TRPC3 and TRPC4 Associate to Form a Redox-sensitive Cation Channel

EVIDENCE FOR EXPRESSION OF NATIVE TRPC3-TRPC4 HETEROMERIC CHANNELS IN ENDOThelial CELLS

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Canonical transient receptor potential proteins (TRPC) have been proposed to form homo- or heteromeric cation channels in a variety of tissues, including the vascular endothelium. Assembly of TRPC multimers is incompletely understood. In particular, heteromeric assembly of distantly related TRPC isoforms is still a controversial issue. Because we have previously suggested TRPC proteins as the basis of the redox-activated cation conductance of porcine aortic endothelial cells (PAECs), we set out to analyze the TRPC subunit composition of endogenous endothelial TRPC channels and report here on a redox-sensitive TRPC3-TRPC4 channel complex. The ability of TRPC3 and TRPC4 proteins to associate and to form a cation-conducting pore complex was supported by four lines of evidence as follows: 1) Co-immunoprecipitation experiments in PAECs and in HEK293 cells demonstrated the association of TRPC3 and TRPC4 in the same complex. 2) Fluorescence resonance energy transfer analysis demonstrated TRPC3-TRPC4 association, involving close proximity between the N terminus of TRPC4 and the C terminus of TRPC3 subunits. 3) Co-expression of TRPC3 and TRPC4 in HEK293 cells generated a channel that displayed distinct biophysical and regulatory properties. 4) Expression of dominant-negative TRPC4 proteins suppressed TRPC3-related channel activity in the HEK293 expression system and in native endothelial cells. Specifically, an extracellularly hemagglutinin (HA)-tagged TRPC4 mutant, which is sensitive to blockade by anti-HA-antibody, was found to transfer anti-HA sensitivity to both TRPC3-related currents in the HEK293 expression system and the redox-sensitive cation conductance of PAECs. We propose TRPC3 and TRPC4 as subunits of native endothelial cation channels that are governed by the cellular redox state.

The TRPC3 subfamily consists of seven isoforms of which several are typically co-expressed in most cell types studied so far, including vascular endothelial cells (1–5). These TRPC proteins assemble to form multimeric pore structures, which enable cation transport across the plasma membrane (6). Multimerization of TRPC species has emerged as a complex issue, because both homo- and heteromeric assemblies appear possible and up to three isoforms may contribute to formation of native pore complexes (7).

TRPC channel activation was found to be triggered by a variety of stimuli, most prominently phospholipase C-derived signals, including classic Ca2+ store depletion as well as mechanical forces and redox processes (8–15). We have previously demonstrated that TRPC3 is involved in a cation conductance that is activated in response to oxidative stress in porcine aortic endothelial cells (PAECs) (14). TRPC channels may sense redox stress due to oxidation-induced changes in the membrane lipid composition that has been recognized as an important regulator of TRPC channel function (16). Accordingly, we identified cholesterol oxidase-induced modification of membrane lipid composition and lipid raft structure, as an effective trigger for activation of TRPC3 channels in HEK293 cells (15). Moreover, a TRPC isoform may exist as a homo- and heteromeric channel complex in a given cell type, and multimeric assembly of TRPC complexes may depend on cellular localization and on the availability of specific interacting protein partners (17). Co-immunoprecipitation and FRET microscopy studies suggested that TRPC3 may form not only homomeric channels but also heteromultimeric pore structures with its close relatives, TRPC6 and TRPC5 (18). Although these investigations excluded heteromers that consist of distant relatives of the TRPC family, other studies provided evidence for heteromerization of TRPC3 with its more distant relative TRPC1 (19, 20) and of TRPC1 with TRPC5 or TRPC4 (7, 21). Moreover, even the presence of a heteromer consisting of TRPC1, TRPC3, and TRPC5 has been proposed (7). For the vascular endothelium the exact subunit composition of native TRPC channels is so far elusive. Here we report evidence for the existence of an endothelial cation channel that represents a TRPC3/C4 heteromer and is sensitive to oxidative modification of plasma membrane cholesterol.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfections—Endothelial cells were isolated from porcine aorta as described previously (14) and cultured in Dulbecco’s modified Eagle’s medium (Sigma-Aldrich) containing 10% heat-inactivated fetal calf serum, 100 units/ml penicillin, 0.1 mg/ml streptomycin, and 1.25 µg/ml amphotericin B. Only the first passage of cells was used for experiments. Cells were grown to ~80% confluence in either 100-mm Petri dishes (6 × 106 cells/dish), for biotinylation and immunoblotting experiments, or in 6-well plates (~106 cells/well) for meas-
urement of membrane currents. Human embryonic kidney (HEK293) cells stably expressing C-terminal HA-tagged human TRPC3 or the wild-type mouse TRPC4α, designated as T3-9 or T4–60 cells (22), respectively, were established and maintained as previously described (13). An electrophysiologically tagged TRPC3 (exoHA-TRPC3) construct was made by inserting a sequence encoding YPYDVPDYA before the coding for Y524A525 of human TRPC3, thus creating a complete HA epitope YPYDVPDYA in the putative second extracellular loop of the channel subunit. Similarly, an exoHA-TRPC4α construct was created by inserting codons for PYDVPDYA after that of Tyr-458 of the wild-type TRPC4α. The N-terminal fragment N292-TRPC4, including two ankyrin-like repeats and the coiled-coil region of the mouse TRPC4β isoform, was obtained by internal restriction site Rsal and the cloning site KpnI. This fragment was subcloned in pTracer-CMV-II for electrophysiology and in pEY/CFP-C1 for both electrophysiology and FRET measurements. T3-9 cells were transiently co-transfected with exoHA-TRPC3 or exoHA-TRPC4 and YFP with Transfast™ Transfection Reagent (Promega Corp., Mannheim, Germany) according to the manufacturer’s instructions. Some experiments were performed with wild-type HEK293 cells, which were cultured in the absence of Geneticin. PAECs were transiently co-transfected with exoHA-tagged TRPC species and YFP as a transfection marker using FuGENE 6 transfection reagent (Roche Applied Science) according to the manufacturer’s instructions. For FRET and competition FRET experiments, CFP and YFP fusions of TRPC proteins were transiently co-transfected into wild-type HEK293 cells or in cells stably expressing TRPC3 or TRPC4 (T3-9 or T4-60 cells), respectively.

Biotinylation of Cell-surface Membrane Proteins—PAECs were washed three times with ice-cold PBS containing 1 mM MgCl₂, 0.8 mM CaCl₂ (DPBS, pH 8) and incubated on ice for 30 min with 1 mg/ml EZ-Link Sulfo-NHS-SS-Biotin (Pierce) in DPBS. The biotinylation reaction was terminated by washing the cells three times with ice-cold DPBS containing 100 mM glycine. Cells were then washed twice with ice-cold PBS, scraped off from the dishes in PBS, and centrifuged at 4°C. The pellets were re-suspended in 0.5 ml of ice-cold lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 60 mM n-octyl-β-D-glucopyranoside, 1 mM phenylmethylsulfonyl fluoride, and the protease inhibitor mixture (Complete™, Roche Applied Science, Basel, Switzerland)) and incubated for 15 min on ice. The cell lysates were sonicated and centrifuged at 10,000 x g for 10 min at 4°C to remove cell debris. Biotinylated proteins were isolated by adding cleared cell lysate (500 µg) to 60 µl of streptavidin beads (Pierce) in a total volume of 0.5 ml of lysis buffer. The samples were gently rotated end over end overnight at 4°C. The beads were recovered by centrifugation for 5 min at 2,000 x g, and the pellets (representing surface proteins) were washed three times with ice-cold lysis buffer. The biotinylated cell-surface proteins were eluted from the streptavidin-agarose beads in 60 µl of 2× Laemmli buffer, incubated at 60°C for 30 min, and subjected to SDS-PAGE.

Immunoprecipitation and Western Blot—The cell lysates from PAECs and T3-9 transiently transfected with YFP-TRPC4, obtained as described above, were sonicated and centrifuged at 10,000 x g for 10 min at 4°C to remove cell debris. Nonspecific bound proteins were removed by preincubation of supernatants (500 µg) with 50 µl of protein A-Sepharose beads (Sigma-Aldrich) and subsequent gently rotated for 1 h at 4°C. Preclearing supernatants were incubated with 3 µg of antibody either against TRPC3, against TRPC4, or against HA overnight at 4°C. Subsequently, 50 µl of protein A-Sepharose beads was added to the immune complex for 3 h at 4°C. The beads were pelleted, washed three times with ice-cold lysis buffer, resuspended in 50 µl of 2× Laemmli buffer, and heated to 95°C for 5 min. Proteins from cell lysates, biotinylated samples, and immunocomplexes were separated by 8% SDS-PAGE and transferred to nitrocellulose sheets. Nitrocellulose membranes were incubated with PBS containing 5% Bio-Rad blocking reagent overnight at 4°C and then incubated with polyclonal antibodies against TRPC4 (1:200, Santa Cruz Biotechnology, Santa Cruz, CA), against TRPC3 (produced in our laboratory), or against GFP (1:1,000, Roche Applied Science) for 2 h at room temperature. After washing with PBS, membranes were incubated with a horseradish peroxidase-conjugated anti-rabbit secondary antibody (1:5,000, 1 h). Membranes were detected via the ChemiGlow™ West detection system and developed using a Herolab RH-5.2 Darkroom Hood, equipped with an E.A.S.Y. 1.3 HC camera (Herolab GmbH, Wiesloch, Germany).

Measurement of Membrane Currents—Patch pipettes were pulled from borosilicate glass (Clark, Electromedical Instruments, Reading, England) and had a resistance of either 3–5 MΩ (whole cell recording) or 8–10 MΩ (single channel recording). Patch pipettes were filled either with a solution containing 120 mM cesium methanesulfonate, 20 mM CsCl, 10 mM HEPES, 3 mM MgCl₂, 1 mM EGTA for whole cell experiments or 137 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 2 mM MgCl₂, and 10 mM HEPES for cell-attached recording experiments. In whole cell recordings, cells were perfused with a solution containing either 137 mM NaCl, 5.4 mM KCl, 10 mM HEPES, 1 mM MgCl₂ (nominally Ca²+ free) or a buffer, additionally including 2 mM CaCl₂ (plus Ca²+). In single channel (cell-attached) experiments, the bath solution contained 145 mM potassium gluconate, 5.3 mM KCl, 3 mM MgCl₂, and 15 mM HEPES. The pH of all solutions was adjusted to 7.4. Cells were stimulated with either 200 mM carbacbol (CCh), 100 µM 1-octyl-2-acetyl-sn-glycerol (OAG) or 0.5 unit/ml cholesteral oxidase. For some experiments cells were incubated with a HA-antibody (for 1 h, 1:200 in bath solution). Currents were recorded using a List EPC7 patch clamp amplifier (List-Medical-Electronic, Darmstadt, Germany). Single channel currents were filtered at 1 kHz (low pass, ~3 dB), digitized at a sampling rate of 5 kHz, and analyzed with pClamp9 software (Axon Instruments, Foster City, CA). Voltage clamp protocols (voltage ramps from ~80 to +50 mV or ~100 to +80 mV/0.6 V/s, 0.2 Hz, holding potential ~70 mV) were controlled by pClamp9 software. Experiments were performed at room temperature.

Fluorescence Resonance Energy Transfer—Transfected HEK293 cells grown on coverslips for 2 days were transferred to an extracellular solution identical to the bath solution used in functional experiments (see above). A MultiSpec Imager (Visitron Systems GmbH, Puchheim, Germany) was used for separating FRET images and was connected to a Photometrics CoolSnap fx-HQ monochrome camera (Roper Scientific, Tucson, AZ). This system was attached to an Axivert 200M microscope (Zeiss, Jena, Germany) in conjunction with a VisiChrome high speed polychromator (Visitron Systems). Used filter sets (Chroma Technology Corp., Rockingham, VT) included a 485 cyan emission filter set (MultiSpec) and a 535 yellow emission filter set. MetaVue software (Universal Imaging Corp., Downingtown, PA) was used to acquire images and to control the system. Images were analyzed using MetaMorph (Universal Imaging Corp.)

The FRET image must be corrected due to cross-talk from one channel to other. For this, the corrected FRET image (nFRET) was calculated after background subtraction using the sensitized emission correction method according to nFRET = rawFRET – corrD1 donor corrA1 acceptor. The calibration factors, representing relative cross-talk fluorescence between the donor and acceptor channels, were obtained from experiments where only CFP or YFP was expressed (corrD = 0.4, corrA = 0.1). For statistical analysis, the mean cellular nFRET intensity in 5–10 cells was determined after background correction.
Statistical Analysis—Results are expressed as mean value ± S.E. Differences were considered statistically significant at \( p < 0.05 \) using Student’s \( t \) test for unpaired values.

RESULTS

Oxidative Stress Activates a Voltage-insensitive, Non-selective Cation Channel in PAECs and in T3-9 Cells—TRPC3 was previously found to contribute to the oxidative stress-induced conductance of PAECs (14). As a further step to analyze the role of TRPC3 as a redox sensor, we compared responses of PAECs and HEK293 cells stably expressing TRPC3 (T3-9) to oxidative stress. Plasma membrane-delimited redox stress was introduced by incubation with cholesterol oxidase (0.5 unit/ml, 45 min). As shown in Fig. 1 \( \text{A and B} \), both cell types displayed a large increase in the membrane conductance in response to cholesterol oxidase. Fairly linear current to voltage relations and reversal potentials of \(-25\text{ mV}\) and \(-10\text{ mV}\) were recorded in PAECs and T3-9 cells, respectively. The observed ability of TRPC3 to generate a redox-sensitive cation conductance upon overexpression supported the view of TRPC3 as a major component of the native redox-sensitive cation channel. A comparison of the redox sensitivity determined for wild-type HEK293 cells and T3-9 cells, using both the membrane permeable oxidant tert-butylhydroperoxide (t-BHP) and cholesterol oxidase to introduce redox stress, is illustrated in Fig. 1 \( \text{C} \). The results demonstrate that TRPC3-overexpressing cells (T3-9) displayed an oxidant stress-induced membrane conductance that was virtually lacking in wild-type cells, suggesting that cation channels formed by TRPC3 in HEK293 cells, which are sensitive to oxidative stress. Notably, the TRPC3-dependent redox-activated membrane conductances displayed a strikingly linear current to voltage relation, which is not perfectly in line with the typical characteristics of classic phospholipase C-regulated TRPC3 conductance observed in HEK293 cells.

Endogenous TRPC3 and TRPC4 Associate in PAECs to Form a Plasma Membrane-targeted Complex—To further analyze the composition of native, redox-sensitive TRPC channels in PAECs, we performed biotinylation and co-immunoprecipitation experiments to screen for plasma membrane-expressed TRPC species and for possible heteromerization. 
In initial immunoblotting experiments immunoreactivity of TRPC1, TRPC3, and TRPC4 and, albeit at a low level, of TRPC6 was detected in PAEC homogenates (data not shown). However, in cell-surface biotinylation experiments only TRPC3 and TRPC4 were detectable as plasma membrane-targeted TRPC species (Fig. 2A). The presence of both TRPC3 and TRPC4 in the plasma membrane of PAECs prompted us to investigate a possible heteromeric assembly of these TRPC species by immunoprecipitation experiments. As shown in Fig. 2B, TRPC4 was detectable in anti-TRPC3 precipitates and vice versa. These data suggest the existence of a TRPC3-TRPC4 heteromer, representing a potential redox-sensitive channel complex in PAECs.

**Association of TRPC3 and TRPC4 Proteins in the HEK293 Expression System**—As a next step to test the ability of TRPC3 and TRPC4 proteins to interact physically, we performed immunoprecipitation experiments with T3-9 cells that were additionally transfected to express YFP-tagged TRPC4. As illustrated in Fig. 3A, immunoprecipitation of the C-terminally HA-tagged TRPC3 from T3-9 homogenates using anti-HA clearly recovered YFP immunoreactivity, displaying the expected size for the YFP-TRPC4 fusion (~130 kDa). Similarly, anti-TRPC3 immunoprecipitates derived from T3-9 cells transfected to express TRPC4 recovered significant TRPC4 immunoreactivity (data not shown), whereas the anti-TRPC3 failed to pull down TRPC4 in the T4–60 cell line, which lacks significant TRPC3 expression (not shown). Controls for specificity of the anti-TRPC4 antibody were performed in both HEK293 wild-type and T3-9 cells and confirmed that proteins with a molecular mass corresponding to that of TRPC4 are not detectable with these antibodies in the HEK293 system (not shown).

In an attempt to further test and analyze TRPC3-TRPC4 association, we used expression of CFP and YFP fusion proteins of TRPC3 and TRPC4 proteins to perform FRET analysis of this interaction. Because a previous study, which tested for TRPC3-TRPC4 interactions, did not detect any FRET signal using C-terminal fusion proteins (17), we tested whether C-N type interactions are detectable. Fig. 3B illustrates the results of FRET microscopy in cells that expressed CFP/YFP fusion proteins in which the fluorophors were linked to the N and C termini of the species, respectively, as outlined in the scheme shown below the micrographs. We observed a significant FRET signal that was localized in the plasma membrane region of the cells (Fig. 3B, upper panel). Moreover, competition FRET experiments were performed to test for the ability of TRPC4 to disrupt the relatively robust homomeric interaction within TRPC3 complexes. As illustrated in Fig. 3B (middle panel), co-expression of YFP- and CFP-tagged TRPC3 fusion resulted in substantial FRET, which was markedly suppressed when TRPC4 was expressed in addition to the TRPC3 FRET partners (Fig. 3, B, lower panel, and C). These results demonstrate interaction of full-length TRPC3 and TRPC4 proteins in the HEK293 expression system.

**The PLC- and Redox-activated TRPC3 Cation Conductances Recorded in the HEK293 Expression System Are Inhibited by Dominant Negative TRPC4 Mutants**—The concept of heteromeric interaction implies that dominant negative mutants of one specie are likely to suppress the channel function generated by the other TRPC species. We therefore set out to study the effect of dominant negative TRPC4 constructs on TRPC3 currents. Two different approaches were employed to test for sensitivity of TRPC3 to dominant negative suppression by TRPC4 proteins. As we have previously found that N-terminal fragments of TRP proteins are highly effective as dominant negative constructs (2, 14), we co-expressed full-length TRPC3 with an N-terminal fragment (1–292 amino acids) of TRPC4. Expression of this N-terminal TRPC4 fragment significantly suppressed the carbachol-activated TRPC3 channel currents typically recorded in the HEK293 expression system (Fig. 4A). In a second approach, we co-expressed TRPC3 with an exoHA-tagged TRPC4(α) protein, which by itself, did not inhibit the CCh-induced conductance mediated by TRPC3. Nonetheless, this construct introduced a significant sensitivity of the CCh-activated TRPC3 channel activity to block by anti-HA antibody. As illustrated in Fig. 4B, TRPC3-related currents in T3-9 cells were virtually lacking in exoHA-TRPC4-
expressing cells incubated with anti-HA antibody. Thus, we observed generation of anti-HA sensitivity of a TRPC3-related conductance by co-expression of an exoHA-TRPC4 construct indicating the ability of the TRPC4 construct to form a pore complex with TRPC3. Similarly, expression of exoHA-TRPC4 generated a significant anti-HA sensitivity of the cholesterol oxidase-induced membrane currents in TRPC3-expressing (T3-9) cells, and in the absence and presence of anti-HA antibody (1:200, 30 min). Current to voltage relations were derived by voltage ramp protocols. D, mean values of current densities measured at -70 mV in controls and in cells challenged with cholesterol oxidase (0.5 unit/ml) in the absence and presence of anti-HA antibody are shown as well as control experiments in sham-transfected T3-9 cells. Mean values ± S.E. (n = 8 - 10) are shown. Asterisks indicate statistically significant difference versus control. The rightmost bar shows cholesterol oxidase-induced response in sham-transfected T3-9 cells in the presence of anti-HA antibody.

**FIGURE 5.** Suppression of the endogenous redox-sensitive cation conductance of PAECs by dominant negative TRPC3 and TRPC4 mutants. A, mean values ± S.E. of cholesterol oxidase-induced membrane conductances in PAECs expressing exoHA-TRPC3 or TRPC4 in the presence or absence of anti-HA-antibody (1:200, 30 min). Asterisks indicate significant difference versus control cells (absence of antibody). B, current to voltage relations of basal (control) and cholesterol oxidase-induced currents measured in PAECs transiently transfected with exoHA-tagged TRPC4 and preincubated with cholesterol oxidase (0.5 unit/ml, 45 min) in the presence or absence of anti-HA-antibody.

The Redox-activated Cation Conductance of PAECs Is Inhibited by Dominant Negative Exo-tagged TRPC3 and TRPC4 Mutants—The involvement of both TRPC3 and TRPC4 in the cholesterol oxidase-activated conductance of PAECs was finally tested by patch-clamp experiments in PAECs, transiently transfected to express either exoHA-TRPC3 or TRPC4.
TRPC3 or exoHA-TRPC4. As shown in Fig. 5A, anti-HA antibody incubation of PAECs transfected to express either exoHA-tagged TRPC species significantly suppressed the redox stress-induced membrane current (Fig. 5A). Fig. 5B shows representative current to voltage relationships derived by voltage ramp protocols in cholesterol oxidase-treated PAECs, which were transiently transfected with exoHA-tagged TRPC4. Expression of exoHA-tagged TRPC species by itself did not prevent the redox-activated conductance but generated anti-HA sensitivity similar to that observed in T3-9 cells (Fig. 4C). These results further corroborated the concept of TRPC3/C4 heteromerization in native endothelial cells and indicate that TRPC3/C4 heteromeric cation channel serves as a sensor for the cellular redox state.

Comparison of Single Channel Properties—The existence of distinct, redox-sensitive pore complexes composed of TRPC3 and TRPC4 subunits was further tested at the single channel level. Fig. 6A illustrates the prominent unitary conductances activated by cholesterol-oxidase in TRPC3- and TRPC3+TRPC4-expressing HEK293 cells as well as in native endothelial cells (PAECs). All conductances reversed close to 0 mV (Fig. 6B), and the gating behavior of the observed channels was highly complex in all three cell systems displaying occasional fast, flickering behavior as well as subconductance levels. The prominent channel observed in T3-9 cells exhibited a chord conductance of $68 \pm 7$ pS ($n = 3$) at 0 mV. This channel type was also activated in response to stimulation of the cells with CCh (200 $\mu$M, $n = 2$, not shown). In T3-9 cells transfected to express TRPC4 in addition to TRPC3, the prominent unitary conductance activated by cholesterol oxidation was significantly smaller $44 \pm 2$ pS ($n = 4$). It is of note, that one cell-attached patch from a cell co-expressing TRPC3-TRPC4 contained a channel of larger conductance ($80$ pS, data not shown). As the unitary conductance of the prominent channel present in TRPC3-TRPC4-expressing cells was close to that reported for TRPC4 channels ($30$ pS (23)), we investigated additional regulatory properties of this channel. Challenge of TRPC3-TRPC4-expressing cells with OAG (100 $\mu$M), which has been reported to activate TRPC3 but not TRPC4 channels (8), induced a dramatic increase in the activity of the 44-pS channel observed in TRPC3-TRPC4-co-expressing cells ($n = 3$, Fig. 6C), substantiating that TRPC3-TRPC4-co-expressing cells display channel activity that is distinctly different from that of HEK293 cells expressing TRPC3 or TRPC4 only. The prominent cation channels observed in cholesterol oxidase-treated PAECs exhibited a unitary conductance that was slightly larger ($81 \pm 3$ pS, $n = 4$, Fig. 6, A and B) than that calculated for TRPC3

![FIGURE 6. Single channel properties of TRPC3-TRPC4 heteromers. A, typical single channel activity recorded at $+30$ mV and $-30$ mV in cell-attached patches of TRPC3-expressing HEK cells (T3-9, left), HEK293 cells expressing TRPC3 plus TRPC4 (middle) and PAECs (right) subsequently to incubation with cholesterol oxidase (45 min, 0.5 unit/ml). Arrows indicate closed state. B, unitary current to voltage relation corresponding to the experiments shown in A, for TRPC3 expressing HEK cells (closed triangles), TRPC3 plus TRPC4-expressing cells (closed circles) and PAECs (open circles). Unitary conductances of 68 pS (TRPC3), 44 pS TRPC3-TRPC4 co-expression, and 81 pS (PAECs) have been calculated. C, response of 44-pS TRPC3-TRPC4 channels in a cell-attached patch held at $-30$ mV to OAG (100 $\mu$M). Upper panel: time course of channel activity. Arrow indicates closed state. Lower panel: amplitude histograms derived from 20 s recordings in the absence (control) and presence of OAG. Closed (c) and first open level ($o_1$) are indicated.]

Redox-sensitive TRPC3-TRPC4 Heteromers
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channels. Activity of this channel typically occurred in bursts displaying fast flickering behavior (Fig. 6A).

These results from single channel recordings demonstrate that co-expression of TRPC3 and TRPC4 in HEK293 cells produces a unique pore structure, clearly different from homomeric TRPC3 or TRPC4 channels. Generation of heteromeric TRPC3-TRPC4 channels in the HEK293 expression system, however, failed to perfectly reconstitute the native redox-sensitive cation conductance of endothelial cells.

**DISCUSSION**

With the present study, we provide evidence for heteromeric assembly of TRPC3 and TRPC4 and indicate that such TRPC3/C4 heteromers are expressed endogenously in PAECs to serve as redox-sensitive channel proteins. Our results support the previously proposed involvement of TRPC3 in the redox-sensitive membrane conductance of PAECs (14, 15).

To investigate redox sensitivity of membrane conductances, cholesterol oxidase incubation was employed as an experimental model that allows for rather selective oxidative modifications of membrane lipids, generating membrane-delimited oxidative stress with less perturbation of overall cellular function as compared with direct administration of peroxides (15). Incubation of cells with cholesterol oxidase was found to induce a non-selective cation conductance in both native endothelial cells and TRPC3-overexpressing HEK293 cells (T3-9 cells). The membrane responses induced by cholesterol oxidase incubation were similar to those obtained with the lipophilic peroxide t-BHP, which was used in previous studies aimed at the characterization of redox-sensitive membrane conductances (24). Cholesterol oxidase activated cation conductances in both native endothelial cells (PAECs) as well as in TRPC3-expressing cells. These currents were similar but not perfectly identical in terms of their biophysical properties, because reversal potentials were slightly more negative in native endothelial cells. Moreover, the currents did not perfectly resemble those typically observed upon phospholipase C activation in TRPC3-expressing HEK293 cells (25). This finding was considered to arise either from overlap of additional redox-sensitive conductances in PAECs or from a different composition of the redox-sensitive pore complexes. Nonetheless, both t-BHP and cholesterol oxidase activated a cation conductance in cells expressing TRPC3 but not in wild-type HEK293 cells, substantiating the potential role of TRPC3 as a component of redox-sensitive cation channels.

The previously proposed concept of TRPC3 as a redox sensor in PAECs (14) was in part based on the observed expression of TRPC3 mRNA in these native endothelial cells. In the present study, we confirmed TRPC3 expression at the protein level and demonstrated that this TRPC species is indeed targeted to the plasma membrane of these endothelial cells. As native TRPC channels are considered to involve heteromultimerization, we screened for plasma membrane expression of potential heteromerization partners of TRPC3. However, only TRPC4, a related protein that has so far not been identified as typical heteromerization partner of TRPC3 (18, 26), was detected in the plasma membrane of PAECs. Nonetheless, Strübing et al. (7) demonstrated the general possibility of the formation of heteromeric channel structures composed of TRPC3 and TRPC4 when TRPC1 was present as the third channel subunit. Our immunoprecipitation experiments in PAECs indicated that endogenous TRPC3 and TRPC4 are able to associate in the membrane of PAECs. Consistent with the results of Strübing et al. (7), we found indeed TRPC1 expression and evidence for a weak interaction between TRPC3 and TRPC1. However, plasma membrane expression of TRPC1 was not detectable in biotinylation experiments. Thus, the role of TRPC1 in the formation of heteromeric TRPC channels in PAECs remains to be clarified. Because TRPC3 and TRPC4 interactions have been excluded by two previous studies using heterologous overexpression combined with immunoprecipitation and FRET experiments (18, 26), we performed several further experimental approaches to test for the existence of pore forming TRPC3/C4 complexes.

The association of TRPC3 with TRPC4 proteins in HEK293 cells was further confirmed by immunoprecipitation experiments and FRET microscopy. Importantly, we detected energy transfer between CFP- and YFP-tagged TRPC3 and TRPC4 proteins when the fluorescence tags were linked to the N- and C-terminal ends of the TRPC4 and TRPC3 protein, respectively. Protein interactions were not detectable when fluorescence probes were linked to only the N or C termini of the interaction partners. These results indicate a high level of structural requirements regarding localization of the fluorophors for the detection of TRPC3/C4 association, which may explain some of the previous negative results obtained with FRET analysis. Our results indicate a close proximity of N and C termini within the TRPC3-TRPC4 complexes. TRPC3-TRPC4 interaction was further confirmed by the ability of wild-type TRPC4 to significantly reduce the homomultimerization between the CFP- and YFP-tagged TRPC3.

Heteromerization of TRPC3 and TRPC4 in terms of formation of a distinct pore structure was further tested in single channel recording experiments, which clearly demonstrated that co-expression of TRPC3 and TRPC4 gives rise to cation channel activity of unique properties. The observed 44 pS, redox-regulated channel was found sensitive to activation by OAG, thereby representing a cation conductance clearly different from the so far described TRPC3 and TRPC4 unitary conductances generated by overexpression of individual channel proteins (23, 27).

As the existence of TRPC3-TRPC4 pore complexes implies mutual dominant negative effects, we investigated the dominant negative effects of TRPC4 constructs on TRPC3-mediated membrane currents in HEK293 cells and in PAECs. Our results clearly demonstrated that both an N-terminal fragment of TRPC4 as well as an antibody-sensitive full-length TRPC4 mutant is able to eliminate TRPC3-related currents in T3-9 cells and PAECs. Thereby, we demonstrated that TRPC mutants, containing a HA epitope in the second extracellular loop, exert a dominant negative inhibition on TRPC3-mediated conductances. This dominant negative effect is “conditional” in terms that it requires the presence of an additional experimental condition, i.e. incubation with anti-HA antibody. Interestingly, co-expression of the full-length, exoHA-tagged TRPC4 construct with TRPC3 in HEK293 cells reconstituted the currents observed in native PAECs fairly well. Moreover, the oxidant-induced current responses were completely blocked in these cells by anti-HA incubation, indicating that this TRPC4 specie is effectively incorporated into the TRPC3 channel complexes. In line with the hypothesis of TRPC3 and TRPC4 association within a redox-sensitive channel complex in PAECs, we found that either expression of exoHA-TRPC3 or exoHA-TRPC4 was equally effective in terms of the antibody-induced dominant-negative suppression of the redox-sensitive current in PAECs. Nonetheless, characterization of the cholesterol oxidase-activated cation channels in PAECs revealed differences regarding unitary conductance and gating behavior between the endothelial redox-sensitive channel and the prominent channel observed in TRPC3-TRPC4-expressing HEK293 cells. The basis of the observed differences in single channel properties is unclear at this point. Nonetheless, it is tempting to speculate about the existence of TRPC3-TRPC4 heteromers with a different stoichiometry, which may explain the discrepancies between native cells and the heterologous expression system. Interestingly, we observed indeed in one patch of a TRPC3-
TRPC4-expressing cell a larger conductance of ~80 pS along with the predominant 44 pS channel, an observation that might be interpreted in terms of generation of a second heteromultimer.

It appears important to note that the redox-sensitive channel observed in porcine aortic endothelium displays properties distinctly different from a previously reported redox-sensitive cation conductance observed in endothelial cells of calf aorta (28), suggesting molecular heterogeneity of endothelial redox-regulated cation channels.

In conclusion, we provide the first evidence for cation channels comprised of both TRPC3 and TRPC4 subunits. We suggest that a TRPC3-TRPC4 heteromeric channel is expressed in porcine aortic endothelial cells and represents a possible candidate responsible for oxidant-induced functional changes in the endothelium and, therefore, may be considered as a potential therapeutic target for novel cardiovascular drug therapies.

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REFERENCES

1. Chang, A. S., Chang, S. M., Garcia, R. L., and Schilling, W. P. (1997) FEBS Lett. 415, 335–340
2. Groschner, K., Hingel, S., Lintschinger, B., Balzer, M., Romanin, C., Zhu, X., and Schreibmayer, W. (1998) FEBS Lett. 437, 101–106
3. Moore, T. M., Brough, G. H., Babal, P., Kelly, J. J., Li, M., and Stevens, T. (1998) Am. J. Physiol. 275, L574–L582
4. Tiruppathi, C., Freichel, M., Vogel, S. M., Paria, B. C., Mehta, D., Flockerzi, V., and Malik, A. B. (2002) Circ. Res. 91, 70–76
5. Kohler, R., Brakemeier, S., Kuhn, M., Degenhardt, C., Buhr, H., Pries, A., and Hoyer, J. (2001) Cardiovasc Res. 51, 160–168
6. Vazquez, G., Wedel, B. J., Aziz, O., Trebak, M., and Putney, J. W., Jr. (2004) Biochim. Biophys. Acta 1742, 21–36
7. Strubing, C., Krapivinsky, G., Krapivinsky, L., and Clapham, D. E. (2003) J. Biol. Chem. 278, 39014–39019
8. Hofmann, T., Obukhov, A. G., Schaefer, M., Harteneck, C., Guedermann, T., and Schultz, G. (1999) Nature 397, 259–263
9. Trebak, M., Bird, G. S., McKay, R. R., and Putney, J. W., Jr. (2002) J. Biol. Chem. 277, 21617–21623
10. Zhang, Z., Tang, J., Tikunova, S., Johnson, J. D., Chen, Z., Qin, N., Dietrich, A., Stefani, E., Birnbaumer, L., and Zhu, M. X. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 3168–3173
11. Kiseliov, K., Xu, X., Mozhayeva, G., Kuo, T., Pessah, I., Mignery, G., Zhu, X., Birnbaumer, L., and Muallem, S. (1998) Nature 396, 478–482
12. Boulay, G., Brown, D. M., Qin, N., Jiang, M., Dietrich, A., Zhu, M. X., Chen, Z., Birnbaumer, M., Mikoshiba, K., and Birnbaumer, L. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 14955–14960
13. Zhu, X., Jiang, M., and Birnbaumer, L. (1998) J. Biol. Chem. 273, 133–142
14. Balzer, M., Lintschinger, B., and Groschner, K. (1999) Cardiovasc. Res. 42, 543–549
15. Groschner, K., Rosker, C., and Lukas, M. (2004) Novartis Found. Symp. 258, 222–230; discussion 231–225, 263–266
16. Lockwich, T. P., Liu, X., Singh, B. B., Jadlowiec, J., Weiland, S., and Ambudkar, I. S. (2000) J. Biol. Chem. 275, 11934–11942
17. Schaefer, M. (2005) Pflugers Arch. 451, 35–42
18. Hofmann, T., Schaefer, M., Schultz, G., and Guedermann, T. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 7461–7466
19. Lintschinger, B., Balzer-Geldsetzer, M., Basaran, T., Graier, W. F., Romanin, C., Zhu, M. X., and Groschner, K. (2000) J. Biol. Chem. 275, 27799–27805
20. Liu, X., Bandopadhyay, B. C., Singh, B. B., Groschner, K., and Ambudkar, I. S. (2005) J. Biol. Chem. 280, 21600–21606
21. Strubing, C., Krapivinsky, G., Krapivinsky, L., and Clapham, D. E. (2001) Neuron 29, 645–655
22. Zhang, Z., Tang, Y., and Zhu, M. X. (2001) Biochem. J. 354, 717–725
23. Schaefer, M., Planat, T. D., Stresow, N., Albrecht, N., and Schultz, G. (2002) J. Biol. Chem. 277, 3752–3759
24. Elliott, S. J., Eskin, S. G., and Schilling, W. P. (1989) J. Biol. Chem. 264, 3806–3810
25. Rosker, C., Graziari, A., Lukas, M., Eder, P., Zhu, M. X., Romanin, C., and Groschner, K. (2004) J. Biol. Chem. 279, 13696–13704
26. Schilling, W. P., and Goel, M. (2004) Novartis Found. Symp. 258, 18–30; discussion 30–43, 98–102, 263–106
27. Kiseliov, K., Mignery, G. A., Zhu, M. X., and Muallem, S. (1999) Mol. Cell 4, 423–429
28. Koliwad, S. K., Elliott, S. J., and Kunze, D. L. (1996) J. Physiol. 495, 37–49