TRP4 (CCE1) Protein Is Part of Native Calcium Release-activated Ca\(^{2+}\)-like Channels in Adrenal Cells*

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Mammalian TRP proteins have been implicated to function as ion channel subunits responsible for agonist-induced Ca\(^{2+}\) entry. To date, TRP proteins have been extensively studied by heterologous expression giving rise to diverse channel properties and activation mechanisms including store-operated mechanisms. However, the molecular structure and the functional properties of native TRP channels still remain elusive. Here we analyze the properties of TRP4 (CCE1) channels in their native environment and characterize TRP expression patterns and store-operated calcium currents that are endogenous to bovine adrenal cells. We show by Northern blot analysis, immunoblots, and immunohistochemistry that TRP4 transcripts and TRP4 protein are present in the adrenal cortex but absent in the medulla. Correspondingly, bovine adrenal cortex cells express TRP4 abundantly. The only other TRP transcript found at considerable levels was TRP1, whereas TRP2, TRP3, TRP5 (CCE2), and TRP6 were not detectable. Depletion of calcium stores with inositol 1,4,5-trisphosphate or thapsigargin activates store-operated ion channels in adrenal cells. These channels closely resemble calcium release-activated Ca\(^{2+}\) (CRAC) channels. Expression of trp4(CCE1) cDNA in antisense orientation significantly reduces both, the endogenous CRAC-like currents and the amount of native TRP4 protein. These results demonstrate that TRP4 contributes essentially to the formation of native CRAC-like channels in adrenal cells.

Receptor-operated Ca\(^{2+}\) signaling has been described in a variety of non-excitable cells and has been attributed to basic and specific cell functions like cell growth, differentiation, and hormone release (reviewed by Refs. 1–3). The receptor-dependent and phospholipase C-mediated formation of inositol 1,4,5-trisphosphate (InsP\(_3\)) leads to the depletion of intracellular Ca\(^{2+}\) stores which in turn activates store-operated Ca\(^{2+}\) channels in the plasma membrane. The mechanisms linking store depletion to channel activation are still unknown but might include direct coupling of store proteins to the plasma membrane channel (1, 4–7), diffusible messengers (8–10), or exocytotic insertion of channels preformed in vesicles (11–13). The current picture and implications of these activation mechanisms have been reviewed recently (14, 15). The best studied store-operated Ca\(^{2+}\) channels in terms of function and biophysical properties are calcium release-activated Ca\(^{2+}\) (CRAC) channels which have been originally described in mast cells and T-lymphocytes (16, 17). This prototype of a store-operated Ca\(^{2+}\) channel is characterized by its inward rectifying currents and by its high selectivity for Ca\(^{2+}\). The molecular structure of CRAC channels, however, remains elusive.

Mammalian homologues of the Drosophila TRP and TRPL ion channels are candidate proteins underlying CRAC channels but also other store-operated and/or receptor-operated Ca\(^{2+}\) permeable ion channels. So far, the cDNAs of seven mammalian TRP genes have been cloned and characterized as functional cation channels after heterologous expression in various cell systems including HEK 293 cells, L-cells, Chinese hamster ovary cells, SF9 insect cells, and Xenopus oocytes (reviewed in Ref. 18). The gating mechanisms for the recombinant mammalian TRP channels are still controversial. The channels formed by heterologously expressed TRP1, TRP4, and TRP5 appear to be activated by maneuvers known to deplete Ca\(^{2+}\) stores and represent store-operated Ca\(^{2+}\) channels (19–23) but store independent activation of cation channels has also been reported after heterologous expression of TRP4 and TRP5 (24). In addition, TRP3 appears to be gated by Ca\(^{2+}\) store depletion via InsP\(_3\) (5, 25, 26) and it has been connected to InsP\(_4\)-dependent channel influx in pontine neurones (27). However, direct activation by diacylglycerol of TRP3 and TRP6 cation channels has also been demonstrated (28). TRP1, TRP3, and TRP6 encode Ca\(^{2+}\) permeable but non-selective cation channels when expressed in eukaryotic cells (19, 29–32), whereas TRP4 and TRP5 form ion channels mainly permeable for Ca\(^{2+}\) (20–23, 33). Obviously, some recombinant mammalian TRP channels are sensitive to store depletion and form ion channels mainly permeable to Ca\(^{2+}\) whereas others appear to be non-selective cation channels insensitive to store depletion. Probably, depletion-insensitive TRP proteins may form store-operated channels through multimerization with a store-sensitive subunit. TRP4 and TRP5 are appropriate candidates to play the role of a dominant subunit of these channels in terms of Ca\(^{2+}\) selectivity.

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¶ The abbreviations used are: InsP\(_3\), inositol 1,4,5-trisphosphate; CRAC, calcium release-activated Ca\(^{2+}\); RT-PCR, reverse transcriptase-polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GST, glutathione S-transferase; TG, thapsigargin; kb, kilo-
To analyze the properties of TRP proteins, their cDNAs have been usually overexpressed in cells which already may contain various endogenous TRP proteins as well as store-operated Ca\(^{2+}\) entry channels. HEK 293 cells, for example, show a significant amount of CRAC currents (34) and do express TRP transcripts to some extent (25, 26, 35, 36). Expression of TRP cDNAs in HEK 293 cells might lead to heterooligomeric channels by multimerization of recombinant and endogenous TRP proteins. In addition, homomultimeric recombinant channels may be formed due to overexpression of the recombinant TRP cDNA in the heterologous system. Consequently, the activity of recombinant TRP channels expressed in a foreign cellular environment does not necessarily correspond to the activity of native store-operated Ca\(^{2+}\) channels.

Further evidence linking TRP proteins to agonist induced Ca\(^{2+}\) entry was obtained by expression of mammalian TRP cDNA fragments in antisense orientation in mouse fibroblasts (37) and salivary gland cells (38). In clones of salivary gland cells stably expressing TRP1 antisense cDNA, Ca\(^{2+}\) influx in the presence of thapsigargin is decreased, suggesting that TRP1 is a candidate protein for store-operated calcium Ca\(^{2+}\) entry mechanisms (38). Using fura-2 Ca\(^{2+}\) measurements, Birnbaumer and colleagues (37) reported that clonal lines of murine Ltk (tk-) cells stably transfected with the muscarinic M5 receptor cDNA together with a mouse TRP4 antisense cDNA fragments showed a lack of M5 receptor stimulated calcium entry. This entry could be due either to the activation of receptor-operated or store-operated channels, which might be selective for Ca\(^{2+}\). It remains to be shown which TRP proteins are expressed in Ltk- cells.

The TRP4 cDNA was originally isolated from bovine adrenal gland mRNA and we show now, that TRP4 transcripts and proteins are abundantly expressed in the cortex but not in the medulla of the adrenal gland. To study TRP4 function in its native environment we used bovine adrenal cortex cells which were found to express significant amounts of TRP4. Analyzing store-operated currents, we show that these cells display endogenous CRAC-like currents after Ins\(_5\)P\(_3\)- or thapsigargin-induced Ca\(^{2+}\) store depletion. In this study we used an antisense strategy to dissect the relation between the TRP4 protein expression and native store-operated Ca\(^{2+}\) entry channels. Transfection of bovine adrenal cortex cells with TRP4 antisense cDNA reduces both, the endogenous CRAC-like current as well as the amount of endogenously expressed TRP4 protein strongly indicating that TRP4 is part of native CRAC-like channels in adrenal cells.

**EXPERIMENTAL PROCEDURES**

**Construction of Bacterial Expression Plasmids**—The pCAGGS expression vector (39) and the pCAGGS-EGFP plasmid containing the cDNA of the green fluorescent protein EGFP (CLONTECH) were kindly provided by Jun-ichi Miyazaki (Osaka University Medical School, Osaka, Japan). To create additional cloning sites for the synthetic cDNAs 5′-CTCGAGGCCCCTGATCATACATCTTATCTGA-TCAAGAGCTGAG-3′ was subcloned in the Xho I-cleaved pCAGGS in both orientations yielding pCAGGS1 and pCAGGS2. The Not I/Asc I cDNA fragment of pEBFP (CLONTECH) encoding the blue fluorescent protein and the Not I/Bsp DI pCAGGS2 fragment were ligated to obtain the reporter plasmid pCAGGS2-EFpB. The complete coding cDNAs of bovine TRP4 (20) (previously CCE or CCE1) and bovine TRP2 (40) were subcloned into pCAGGS2 in antisense orientation downstream of the 5′-aCT-p promoter to obtain pCAGGS2-TRP4 antisense and pCAGGS2-TRP2 antisense.

**Culture and Transfection of SBAC Cells and HEK 293 Cells**—Bovine adrenocortical cells (SBAC, ATCC CRL 1796) were obtained seven passages after primary culture from the American Tissue and Cell Collection as frozen stocks. On thawing, cells were grown in Ham's F-12 medium supplemented with 10% fetal calf serum (Life Technologies Lot No. 40F4566J), 50 units/ml penicillin, 50 μg/ml streptomycin at 37 °C in a 5% CO\(_2\) humidified atmosphere. Cells were subcultured every third day and grown to ~90% confluence. Cells were detached from plates in the presence of 0.5 g/liter trypsin (Life Technologies) and thereafter, trypsin was neutralized by addition of 10 volumes of serum-supplemented medium. Stocks were stored in 5% dimethyl sulfoxide in liquid nitrogen until required for experiments. A transfection of SBAC cells (grown in 35-mm dishes to ~80% confluence) was carried out essentially as described by the manufacturer of Superfect reagent (Qiagen) using 1 μg of pCAGGS-EGFP together with 3 μg of pCAGGS2-TRP4 antisense or 3 μg of pCAGGS2-EBFP or pCAGGS2-TRP2 antisense. Electroporation was carried out as follows: 10⁷ cells were suspended in 300 μl of fresh medium and co-transfected with 5 μg of pCAGGS-EGFP and 15 μg of pCAGGS2-TRP4 antisense or 15 μg of pCAGGS2-EBFP, respectively, using a gene pulser transfection apparatus (Bio-Rad) at a setting of 0.23 kV and 960 microfarads. Transfection of HEK 293 cells with pCCE were carried out as described (20).

**Cloning of Bovine TRP5 and TRP6 and Human TRP6 cDNA**—A 365-bp bovine TRP5 cDNA fragment was amplified from 5 μg of DNase I-treated bovine brain total RNA using degenerated oligonucleotides corresponding to amino acids EKWEEMWHL (5′-GARGATTGGGARAGTTGGCA-3′) and M\(^{600}\)HKWGDG (5′-CCRTCNCCTCCCAAYRTTGCATG-3′) and nested primers corresponding to amino acids L\(^{131}\)DILKFL (5′-CTTGATATCTCAAAATTTCTC-3′) and L\(^{326}\)AJADH (5′-TCCAGCATG-ATCCAGCAATG-3′) of mouse TRP5 (21). The alignment with the corresponding mouse and rabbit TRP5 sequences revealed 91 and 100% sequence and amino acid identity, respectively. Transcripts encoding TRP6 cDNA fragments were amplified from DNase I-treated bovine brain total RNA using primers corresponding to D\(^{85}\)IKPMV (5′-GATACTT-CATAATCTTTGTCG-3′) and S\(^{267}\)FQIE (5′-CTAATTTCTGGAATGAC-3′) of mouse TRP6 (30). The alignment with the corresponding mouse and human TRP6 sequences revealed 89.9 and 94.7% nucleotide and 100% amino acid sequence identity. The complete coding sequence of human TRP6 (2796 bp, Genbank accession number AJ006276) was amplified from human placenta poly(A)+ RNA. We found two alternatively spliced transcripts encoding hTRP6 variants now called hTRP6\(_{\text{A16-431}}\) and hTRP6\(_{\text{316-431}}\), both lacking the first of seven hydrophobic regions predicted to span the plasma membrane. The hTRP1, hTRP6, hTRP6\(_{\text{A16-431}}\), and hTRP6\(_{\text{316-431}}\) sequences have been deposited in the GenBank database under GenBank accession numbers AJ271067, AJ271068, and AJ271068, respectively.

**Analysis of the TRP Expression Pattern by RT-PCR**—5 μg of SBAC total RNA was treated with DNase I for 30 min and reverse transcribed using random primers and SuperScript II reverse transcriptase (Life Technologies). One-seventh of the resulting single strand cDNA or of PCR control reactions performed without reverse transcriptase were used for PCR in the presence of 0.2 μm primers specific for each bovine TRP sequence. The following primers were used: bTRP1 (GenBank accession number AF012900): 5′-GACAGATGTCGGTTACCC-3′ and 5′-ATTCTTTTCTAAGGCTGTCG-3′; bTRP2 (GenBank accession number AJ006304): 5′-CAGCTCTTGCGAAAACCC-3′ and 5′-GACCTTCTGATCTTCTGGT-3′; bTRP5 (GenBank accession number AJ006781): 5′-TGCAATGACACGACGAC-3′ and 5′-TACGCGAATCTAGTCCTG-3′; bTRP4 (GenBank accession number X99792): 5′-TGGCGTCTCC-GTGAGC-3′ and 5′-AGGCCACCTGGTCATATC-3′; bTRP6 (this manuscript): 5′-GATCTGCTGCTTGGTCTCCG-3′ and 5′-ATGCAGCAGTACACCC-3′. PCR products were analyzed by electrophoresis in 2% agarose gels. The identity of the amplified cDNA fragments was confirmed by restriction analysis in 5% polyacrylamide gels and by sequence analysis.

**Northern Blot Analysis**—Northern blots were performed using 10 μg of poly(A)+ RNA as described (20). For the analysis of TRP transcripts in adrenal gland, cortex and medulla were separated macroscopically. The following probes were labeled by random priming using 5′-[\(\alpha\)\(^{32}P\)]dCTP (6000 Ci/mmol): TRP1, a 2289-bp cDNA fragment corresponding to the complete coding region of human TRP1 (19); TRP3, a 395-bp fragment of bovine TRP3 (32) and a 2544-bp cDNA fragment spanning the complete coding region of human TRP3 (25); TRP5, a 2865-bp fragment corresponding to the coding region of bovine TRP5, a 365-bp cDNA fragment of bovine TRP5 (see above), a 2895-bp cDNA fragment encoding mouse TRP5, and a 1630-bp cDNA fragment of rabbit TRP5 (21); TRP6, a 2786-bp fragment encoding hTRP6 (see above). The filters were exposed to x-ray films with intensifying screens at ~80 °C for the times indicated. The filters used for analysis of TRP3 and TRP5 expression in SBAC cells were reused to analyze of TRP1 and TRP6 expression. For that purpose filters were stripped, exposed to
x-ray films to control residual signals, hybridized with the second probe and, finally, with a 239-bp cDNA fragment of the human glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

In Situ Hybridization—8-μm thick sections of freshly frozen adult bovine adrenal glands were hybridized to bovine TRP4 cDNA as described. Sections were hybridized to bovine TRP4 cDNA into pGEX-T4-2 (Amer sham Pharmacia Biotech) to yield pGST-TPR4e. Escherichia coli BL21 (DE3) cells transformed with pGST-TPR4e or pGEX-T4-2 were grown in LB medium containing 2.5 mM betaine and 440 mM sorbitol. Protein expression was induced by addition of 0.5 mM isopropyl-1-thio-β-D-galactopyranoside and then the cultures were incubated for 3 h at 29 °C. The following steps were carried out at 4 °C: Cells were harvested, resuspended in TBS/phenylmethylsulfonyl fluoride buffer (50 mM Tris-HCl, 150 mM NaCl, pH 7.5, containing 1 mM phenylmethylsulfonyl fluoride), sonicated 3 times for 30 s and incubated 1 h in TBS/phenylmethylsulfonyl fluoride buffer containing 1% Triton X-100. The clear supernatant obtained after centrifugation for 15 min at 12,000 × g, was incubated with glutathione-Sepharose beads washed with TBS/phenylmethylsulfonyl fluoride buffer. GST and GST-TPR4e fusion proteins were eluted with 50 mM Tris-HCl, pH 8.0, containing 10 mM reduced glutathione. Proteins were analyzed by SDS-polyacrylamide gel electrophoresis and stained with Coomassie Blue.

Preparation of a TRP4 Antibody—The synthetic peptide DTP5TTHEDYFYTRL of bovine TRP4 was coupled to keyhole limpet hemocyanine activated with N-succinimidyl-3-maleimidopropionate (Fluka) via an introduced amino-terminal cysteine residue and injected into rabbit 236 together with complete and incomplete Antibody-Multiplier-Adjuvans (Linaris). Serum was affinity purified using the synthetic peptide, which was coupled to epoxy-activated Sepharose (Amer sham Pharmacia Biotech).

Preparation of Bovine Adrenal Cortex Microsomes—All steps were performed at 4 °C. Bovine adrenal glands were obtained from the local slaughterhouse and the cortex was homogenized in TBSP buffer (TBS, 3.5 mM CaCl2, 0.5 mM MgCl2, 1% Triton X-100, 1% Nonidet P-40, 20% methanol) and, finally, with a 239-bp cDNA fragment of the human glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The homogenate was sedimented (15 min, 10,000 × g) and the combined supernatants were re-extracted with TBSP buffer, and the combined supernatants were centrifuged for 60 min at 90,000 × g. The resulting microsomal pellets were resuspended in TBS/phenylmethylsulfonyl fluoride buffer containing 1% Triton X-100 and washed five–seven times with 10 volumes of TBS/phenylmethylsulfonyl fluoride buffer.

Preparation of Bovine Adrenal Cortex Microsomes—All steps were performed at 4 °C. Gels were stained with Coomassie Blue or transferred to nitrocellulose membranes (0.45 μm thick sections of freshly frozen adult bovine adrenal glands were hybridized to bovine TRP4 cDNA as described. Sections were hybridized to bovine TRP4 cDNA into pGEX-T4-2 (Amer sham Pharmacia Biotech) to yield pGST-TPR4e. Escherichia coli BL21 (DE3) cells transformed with pGST-TPR4e or pGEX-T4-2 were grown in LB medium containing 2.5 mM betaine and 440 mM sorbitol. Protein expression was induced by addition of 0.5 mM isopropyl-1-thio-β-D-galactopyranoside and then the cultures were incubated for 3 h at 29 °C. The following steps were carried out at 4 °C: Cells were harvested, resuspended in TBS/phenylmethylsulfonyl fluoride buffer (50 mM Tris-HCl, 150 mM NaCl, pH 7.5, containing 1 mM phenylmethylsulfonyl fluoride), sonicated 3 times for 30 s and incubated 1 h in TBS/phenylmethylsulfonyl fluoride buffer containing 1% Triton X-100. The clear supernatant obtained after centrifugation for 15 min at 12,000 × g, was incubated with glutathione-Sepharose beads washed with TBS/phenylmethylsulfonyl fluoride buffer. GST and GST-TPR4e fusion proteins were eluted with 50 mM Tris-HCl, pH 8.0, containing 10 mM reduced glutathione. Proteins were analyzed by SDS-polyacrylamide gel electrophoresis and stained with Coomassie Blue.

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Western Blot Analysis—HEK 293 cells and SBAC cells were washed with phosphate-buffered saline and resuspended in 50 mM Tris-HCl, pH 6.8, 4 μl urea, 1% SDS, 15% glycerol, 0.005% bromphenol blue, and 2% (v/v) phenylmethylsulfonyl fluoride (PMSF) buffer. Chromosomal DNA was sheared by pipetting through a 26-gauge needle. Reduced, denatured adrenal cortex microsomal proteins (see above), HEK 293 proteins, GST fusion proteins, and SBAC proteins were separated by SDS-polyacrylamide gel electrophoresis. Gels were stained with Coomassie Blue or transferred to nitrocellulose membranes (0.45 μm) by tank blotting (Bio-Rad) in the presence of 1 mM phenylmethylsulfonyl fluoride (PMSF) and 10% nonfat dry milk. The primary antibody 236 (4 μg/ml) was incubated on ice with the membranes for 1 h. After washing, the membranes were incubated with the secondary antibody, which contained 1% nonfat dry milk, at room temperature. Visualization of the antigen-antibody complexes was done by incubating the tissue sections in the presence of the alkaline phosphatase-conjugated goat anti-rabbit IgG antibody (Dako Diagnostika, Fast-Blue RR salt (Sigma) as chromogen, and naphtho-AS-MX-phosphate (Sigma) as substrate.

Patch Clamp Recordings—Patch clamp experiments were performed at 22–25 °C in the tight seal whole cell configuration using fire-polished patch pipettes (3–7 MΩ uncompensated series resistance). Pipette and cell capacitance were electronically compensated before each voltage ramp in case the EPC-9 amplifier was used. Membrane currents were filtered at 1.5–2.3 kHz and digitized at a sampling rate of 5–10 kHz.

To characterize store-operated Ca2+ channels the following protocols were used. Baseline currents were elicited with an EPC-9 amplifier (LIST electronic) controlled by pClamp 6.0 software (Axon Instruments). The pipette solution contained (mM): 115 CaCl2, 10 EGTA, 4 CaCl2 (calculated free internal Ca2+ 100 nm), 4 MgCl2, 10 HEPES (pH 7.2, with CsOH). When indicated 10 μM InsP3 (Calbiochem) was added. The bath solution contained (mM): 115 NaCl, 5 KCl, 2 MgCl2, 10 CaCl2, 10 HEPES (pH 7.2, with NaOH). In some experiments, Na+ or Ca2+ were replaced by NMDG in the presence of Mg2+. In divalent free solutions, Ca2+ and Mg2+ were replaced by NMDG (1 mM EDTA added). Bath solutions were exchanged using a local perfusion system. When indicated 1 μM thapsigargin (TQ, Calbiochem) was added. Whole cell currents were elicited by 400 ms voltage-clamp ramps from −100 to +100 mV from a holding potential of 0 mV. All experiments analyzed on the same day and measured on the same day. Data were analyzed in a blinded fashion and corrected for leak currents.

Statistical Analysis—According to one-way ANOVA, immunostaining intensities, as well as patch clamp data were not significantly different between groups (p < 0.001) except between the group of TRP4 antisense transfected cells and the control groups. Further analysis by the least significant difference and Bonferroni tests at the 95% level of significance demonstrate that the TRP4-anti group was significantly different from the control groups, which, again, were not significantly different from each other. The nonparametric Kruskal/Wallis and Mann-Whitney tests gave identical results.

RESULTS

The TRP4 mRNA Is Abundantly Expressed in the Bovine Adrenal Cortex—Originally we have cloned the CRAC-like channel TRP4 from bovine retina and bovine adrenal gland mRNA (20). In the following we analyzed TRP4 expression in adrenal gland in more detail using Northern blot and in situ hybridization analysis (Fig. 1). Transcripts of 7.0, 4.8, 3.8, and 1.9 kb in length are present in mRNA isolated from the whole adrenal gland and in mRNA isolated from adrenal cortex but

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Expression Pattern of Mammalian TRP Transcripts in Adrenal Cortex Cells—In the following we used the adrenal cortex-derived cell line SBAC to study a possible correlation between TRP4 expression and the occurrence of endogenous store-operated Ca\(^{2+}\) entry channels. First, we studied the endogenous expression of TRP4 and related TRP genes by RT-PCR, Northern blot analysis, and in situ hybridization experiments. To amplify cDNA fragments of bovine TRP1, TRP2, TRP3, TRP4, TRP5, and TRP6 we used specific primers derived from the bovine sequences which have already been described (TRP1, 2, 3, and 4) or which were cloned for that purpose (TRP5 and TRP6; see “Experimental Procedures”). Using RT-PCR, cDNA fragments of TRP4 and TRP1 could be amplified consistently and, to a minor extend, also fragments of TRP3 indicating the presence of TRP4, TRP1, and TRP3 mRNA in SBAC cells (Fig. 2a). In contrast, no TRP2, TRP5, and TRP6 transcripts were detectable.

To get an estimate of the relative abundance of TRP transcripts present in SBAC cells we performed Northern blot analysis. TRP4-specific transcripts of 7.0, 4.0, and 3.8 kb were detected at high abundance which correspond to the transcripts expressed in bovine adrenal cortex (Fig. 2b). Interestingly, SBAC cells lack the 1.9-kb mRNA encoding the truncated TRP4 variant bCCE1\(_{1-314}\) (41). In addition to TRP4, TRP1 transcripts are present in the bovine adrenal cortex whereas TRP2 (40), TRP3, TRP5, and TRP6 could not be detected (Fig. 1c).

Expression of TRP4 transcripts in the bovine adrenal gland. a, Northern blot analysis of TRP4 expression in bovine adrenal gland. The filter was exposed to x-ray film for 24 h. The lower panel shows signals after hybridization of the same filter with human GAPDH cDNA. b, in situ hybridization of a sagittal adrenal gland section with TRP4 antisense cRNA (autoradiographic film image, upper panel). A rectangle indicates the location of the magnification shown in the lower panel (dark-field microscopy). Bars represent 1 mm and 100 \(\mu\)m, respectively. oc, outer cortex; ic, inner cortex. c, TRP expression pattern in bovine adrenal gland (upper panels, expression time 14 days). Lower panels, GAPDH cDNA (expression time 7 days).

Fig. 1. Expression of TRP4 transcripts in the bovine adrenal gland. a, Northern blot analysis of TRP4 expression in bovine adrenal gland. The filter was exposed to x-ray film for 24 h. The lower panel shows signals after hybridization of the same filter with human GAPDH cDNA. b, in situ hybridization of a sagittal adrenal gland section with TRP4 antisense cRNA (autoradiographic film image, upper panel). A rectangle indicates the location of the magnification shown in the lower panel (dark-field microscopy). Bars represent 1 mm and 100 \(\mu\)m, respectively. oc, outer cortex; ic, inner cortex. c, TRP expression pattern in bovine adrenal gland (upper panels, expression time 14 days). Lower panels, GAPDH cDNA (expression time 7 days).
infecting a KHL-coupled peptide into rats. Antibody 236 recognizes GST-bTRP4c fusion proteins (Fig. 3a), recombinant bTRP4 expressed in HEK 293 cells (Fig. 3, b and d) as well as native bovine TRP4 in SBAC cells (Fig. 3, b, c, and e) and in microsomal membrane proteins prepared from bovine adrenal cortex (Fig. 3c). The antibody detected a ~105-kDa protein in SBAC cells and in bovine adrenal cortex identical in size to the recombinant protein expressed in HEK cells (Fig. 3, b and c). Its specificity for TRP4 is shown by its detection of only the GST-bTRP4c fusion protein but not GST alone (Fig. 3c). In addition, TRP4 antibody recognizes the recombinant TRP4 protein of the expected size in transfected HEK cells, but not in non-transfected control cells (Fig. 3, b and d). The latter finding shows that HEK 293 cells do not express endogenous TRP4 as already described previously (20). Finally, excess antigenic peptide inhibited binding of antibody 236 to the GST-bTRP4c fusion protein (Fig. 3a), to the recombinant TRP4 protein expressed in HEK cells (Fig. 3d, right panel) as well as to the native TRP4 protein present in SBAC cells (Fig. 3e, right panel). As expected from the mRNA expression pattern (Fig. 1) TRP4 is expressed in the adrenal cortex, but it is not detectable in the adrenal medulla (Fig. 3f).

**SBAC Cells Display Endogenous CRAC-like Currents**—To characterize store-operated Ca\(^{2+}\) currents in SBAC cells, Ca\(^{2+}\) stores were depleted by two different methods during whole cell patch clamp experiments: activation of the InsP\(_3\) receptor through perfusion of cells with 10 \(\mu\)M InsP\(_3\) (a) or in the presence of 1 \(\mu\)M thapsigargin (b) at 0 mV holding potential. The external solution was changed during the experiment from Ca\(^{2+}\) + Na\(^+\) (10 mM Ca\(^{2+}\), 115 mM Na\(^+\), 2 mM Mg\(^{2+}\)) to Na\(^+\) (115 mM Na\(^+\), 20 mM NMDG\(^+\), 2 mM Mg\(^{2+}\)) to Ca\(^{2+}\) (10 mM Ca\(^{2+}\), 115 mM NMDG\(^+\), 2 mM Mg\(^{2+}\)); leak, leak currents obtained at the beginning (∼60 s) of dialysis with InsP\(_3\) (a) or before application of thapsigargin (b), c, leak-subtracted currents activated by InsP\(_3\) were recorded in the presence Ca\(^{2+}\) and Na\(^+\) (10 mM Ca\(^{2+}\), 115 mM Na\(^+\), 2 mM Mg\(^{2+}\)) and subsequently in the absence of divalent cations (115 mM Na\(^+\), 20 mM NMDG\(^+\), 1 mM EDTA).

**Western blot analysis of TRP4 expression in bovine adrenal gland and in SBAC cells.** a, the purified GST-bTRP4c fusion protein (1 \(\mu\)g, ~42 kDa) and GST (1 \(\mu\)g, ~29.5 kDa) were separated on a 8% SDS-polyacrylamide gel. The gels were either stained (not shown) or immunoblotted with affinity purified antibody 236. The antibody recognizes the TRP4 protein of the expected size in transfected HEK cells, but not in SBAC cell extracts (200 \(\mu\)g/ml). b, equal amounts of HEK 293 cell extracts (60 \(\mu\)g/lane) and SBAC cell extracts (20 \(\mu\)g) were separated on 6.5% SDS-polyacrylamide gels and immunoblotted as described. c, immunocytochemistry using antibody 236 in nontransfected HEK cells (d, left panel), in bTRP4 transfected HEK cells in the absence (d, middle panel) or presence (d, right panel) of excess antigenic peptide. Bar, 10 \(\mu\)m. e, immunocytochemistry using antibody 236 in bTRP4 transfected cells (HEKbTRP4) and in SBAC cells but not in non-transfected cells (HEK). The antibody 236 also recognizes the ~112-kDa TRP5 protein in HEK cells stable expressing native bovine TRP4 in SBAC cells (Fig. 3). The antibody detected a 112-kDa TRP5 protein in HEK cells stable expressing bTRP4 expressed in HEK 293 cells (Fig. 3, and e, right panel). The latter finding is not surprising as part of the antigenic peptide is common to both bTRP4 and rabbit TRP5. c, the antibody 236 recognizes the TRP4 protein (~105 kDa) in SBAC cell extracts (200 \(\mu\)g) and bovine adrenal cortex microsomal proteins (200 \(\mu\)g), separated on 6.5% SDS-polyacrylamide gels and immunoblotted as described. d, immunocytochemistry using antibody 236 in nontransfected HEK cells (d, left panel), in bTRP4 transfected HEK cells in the absence (d, middle panel) or presence (d, right panel) of excess antigenic peptide. Bar, 10 \(\mu\)m. f, immunostaining of a bovine adrenal gland section in the presence or absence (inset) of the primary antibody 236. Bar, 200 \(\mu\)m.

**Activation of Ca\(^{2+}\)-release-activated Ca\(^{2+}\) current in SBAC cells.** I/V relationships obtained after perfusion of cells with 10 \(\mu\)M InsP\(_3\) (a) or in the presence of 1 \(\mu\)M thapsigargin (b) at 0 mV holding potential. The external solution was changed during the experiment from Ca\(^{2+}\) + Na\(^+\) (10 mM Ca\(^{2+}\), 115 mM Na\(^+\), 2 mM Mg\(^{2+}\)) to Na\(^+\) (115 mM Na\(^+\), 20 mM NMDG\(^+\), 2 mM Mg\(^{2+}\)) to Ca\(^{2+}\) (10 mM Ca\(^{2+}\), 115 mM NMDG\(^+\), 2 mM Mg\(^{2+}\)); leak, leak currents obtained at the beginning (∼60 s) of dialysis with InsP\(_3\) (a) or before application of thapsigargin (b), c, leak-subtracted currents activated by InsP\(_3\) were recorded in the presence Ca\(^{2+}\) and Na\(^+\) (10 mM Ca\(^{2+}\), 115 mM Na\(^+\), 2 mM Mg\(^{2+}\)) and subsequently in the absence of divalent cations (115 mM Na\(^+\), 20 mM NMDG\(^+\), 1 mM EDTA).

**Fig. 3.** Western blot analysis of TRP4 expression in bovine adrenal gland and in SBAC cells. a, the purified GST-bTRP4c fusion protein (1 \(\mu\)g, ~42 kDa) and GST (1 \(\mu\)g, ~29.5 kDa) were separated on a 8% SDS-polyacrylamide gel. The gels were either stained (not shown) or immunoblotted with affinity purified antibody 236. The antibody recognizes the TRP4 protein of the expected size in transfected HEK cells, but not in SBAC cell extracts (200 \(\mu\)g/ml). b, equal amounts of HEK 293 cell extracts (60 \(\mu\)g/lane) and SBAC cell extracts (20 \(\mu\)g) were separated on 6.5% SDS-polyacrylamide gels. The gels were either stained (not shown) or immunoblotted with affinity purified antibody 236. The antibody recognizes the TRP4 protein of the expected size in transfected HEK cells, but not in SBAC cell extracts (200 \(\mu\)g/ml). b, equal amounts of HEK 293 cell extracts (60 \(\mu\)g/lane) and SBAC cell extracts (20 \(\mu\)g) were separated on 6.5% SDS-polyacrylamide gels. The gels were either stained (not shown) or immunoblotted with affinity purified antibody 236. The antibody recognizes the TRP4 protein of the expected size in transfected HEK cells, but not in SBAC cell extracts (200 \(\mu\)g/ml).
TRP4 Is Part of CRAC-like Channels

The values given are leak-subtracted current densities (ΔCD, in pA/pF) obtained after activation with InsP3 or thapsigargin (TG) and determined in different bath solutions at a potential of −80 mV. The standard deviation and the number of cells analyzed (in parentheses) are indicated.

|          | ΔCD (pA/pF) ± S.D. |
|----------|--------------------|
| InsP3    | TG                 |
| Ca++ + Na+ | 0.50 ± 0.26 (21)    | 0.71 ± 0.22 (8) |
| Na<sup>+</sup> | <0.1 (8)            | <0.1 (6)         |
| Ca++     | 0.54 ± 0.13 (3)     | 0.62 ± 0.16 (3)  |

Na<sup>+</sup> because currents were fully recovered when changing the extracellular solution to Na<sup>+</sup>-free but Ca<sup>2+</sup>-containing conditions (Fig. 4, a and b). Thus, Na<sup>+</sup> does not act as a charge carrier in the presence of external Ca<sup>2+</sup> ions and the current is carried by Ca<sup>2+</sup> under these conditions. The permeation properties and current densities were found to be indistinguishable between InsP3- and TG-treated cells (summarized in Table I). It is concluded that both, InsP<sub>3</sub> and TG, activate the same properties and current densities were found to be indistinguishable between InsP<sub>3</sub>- and TG-treated cells (summarized in Table I).

It is concluded that both, InsP<sub>3</sub> and TG, activate the same properties and current densities were found to be indistinguishable between InsP<sub>3</sub>- and TG-treated cells (summarized in Table I).

Na<sup>+</sup> permeability in the complete absence of divalent cations (42). Therefore Mg<sup>2+</sup> was removed from the bath and Ca<sup>2+</sup> was chelated below 50 nM with EDTA. Under these conditions we observed Na<sup>+</sup> currents at least 2–3 times bigger then the Ca<sup>2+</sup> currents (Fig. 4c). Hence, store-operated channels in SBAC display the characteristic properties of CRAC channels and in the following, we therefore refer to these channels as CRAC-like channels.

**TRP4 (CCE1) Is Part of CRAC-like Channels in Adrenal Cells**—To test the hypothesis whether TRP4 is part of the endogenous CRAC channels in adrenal cells, we employed an antisense strategy to inhibit endogenous TRP4 expression. For that purpose we tested the suitability of several eukaryotic expression vectors for transfection of SBAC cells. Transfection efficiencies were studied by the expression of the green fluorescent protein (GFP). The most consistent results were obtained with a β-actin promoter-driven vector construct (see “Experimental Procedures”). Next, we analyzed the efficiency of co-transfection of two independent cDNAs, the cDNA of GFP and the cDNA of the blue fluorescent protein (BFP). As shown in Fig. 5a, SBAC cells can be consistently co-transfected. In five experiments 72 out of 73 transfected SBAC cells expressed both, GFP and BFP, indicating that coexpression occurs in nearly all transfected cells. In the next series of experiments SBAC cells were co-transfected with the GFP cDNA and the bovine TRP<sub>4</sub> cDNA in antisense orientation (Fig. 5, b and c). As control we analyzed cells transfected with GFP cDNA alone or in combination with bovine TRP<sub>2</sub> antisense cDNA (40) (Fig. 5c), which shares 45% nucleotide sequence identity with TRP<sub>4</sub>. Four to seven days after transfection endogenous CRAC-like currents were recorded after perfusing the cells with 30 μM InsP<sub>3</sub>. Maximal current density at −80 mV did not significantly differ between non-transfected SBAC cells (−0.85 ± 0.16 pA/pF, n = 6), SBAC cells expressing GFP (−0.76 ± 0.06 pA/pF, n = 13), and SBAC cells expressing GFP and TRP<sub>2</sub> antisense cDNA (−0.74 ± 0.06 pA/pF, n = 7). However, as is evident from the IV-relationships shown in Fig. 5b, the density of endogenous CRAC-like currents is significantly reduced by −50% in SBAC cells co-transfected with GFP cDNA and TRP<sub>4</sub> cDNA in antisense orientation. The current density of those cells at −80 mV was −0.34 ± 0.05 pA/pF (n = 14; Fig. 5c).

Obviously, transfection of SBAC cells with TRP<sub>4</sub> cDNA in

antisense orientation but not with GFP or TRP2 antisense cDNA leads to reduced CRAC-like currents.

These results are consistent with the hypothesis that TRP4 encodes a channel subunit which participates as an obligatory element of CRAC-like channels. According to this hypothesis, expression of TRP4 proteins should be reduced after transfection of the cells with the antisense TRP4 cDNA. Therefore, we designed experiments to quantify possible changes of the amount of expressed TRP4 protein on plated SBAC cells using antibody 236 (Fig. 3). For that purpose, cells were first plated on a microgrid which allowed the reidentification of green cells following the immunocytochemical staining (Fig. 6a). After five and six days of culture the amount of specifically bound antibody was monitored in (i) non-transfected cells, which served as positive control for maximal staining intensity (Fig. 6b, pos.); (ii) cells co-transfected with GFP and BFP cDNAs as control of possible effects of transfection and the presence of unrelated and nonspecific sequences (nsg, Fig. 6b); and (iii) cells co-transfected with TRP4 antisense cDNA and GFP cDNA (Fig. 6, TRP4-anti). There was little nonspecific staining of negative control cells, treated the same way as positive controls with the exception that the primary antibody was omitted (in arbitrary units: 0.92 ± 0.05, n = 100). The staining intensities determined on 121 positive control cells (6.71 ± 0.28) did not signifi-
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Fig. 6. Expression of TRP4 antisense cDNA reduces the amount of TRP4 protein in SBAC cells. a, a SBAC cell co-transfected with TRP4 antisense cDNA together with GFP cDNA shows green fluorescence (upper panel) and reduced immunostaining (lower panel) in comparison to two nonfluorescent cells. Bars, 10 μm. b, immunostaining intensity (given as arbitrary units) of SBAC cells (pos., positive control) or SBAC cells transfected with nonspecific cDNA sequences (nss, i.e. GFP and BFP cDNA) or TRP4 antisense cDNA (TRP4-anti) together with GFP cDNA. Cells were analyzed on three consecutive days after two independent transfections. Data were corrected for nonspecific staining (i.e. staining in the absence of antibody 236; 0, 92 ± 0.05; n = 100) and are given as mean ± S.E. (n as indicated).

DISCUSSION

The major aim of this study was to test the hypothesis that TRP proteins contribute to native store-operated Ca\(^{2+}\) entry channels. The first evidence connecting the TRP protein to store-operated Ca\(^{2+}\) entry channels came from heterologous expression of the TRP4 cDNAs in mammalian cells lines like HEK 293. HEK cells show an endogenous CRAC current (34) and expression of TRP4 cDNA in these cells enhances this endogenous current (20). HEK cells do not express endogenous TRP4 (Fig. 3 and (20) or TRP5 (Fig. 3 and (21)) implicating that the enhanced current originally observed in these cells might be due to the formation of additional TRP4 channels displaying similar properties as the endogenous channels, or of heterologous channels composed of recombinant TRP4 and endogenous channel subunits. In a later study, TRP4-induced currents in Chinese hamster ovary and RBL cells were measured under conditions which were similar to those used to measure I\(_{CRAC}\) in mast cells and Jurkat cells (22). In both, Chinese hamster ovary and RBL cells, expression of TRP4 cDNA enhanced the density of store-operated currents and we concluded that these currents reflect the phenotype of TRP4 currents. Nevertheless, heterologous expression of TRP4 cDNA also led to the appearance of store-independent cation currents (24, 43, 44). These contradictions could be due to the background of TRP proteins present in all expression systems because it cannot be excluded that heteromultimeric channels with very different properties are formed between expressed and endogenous channels. For that reason, heterologous expression studies do not show unambiguously that TRP4 proteins are able to contribute to store-operated channels and do not give any information about the composition of native channel complexes.

We now followed a different experimental approach to investigate the role of TRP4 for Ca\(^{2+}\) entry. First we looked for tissues and finally for cells which abundantly express TRP4 and found that adrenocortical cells of the SBAC line have this property. To investigate the presence of other putative Ca\(^{2+}\) entry channels in SBAC cells we analyzed the expression pattern of other TRP genes and show that in addition to TRP4 only TRP1 transcripts are present in these cells. Therefore, SBAC cells provide a native system to study molecular and functional properties of TRP4 channels in their native environment. We investigated store-operated Ca\(^{2+}\) entry in SBAC cells and as expected for a non-excitable cell type, SBAC cells express store-operated Ca\(^{2+}\) channels which we found to be very similar to the well studied highly Ca\(^{2+}\) selective CRAC channels in mast and Jurkat cells. Finally, our antisense strategy reduced the amount of TRP4 protein as well as CRAC-like channel activity and provides electrophysiological and biochemical evidence that TRP4 is part of native CRAC-like channels in SBAC cells either as an essential constituent for channel activation and/or as a channel forming subunit.

This result, however, may not necessarily be extended to all other cell types. Store-operated (CRAC) channels have been most extensively studied in mast cells and Jurkat T-lymphocytes (16, 17, 45). Relative to these two cell types, the amplitude of CRAC-like channels in SBAC cells is smaller despite the fact that TRP4 transcripts and proteins are highly expressed in SBAC cells. We do not know how much protein is really present in the plasma membrane, but it is hard to imagine that in RBL or Jurkat cells TRP4 is more efficiently shuttled to the plasma membrane than in SBAC cells. Most importantly, TRP4 does not appear to be present in RBL cells (36) but was found on the RNA level in Jurkat cells (36).2 Putting these findings together, one can make two important conclusions: 1) TRP4 is most likely not part of CRAC channels in every cell type, and as a corollary; 2) CRAC channels are not identical in their molecular structure. In line with these conclusions it has been shown that CRAC channel permeation properties of Ba\(^{2+}\) are not identical between RBL and Jurkat cells (45) indicating that the molecular structures of CRAC channels are different even in these cell types. In RBL cells the closest TRP4 relative TRP5 is expressed (36)2 and may substitute for TRP4 in these cells.

Considering the molecular structure of TRP proteins it is reasonable to assume that one channel complex is made of more than one subunit similar to voltage-gated potassium channels (46). As for potassium channels, TRP proteins could form both, homomultimeric or heteromultimeric ion channels. So far, only few data have been presented on the molecular assembly of mammalian TRP proteins but for the Drosophila TRP and TRPL proteins, it has been shown in an expression system that they can form heteromultimeric channels (47). Considering the enormous variety of TRP proteins (18) and the functional data obtained from heterologous expression it is reasonable to assume that different combinations of TRP proteins underlie the many store-operated and receptor-operated Ca\(^{2+}\) entry channels described so far. In SBAC cells, TRP4 is coexpressed with TRP1 and both proteins together might be subunits of CRAC-like channel in these cells. In this context it

\(^{2}\) S. Philipp, unpublished observations.
is important to consider the results recently published by Liu and co-workers (38). Using an antisense strategy they concluded that TRP1 is part of store-operated channels in salivary gland cells. Unfortunately it is not known which other TRPs are expressed in those cells and what kind of store-operated channel in terms of its selectivity is responsible for TRP1-dependent Ca\(^{2+}\) influx. Additional studies are required to determine if TRP1 interacts with TRP4 to form heteromultimeric channel complexes. Because store-operated Ca\(^{2+}\) entry is of great physiological importance, it is necessary to understand the molecular basis of endogenous Ca\(^{2+}\) entry channels. Our result linking a native CRAC-like channel to a certain TRP channel in terms of its selectivity is responsible for TRP1-de-
cluded that TRP1 is part of store-operated channels in salivary and co-workers (38). Using an antisense strategy they con-
cluded that TRP1 is part of store-operated channels in salivary

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