Functional Differences between TRPC4 Splice Variants*

Received for publication, October 11, 2001, and in revised form, November 15, 2001
Published, JBC Papers in Press, November 16, 2001, DOI 10.1074/jbc.M109850200

Michael Schaefer‡, Tim D. Plant, Nicole Stresow, Nadine Albrecht, and Günter Schultz

From the Institut für Pharmakologie, Freie Universität Berlin, Thielallee 67-73, 14195 Berlin, Germany

Functional characterizations of heterologously expressed TRPC4 have revealed diverse regulatory mechanisms and permeation properties. We aimed to clarify whether these differences result from different species and splice variants used for heterologous expression. Like the murine β splice variant, rat and human TRPC4β both formed receptor-regulated cation channels when expressed in HEK293 cells. In contrast, human TRPC4α was poorly activated by stimulation of an H1 histamine receptor. This was not due to reduced expression or plasma membrane targeting, because fluorescent TRPC4α fusion proteins were correctly inserted in the plasma membrane. Furthermore, currents through both human TRPC4α and TRPC4β had similar current-voltage relationships and single channel conductances. To analyze the assembly of transient receptor potential channel subunits in functional pore complexes in living cells, a fluorescence resonance energy transfer (FRET) approach was used. TRPC4α and TRPC4β homomultimers exhibited robust FRET signals. Furthermore, coexpressed TRPC4α and TRPC4β subunits formed heteromultimers exhibiting comparable FRET signals. To promote variable heteromultimer assemblies, TRPC4α/TRPC4β were coexpressed at different molar ratios. TRPC4β was inhibited in the presence of TRPC4α with a cooperativity higher than 2, indicating a dominant negative effect of TRPC4α subunits in heteromultimeric TRPC4 channel complexes. Finally, C-terminal truncation of human TRPC4α fully restored the channel activity. Thus, TRPC4β subunits form a receptor-dependently regulated homomultimeric channel across various species, whereas TRPC4α contains a C-terminal autoinhibitory domain that may require additional regulatory mechanisms.

In addition to their well defined ability to release Ca2+ from internal stores, phospholipase C-coupled receptors activate at least two different classes of cation entry. Capacitative calcium entry (CCE)3 channels are activated as a consequence of the depletion of intracellular Ca2+ stores and receptor-operated channels are gated independently of the filling-state of the endoplasmic reticulum. Although both Ca2+-selective and non-selective CCE channels have been described, their common feature is a receptor-independent activation by thapsigargin, an inhibitor of sarcoplasmic reticulum ATPases. By contrast, receptor-operated channels are activated by poorly defined mechanisms that include the formation of diacylglycerols or other membrane-resident second messengers such as arachidonic acid. Several members of the recently cloned transient receptor potential channel (TRPC) superfamily are candidate molecular substrates for these mechanisms (1, 2). Despite a number of studies that have been conducted to define regulatory and biophysical properties of these channels, no clear picture has emerged. Most prominently, members of the first characterized classical, canonical, or short TRPC family (TRPC1–7) have been implicated in both CCE and store-independent cation entry (3–15).

For almost each of the TRPCs both modes of activation have been proposed. In the case of TRPC4, the initial characterization reported an increased CCE activity in human embryonic kidney (HEK) 293 cells expressing the bovine TRPC4α splice variant (6). Murine TRPC4 and TRPC5 were shown to be nonselective cation channels that share the characteristics of receptor-operated channels (12). In contrast, human TRPC4 (hTRPC4) displayed only basal activity without acute regulation (16). A rat TRPC4 splice variant, also referred to as TRP-R (17), has been reported to enhance store-operated Ca2+ entry when expressed in Xenopus laevis oocytes (18, 19). Furthermore, Strübing et al. (20) described the formation of heteromultimeric channel complexes of TRPC1 with either murine TRPC4α or TRPC5. These channel complexes behave like a receptor-operated channel and display biophysical properties distinct from those of homomultimeric TRPC4α or TRPC5. Thus, the assembly of TRPC4 splice variants in heteromultimeric TRPC complexes might add an additional level of complexity.

In this study, we have attempted to clarify the contradictory findings with heterologously expressed TRPC4 by directly comparing the regulation and biophysical properties of different species and splice variants. In addition, a fluorescence resonance energy transfer (FRET)-based approach was established to investigate the multimerization of TRPC subunits. Our data provide evidence that TRPC4α and TRPC4β subunits form receptor-dependently but store-independently regulated homomultimeric cation channels. TRPC4β shows cation fluxes of similar amplitudes across various species. In contrast, human TRPC4α displays a markedly lower efficiency of receptor-induced activation and exerts a dominant negative effect in heteromultimeric complexes with TRPC4β.

EXPERIMENTAL PROCEDURES

Materials—Culture media and trypsin were purchased from Invitrogen. Fetal calf serum and phosphate-buffered saline were obtained from Biochrom (Berlin, Germany). Thapsigargin was from Calbiochem (Bad Soden, Germany). The rabbit anti-TRPC4 antibody was from Alomone Labs (Jerusalem, Israel). Unless otherwise stated, all other reagents were purchased from Sigma.

3752 This paper is available on line at http://www.jbc.org

‡ To whom correspondence should be addressed. Tel.: 49-30-84451863; Fax: 49-30-84451818; E-mail: schae@zedat.fu-berlin.de.

1 The abbreviations used are: CCE, capacitative calcium entry; TRPC, transient receptor potential channel; FRET, fluorescence resonance energy transfer; hTRPC4, human TRPC4; rTRPC4, rat TRPC4; HEK, human embryonic kidney; CFP, cyan fluorescent protein; YFP, yellow fluorescent protein; InsP3, inositol 1,4,5-trisphosphate.

* This work was supported by the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed. Tel.: 49-30-84451863; Fax: 49-30-84451818; E-mail: schae@zedat.fu-berlin.de.

1 The abbreviations used are: CCE, capacitative calcium entry; TRPC, transient receptor potential channel; FRET, fluorescence resonance energy transfer; hTRPC4, human TRPC4; rTRPC4, rat TRPC4; HEK, human embryonic kidney; CFP, cyan fluorescent protein; YFP, yellow fluorescent protein; InsP3, inositol 1,4,5-trisphosphate.

This paper is available on line at http://www.jbc.org
Cell Culture, Transfections, and Fluorometric Techniques—HEK 293 cells were grown in minimal essential medium supplemented with 10% fetal calf serum, 50 units/ml penicillin, and 50 units/ml streptomycin. For transient transfections, the cells were seeded on 6-cm dishes and transfected the following day at 50–70% confluence as described (12). HEK 293 cells were transfected with the patch pipette (Roche Molecular Biochemicals) according to the manufacturer’s recommendations. The transfected cDNA plasmid concentration was kept constant by cotransferring 0.2 μg/dish of the rat histamine H1 receptor in pcDNA3 and 3.5 μg/dish of the indicated TRPC subunit in pcDNA3 or the corresponding amount of the empty expression vector (vector-transfected controls). After 1–2 days, the cells were trypsinized and seeded on glass coverslips. All fluorometric experiments were conducted 48–72 h after transfection, as described previously (12). Statistical analysis was done with the Mann-Whitney test, and significance was accepted at p < 0.05.

Cloning of TRPC4 Splice Variants and Generation of Fluorescent Fusion Proteins—Total RNA was prepared from Wistar rat brain, embryonic rat vascular smooth muscle cells (21), pooled human testis, and bovine brain using the Trizol reagent (Invitrogen). The first strand synthesis (Superscript II, Invitrogen) was primed with gene-specific oligonucleotides (rat TRPC4, 5′-GTC CAG TAC AAT AAA ACA AA; human TRPC4, 5′-GCA GGC TAC AAA ACA AA; and bovine TRPC4, 5′-GGG GTT AAC AAA ACA A) and allowed to proceed for 80 min at 42 °C. TRPCR was amplified by 36 cycles of PCR Expand-HF; Roche Molecular Biochemicals). Annealing temperature was 58 °C (30 s), and the extension time (3.5 min) was further prolonged by 10 s/cycle in cycles 11–36. The forward primers were 5′-GGC AGC ATG GCT CTC TAT TAC AAA (rat) or 5′-GCA ACC ATG GCT CTC TAT TAC AAA AG (human). Reverse primers were 5′-GGA GCC GAC TAA AGA GGA GGG TT (rat) or 5′-GCC ACC CAG ACC ACT ACG GAA A (human). PCR products were subcloned in a pcDNA3-V5-His vector (eukaryotic TA-TOPO cloning system, Invitrogen) and confirmed by sequencing (ABI-Prism 377, Perkin Elmer). Human TRPC4α was C-terminally truncated (hTRPC4α<sub>344-977</sub>) by PCR with a reverse primer 5′-ATC GGT CAC AAA ATT CAC TTT. C-terminal truncation at the cytosolic C terminus of TRPC4 (22) resulted in the fusion vector without insert, the addition of histamine induced an increase in current amplitude which was not significantly higher in HEK 293 cells expressing acceptor site but is shortened by an additional amino acid. The functional properties of rat TRPC4α and human TRPC4β, differ in an 84-amino acid domain in the cytosolic C terminus of TRPC4α that is absent in TRPC4β. A TRPC4β-like rat TRPC4β<sub>3</sub> (AF421365) shares the same splicing acceptor site but is shortened by an additional amino acid. Our data indicate that the functional properties of rat TRPC4β<sub>3</sub> are similar to rat TRPC4β<sub>4</sub> (data not shown). A number of rat and human splice variants (h[TRPC4<i>α</i>]; AF421361; r[TRPC4<i>α</i>]; AF421366; r[TRPC4<i>β</i>]; AF421367; and r[TRPC4<i>β</i>]; AF421368) are shortened at a position that corresponds to the predicted second transmembrane domain. Because these alternative splice events result in a shifted reading frame and premature stop codons, we did not characterize these truncated proteins.

When coexpressed with the rat H<sub>1</sub> histamine receptor in HEK 293 cells, r[TRPC4<i>α</i>], and more efficiently, r[TRPC4<i>β</i>], could be activated by histamine (100 μM; Fig. 1). Only a weak activation was observed for the human TRPC4<i>α</i> (Fig. 2). In control cells that were cotransfected with the H<sub>1</sub> receptor and the expression vector without insert, the addition of histamine induced an immediate [Ca<sup>2+</sup>]<sub>i</sub> elevation from 94 ± 3 to 455 ± 31 nm (means ± S.E. of five independent transfection experiments). The basal [Ca<sup>2+</sup>]<sub>i</sub> was not significantly higher in HEK 293 cells that expressed any of the investigated TRPC4 variants. How...
however, markedly higher peak values for the histamine-induced [Ca\(^{2+}\)] were transiently cotransfected with the H1 receptor and the empty expression vector instead of TRPC4, the quench rate of the fura 2 fluorescence in the presence of extracellular Mn\(^{2+}\) (1 mM) to the bath solution as indicated by the bars. Depicted are the time courses of [Ca\(^{2+}\)], and the Ca\(^{2+}\)-independent total fura 2 fluorescence. The black lines represent the calculated means, whereas the gray lines depict single cell traces. The background-corrected total fura 2 fluorescence were recorded by exciting fura 2 at its isobestic wavelength (358 nm) and normalized to the initial values of each single cell. Mn\(^{2+}\) entry into the cells results in a quenching of the fura 2 signal recorded at 358 nm.

Histamine-induced activation of human TRPC4 and TRPC4β. The human TRPC4 splice variants hTRPC4α and hTRPC4β were transiently expressed in HEK 293 cells. [Ca\(^{2+}\)] and Mn\(^{2+}\) entry were recorded as described in the legend to Fig. 1.

Because the peak [Ca\(^{2+}\)] results from both Ca\(^{2+}\) mobilization and Ca\(^{2+}\) influx, we used Mn\(^{2+}\) as a Ca\(^{2+}\)-surrogate that permeates through TRPC4 and allows a differentiation between Ca\(^{2+}\) release and cation entry (12). In control cells that were cotransfected with the H1 receptor and the empty expression vector instead of TRPC4, the quench rate of the fura 2 fluorescence in the presence of extracellular Mn\(^{2+}\) (1 mM) was 1.2 ± 0.7%/30 s before and 2.4 ± 1.0%/30 s after the addition of histamine (100 μM), respectively. In rat or human TRPC4β-expressing cells, only a marginal and not statistically significant acceleration of Mn\(^{2+}\) entry was evident in the absence of the agonist. Stimulation with histamine, however, resulted in 15- and 17-fold increases in Mn\(^{2+}\) entry for rat and human TRPC4β, respectively (Figs. 1 and 2). Regarding the α splice variants, we observed a difference between rat and human clones; histamine application accelerated the Mn\(^{2+}\) entry rate through rTRPC4α by more than 10-fold (Fig. 1), but hTRPC4α was only weakly (3.8-fold; Fig. 2) yet significantly (p < 0.05 versus vector-transfected controls) activated by histamine.

Because a store-dependent mode of activation has been described for bovine TRPC4α (6), we tested the effects of thapsigargin, an inhibitor of sarcoendoplasmic Ca\(^{2+}\)-ATPases, on human TRPC4 splice variants. Thapsigargin (2.5 μM) did not induce an acceleration of Mn\(^{2+}\) entry through hTRPC4α (Fig. 3A), hTRPC4β (Fig. 3B), or the corresponding rat orthologues (data not shown). The subsequent addition of histamine (100 μM) activated only hTRPC4β but not hTRPC4α (Fig. 3). The absence of a histamine-induced Ca\(^{2+}\) mobilization in hTRPC4α-expressing cells (Fig. 3A) indicates that the thapsigargin treatment effectively depleted InsP\(_3\)-sensitive intracellular Ca\(^{2+}\) stores. In hTRPC4β-expressing cells, however, a histamine-induced increase in [Ca\(^{2+}\)] indicative of cation entry paralleled the Mn\(^{2+}\) quench (Fig. 3B). Thus, store depletion is not sufficient to activate any of the investigated TRPC4 variants. Likewise, the addition of the membrane-permeable diacylglycerols 1,2-dioctanoyl-sn-glycerol or 1-oleoyl-2-acetyl-sn-glycerol at concentrations up to 200 μM did not induce Ca\(^{2+}\) or Mn\(^{2+}\) entry through human or rat TRPC4α and TRPC4β variants.

Electrophysiological Whole Cell Recordings—We studied the
properties of human and rat TRPC4α and TRPC4β expressed in HEK 293 cells in whole cell patch clamp recordings. Following patch rupture, no constitutive activity was observed at a holding potential of −60 mV for any of the clones (Fig. 4, A and B). Currents at the start of whole cell recording were in the range of −0.72 to −1.45 pA/pF for each of the investigated TRPC4 variants (n = 5–34). These values are not significantly different from those in vector-transfected control cells (−0.80 ± 0.22 pA/pF, n = 7), indicating the low basal activity of the TRPC4 variants studied. Again, we also tested whether hTRPC4α could be stimulated by a store depletion protocol. Four hTRPC4α-expressing cells showed no response to infusion with 100 µM InsP3 in a solution containing 10 mM EGTA with 100 mM free Ca2+ but responded to subsequent application of histamine. At a holding potential of −60 mV, histamine evoked transient inward currents in cells expressing any of the splice variants (Fig. 4C). Despite the continuous presence of the agonist, currents rapidly reached a maximum and then decayed with an initial rapid phase followed by a slow phase. Not all cells displayed the rapid phase of current decay, particularly when the current amplitude was very small. Histamine-induced inward currents were completely abolished by the removal of Na+ and Ca2+ from the extracellular solution (Fig. 4, A and B). Upon removal of histamine, the currents returned to the prestimulation level.

As reported previously for murine TRPC4β (12), the current-voltage relations of all of the splice variants had a typical doubly rectifying form (Fig. 4, A and B) and reversal potentials close to 0 mV (7.8 ± 0.5 for hTRPC4α, n = 31; 6.3 ± 0.6 for hTRPC4β, n = 22; 2.8 ± 0.4 for rTRPC4α, n = 8; and 4.6 ± 1.2 for rTRPC4β, n = 5), indicative of poor cation selectivity. In a minority of cells expressing hTRPC4α or hTRPC4β, we observed current-voltage relations that displayed a clear minimum in the inward direction and no plateau in the outward direction. These current-voltage relations closely resembled those of heteromultimers of TRPC1 and TRPC4 (20). In vector-transfected control cells, this type of cation current was not observed upon histamine application. Consistent with the low rates of Mn2+ influx in fluorometric measurements (Fig. 2), the histamine-induced increases in current density were much smaller in cells that expressed hTRPC4α than in cells expressing other TRPC4 variants (Fig. 4C).

A characteristic feature of murine TRPC4β and TRPC5 is their stimulation by micromolar concentrations of La3+ (12, 20). Likewise, extracellular La3+ rapidly and reversibly potentiated inward currents through hTRPC4α (Fig. 4D) and the other TRPC4 variants investigated (data not shown). The mean increases in current at −60 mV with 100 µM La3+ were typically 3–4-fold (n = 3–5 for each TRPC4 variant). At a concentration of 10 µM, La3+ had weaker stimulatory effects (about 50% augmentation of inward currents). The low concentrations at which it is effective and the absence of a shift in reversal potential indicate that La3+ potentiates Na+ currents rather than carrying the additional inward currents. Furthermore, some potentiation, although less than for inward currents, was also observed at positive potentials where Cs+ is the charge carrier.

Single Channel Recordings—The single channel properties of hTRPC4α and hTRPC4β were characterized in outside-out patches. Under the same conditions used for whole cell recordings, single channel events with an amplitude of around −2 pA at −60 mV were observed in outside-out patches from cells expressing either of the hTRPC4 splice variants. Levels of single channel activity were higher in patches from cells to which histamine had been applied during the whole cell recording than in patches from cells that had not previously been exposed to the agonist. Application of histamine to outside-out patches resulted in a transient, reversible stimulation of single channel activity (Fig. 5A). The potential dependence of the current amplitude is shown in Fig. 5B. Because patches were unstable at positive membrane potentials under the conditions used, we limited the analysis to potentials below 0 mV. Current amplitudes were similar at all negative potentials for both hTRPC4α and hTRPC4β (Fig. 5B). The chord conductances at −60 mV were 30.3 ± 0.6 pS (n = 7) and 29.7 ± 1.0 pS (n = 6).
for hTRPC4α and hTRPC4β, respectively. Under similar conditions, rTRPC4α displayed similar properties to the human clones (data not shown). The chord conductance was 27.5 ± 0.7 pS (n = 4) at −60 mV.

Membrane Targeting and Multimerization of TRPC4 Splice Variants—The reason for the weak receptor-induced cation entry through hTRPC4α was obscure. Because expression plasmids for both human splice variants contain the same 5′-untranslated regions and ribosomal docking sequences preceding the start codon (GCC ACC ATG), the low-receptor-induced Ca2+ and Mn2+ entry through hTRPC4α is unlikely to be caused by different expression levels of these clones. The Western blot analysis of heterologously expressed human TRPC4 variants demonstrated comparable expression levels for both TRPC4α and TRPC4β (Fig. 6A). Moreover, the expression of the C-terminally YFP-fused subunits resulted in similar fluorescence intensities of the TRPC4 splice variants in fluorescence imaging experiments (data not shown).

A retention in intracellular compartments such as the endoplasmic reticulum or the Golgi apparatus has recently been demonstrated for murine TRPC2 (23). The targeting of the splice variants was therefore studied with TRPC4 fusion proteins C-terminally tagged with the YFP. Both human and rat TRPC4 variants were integrated into the plasma membrane with similar efficiency (Fig. 6B). The clustered plasma mem-

brane distribution of rat TRPC4α resembles that of human TRPC4α (15) or murine TRPC4β (12). The lateral mobility of these clusters was highly restricted, indicating a tight connection to rigid structures such as elements of the cytoskeleton. When coexpressed, CFP-fused TRPC4α and YFP-fused TRPC4β strictly colocalize within the same clusters (data not shown).

Despite correct membrane targeting, hTRPC4α might still fail to assemble into functional channel complexes presumably requiring assembly of four channel subunits. Thus, the formation of multimeric channel complexes was analyzed. Because of the tight coupling to large immobile clusters harboring a large number of other proteins, communoprecipitation was considered as inappropriate to prove direct interactions between channel subunits. Channel multimerization was therefore assessed with a FRET approach. Static FRET efficiencies between fluorescent TRPC fusion proteins were determined by measuring the donor recovery during acceptor bleach. The maximal FRET efficiency in an intramolecularly coupled CFP-YFP tandem protein was 53.6 ± 0.5%. Because the Förster radius (R0) at which the CFP/YFP combination exhibits a FRET efficiency of 50% was assumed to be 5–6.5 nm (24), an additional 5-nm distance of the tags results in a drop of the FRET signals below 1%. Correspondingly, no significant FRET (FRET efficiency, 0.07 ± 0.2%) could be observed when CFP and YFP were cotransfected on separate plasmids (data not shown). The applicability of the FRET technique was verified using the rat vanilloid receptor (VR1), which has been demonstrated to form homomultimers (25). Coexpressed C-terminal fusion proteins of VR1 (VR1-CFP and VR1-YFP) yielded FRET efficiencies of 18.5 ± 0.5% (Fig. 7A). For coexpressed hTRPC4α-CFP/VR1-YFP or hTRPC4β-CFP/VR1-YFP, no FRET signals were detectable (Fig. 7A) confirming the sensitivity and specificity of the FRET-based analysis of TRPC multimerization. For coexpressed CFP- and YFP-tagged hTRPC4α, a FRET efficiency of 9.4 ± 0.04% was observed (Fig. 7B). Similarly, a FRET efficiency of 7.7 ± 0.3% was detected for hTRPC4β. Thus, both hTRPC4 subunits appear to form homomultimers with similar efficiencies. Interestingly, a heteromultimeric assembly of hTRPC4α-CFP and hTRPC4β-YFP (FRET efficiency, 8.3 ± 0.2%) or hTRPC4β-CFP and hTRPC4α-YFP (FRET efficiency, 7.9 ± 0.3%) resulted in FRET signals similar to their respective homomultimers (Fig. 7, B and C).

TRPC4α Is a Dominant Negative Regulator in Heteromultimeric Channel Complexes—The weak activation of hTRPC4α may be compensated by neighboring TRPC4β subunits in the same channel complex. The receptor-induced activation of heteromultimeric TRPC4 complexes was assessed by cotransfected different cDNA plasmid amounts of hTRPC4α and hTRPC4β. Unexpectedly, when HEK 293 cells were cotransfected with hTRPC4α and hTRPC4β at a 1:1 plasmid ratio, the histamine-induced Mn2+ entry was almost indistinguishable from cells that only expressed hTRPC4α (Fig. 8). A further reduction of the TRPC4α plasmid concentration in favor of hTRPC4β (1:4 plasmid ratio) revealed a reduction in histamine-stimulated Mn2+ entry of about 40% when compared with hTRPC4β expressed alone (Fig. 8). Thus, hTRPC4α subunits inhibit the conductance of heteromultimeric TRPC4 channel complexes with a high cooperativity. These data are in agreement with a model in which a single hTRPC4α channel subunit is sufficient to block a heterotetrameric TRPC4 channel complex. Because the pore region is not affected by differential splicing of TRPC4α or TRPC4β, either binding of the channel-activating agent or the subsequent allosteric movement may be prevented by the hTRPC4α subunit. To clarify whether the 84-amino acid domain that is unique for hTRPC4α
contains an autoinhibitory domain, we constructed expression plasmids encoding C-terminally truncated hTRPC4 subunits. A C-terminal truncation that contains only a part of the hTRPC4α-specific domain (hTRPC4αΔ842–977) partially recovered the activation by histamine (Fig. 9A). A deletion at the

Fig. 7. Homo- and heteromultimerization of TRPC subunits in living cells. The multimerization of TRPC subunits in living HEK 293 cells was tested by a FRET approach. Static FRET signals are detected by recording the donor recovery ($\Delta F_{\text{CFP}}$, black lines) during acceptor bleach ($F_{\text{YFP (gray lines)}}$. A, left panel, FRET signals for coexpressed rat vanilloid receptor 1 C-terminally fused to CFP (rVR1-CFP) or YFP (rVR1-YFP). B, right panel, absence of FRET signals in cells coexpressing hTRPC4α-CFP and rVR1-YFP or hTRPC4β-CFP and rVR1-YFP. FRET between TRPC4 homo- (B) and heteromultimers (C) were recorded after coexpression of the indicated fluorescent human TRPC4 subunits.

splicing donor site (Glu85 in hTRPC4α) resulted in hTRPC4αΔ842–977, which is shortened by 192 C-terminal amino acids. This gross deletion fully restored the histamine-induced Mn2⁺ entry (Fig. 9B) and whole cell currents (Fig. 9C) to the same levels as observed for hTRPC4β.

DISCUSSION

In this study we show that splice and species variants of TRPC4 exhibit major differences in their regulation despite similarities in their mechanism of activation and biophysical properties. In particular, human TRPC4β showed much stronger responses to phospholipase C-coupled receptor activation than the α splice variant. Neither reduced expression levels, nor a defect in the plasma membrane targeting account for the weak receptor-induced activation of hTRPC4α. Furthermore, we have successfully applied the FRET technique to study TRPC homo- and heteromultimerization. The similar FRET signals observed for coexpressed fluorescent TRPC4α and TRPC4β subunits indicate that homo- and heteromultimers form with comparable efficiencies. The functional properties of heteromultimeric hTRPC4 channel complexes revealed a dominant negative effect of the hTRPC4α subunit. C-terminal truncation experiments confirmed the presence of an autoinhibitory domain in hTRPC4α.

Our functional data on heterologously expressed TRPC4α or TRPC4β species variants are consistent with a receptor-operated but store-independent mode of activation. By contrast, either store-dependent activation or basal activity without
acute stimulation have been reported for bovine and human TRPC4α (6, 16). In our hands, hTRPC4α is only poorly activated by histamine and not responsive to store depletion protocols. Earlier reports that a frameshifted splice variant that ends at the second transmembrane domain and corresponds to our rat TRPC4β might act as a store-operated CCE channel (18, 19) are in contrast with our findings for the full-length rTRPC4 variants. Interestingly the N-terminal 765 amino acids of human, bovine, mouse, or rat TRPC4 display a similarity of more than 99% whereas the C-terminal 212 amino acids are significantly less conserved (76% similarity). The intact receptor-induced gating of rTRPC4α is in agreement with recent data obtained with the phylogenetically more closely related murine TRPC4α (20). Although this does not exclude the presence of functionally relevant domains in the C terminus, its structural constraints appear to be less conserved during evolution. In a number of human tissues, TRPC4α transcripts appear to be more abundant than the TRPC4β splice variant (26). Another study, however, reported the abundant expression of the hTRPC4β in gastrointestinal myocytes (27). Interestingly, the biophysical and regulatory properties of endogenous receptor-activated currents in ileal smooth muscle cells (28) display a striking similarity to those of heterologously expressed TRPC4. Furthermore, the requirement for intracellular Ca\(^{2+}\) and a potentiation of inward currents by external La\(^{3+}\) has been also reported for the endogenous acetylcholine-induced currents in ileal myocytes (29, 30).

Our data favor a model in which human TRPC4α subunits contain an autoinhibitory domain that inhibits the receptor-induced activation of homo- or heteromultimeric TRPC4 channel complexes. To date, a dominant negative effect between TRPC subunits has only been described for artificial N-terminal TRPC3 or TRPC6 fragments, which might interfere with the multimerization process (31, 32). However, the mechanism as well as the selectivity of those dominant negative effects are not substantiated by experimental data. In contrast to the dominant negative effects of a MaxiK channel splice variant (33) or Kv1.1 channels (34), the surface targeting and multimerization of hTRPC4α subunits are not affected. Alternative concepts like the inactivation by a ball-and-chain domain in voltage-gated “Shaker” potassium channels (35) or a phosphorylation-sensitive autoinhibitory domain as described in the CFTR (36) will have to be tested.

The store-independent gating and the biophysical properties of TRPC4 variants argue against previous findings that TRPC4 by itself may be a part of Ca\(^{2+}\)-selective, store-operated channels. Nonetheless, our data are not in conflict with other concepts how TRPC4 variants may be linked to CCE. Because the autoinhibitory domain of TRPC4α overlaps with recently described binding motifs for calmodulin (37, 38) and/or Ins\(_P_3\) receptors (26), binding of these proteins may serve additional regulatory mechanisms. There is evidence for the existence of two independent calmodulin-binding sites on TRPC4α. The first is present in both TRPC4α and TRPC4β and alternatively binds to a N-terminal F2q segment of Ins\(_P_3\) receptors (37, 38). A second calmodulin-binding site is present only in TRPC4α and does not bind the F2q segment (38) but a C-terminal portion of Ins\(_P_3\) receptors (26). Although infusion of Ins\(_P_3\) was not sufficient to activate any of the TRPC4 variants investigated in this study, we do not want to exclude the possibility that the binding site for Ins\(_P_3\) receptors may play a role in TRPC4 activation at low expression levels. Alternatively, TRPC4α-containing complexes may anchor the endoplasmic reticulum via Ins\(_P_3\) receptors. Thus, it is plausible that TRPC4 channels may act as a scaffold to attach the endoplasmic reticulum in the vicinity of store-operated channels and thereby optimize CCE. Although Ins\(_P_3\) receptors, calmodulin, and the Na\(^{+}/H^+\) exchanger regulatory factor (39) are proteins currently known to interact with TRPC4, the identification of other proteins that reside in the same clusters as TRPC4 may reveal the composition of TRPC4 signaling complexes in vivo.

The investigation of TRPC4 subunit multimerization is complicated by its tight docking to rigid supramolecular structures as inferred from the clustered appearance of TRPC4 and the very low lateral mobility of these patches within the plasma membrane. Attachment of TRPC4 to supramolecular structures precludes the identification of directly interacting proteins with standard coimmunoprecipitation approaches. Therefore, the TRPC4 subunit assembly was studied in living cells using the FRET technique. The vanilloid receptor 1, which is an essential component of the pain pathway (40) and which is known to form homotramers (25), was chosen as a positive control. The robust FRET signals for coexpressed CFP- and YFP-tagged VR1 support these findings. The inability of CFP-fused hTRPC4 splice variants to generate a FRET signal with VR1-YFP further confirmed the sensitivity and specificity of the FRET-based multimerization assay. This first application of the FRET technique to detect the assembly of ion channel subunits in living cells will be extended to clarify the specificity and promiscuity of TRPC heteromultimerization in future studies.

Considering that hTRPC4α is a dominant negative modulator of hTRPC4β, it may predominantly function in TRPC complexes that contain other TRPC subunits such as the ubiquitously expressed TRPC1 (20). Our preliminary data, however, failed to demonstrate a receptor-induced activation of channel complexes formed by TRPC1 and the α splice variant of hTRPC4. Although coexpressed in selected structures in the central nervous system, the restricted expression pattern of TRPC5, the closest relative of TRPC4, makes it unlikely that these TRPC subunits combine in other tissues. Taken together, the regulatory properties of TRPC4α cloned from different species are inconsistent, presumably because of the variability of the C-terminal cytosolic domain. We provide evidence that some of the apparently conflicting findings for TRPC4 regulation rely on the different function of TRPC4 species and splice variants. In addition, we show that FRET between fluorescent subunits is a valuable tool for studying TRPC homo- and heteromultimerization in living cells. Our data support a concept in which TRPC4β is a functional cation channel across various species, whereas hTRPC4α subunits prevent full channel activation but offer additional sites for protein-protein interactions.

Acknowledgments—We thank Peter Reusch for critical reading of the manuscript. We are also grateful to Prof. Eberhard Nieschlag and Ching-Hei Yeung (Institut für Reproduktionsmedizin, Universität Münster, Germany) for providing human testis biopsies.

REFERENCES

1. Harteneck, C., Plant, T. D., and Schultz, G. (2000) Trends Neurosci. 23, 159–166
2. Clapham, D. E., Runnels, L. W., and Strubing, C. (2001) Nat. Rev. Neurosci. 2, 387–396
3. Zhu, X., Jiang, M., Peyton, M., Boulay, G., Hurst, S., Stafini, E., and Birnbaumer, L. (1996) Cell 85, 661–671
4. Boulay, G., Zhu, X., Peyton, M., Jiang, M., Hurst, S., Stafini, E., and Birnbaumer, L. (1997) J. Biol. Chem. 272, 29672–29680
5. Zitt, C., Zobel, A., Obukhov, A. G., Hartzenecker, C., Kaufkrenner, F., Lückhoff, A., and Schulz, G. (1996) Neuron 16, 1189–1196
6. Philipp, S., Cavalié, A., Freichel, M., Wissenbach, U., Zimmer, S., Trost, C., Marquart, A., Murakami, M., and Flockerzi, V. (1996) EMBO J. 15, 6166–6171
7. Zitt, C., Obukhov, A. G., Strubing, C., Zobel, A., Kaufkrenner, F., Lückhoff, A., and Schulz, G. (1997) J. Cell Biol. 138, 1333–1341
8. Okada, T., Shimizu, S., Wakanomi, M., Maeda, A., Kurosaki, T., Takada, N., Imoto, K., and Mori, Y. (1998) J. Biol. Chem. 273, 10279–10287
9. Philipp, S., Hambrecht, J., Braslavski, L., Schroth, G., Freichel, M., Murakami, M., Cavalié, A., and Flockerzi, V. (1998) EMBO J. 17, 4274–4282
10. Hofmann, T., Obukhov, A. G., Schaefer, M., Harteneck, C., Gudermann, T., and Schultz, G. (1999) *Nature* **397**, 259–263
11. Vannier, B., Peyton, M., Boulay, G., Brown, D., Qiu, N., Jiang, M., Zhu, X., and Birnbaumer, L. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96**, 2060–2064
12. Schaefer, M., Plant, T. D., Obukhov, A. G., Hofmann, T., Gudermann, T., and Schultz, G. (2000) *J. Biol. Chem.* **275**, 17517–17526
13. Vazquez, G., Lievremont, J. P., St. J. Bird, G. S. J., and Putney, J. W., Jr. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98**, 11777–11782
14. Schaefer, M., Plant, T. D., Obukhov, A. G., Hofmann, T., Gudermann, T., and Schultz, G. (2000) *J. Biol. Chem.* **275**, 17517–17526
15. Venkataraman, K., Ma, H. T., Ford, D. L., and Gill, D. L. (2001) *J. Biol. Chem.* **276**, 33980–33985
16. Reusch, H. P., Schaefer, M., Plum, C., Schultz, G., and Paul, M. (2001) *J. Biol. Chem.* **276**, 19540–19547
17. Heim, R. (1999) *Methods Enzymol.* **302**, 408–423
18. Kedei, N., Szabo, T., Lile, J. D., Treanor, J. J., Olah, Z., Iadarola, M. J., and Blumberg, P. M. (2001) *FEBS Lett.* **286**, 28613–28619
19. Mery, L., Magnino, F., Schmidt, K., Krause, K. H., and Dufour, J. F. (2001) *FEBS Lett.* **286**, 28613–28619
20. Zhang, L., and Saffen, D. (2001) *J. Biol. Chem.* **276**, 13331–13339
21. Manganas, L. N., and Trimmer, J. S. (2000) *J. Biol. Chem.* **275**, 29685–29693
22. Tang, Y., Tang, J., Chen, Z., Trost, C., Flockerzi, V., Li, M., Ramesh, V., and Zhu, M. X. (2000) *J. Biol. Chem.* **275**, 37559–37564
23. Caterina, M. J., Leffler, A., Malmberg, A. B., Martin, W. J., Tranter, J., Petersen-Verzijl, K. R., Koltzenburg, M., Basbaum, A. I., and Julius, D. (2000) *Science* **288**, 306–313
