Coassembly of Trp1 and Trp3 Proteins Generates Diacylglycerol- and Ca\(^{2+}\)-sensitive Cation Channels*

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To analyze the functional consequences of coassembly of transient receptor potential 1 (Trp1) and Trp3 channel proteins, we characterized membrane conductances and divergent cation entry derived by separate overexpression and by coexpression of both Trp isoforms. Trp1 expression generated a 1-oleoyl-2-acetyl-sn-glycerol (OAG)-activated conductance that was detectable only in Ca\(^{2+}\)-free extracellular solution. Trp3 expression gave rise to an OAG-activated conductance that was suppressed but clearly detectable at physiological Ca\(^{2+}\) concentrations. Coexpression of both species resulted in a constitutively active, OAG-sensitive conductance, which exhibited distinctive cation selectivity and high sensitivity to inhibition by intracellular Ca\(^{2+}\). Trp1-expressing cells displayed only modest carbachol-induced Ca\(^{2+}\) entry and lacked OAG-induced Sr\(^{2+}\) entry, whereas Trp3-expressing cells responded to both agents with a substantial divalent cation entry. Coexpression of Trp1 plus Trp3 suppressed carbachol-induced Ca\(^{2+}\) entry compared with Trp3 expression and abolished OAG-induced Sr\(^{2+}\) entry signals. We concluded that coassembly of Trp1 and Trp3 resulted in the formation of oligomeric Trp channels that are subject to regulation by phospholipase C and Ca\(^{2+}\). The distinguished Ca\(^{2+}\) sensitivity of these Trp1/Trp3 hetero-oligomers appeared to limit Trp-mediated Ca\(^{2+}\) signals and may be of importance for negative feedback control of Trp function in mammalian cells.

Receptor-mediated stimulation of phospholipase C is associated with various alterations in cellular cation conductances. Trp1 and Trp-related genes, originally cloned from Drosophila melanogaster (1, 2), have been suggested to encode ion channel proteins that function in terms of a link between cellular phospholipase C activity and cation transport across the plasma membrane (3–6). The ability of mammalian Trp proteins to produce cation conductances has been demonstrated in a number of heterologous expression systems. Some Trp species were found capable of forming highly Ca\(^{2+}\)-selective channels, which may provide the basis of Ca\(^{2+}\) entry and refilling of intracellular Ca\(^{2+}\) stores after IP\(_3\)-mediated store depletion (7, 8). In contrast, other Trp isoforms appear to form rather nonspecific cation channels that allow permeation of both mono- and divalent cations (9–12). These nonspecific, Trp-mediated conductances are likely to play a significant physiological role not only because of their substantial impact on membrane potential and homeostasis of monovalent ions such as Na\(^+\) but also because of a small but nonetheless detectable Ca\(^{2+}\) entry mediated by these ion channels (9, 10, 12). Albeit receptor-mediated stimulation of phospholipase C is unequivocally a key event in cellular regulation of Trp channels, the different species appear to be linked to this event via divergent mechanisms. For Trp3, a direct conformational coupling to activated IP\(_3\) receptors has recently been demonstrated (4, 6), whereas other species such as Trp1, -4, and -5 appear to be sensitive to depletion of the cellular Ca\(^{2+}\) stores without activation of the IP\(_3\) receptor (7, 8, 9). Alternatively, activation by diacylglycerols has been suggested as a central mechanism of regulation for Trp3 and related species such as Trp6 (5). Thus, overexpression of individual Trp isoforms results in the formation of cation channels that display divergent regulatory and biophysical properties. The cation channels produced by overexpression of a Trp isoform are considered to be multimeric, probably tetrameric, complexes based on the current models of Trp channel structure (13, 14). The existence of heteromultimeric Trp channels in tissues that express multiple isoforms appears likely because of a recent report demonstrating strong protein-protein interactions between Trp1 and Trp3 proteins in the human embryonic kidney (HEK) 293 expression system (14). Moreover, the latter study provided evidence for a substantial modification of the conductance provided by Drosophila Trp in conditions that favor coassembly of Drosophila Trp and Trp-like (Trp1) proteins.

The present study was designed to investigate the functional consequences of hetero-oligomerization of two mammalian Trp species, i.e. hTrp1 and hTrp3. We present evidence for functional interaction of Trp1 and Trp3 proteins, resulting in the formation of a heteromultimeric cation channel of distinct biophysical and regulatory properties.

**EXPERIMENTAL PROCEDURES**

*Cell Culture and Transfection—*HEK293 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and, in the case of stably transfected cells, with 0.178 g/liter Geneticin. The cells were either stably or transiently transfected with the coding region of hTrp1 (GenBankTM accession number X89066) or hTrp3 (GenBankTM accession number U47050) as described (3). These cell lines were designated as HEKTrp1 and HEKTrp3, respectively. To
study the function of channels derived by hetero-oligomerization, Trp isoforms were coexpressed by transient expression of hTrp1 in HEK-Trp3 cells. The cells were additionally transfected to express green fluorescent protein as a marker for successful transfection (15). Electrophysiological recordings and the measurement of intracellular Ca\textsuperscript{2+} signals were performed 2–3 days after transfection in green fluorescent protein-positive cells.

**Measurement of Membrane Currents**—Extracellular solution contained 137 mM NaCl, 2.5 mM methanesulfonic acid cesium salt, 10 mM HEPES, 2.0 mM CaCl\textsubscript{2}, or 0.1 mM EGTA; pH was adjusted to 7.4 with N-methyl-d-glucamine. In some experiments, NaCl or CaCl\textsubscript{2} was omitted in the bath solution and substituted by choline chloride. The pipette solution contained 130 mM methanesulfonic acid cesium salt, 10 mM HEPES, 5 mM EGTA or 10 mM BAPTA, respectively; pH was adjusted to 7.4 with N-methyl-d-glucamine, and pCa was measured using a Ca\textsuperscript{2+}-sensitive electrode.

Membrane currents were recorded in conventional whole-cell patch clamp mode using an Axopatch 200A amplifier (Axon Instruments, Foster City, CA). Patch pipettes were fabricated from borosilicate glass (Clark Electromedical Instruments, Pangbourne, UK) and had resistances of 1–2 M\textOmega. To induce rapid depletion of intracellular Ca\textsuperscript{2+} stores upon obtaining conventional whole-cell configuration, 100 \muM IP\textsubscript{3} was included in the pipette solution in some experiments (as indicated). Changes in membrane conductances were studied by applying slow voltage ramps (0.06 V s\textsuperscript{-1}; 0.2 Hz). All experiments were performed at room temperature.

**Ca\textsuperscript{2+} Measurement**—The intracellular free Ca\textsuperscript{2+} concentration was determined by measurement of fura-2 fluorescence in single cells as described previously (16). Briefly, cells were harvested by use of trypsin, centrifuged, resuspended in Dulbecco’s modified Eagle’s medium, and loaded with fura-2/AM (2 \muM) at 37 °C in serum-free medium for 45 min. Fura-2-loaded cells were centrifuged, washed twice, and resuspended in extracellular solution (see above). Fura-2 fluorescence was measured in single cells by excitation at 360 and 380 nm (alternatively every 50 ms), and collection of the emission was made at 510 nm. Changes in intracellular Ca\textsuperscript{2+} concentration were expressed as the ratio of emission recorded at 360 nm/380 nm excitation ($F_{360/380}$) (16, 17).

**Statistics**—Averaged results are given as mean ± S.E. for the indicated number of experiments. Two-tailed t tests were used for statistical comparisons, using either the unpaired or paired format. Differences were considered statistically significant at p < 0.05.

**Materials**—Tissue culture medium was from Life Technologies, Inc. (Vienna, Austria); all other chemicals were from Sigma Chemical Co. (Vienna, Austria). Solutions containing OAG were sonicated for 5 min before use.

**RESULTS**

**Trp1 and Trp3 Cation Channels Are Activated by Diacylglycerol and Inhibited by Extracellular Ca\textsuperscript{2+}**—In an initial set of experiments, we characterized the membrane conductances produced by overexpression of either Trp1 or Trp3 alone in HEK293 cells (HEKTrp1 and HEKTrp3). In conventional whole-cell experiments using a pipette solution that contained 10 mM BAPTA to buffer free Ca\textsuperscript{2+} below 10 nM, i.e. conditions that are expected to deplete intracellular Ca\textsuperscript{2+} stores, membrane conductances of either HEKTrp1 or HEKTrp3 cells were not significantly affected even during prolonged dialysis of cells. Similarly, active depletion of stores by inclusion of 100 \muM IP\textsubscript{3} into the pipette failed to induce appreciable membrane conductances in HEKTrp1 or HEKTrp3 cells. In HEKTrp3 cells, the membrane currents, measured at −80 mV after a dialysis of 5–8 min, were −18 ± 4 pA (n = 5) in the absence and −28 ± 8 pA (n = 8) in the presence of 100 \muM IP\textsubscript{3}. Moreover, insensitivity of both Trp1 and Trp3 channels to store depletion was also indicated in experiments using thapsigargin to deplete intracellular Ca\textsuperscript{2+} stores. Thapsigargin failed to induce significant changes in membrane conductance of HEKTrp1 (n = 3) as well as HEKTrp3 (n = 4; not shown). Typical experiments performed in nominally Ca\textsuperscript{2+}-free bath solution and with a pipette solution containing both 10 mM BAPTA plus 100 \muM IP\textsubscript{3} are illustrated in Fig. 1. Under these conditions, dialysis of HEKTrp1 or HEKTrp3 cells resulted in a slow, minute increase in membrane conductance as evident from the time course of membrane current measured at −80 mV shown in the upper panels of Fig. 1. By contrast, the potential Trp channel activator OAG (100 \muM) induced a substantial membrane conductance in both HEKTrp1 and HEKTrp3 cells, resulting in inward currents of −111 ± 56 pA (n = 5) and −146 ± 43 pA (n = 6) at −80 mV, respectively. A similar activation of membrane currents in cells by OAG was also observed in both HEKTrp1 and HEKTrp3 when IP\textsubscript{3} was omitted from the pipette solution (not shown), whereas vector-transfected control cells did not respond to OAG (current level at −80 mV = −13.4 ± 4 pA, n = 8). The lower panels of Fig. 1 illustrate the current to voltage relationship of the OAG-induced currents in HEKTrp1 and HEKTrp3. It is clearly evident that the Trp1-mediated current displayed an almost linear current to voltage relation with some inward rectification, whereas Trp3-mediated currents showed slight outward rectification. With Na\textsuperscript{+} and Ca\textsuperscript{2+} as the main charge carriers, the currents reversed in both HEKTrp1 and HEKTrp3 cells, close to neutral potential (+13 mV and +12 mV, respectively), indicating a nonselective conductance. The OAG-activated currents recorded in either Trp1- or Trp3-expressing cells were effectively suppressed when extracellular Ca\textsuperscript{2+} was elevated to physiological levels (2 mM) (Fig. 1, upper panels). Addition of Ca\textsuperscript{2+} blocked OAG-induced currents in Trp1-expressing cells fairly completely, i.e. to a level close to the initial membrane conductance measured at the beginning of the experiments. In Trp3-expressing cells a small but significant level of current remained in the presence of extracellular Ca\textsuperscript{2+}. This incomplete suppression of Trp3-mediated currents by extracellular Ca\textsuperscript{2+} was clearly evident in experiments performed in the presence of physiological Ca\textsuperscript{2+} concentrations. As illustrated in Fig. 2, OAG failed to elicit any current response in HEKTrp1 cells. However, it induced a small but significant increase in membrane conductance of HEKTrp3 cells when 2 mM Ca\textsuperscript{2+} was present in the extracellular solution. The current was sensitive to block by La\textsuperscript{3+} and carried in part by Na\textsuperscript{+} as exchange of extracellular Na\textsuperscript{+} by choline shifted the current to voltage relationship (Fig. 2, lower panels) by approximately 20 mV in hyperpolarizing direction. Thus, under physiological conditions, i.e. in the presence of millimolar concentrations of extracellular Ca\textsuperscript{2+}, OAG was able...
to activate Trp3 but not Trp1 currents. These results demonstrated that overexpression of Trp1 or Trp3 alone in HEK293 cells generated different membrane conductances that share the principle sensitivity to activation by diacylglycerols and to inhibition by extracellular Ca\(^{2+}\).

**Coexpression of Trp1 and Trp3 Proteins Generates a Unique Cation Conductance**—Because the presence of Trp1/Trp3 hetero-oligomers in cells that coexpress both isoforms has recently been demonstrated (14), it appeared of interest to investigate the functional consequences of this protein-protein interaction. To promote the formation of such heteromultimeric Trp1/Trp3 complexes we overexpressed Trp1 in HEKTrp3 cells and characterized the membrane properties of these HEKTrp1/3 cells. Fig. 3 shows an overview of the levels of basal membrane currents measured at −80 mV in HEKTrp1, HEKTrp3, and HEKTrp1/3 cells. The most striking effect of coexpression of Trp1 and Trp3 proteins was an unusually high level of constitutive, i.e. basal, activity. HEKTrp1/3 displayed a large basal inward current at −80 mV, which was inhibited by extracellular Ca\(^{2+}\).

As illustrated in Fig. 4, HEKTrp1/3 cells responded to OAG with a clear increase in membrane conductance, and the Trp1 plus Trp3 (Trp1/3)-mediated currents were again sensitive to inhibition by extracellular Ca\(^{2+}\) as well as La\(^{3+}\). A comparison of the current to voltage relationship of the OAG-induced membrane conductances in HEKTrp3 and Trp1/Trp3 cells with Na\(^{+}\) and Ca\(^{2+}\) as the main charge carrier revealed a moderate but significant difference in the cation selectivity as evident from a about a 12-mV more positive reversal potential in HEKTrp3 cells (Fig. 5).

In both HEKTrp3 and HEKTrp1/3 cells, basal as well as OAG-stimulated membrane currents were suppressed but still clearly detectable in the presence of physiological extracellular Ca\(^{2+}\). Fig. 6 shows a comparison of the currents measured in (i) nominally Ca\(^{2+}\)-free bath solution, (ii) after instantaneous elevation of extracellular Ca\(^{2+}\) from nominally free to a physiological level (acute elevation), and (iii) in the continuous presence of extracellular Ca\(^{2+}\). Trp1-mediated currents were detectable only when extracellular Ca\(^{2+}\) was low (nominally Ca\(^{2+}\)-free = approximately 1–5 μM). Trp3 currents were also largest in nominally Ca\(^{2+}\)-free solution, but Ca\(^{2+}\)-induced inhibition of the Trp3 conductance was more prominent when Ca\(^{2+}\) was elevated instantaneously. In contrast, the suppression of Trp3/1 currents by continuously elevated Ca\(^{2+}\) was even slightly more pronounced than in the initial phase after Ca\(^{2+}\) elevation. Thus, the behavior of Trp3/1 currents was in clear contrast to the characteristics of Trp3-mediated currents and also to that of Trp1 currents, which were completely occluded in the presence of extracellular Ca\(^{2+}\). This divergent regulation by changes in extracellular Ca\(^{2+}\) was even more pronounced when a moderate intracellular Ca\(^{2+}\) buffer was used. In the experiments shown in Fig. 7, intracellular Ca\(^{2+}\) was buffered moderately using 5 mM EGTA. Trp3 currents were rapidly suppressed during acute elevation of extracellular Ca\(^{2+}\) but recovered substantially during the continuous presence of Ca\(^{2+}\), whereas Trp1/3 currents were persistently suppressed by extracellular Ca\(^{2+}\).

The phenomenon of Trp currents being effectively blocked by acute administration of extracellular Ca\(^{2+}\) but less in the continuous presence of Ca\(^{2+}\) has been explained previously by intracellular Ca\(^{2+}\)-mediated stimulation of channel activity (18). Thus, it appeared of interest to test for a dependence of currents on the intracellular Ca\(^{2+}\) buffer system, which determines the level of intracellular Ca\(^{2+}\) during the Ca\(^{2+}\) entry associated with elevation of extracellular Ca\(^{2+}\). Therefore, we performed experiments with pipette solutions containing different Ca\(^{2+}\) buffer systems. Indeed, Trp3- and Trp1/Trp3-me-
results demonstrate a divergent sensitivity to intracellular difference versus basal currents from OAG-induced currents, using NaTrp3 and HEKTrp1/3 cells. Difference currents derived by subtraction of basal currents from OAG-induced currents, using Na− and Ca2+ as the main charge-carrying cations in the extra- and intracellular solutions, respectively, are shown. Reversal potentials are indicated by arrows. Lower panel shows mean reversal potentials of OAG-induced Trp1, Trp3, and Trp1/3. Mean values ± S.E. (n = 6–9) are given. * denotes significant difference versus Trp1 and Trp3.

**FIG. 6. Effects of extracellular Ca2+ on Trp currents.** Mean-induced inward currents recorded in the presence of OAG (100 µM, at -80 mV) in cells expressing Trp1, Trp3, and Trp1/3, respectively, are shown. Values measured in the absence of extracellular Ca2+ (black columns), during acute elevation of extracellular Ca2+ (open columns; a), and in the continuous presence of extracellular Ca2+ (gray columns; c) are shown. Experiments were performed with 10 mM BAPTA in the pipette. Mean values ± S.E. (n = 3–8) are given. * denotes significant difference versus acute administration.


diated conductances were dierently affected by variation of the intracellular Ca2+ buffer capacity (Fig. 8). OAG-induced Trp3 currents, recorded in physiological Ca2+ solutions, were barely affected or were even slightly promoted by lowering the intracellular Ca2+ buffer capacity, whereas OAG-induced Trp1/3 currents were markedly suppressed when intracellular BAPTA (10 mM) was exchanged with EGTA (5 mM). These results demonstrate a divergent sensitivity to intracellular Ca2+ of the cation channels present in HEKTrp3 and HEK-Trp1/3 cells. The coexpression of both Trp species creates a cation conductance that appears to be effectively inhibited during Ca2+ entry-induced elevation of cytoplasmic Ca2+ and in turn promoted when cytoplasmic Ca2+ levels are low.

**FIG. 7. Coexpression of Trp1 and Trp3 creates a membrane conductance of distinct sensitivity to extracellular Ca2+.** Time courses of membrane current at -80 mV recorded in HEK cells expressing Trp3 (left) and Trp1/3 (right) are shown. Cells were dialyzed with a pipette solution that contained 5 mM EGTA starting with the rupture of the patch at time 0, and membrane currents were recorded in nominally Ca2+-free solution. Administration of OAG (100 µM) and Ca2+ (2 mM) is indicated.

**FIG. 8. Coexpression of Trp1 and Trp3 creates a membrane conductance of distinct sensitivity to intracellular Ca2+.** Upper panels show the current to voltage relation of membrane currents recorded in the presence of OAG (100 µM) in HEKTrp3 and HEKTrp1/3 cells using different intracellular solutions. Cells were dialyzed with pipette solutions that contained either 5 mM EGTA or 10 mM BAPTA, and membrane currents were recorded in nominally Ca2+-free solution. Administration of OAG (100 µM) and Ca2+ (2 mM) is indicated. Lower panels show mean-induced inward currents recorded at -80 mV in the absence (black columns) and presence of OAG (100 µM) (gray columns) using EGTA and BAPTA in the pipette solution, respectively. Values were measured in the presence of extracellular Ca2+ (2 mM). Mean values ± S.E. (n = 6–9) are given. * denotes significant difference versus experiments with BAPTA-containing pipette solution.

**Coexpression of Trp Isoforms Modifies Trp-mediated Ca2+ Signals**—Because coexpression of Trp1 and Trp3 proteins was found to generate a cation conductance with unique properties, including a Ca2+ sensitivity distinctly different from that of Trp3 currents, we studied the consequences of coexpression of these Trp isoforms for divergent cation entry. Fig. 9A shows a comparison of the Ca2+ entry into carbachol-stimulated HEKTrp1, HEKTrp3, and HEKTrp1/3 cells. The cells were challenged with 100 µM carbachol in nominally Ca2+-free solution, and Ca2+ entry was initiated by elevation of extracellular Ca2+ to 1 mM. Ca2+ entry into Trp1-expressing cells was not significantly different from that recorded in vector-transfected controls (not shown), whereas Ca2+ entry into HEKTrp3 cells was significantly larger. Coexpression of Trp3 and Trp1 proteins resulted in a Ca2+ entry signal that was comparable
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Fig. 9. Carbachol- and OAG-induced divalent cation entry into HEK293 cells that overexpress Trp1, Trp3, or Trp1/3. Fura-2-loaded HEKTrp1, HEKTrp3, and HEKTrp1/3 cells were stimulated to induce divalent cation entry. A, cells were challenged with 100 μM carbachol (CCC) in nominally Ca²⁺-free solution and extracellular Ca²⁺ was elevated to initiate Ca²⁺ entry as indicated. B, cells were stimulated with 100 μM OAG in the presence of 1 mM Sr²⁺ as indicated. All cells expressed green fluorescent protein that was used as a marker for positive transfection. Mean values ± S.E. for the divalent sensitive fluorescence ratio (F₃₈₀/F₃₄₀) obtained during divalent cation entry are given (n = 6–9). * denotes significant difference versus Trp3.

with that recorded in HEKTrp1 cells and was significantly smaller than that of HEKTrp3 cells, indicating a profound inhibitory effect of Trp1 on Trp3-mediated Ca²⁺ signaling. In a second set of experiments, we recorded OAG-induced divalent cation entry into these HEK293 cells with different Trp expression. We used Sr²⁺ as a divalent cation, which allows for a rather selective analysis of Trp3 channel function (19). In the presence of extracellular Sr²⁺, OAG (100 μM) induced a clear increase in fura-2 fluorescence in HEKTrp3 cells but not in HEKTrp1 or HEKTrp1/3 cells (Fig. 9B). Thus, overexpression of the Trp3 alone promotes carbachol-induced Ca²⁺ signals and generates a significant OAG-activated Sr²⁺ entry, whereas overexpression of Trp1 together with Trp3 exerts an inhibitory modulation on the Trp3-mediated divalent cation entry.

**DISCUSSION**

The present study suggests that hetero-oligomerization is a crucial determinant of Trp channel function and regulation. We observed that coexpression of the Trp1 and the Trp3 isoform resulted in the formation of cation channels that exhibited distinctive biophysical and regulatory properties. It is suggested that hetero-oligomerization determines the function and physiological role of Trp cation channels in tissues that express multiple Trp isoforms.

**Regulation of Trp1 and Trp3 Channels in HEK293 Cells**—Trp proteins currently appear as most attractive candidates for cation channels that mediate capacitative Ca²⁺ entry, i.e., Ca²⁺-permeable channels that are activated in response to a depletion of intracellular Ca²⁺ stores (20). All mammalian Trp isoforms are apparently sensitive to activation via the phospholipase C/IP₃/store depletion pathway. The molecular mechanism of channel activation in response to stimulation of phospholipase C may vary for different Trp species and is likely to involve interaction of the channels with activated IP₃ receptors (4, 6), IP₃-induced store depletion (7–9), or alternatively direct activation by diacylglycerols (5, 16). Trp3 has been reported to provide some constitutive activity and to be activated both by IP₃ (4, 6) and diacylglycerols (5). The available reports on regulatory properties of Trp1 are highly discrepant. Expression of Trp1 in Chinese hamster ovary cells was reported to provide a cation conductance that is sensitive to store depletion by thapsigargin as well as IP₃ (9). Trp1 channels expressed in Sf9 cells, in contrast, were found to be constitutively active but insensitive to store depletion (10). Our present experiments with HEK293 cells overexpressing Trp1 showed that this Trp species failed to produce any constitutively active conductance and was barely sensitive to passive store depletion in this expression system. Similarly, active depletion of Ca²⁺ stores by intracellular administration of IP₃ did not promote Trp1 currents. For Trp3, recent evidence suggests that IP₃ receptors play a pivotal role in activation of these Trp channels (4). In the present study, intracellular administration of IP₃ exerted only modest effects on the membrane currents in Trp3-expressing HEK293 cells. The reason for this low IP₃ sensitivity of Trp3 in the present study is unclear. Our results suggest that the channel derived by overexpression of both Trp species in HEK293 cells is barely sensitive to passive as well as active store depletion.

Nonetheless, Trp1 as well as Trp3 expression gave rise to a marked diacylglycerol-sensitive cation conductance in HEK293. Large OAG-induced currents were observed in all Trp3- and Trp1-expressing cells when measurements were performed in nominally Ca²⁺-free solution. Activation of the Trp3/6 subfamily by diacylglycerols has recently been demonstrated, whereas Trp1 was suggested to be insensitive to diacylglycerols (5). Here we demonstrated that OAG-induced activation of Trp1 currents was unequivocally detectable in the HEK293 expression system. This discrepancy is well explained by the fact that so far activation by diacylglycerols of Trp currents was studied exclusively in the presence of divalent cations (5). In the present study, OAG-induced activation of Trp1 currents was observed only when extracellular Ca²⁺ was reduced to low micromolar levels (nominally Ca²⁺-free). Thus we present for the first time evidence for OAG sensitivity of the Trp1 protein, demonstrating that Trp1 and Trp3 share the principle property of activation by diacylglycerols. These results also suggest that the elucidation of the ion channel function of Trp proteins requires a careful analysis of the Ca²⁺ sensitivity of these channels and that Trp channel activity may easily be missed because of unfavorable experimental conditions.

So far, Ca²⁺ ions have been reported to exert variable effects on Trp channel function (8, 12, 18, 21). Nonetheless, inhibition of Trp currents by extracellular divalents is a commonly observed phenomenon. In the present study, Trp1- and Trp3-mediated conductances were substantially inhibited in the presence of physiological levels of Ca²⁺. Trp1 currents were completely occluded in Ca²⁺-containing solutions, whereas Trp3 currents showed a tendency to recover slowly after elevation of extracellular Ca²⁺ to physiological levels. This phenomenon was prominent when a low intracellular Ca²⁺ buffer capacity was used and may be explained by an as yet unidentified Ca²⁺-dependent cellular mechanism that promotes Trp3 activity. A similar phenomenon has recently been reported for the structurally related Trp7 protein (18). Moreover, evidence has been presented for activation of Trp3 by intracellular Ca²⁺.
Properties of Heteromultimeric Trp1/Trp3 Cation Channels—Protein-protein interactions between different Trp proteins have recently been demonstrated by coimmunoprecipitation (14). Similarly, an interaction between the Drosophila Trp and Trp1 proteins has been reported, and this Trp/Trp1 hetero-oligomerization was found to result in the generation of cation channels with unique properties (14). A coexpression of multiple Trp species has been observed in various mammalian tissues (15, 23, 24). Thus, it appears reasonable to speculate that specific membrane conductances in these tissues are based on heteromultimeric Trp complexes. In this study, we obtained evidence for a profound functional consequence of Trp1/Trp3 hetero-oligomerization. The membrane conductance of cells that overexpressed both Trp species exhibited properties distinctly different from the membrane conductance of cells that overexpressed only a single species. In striking contrast to Trp1 or Trp3 channels, Trp1/Trp3 channels displayed a high constitutive activity that was inhibited by extracellular Ca\(^{2+}\). Thus, coexpression of Trp1 plus Trp3 resulted in a significant Ca\(^{2+}\)-dependent basal cation conductance that was not obtained by overexpression of either isoform alone. The Trp1/3 conductance was clearly sensitive to stimulation by the diacylglycerol OAG and inhibited by physiological levels of extracellular Ca\(^{2+}\). Experiments with different intracellular Ca\(^{2+}\) buffer systems showed that strong intracellular Ca\(^{2+}\) buffering slightly suppressed Trp3 currents but clearly promoted Trp1/3 currents. Trp1/3 currents were significantly facilitated when intracellular Ca\(^{2+}\) was strongly buffered with BAPTA as compared with experiments in which EGTA was used. It is of importance to note that the calculated free Ca\(^{2+}\) levels were in both cases below 10 nM. Because regulation of the channels at such low Ca\(^{2+}\) concentration appears rather unlikely, it is tempting to speculate that the regulation takes place in proximity to the inner mouth of the Trp channel where local Ca\(^{2+}\) concentration may be considerably higher upon channel opening and Ca\(^{2+}\) entry. Subplasmalemmal Ca\(^{2+}\) levels may rise to levels significantly higher than those calculated for the buffer systems, and the dynamic changes in subplasmalemmal Ca\(^{2+}\) are expected to be buffered better with BAPTA than with EGTA. A summary on the observed Ca\(^{2+}\)-dependence of Trp3 and Trp1/3 channels together with average membrane currents recorded at different intra- and extracellular Ca\(^{2+}\) is shown in Fig. 10. We suggest that a tight regulatory cross-talk exists between intra- and extracellular Ca\(^{2+}\) at Trp channel proteins. The coassembly of Trp1 and Trp3 appears to eliminate the permissive effect of intracellular Ca\(^{2+}\) on Trp3 homomultimers and in turn generates additional sensitivity to inhibition by intracellular Ca\(^{2+}\). The regulation of Trp channels by both extracellular and subplasmalemmal Ca\(^{2+}\) may represent a crucial determinant of the physiological role of a specific type of Trp channel. Although it is tempting to speculate that inhibitory as well as permissive effects of Ca\(^{2+}\) may be based on a direct interaction of Ca\(^{2+}\) with the channel proteins, other mechanisms involving more indirect and complex Ca\(^{2+}\)-dependent regulation of the channels cannot be excluded at present.

In line with a number of previous reports on the permeability properties of Trp channels, Trp1/3 channels proved to be nonselective in that they allow for membrane currents carried by various mono- and divalent cations (9–12, 19). Consistent with previous reports, we calculated Na\(^+/\text{Cs}^{+}\) permeability ratios for Trp1 and Trp3 channels that were approximately 1.5 (10, 12). Nonetheless, Trp1/3 channels displayed a significantly different (more positive) reversal potential than Trp3 currents, and a Na\(^+/\text{Cs}^{+}\) permeability ratio of 2.6 was calculated. This result indicates that coassembly of the different isoforms results in a modest but significant change of the permeability properties, probably because of a significant modification of the pore structure in Trp heteromultimers.

Physiological Significance of the Hetero-oligomerization of Trp Proteins—It appears important to note that the observed Trp1/3 currents do not perfectly resemble any nonselective cation current that has so far been described in native tissues. Nonetheless, this type of current, which is sensitive to activation by diacylglycerol and to facilitation by low subplasmalemmal Ca\(^{2+}\) concentrations, may contribute to agonist-induced activation of excitatory as well nonexcitable cells even at current densities much lower than those obtained in the HEK293 expression system. In cells that exhibit a high input resistance even small inward currents are sufficient to cause profound changes in membrane potential and to affect cellular functions because of activation of voltage-dependent ion channels and/or ion exchange mechanisms. Thus, Trp heteromultimers may in principle serve in regulation of cell functions even at low levels of expression that give rise to small agonist-regulated, nonselective ion conductances.

The relation between such nonselective cation conductances and cellular Ca\(^{2+}\) signals is highly complex because of the simultaneous impact of nonselective cation conductances on a
variety of factors that govern Ca\(^{2+}\) entry, such as Ca\(^{2+}\) permeability, intracellular Na\(^+\) concentration, and membrane potential. Our observation that overexpression of Trp1 in cells that also express Trp3 results in a gain of negative feedback regulation of Trp channels by intracellular Ca\(^{2+}\) suggests that the formation of Trp1/3 heteromultimers may lead to diminished overall Ca\(^{2+}\) signals during agonist stimulation. Indeed, we observed that carbacohol-stimulated Ca\(^{2+}\) entry into HEK-Trp1/3 cells was significantly smaller than that into HEKTrp3 cells. Similarly, OAG-induced Sr\(^{2+}\) entry was detectable only in HEKTrp3 cells but not in HEKTrp1/3 cells. This is in line with the observation that OAG-induced Trp1/3 currents were smaller than Trp3 currents when intracellular Ca\(^{2+}\) was buffered moderately and Ca\(^{2+}\) was present in the extracellular solution. By contrast, Trp1/3 inward currents were even larger than Trp3 currents when intracellular Ca\(^{2+}\) was kept low by effective Ca\(^{2+}\) buffering, indicating that the reduced Ca\(^{2+}\) entry into HEKTrp1/3 cells is not because of reduced density of functional channels but rather because of altered channel regulation. It is tempting to speculate that Trp1/3 channels allow Ca\(^{2+}\) entry only when subplasmalemmal Ca\(^{2+}\) is essentially low, thereby providing a pathway for the refilling of intracellular Ca\(^{2+}\) stores at low cytoplasmic Ca\(^{2+}\) concentrations. Moreover, it has recently been demonstrated that a reduction of the overall intracellular Ca\(^{2+}\) signal is not necessarily associated with blunted regulation of cellular functions (25). Even a modest entry of Na\(^+\) ions may well be sufficient to depolarize the cell and/or to elevate subplasmalemmal Ca\(^{2+}\) because of Na\(^+\)/Ca\(^{2+}\) exchange despite detectable elevation of overall Ca\(^{2+}\) (25, 26). Thus the physiological role of specific Trp channels, which depend on the biophysical and regulatory properties, remains to be elucidated. Nonetheless, the present results unequivocally demonstrate that the isofrom expression pattern of Trp protein is a crucial determinant of both the biophysical and regulatory properties of Trp cation channels. We suggest that a considerable variability of Trp channel function and consequently of the physiological roles of these channels may arise from the formation of hetero-oligomers of variable properties.

In summary, we observed that coexpression of Trp1 and Trp3 proteins in HEK293 cells generates a membrane conductance of unique regulatory as well as biophysical properties. Our results suggest that coassembly of different mammalian Trp species into multimeric channel complexes creates cation channel properties that are substantially different from those of the individual Trp species. So far, Trp channels have been characterized extensively by separate overexpression of specific isoforms, and the results were interpreted in terms of physiological relevance of these Trp species. However, these experiments may provide only a small amount of information on the physiological role of a specific Trp isofrom because physiological Trp channels may be heteromultimers depending on the isofrom expression pattern of a given tissue. Trp1/3 hetero-oligomerization creates a nonselective cation permeability that is tightly controlled by both phospholipase C activity and the cytoplasmic Ca\(^{2+}\) level and may play a physiological role by providing an important negative feedback control of Trp function.

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REFERENCES
1. Montell, C., and Rubin, G. M. (1989) Neuron 2, 1313–1323
2. Phillips, A. M., Bull, A., and Kelly, L. E. (1992) Neuron 8, 631–642
3. Zhu, X., Jiang, M., and Birnbaumer, L. (1996) J. Biol. Chem. 271, 143–142
4. Kiselev, K., Mignery, G. A., Zhu, M. X., and Mualem, S. (1999) Mol. Cell. Biol. 19, 423–429
5. Hofmann, T., Obukhov, A. G., Schaefer, M., Guder, T., and Schultz, G. (1999) Nature 397, 409–416
6. Kiselev, K., Xu, C., Moyle, G., Ko, T., Pessah, I., Mignery, G., Zhu, X., Birnbaumer, L., and Mualem, S. (1999b) Nature 397, 409–416
7. Philipp, S., Cavaillé, A., Freichel, M., Wissenbach, U., Zimmer, S., Trost, C., Marquart, A., Murakami, M., and Fleckner, V. (1999) EMBO J. 18, 6161–6171
8. Warnat, J., Philipp, S., Zimmer, S., Fleckner, V., and Cavaillé, A. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 5243–5247
9. Zitz, C., Zobel, A., Obukhov, A. G., Hartenberg, C., Kalkbrenner, F., Lückhoff, A., and Schultz, G. (1999) Neuron 16, 1189–1196
10. Sinkins, W. G., Estacion, M., and Schilling, P. (1998) Biochem. J. 331, 333–339
11. Hurst, R. S., Zhu, M., Boulay, G., Birnbaumer, L., and Stefani, E. (1998) FEBS Lett. 422, 333–338
12. Kamisuki, M., Philipp, S., Fleckner, V., Wissenbach, U., Mamin, A., Rasymaera, L., Eggermont, J., Droogmans, L., and Nilius, B. (1999) J. Physiol. (Lond.) 518, 345–358
13. Birnbaumer, L., Zhu, X., Jiang, M., Boulay, G., Peyton, M., Vannier, B., Brown, D., Platano, D., Sadeghi, H., Stefani, E., and Birnbaumer, L. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 15195–15202
14. Xu, X.-Z., Li, H., Guggino, W. B., and Montell, C. (1997) Cell 89, 1155–1164
15. Groschner, K., Hingel, S., Linschinger, B., Balzer, M., Romanin, C., Zhu, X., and Schenmbayer, W. (1998) FEBS Lett. 437, 101–106
16. Graier, W. F., Simicek, S., and Sturek, M. (1995) J. Physiol. (Lond.) 492, 579–594
17. Graier, W. F., Paltauf-Doburzynska, J., starz, K., and Paltauf, G. (1998) J. Physiol. (Lond.) 513, 369–379
18. Okada, T., Itoe, R., Tanazaki, K., Maeda, A., Kurosaki, T., Yamazaki, T., Tanaka, I., Shimagiri, S., Ikegami, K., Imoto, K., and Mori, Y. (1999) J. Biol. Chem. 274, 27359–27370
19. Ma, H., Patterson R., L., van Rossum, D. B., Birnbaumer, L., Mikoshiba, K., and Gill, D. G. (2000) Science 287, 1674–1681
20. Putney, J. W., and McKay, R. R. (1999) Bioessays 21, 38–46
21. Hardie, R. C., and Minke, B. (1994) J. Gen. Physiol. 103, 409–427
22. Zitz, C., Obukhov, A. G., Strubing, C., Zobel, A., Kalkbrenner, F., Lückhoff, A., and Schultz, G. (1997) J. Cell Biol. 138, 1333–1341
23. Chang, A. S., Chang, S. M., Garcia, R. L., and Schilling, W. P. (1997) FEBS Lett. 415, 335–340
24. Garcia, R. L., and Schilling, W. P. (1997) Biochem. Biophys. Res. Commun. 239, 279–283
25. Teubl, M., Groschner, K., Kehlwein, S. D., Mayer, B., and Schmidt, K. (1999) J. Biol. Chem. 274, 29529–29535
26. Paltauf-Doburzynska, J., Frieden, M., Spitaler, M., and Graier, W. F. (2000) J. Physiol. (Lond.) 524, 707–713
Coassembly of Trp1 and Trp3 Proteins Generates Diacylglycerol- and Ca^{2+}-sensitive Cation Channels

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