell recording and RT-PCR...
were located within an ~20-cell area at the central circumference of the tUB.

Patch-clamp recordings. Branching UBs were rinsed with NaCl bath solution (in mM: 150 NaCl, 10 D-glucose, 10 HEPES, 5 KCl, 1.6 CaCl₂, and 0.8 MgCl₂, pH 7.2), and dishes were mounted on the stage of an inverted microscope equipped with differential-interference contrast optics (Zeiss, Oberkochen, Germany). Dishes were constantly superfused (1 ml/min) with NaCl bath solution or with a solution containing 180 mM N-methyl-D-glucamine (NMDG) titrated with 5 mM HEPES and ~130 mM HCl to pH 7.2 through a flow system inserted into the dish to reduce the bath volume to 50 µl. After an initial 15-min period of superfusion, patch-clamp experiments were performed, using 1.5-mm borosilicate glass pipettes with 2- to 5-MΩ tip resistance (GC 150 TF-10; Clark Medical Instruments, Pangbourne, UK) and a WR-88 water hydraulic micromanipulator (Narishige, Tokyo, Japan). Pipettes were manufactured by a microprocessor-driven DMZ puller (Zeitz, Augsburg, Germany) and filled with 6.5 µl of pipette solution (in mM: 135 KCl, 5 HEPES, 4 MgCl₂, and 1 EGTA, pH 7.2). Currents were recorded in the whole cell and the outside-out mode and 1-kHz low-pass filtered by an Axopatch 200A amplifier (Axon Instruments, Foster City, CA). Whole cell voltage-clamp pulse protocols were applied, and data were acquired with a rate of 5 kHz by a microcomputer, using pCLAMP software and an TL1 DMA interface (Axon Instruments). Single cell data were stored on digital audiotape (DTR 1204 Recorder; Bio-Logic, Clai, France) and analyzed offline with a sampling rate of 10 kHz. Pipettes and Ag-AgCl wire were baked (220°C for 5 h), tubings and the dish insert were rinsed with 0.1% diethyl pyrocarbonate (DEPC) water before use, and 0.1% DEPC was mixed to bath and pipette solutions before autoclaving. For data analysis, applied voltages were corrected for estimated liquid junction potentials, as previously described (5). All data are expressed as mean ± SE.

Harvesting of cytoplasm. During formation of gigaohm seal and whole cell recording mode, large portions of cytoplasm were clearly visible as they were aspirated several micrometers into the pipette tip by negative pressure applied to the pipette lumen (Fig. 4A). Disrupting the aspirated membrane ensured the electrophysiological access to the whole cell and, specifically, the dialysis of cytoplasm by pipette solution. This was monitored by the large increase in capacitive current evoked by a 5-mV square pulse. When the pipette was retracted, the aspirated cytoplasm was pulled from the cell, thus trapping a considerable fraction of cytoplasm within the pipette, whereas the nucleus always remained in the cell. At this point, the pipette was rapidly withdrawn from cell and bath, and the total pipette volume was directly expelled into a tube (filled with 3.5 µl of reverse transcriptase mixture) by applying gentle positive pressure and breaking the glass tip at the tube bottom. The whole cell current recording protocol was carried out in 35 out of 57 cells. In 22 experiments, the pipette was withdrawn immediately after formation of the whole cell recording mode, and its contents were expelled into the reverse transcriptase mixture, so that cDNA synthesis began already ~10 s after sealing and rupturing of the cell membrane.

Reverse transcription. The RT mixture contained 1 µl dithiothreitol (0.1 mM), 0.1 µl single-strand buffer (5-fold), 0.5 µl RNase inhibitor (RNasin, 40 U/µl) and 0.5 µl dNTP mixture (25 mM each) (both Promega, Eggenstein, Germany); 0.5 µl SuperScript RT (200 U/µl) (both Life Technologies, Ingelheim, Germany); and 1 µl random hexamer oligonucleotide primer (Boehringer, Mannheim, Germany). The RNA was transcribed for 1 h at 37°C. From the total volume of ~10 µl cDNA, a 2-µl aliquot was placed in a second tube, and both aliquots were stored at -25°C.

Polymerase chain reaction. cDNA specific for the Cl channel CIC-2 (GenBank accession no. X64139) and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH; GenBank accession no. M17701) were amplified by 50 PCR cycles, using sequence-specific oligonucleotide primers with products of 375- and 340-bp lengths, respectively (CIC-2: sense: 5’-CAA GCC TCC AGG AAG GTA C-3’ (bp 2306–2324), CIC-2 antisense: 5’-TCC CAA TGA GTC TGC-3’ (bp 707–727); GAPDH: sense: 5’-TCC GCC CCT TCC GCT GAT G-3’ (bp 388–406), GAPDH antisense: 5’-CAC GGA AGG CCA TGC CAG TGA-3’ (bp 707–727); primers were purchased from Life Technologies). The CIC-2 cDNA was amplified in the original RT tube by adding 48 µl of PCR reaction mixture to the remaining 8 µl cDNA. In parallel, GAPDH-PCR was started by adding 24 µl of PCR reaction mixture...
mixture to the 2-µl aliquots. In 24 µl of the reaction mixture were 20.6 µl double-distilled H₂O, 0.2 µl dNTPs (25 mM, Promega), 0.25 µl sense primer, 0.25 µl antisense primer (both 10 µM), 0.125 µl AmpliTaq gold polymerase (5 U/µl), and 2.5 µl 10-fold buffer (15 mM MgCl₂) (both from PerkinElmer, Weiterstadt, Germany). Samples were incubated in a MJ Research thermal cycler (DNA Engine PTC 200; Biozym, Oldendorf, Germany), first for 10 min at 95°C to activate AmpliTaq gold polymerase, followed by 50 cycles for 45 s at 94°C, 1 min at 56°C, 1 min at 72°C, and a final extension at 72°C for 7 min. Starting at cycle 31, the extension time was prolonged by 3 s every cycle to compensate for degradation of the polymerase. Twelve milliliters of each PCR reaction were separated by electrophoresis in a nondenaturating 5% acrylamide gel. PCR product bands were stained with a fluorescence dye (Vistra green; Amersham, Braunschweig, Germany) and visualized with ImageQuant Software on a Storm Fluorophosphorimager (both from Molecular Dynamics, Krefeld, Germany). A specific CIC-2 product was not detectable after the first round of PCR amplification. Therefore, the products were purified by QIA quick-spin columns (Qiagen, Hilden, Germany), eluted in 30 µl double-distilled H₂O, and reamplified (15-µl PCR product, 48-µl PCR reaction mixture, 40 cycles), using a nested primer pair with a product length of 326 bp [CIC-2 sensenested 5'-ATG GAA TCA GCA GGC ATT GC 3' (bp 2335–2354) and CIC-2 antisensenested 5'-CTG GTG ACA TAA GCA TGG TC 3' (bp 2641–2660) (LifeTechnologies)].

PCR-negative controls. Aliquots of bath solution aspirated in the patch pipette just above the cells were processed identically to the cytoplasmic samples and served as extracellular controls, which were performed after every second cytoplasm harvested. Aliquots of pipette and bath solution were treated identically. For every PCR run, a water control was made by adding water instead of cDNA to the PCR reaction mixture. These solution controls, as well as the PCR water controls, were consistently negative. Fifteen cytoplasmic samples were processed for RT− controls by incubating the RT step in the presence or absence of heat-inactivated reverse transcriptase enzyme.

PCR-positive controls. RNA from UB cultures was used after oligo-dT₁₂-₁₈-primed reverse transcription and serial dilution of the cDNA for selection of oligonucleotide primer with maximal sensitivity, optimal annealing temperature, Mg²⁺ concentration, and pH. The cDNA also served as positive control for the PCR reaction in the single cell experiments. RNA was isolated (see Ref. 8) from primary monolayer cultures of embryonic day 17 (E17) rat UB (12), using a commercially prepared phenol guanidine isothiocyanate reagent (TRISolve Reagent; Molecular Research Center, Cincinnati, OH).

Ultrastructure. For standard scanning electron microscopy, UBs were fixed at room temperature by modified Karnovsky reagent (2% paraformaldehyde and 2.5% glutaraldehyde in 80 mM phosphate buffer pH 7.4), postfixed by 1%OsO₄ (in 100 mM phosphate buffer, pH 7.4), dehydrated by increasing ethanol, and critical-point dried.

RESULTS

Ultrastructure. Scanning electron micrographs of microdissected UBs show the basolateral membrane of the cells at the tip (Fig. 2A). The basal lamina had been removed by dissection to provide free access for the patch pipette. Basolateral membranes of these tip cells, strikingly, have long cilia and are folded to short microvilli (Fig. 2B), as has been described previously for the apical membrane of cortical collecting duct (CCD) principal cells (9). The tip cells are small (~5 × 5 × 8 µm) and cubical to columnar in shape, and they enclose a small bud lumen (Fig. 4A).

Electrophysiology. Gigaohm seal formation between the basolateral membrane of tip cells and the patch pipette occurred in ~1 of 10 approaches. Whole cell membrane capacity was low (6.62 ± 0.67 pF; n = 35), due to the small cell size. Whole cell conductance, in contrast, was very high (13.8 ± 2.1 nS/10 pF; n = 8; calculated for the outward currents in physiological NaCl bath solution, Fig. 3, A and B). The reversal potentials (Vₐ) of KCl in the pipette of the current-voltage relations, recorded with sodium or the impermeant NMDG as principal cation in the bath, were both near their Cl equilibrium potentials [E_Cl = −5 mV (NaCl) and E_Cl = −2 mV (NMDG-Cl); Fig. 3B)], suggesting Cl selectivity for the major fraction of whole cell conductance. In both bath solutions, whole cell currents rectified outwardly and activated time dependently at positive (more than or equal to +40 mV) and inactivated at negative voltages (less than or equal to −40 mV; Fig. 3, A and B), indicating Cl selectivity at least for the inactivating inward currents. Time constants, as calculated in the eight cells recorded in physiological NaCl bath solution, were τ = 38 ± 9 ms for activation and τ = 20 ± 2 ms for inactivation.

A second whole cell current component, which activated slowly at strong hyperpolarizing voltages, was
seen in 20 of the 35 recorded cells (Fig. 3C). This slowly activating fraction \((\Delta I_{\text{activating}})\) of inward currents was identical in NaCl (5 cells) and NMDG-Cl bath solution (15 cells), indicating Cl selectivity (Fig. 3D). The time course of activation was \(t = 0.1 \pm 0.008\) s, as calculated in 10 cells, in which \(\Delta I_{\text{activating}} > 30\) pA. The general characteristics of this current fraction, i.e., its strong hyperpolarization-induced activation, resembled that of ClC-2 Cl channels (30). To confirm the mRNA expression of this channel type, ClC-2 mRNA was evaluated in single cells at the tUB by RT-PCR (see below).

At the single channel level, two channel types with low (28 and 26 pS) and intermediate (63 pS) conductances, respectively, were identified in two outside-out patches of the basolateral membrane (Fig. 3F). The (extrapolated) \(V_{\text{rev}}\) of their current voltage relations was near the K equilibrium potential, which suggested K selectivity (Fig. 3G).

Single cell RT-PCR. As a prerequisite to interpret the negative controls, the highly abundant (and presumably high-yield) template encoding the GAPDH gene was amplified from a 2-µl aliquot of the 10-µl RT product. After 50 PCR cycles, the 340-bp GAPDH sequence-specific product was identified in 23 out of 57 cytoplasmic samples (Fig. 4B). Cells in which the whole cell recording protocol was run yielded fewer GAPDH products (11 out of 35), compared with those where RT started immediately after rupturing of the cell membrane (12 out of 22 cells). Contamination by genomic DNA could be excluded by identical processing of the cytoplasm samples (Fig. 4C). Identical in NaCl (5 cells) and NMDG-Cl bath solution (60 ms from that at 380 ms. Data shown in D regrouped in those cells which were positive \((n = 4)\) or negative \((n = 16)\) for ClC-2 mRNA, as assessed by single cell RT-PCR. F: K-selective channels in the basolateral membrane of UB cells recorded in the outside-out mode. Original recording traces of 3 different channel types at different voltages (KCl pipette and NaCl bath solution). G: calculated current-voltage relations of 3 channels from 2 outside-out patches recorded as in E.

mRNA of the volume-regulatory Cl channel ClC-2 was assumed to be much less abundant compared with GAPDH in tUB cells. Therefore, a nested PCR strategy was employed to detect the ClC-2 message. After amplification by 50 PCR cycles, purification of the PCR product, and reamplification by 40 cycles, ClC-2 sequence-specific PCR products were observed in 8 out of 57 cytoplasmic samples. No product could be detected in any of the extracellular controls \((n = 25)\) or in the RT controls \((n = 15; \text{Fig. 4B})\).

Two PCR products were identified. Besides the expected 326-bp band seen in four samples, a 260-bp band was apparent in five experiments (in one sample, both products were amplified). The sequence of the longer PCR product was 99% identical to the published ClC-2 cDNA. In the shorter product, 63 bp (bp 2398–2460) were deleted by suspected alternative splicing, and the following CTG GCG (bp 2461–2466) was exchanged to
TCA GAA, resulting in a loss of 21 amino acids and an exchange of two AA at the cytoplasmic COOH terminus of the channel between the putative transmembrane domain D12 and region D13.

In an attempt to simultaneously determine whole cell recordings and then do single cell PCR on these same cells, cytoplasm was aspirated from 35 cells after recording. Four of the 35 cells yielded CIC-2 PCR products. All four of these CIC-2-positive cells exhibited $\Delta I_{\text{activating}}$ at $-100 \text{ mV}$ voltage. This current fraction, however, was not different from that of the 16 cells that expressed $\Delta I_{\text{activating}}$ but happened to be negative for CIC-2 mRNA (Fig. 3E).

**DISCUSSION**

This study presents for the first time a combined analysis by patch-clamp and single cell RT-PCR of isolated ureteric buds demonstrating CIC-2 mRNA expression in cells at the tUB. The tUB cell denotes a unique, complex, and transitory biological situation. 1) The cells maintain a high mitotic activity as long as branching morphogenesis (4) continues, i.e., until the distal end of the ureteric bud has fused with the mesenchyme-derived nephron, most likely in the region of the connecting tubule. 2) tUB cells represent the least differentiated progeny during branching morphogenesis. The tUB ("ampullary") cells in the rabbit have been shown to express the $\alpha$-subunit of the Na-K-ATPase in the entire plasma membrane, indicating that the pump is not yet distributed in the polar pattern characteristic for differentiated epithelial cells (21). 3) tUB cells face the cap of condensed mesenchymal cells across a small interspace (17), and they most likely produce two distinct signals to induce nephrogenesis (3). Moreover, the tUB cell itself is the target for mesenchyme-derived ligands of receptor tyrosine kinases (28).

In the present study, cells from the very tip of the ureteric bud were analyzed to ultimately compare this cell population with those located in other segments of the branching ureteric tree. The electrophysiological characterization of tUB revealed that the main fractional whole cell current rectified outwardly, and it is activated at depolarizing and inactivated at hyperpolarizing voltages (Fig. 3A). Conductance properties of rat UB cells (day E17) have been studied previously in monolayer cultures derived from microdissected UB (14). Whole cell currents of the tUB cells are identical to those of the cultured cells. The outwardly rectifying, depolarization-activated main current fraction of the latter has been demonstrated to be Cl selective (14) and resembles a Ca-activated type by its voltage and time dependence, which is typical for nonepithelial cells or for epithelia grown in monolayer on impermeable support but not for differentiated, i.e., highly polarized cells (1). Microdissected tUB cells, therefore, expressed a nonpolarized phenotype, as judged by their conductive properties. This nonpolarized phenotype is gradually downregulated with developmental differentiation of the early postnatal principal cell, whereas the epithelial phenotype is increasingly expressed by acquisition of the typical pattern of apical ion channels (13, 14). Taken together, these data suggest a specifically embryonic role for the outwardly rectifying, depolarization-activated conductance in tUB cells.

K channels with low and intermediate conductance were identified in two outside-out patches of tUB cells. Interestingly, their conductance resembled the basolateral K channels of mature CCD principal cells (11), suggesting that essential membrane proteins in the basolateral membrane are expressed earlier in epitheliogenesis than those in the apical membrane (13).

Another minor fractional whole cell current was measured in 20 out of 35 tUB cells. This current fraction, which activated slowly at strong hyperpolarizing voltages (Fig. 3C), was Cl selective, and, by these properties, it resembled a CIC-2-generated current expressed in oocytes (30) (except for the fact that activation kinetics of CIC-2 expressed in oocytes appear to be lower by more than a factor of 20). Importantly,
activation kinetics of the slowly activating inward currents in tUB cells were quite similar to those of endogenous CIC-2-like whole cell currents in other epithelial cells (2, 7, 18). CIC-2 Cl channels were suggested to play an important role in lung development (22), and it is of interest that CIC-2 mRNA is differentially expressed early in collecting duct embryogenesis (12), as demonstrated in primary monolayer cultures of UB cells by semiquantitative RT-PCR.

Considering the data on single cell mRNA of the present work, it must be mentioned that the single cell RT-PCR technique (20) includes several steps, each with the potential for multiple errors. The consistently successful application of the method, therefore, requires a set of positive and negative controls that verify the variability and provide an estimate of relative efficiency. False-positive PCR amplifications were ruled out by special precautions (19). All solutions were negatively screened for template contamination by RT and PCR amplification. The cytoplasmic origin of a PCR product was proven by negative extracellular controls (see METHODS). The amplification of genomic PCR product was proven by negative extracellular and PCR amplification. The cytoplasmic origin of a negatively screened for template contamination by RT efficiency. False-positive PCR amplifications were ruled

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