Expression of Trp3 Determines Sensitivity of Capacitative Ca\textsuperscript{2+} Entry to Nitric Oxide and Mitochondrial Ca\textsuperscript{2+} Handling

EVIDENCE FOR A ROLE OF Trp3 AS A SUBUNIT OF CAPACITATIVE Ca\textsuperscript{2+} ENTRY CHANNELS

The role of Trp3 in cellular regulation of Ca\textsuperscript{2+} entry by NO was studied in human embryonic kidney (HEK) 293 cells. In vector-transfected HEK293 cells (controls), thapsigargin (TG)-induced (capacitative Ca\textsuperscript{2+} entry (CCE)-mediated) intracellular Ca\textsuperscript{2+} signals and Mn\textsuperscript{2+} entry were markedly suppressed by the NO donor 2-(N,N-diethylamino)diazenolate-2-oxide sodium salt (\(3 \mu\)M) or by authentic NO (100 \(\mu\)M). In cells overexpressing Trp3 (T3-9), TG-induced intracellular Ca\textsuperscript{2+} signals exhibited an amplitude similar to that of controls but lacked sensitivity to inhibition by NO. Consistently, NO inhibited TG-induced Mn\textsuperscript{2+} entry in controls but not in T3-9 cells. Moreover, CCE-mediated Mn\textsuperscript{2+} entry into T3-9 cells exhibited a striking sensitivity to inhibition by extracellular Ca\textsuperscript{2+}, which was not detectable in controls. Suppression of mitochondrial Ca\textsuperscript{2+} handling with the uncouplers carbonyl cyanide m-chlorophenyl hydrazine (300 \(\mu\)M) or antimycin A\textsubscript{1} (AA\textsubscript{1}) mimicked the inhibitory effect of NO on CCE in controls but barely affected CCE in T3-9 cells. T3-9 cells exhibited enhanced carbachol-stimulated Ca\textsuperscript{2+} entry and clearly detectable cation currents through Trp3 cation channels, NO as well as carbonyl cyanide m-chlorophenyl hydrazone slightly promoted carbachol-induced Ca\textsuperscript{2+} entry into T3-9 cells. Simultaneous measurement of cytoplasmic Ca\textsuperscript{2+} and membrane currents revealed that Trp3 cation currents are inhibited during Ca\textsuperscript{2+} entry-induced elevation of cytoplasmic Ca\textsuperscript{2+}, and that this negative feedback regulation is blunted by NO. Our results demonstrate that overexpression of Trp3 generates phospholipase C-regulated cation channels, which exhibit regulatory properties different from those of endogenous CCE channels. Moreover, we show for the first time that Trp3 expression determines biophysical properties as well as regulation of CCE channels by NO and mitochondrial Ca\textsuperscript{2+} handling. Thus, we propose Trp3 as a subunit of CCE channels.

Depletion of intracellular Ca\textsuperscript{2+} stores by IP\textsubscript{3}-dependent\textsuperscript{1} or IP\textsubscript{3}-independent pathways stimulates a Ca\textsuperscript{2+} influx phenomenon that is termed Ca\textsuperscript{2+} release-activated Ca\textsuperscript{2+} entry, store-operated Ca\textsuperscript{2+} entry, or capacitative Ca\textsuperscript{2+} entry (CCE) (1). The molecular nature of CCE channels is still not clearly resolved. Nonetheless, increasing evidence suggests a role of members of the Trp protein family in the formation of CCE channel complexes (2, 3). Various cellular mechanisms have been proposed for activation of CCE channels (4), including a conformational coupling between IP\textsubscript{3} receptors and CCE channels (5). A direct gating by interaction with a specific cytosolic domain of the IP\textsubscript{3} receptor has been demonstrated for cation channels derived by overexpression of Trp3 protein, and activation of this Trp channel was found to depend on IP\textsubscript{3} (6, 7). However, recent studies with cells lacking expression of all three types of IP\textsubscript{3} receptors question a general role of IP\textsubscript{3} in activation of CCE channels but confirm requirement of basal PLC activity (8, 9). Therefore, Trp species that are sensitive to PLC-derived signals such as IP\textsubscript{3} and diacylglycerol appear as attractive candidates for proteins forming CCE channel complexes. Although heterologous overexpression of Trp3 or Trp6 clearly generates phospholipase C/IP\textsubscript{3}-dependent Ca\textsuperscript{2+} entry pathways (6, 7, 10) and sensitivity to activation by diacylglycerols (11, 12), these proteins fail to promote the classical CCE phenomenon upon overexpression (10, 12). Thus, the cation channels derived by overexpression of Trp3 or Trp6 resemble native CCE channels in terms of their principle PLC dependence, but apparently lack the distinctive ability of CCE channels to sense the filling state of the Ca\textsuperscript{2+} stores. Nonetheless, Trp3 or Trp6 proteins may function as subunits of multimeric CCE channel complexes. This hypothesis has been put forward by the observation that Trp3 overexpression not only increases phospholipase C/IP\textsubscript{3}-dependent Ca\textsuperscript{2+} entry but in addition changes its pharmacological properties, leading to loss of sensitivity to block by Go\textsubscript{3+} (10). These results were interpreted as the ability of Trp3 proteins to serve as functional subunits of CCE channel complexes (10, 13).

The present work was designed to test whether Trp3 interacts with the regulatory properties of CCE channels such as sensing local intracellular Ca\textsuperscript{2+} concentrations that are controlled by mitochondria (14). Another important mechanism of

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\¶ Austrian Academic Exchange Services fellow.

** To whom correspondence should be addressed: Dept. of Pharmacology and Toxicology, Karl Franzens University, Universitaetsplatz 2, A-8010 Graz, Austria. Tel.: 43-316-380-5577; Fax: 43-316-380-8980; E-mail: klaus.groschner@kfunigraz.ac.at.

\(1\) The abbreviations used are: IP\textsubscript{3}, inositol 1,4,5-trisphosphate; AA\textsubscript{1}, antimycin A\textsubscript{1}; [Ca\textsuperscript{2+}]\textsubscript{i}, intracellular Ca\textsuperscript{2+} concentration; CCE, capacitative Ca\textsuperscript{2+} entry; CCh, carbachol; CCCP, carbonyl cyanide m-chlorophenyl hydrazine; DEANO, 2-(N,N-diethylamino)diazenolate-2-oxide sodium salt; DMEM, Dulbecco’s modified Eagle’s medium; ER, endoplasmic reticulum; HEK, human embryonic kidney; PLC, phospholipase C; TBST, Tris-buffered saline with Tween 20; TG, thapsigargin; Trp, transient receptor potential.
CCE regulation is its inhibition by nitric oxide (NO), which has been proposed to promote refilling of the endoplasmic reticulum resulting in reduced CCE (15). NO donors such as DEANO have been demonstrated to inhibit CCE into platelets (16) and smooth muscle cells (15, 17). Interestingly, the lipophilic NO donor GEA3162 has been demonstrated to enhance CCE into mouse parotid acini (18) and to activate Ca2+ entry into human embryonic kidney (HEK) 293 cells stably expressing Trp3 (19). Thus, it appears reasonable to speculate that variable NO sensitivity might exist among CCE channel complexes, depending on the subunit stoichiometry and contribution of specific Trp species. With this study, we demonstrate for the first time that the expression level of Trp3 affects two distinctly different Ca2+ entry pathways. Trp3 overexpression in HEK293 cells promotes a phospholipase C-dependent Ca2+ entry pathway and eliminates the sensitivity of classical CCE channels to inhibition by NO as well as mitochondrial Ca2+ handling.

MATERIALS AND METHODS

Cell Culture—HEK293 cells were stably transduced cells with either the coding region of hTrp3 (GenBank™ accession no. U47050) (10), designated as T3-9 cells, or with the neomycin resistance cassette, designated as HEK7 (controls). Cells were cultured in DMEM supplemented with 10% fetal calf serum and 0.178 g/liter Gentamicin. In case of control cells, different clones were pooled together and cultured as specified above.

SDS-PAGE and Immunoblotting—T3-9 cells were grown to confluency. Carbonate-extracted cell lysates were separated on discontinuous sucrose gradients, and fractions were collected after ultracentrifugation. 25% of the fraction was used for Bradford assay, and the remainder 75% of the fraction was used for Western blot analysis. The fraction was separated on SDS-PAGE (8 or 12% SDS gels; 50 min, 180 mV) and afterward transferred to nitrocellulose membranes (90 min, 200 mA) using the Bio-Rad Protein III and Mini Transblot. The blots were incubated in TBST buffer (pH 7.4) containing 1% Bio-Rad Blocking solution (anti-hemagglutinin, 1:1000), and blots were incubated for 2 h with the first antibody, washed with TBST buffer, followed by 60-min incubation with the second antibody (horseradish-conjugated anti-rabbit from Sigma). After washing the blot with TBST buffer, immuneactivity was detected using the ECL detection system (Amersham Pharmacia Biotech).

Measurement of Cytoplasmic Ca2+—Intracellular Ca2+ was measured with fura-2. Fura-2/AM was initially dissolved in Me2SO at 2 mM and used at a final concentration of 2 ¿M. Confluent cells were harvested by enzymatic digestion (0.25% trypsin), suspended in 5 ml of culture medium (Dulbecco’s modified Eagle’s medium) without serum, and loaded with fura-2/AM for 60 min at 37 °C and 5% CO2. Thereafter, cells were washed once with Ca2+-free TRIS medium. Mn2+-containing Tris buffer (50 mM Tris, 2.5 mM CaCl2, 1 mM MgCl2, pH 7.4), incubated for 20 min, and washed in Tris buffer without Ca2+ (50 mM Tris, 1 mM MgCl2, 100 ¿M EGTA, pH 7.4). Fluorescent measurements were carried out with a dual wavelength spectrofluorimeter (Hitachi F2000). Cells were maintained at 37 °C, and emission was collected at 510 nm at excitation of 340 and 380 nm, respectively. For store depletion 100 nM thapsigargin (TG) or 200 ¿M Ca2+-chelated condition as specified. Mn2+-induced quench in fluorescence was measured at 360 nm (isosbestic point), and Mn2+ entry was initiated by addition of 100 ¿M Mn2+ at 60 s. 3 ¿M DEANO or 300 mM CCCP was added 200 s before stimulation of Mn2+ entry by thapsigargin (100 mM). Control experiments were performed with vehicle only. Mn2+ quench was calculated by measuring the quench in fluorescence per second caused by Mn2+ entry and represented as change on fluorescence (Fm/F0) per second.

Measurement of Mitochondrial Ca2+—For measurements of intramitochondrial Ca2+, cells were seeded on poly-l-lysine-coated coverslips (6 × 6 mm) and loaded with rhod-2/AM in DMEM without fetal calf serum, at a concentration of 2 ¿M for 45 min. at 37 °C. Cells were illuminated with a Polychrome II at 540 nm, and fluorescence emission was collected at 660 nm. The rhod-2 fluorescence was monitored on a Nikon Diaphot microscope equipped with an photomultiplier (Sensicam) and analyzed using an Axon Imaging Workbench 2.1 software.

Electrophysiology—All patch-clamp experiments were performed using a conventional extracellular solution containing (in mM) 50 Tris, 137 NaCl, 65 KCl, and 0.1 EGTA or 2.5 CaCl2 pH adjusted to 7.4 with HCl. The pipette solution for whole cell experiments contained (in mM) 145 potassium gluconate, 15 KCl, 1 M EGTA, pH adjusted to 7.4 with N-monomethyl tris (NMTES) and 0.5% trimethylamine. For perforated patch experiments and simultaneous current/frequency measurement, amphotericin B was added to the pipette solution to a final concentration of 100 µg/ml (0.5% Me2SO). Measurement of Ca2+ in fluo-3-loaded cells was performed simultaneously with current measurements in voltage clamped cells as described above. Pipettes were pulled from borosilicate glass (Clark Electro-Physiology, Unist, Pungdei, Unilabs, United Kingdom) and finally fire-polished. The pipette resistance (filled) was in the range of 2–5 megohms. Recordings were performed in a 200-µl bath chamber and with continuous perfusion by a gravity-driven system.

Voltage clamp and current amplification was performed with a List EPC-7 (List, Darmstadt, Germany) patch-clamp amplifier. Voltage ramp protocols covering a voltage range from −100 to +100 mV with a frequency of 0.2 Hz was applied. Signals were low pass filtered at 1 kHz and digitized with a 5 kHz. Stimulation protocols and recordings were controlled by Axon pCLAMP software (Axon Instruments, Foster City, CA) via an Axon Digidata 1200 computer interface.

Chemicals—Chemicals were purchased from the following suppliers: Tissue culture medium was from Life Technologies, Inc. (Vienna, Austria); fura-2/AM and rhod-2/AM were from Molecular Probes (Leiden, Netherlands); DEANO was from Alexis (Switzerland) and all other chemicals were purchased from Sigma (Vienna, Austria).

Statistical Analysis—All the individual experiments were averaged, and the mean of the time courses is represented as trace. Results obtained at specific time points were expressed as mean value ± S.E. Differences were considered statistically significant at p < 0.05 using Student’s t test for unpaired values.

RESULTS

Trp3 Overexpression Fails to Promote Thapsigargin-induced CCE but Generates a Carbachol-stimulated Ca2+ Entry Pathway in HEK293 Cells—Fig. 1 shows a comparison of TG- and CCh-induced Ca2+ entry into transduced (HEK7) and Trp3 expressing HEK293 cells (T3-9) based on fura-2 fluorescence measurements in cell populations. Expression of the hemagglutinin-tagged Trp3 channel protein in the cells was verified by Western blot experiments, which demonstrated the presence of the ~100-kDa hemagglutinin-tagged protein in membranes of T3-9 cells (data not shown). To activate the classical CCE pathway, we depleted intracellular stores with thapsigargin (100 nM) in Ca2+-free solution. Alternatively, carbachol (200 µM) was used to induce phospholipase C-mediated release of Ca2+ from intracellular stores. Ca2+ entry was
Ca\(^{2+}\)

initiated by re-addition of extracellular Ca\(^{2+}\). As shown in Fig. 1A, elevation of extracellular Ca\(^{2+}\) induced a peak intracellular Ca\(^{2+}\) level of 1156 ± 36 nM \((n = 6)\) in TG-stimulated cells, which was 3-fold higher than that measured in carbachol-stimulated cells \((373 ± 14 \text{ nM}; n = 7)\). In Trp3-overexpressing cells, this relationship was distorted, in that TG-induced Ca\(^{2+}\) signals were slightly reduced \((1064 ± 64 \text{ nM, } n = 10)\) whereas Ca\(^{2+}\) entry in carbachol-stimulated cells \((750 ± 32 \text{ nM, } n = 12)\) was enhanced as shown in Fig. 1B. Thus, Trp3 overexpression clearly promoted or generated a phospholipase C-dependent Ca\(^{2+}\) entry pathway but not a classical CCE.

**NO Inhibits Thapsigargin-induced CCE in Vector-transfected HEK293 Cells but Not in T3-9 Cells**—Fig. 2 (A–D) illustrates the effects of the NO donor DEANO \((3 \mu\text{M})\) on thapsigargin-induced CCE in HEK\(^{VT}\) and T3-9 cells in suspension. CCE into HEK\(^{VT}\) was markedly reduced in the presence of the NO donor DEANO \((3 \mu\text{M})\), resulting in a reduction of peak intracellular Ca\(^{2+}\) levels from 1156 ± 36 nM to 425 ± 41 nM \((n = 6)\), as shown in Fig. 2A. By contrast, thapsigargin-induced CCE into T3-9 cells was completely insensitive to DEANO \((988 ± 223 \text{ nM, } n = 8\), Fig. 2B). Similar results were obtained with authentic NO at a concentration of 100 \(\mu\text{M}\) (data not shown).

8-Bromo-cGMP, a cell permeant cGMP analogue, did not mimic the effects of NO in respect to Ca\(^{2+}\) mobilization and inhibition of CCE \((1146 ± 41 \text{ nM, } n = 5)\), suggesting that the effect of NO was independent of cGMP. The action of NO was typically associated with a slight increase in cytoplasmic Ca\(^{2+}\) that was transient in nature and did not require extracellular Ca\(^{2+}\) (see Fig. 2A).

Fig. 2 (C and D) illustrates the effects of DEANO on TG-induced Mn\(^{2+}\) entry in HEK\(^{VT}\) and T3-9 cells, respectively. DEANO clearly suppressed TG-induced Mn\(^{2+}\) entry into HEK\(^{VT}\) cells (Fig. 2C) but not in T3-9 cells (Fig. 2D). The observed lack of sensitivity to NO in T3-9 cells indicates that Trp3 is able to modify the regulatory property of CCE channels. Trp3-induced elevation of NO sensitivity of CCE was confirmed in single fluo-3-loaded HEK293 cells, as shown in Fig. 2 (E and F). Thapsigargin-stimulated Ca\(^{2+}\) entry was again markedly inhibited by the NO donor in vector-transfected controls but not in T3-9 cells.

**Trp3 Overexpression Generates Sensitivity of TG-induced Mn\(^{2+}\) Entry to Extracellular Ca\(^{2+}\)**—The Mn\(^{2+}\) quench experiments illustrated in Fig. 2 were performed in a buffer containing EGTA \((100 \mu\text{M})\), resulting in free Ca\(^{2+}\) levels below 1 \(\mu\text{M}\). T3-9 cells exhibited reduced TG-induced Mn\(^{2+}\) quench in nominally Ca\(^{2+}\)-free solutions. As shown in Fig. 3, a clear dependence of TG-induced Mn\(^{2+}\) entry on extracellular Ca\(^{2+}\) was observed in T3-9 cells but not in HEK\(^{VT}\) cells. Chelation of extracellular Ca\(^{2+}\) with EGTA restored Mn\(^{2+}\) entry in T3-9 cells to a level comparable with that of controls, indicating that Mn\(^{2+}\) permeation through CCE channels is affected by overexpression of Trp3 in that the TG-sensitive Mn\(^{2+}\) entry pathway gained sensitivity to extracellular Ca\(^{2+}\).

**Inhibition of Thapsigargin-induced CCE by Mitochondrial Uncoupling Is Impaired in T3-9 Cells**—The inhibitory action of NO was mimicked by the mitochondrial uncoupler CCCP. TG-stimulated CCE into control cells (HEK\(^{VT}\)) was reduced from 1156 ± 36 nM to 416 ± 38 nM in the presence of CCCP \((300 \text{ nM}; \text{Fig. 4A})\), corresponding to an inhibition of CCE by 64\%. Similar to NO, CCCP by itself induced a modest, transient elevation of cytoplasmic Ca\(^{2+}\). CCE into T3-9 cells was less sensitive to inhibition by CCCP as compared with control cells (Fig. 4B). CCCP-induced inhibition amounted to 28\% in T3-9 cells. CCCP inhibited TG-induced Mn\(^{2+}\) entry into control as well as in T3-9 cells (Fig. 4, C and D). Nonetheless, inhibition of Mn\(^{2+}\) entry into T3-9 cells was less pronounced than in vector-transfected controls (41\% versus 63\%, respectively).

Fig. 5 (A and B) illustrates the effects of the mitochondrial uncouplers antimycin A\(_1\) \((2 \mu\text{M})\) and oligomycin \((6 \mu\text{M})\) on TG-induced Ca\(^{2+}\) signals in single fluo-3-loaded cells. Interestingly, antimycin A\(_1\), which prevents the generation of mitochondrial H\(^{+}\) gradients and rapidly disturbs Ca\(^{2+}\) uptake into mitochondria \((21)\), was most effective as an inhibitor of CCE, whereas oligomycin, which primarily inhibits ATP synthase \((22)\), failed to inhibit CCE significantly, indicating a key role for mitochondrial Ca\(^{2+}\) handling. Fig. 5C compares the sensitivity of Ca\(^{2+}\) entry into HEK\(^{VT}\) and T3-9 cells to inhibition by DEANO, CCCP, antimycin A\(_1\), and oligomycin. In T3-9 cells, the sensitivity of CCE to inhibition by the NO donor as well as by the mitochondrial uncouplers CCCP and antimycin A\(_1\) were strongly reduced or eliminated.

The similarities between the effects of NO and those of mitochondrial uncouplers supported the hypothesis of mito-
**Fig. 2.** DEANO inhibits TG-induced divalent cation entry in HEKV<sup>T</sup> cells but not in T3-9 cells. Time courses of $[Ca^{2+}]_i$ (A and B) and Mn<sup>2+</sup> quench (C and D) in HEKV<sup>T</sup> and T3-9 cells. E and F represent experiments performed with fluo3 loaded single HEKV<sup>T</sup> (E) and T3-9 (F) cells. Mean time courses derived from 6–44 individual experiments are shown. Thin lines denote experiments performed in the absence of DEANO and thick lines represent experiments performed in the presence of DEANO. DEANO (3 μM) was administered 140 s before stimulation with thapsigargin.

**Fig. 3.** TG-stimulated Mn<sup>2+</sup> entry into T3-9 cells is dependent on extracellular Ca<sup>2+</sup>. Columns represent the initial slope of Mn<sup>2+</sup> entry-induced reduction of fura-2 fluorescence initiated by administration of TG (100 nM) in HEKV<sup>T</sup> and T3-9 cells in nominally Ca<sup>2+</sup> free solution (free Ca<sup>2+</sup> ~ 10 μM) and in an EGTA containing solution (free Ca<sup>2+</sup> < 1 μM). Mean values ± S.E. are given for n = 8–10. * denotes statistically significant difference at p < 0.05 versus 10 μM extracellular Ca<sup>2+</sup> concentration.
chondria as a primary target of NO action. Therefore, we tested
the effects of NO on Ca\(^{2+}\) handling by mitochondria.

**NO Prevents Ca\(^{2+}\) Sequestration by Mitochondria**—DEANO and CCCP induced a moderate rise in basal intracellular Ca\(^{2+}\) concentrations in the absence of extracellular Ca\(^{2+}\), as evident from the experiments shown in Figs. 2 and 4. The mobilization of intracellular Ca\(^{2+}\) by CCCP is known to result from
depolarization of mitochondria and the consequent depletion of
mitochondrial Ca\(^{2+}\). To test whether NO mimics CCCP in
modulation of mitochondrial Ca\(^{2+}\) handling, we performed ex-
periments with rhod-2 to measure changes in mitochondrial Ca\(^{2+}\). As shown in Fig. 6A, rhod-2 fluorescence was localized
within the cells in discrete spots and increased upon stimulation
with CCh (200 \(\mu\)M), whereas rhod-2 fluorescence decreased
significantly upon administration of either DEANO (3 \(\mu\)M) or
CCCP (300 \(\mu\)M). Moreover, CCh-induced increase in rhod-2
fluorescence was effectively prevented by both agents. Aver-
ged time courses of mitochondrial rhod-2 fluorescence during
exposure of cells to DEANO, CCCP, and CCh are given in Fig.
6 (C, F, and I), illustrating CCh-induced changes in rhod-2
fluorescence in the absence of drugs (\(n = 55\)) and in the
presence of DEANO (\(n = 66\)) or CCCP (\(n = 59\)), respectively.

**NO as Well as CCCP Promote CCh-activated Ca\(^{2+}\) Entry into
T3-9 Cells**—Trp3 overexpression has been demonstrated pre-
viously to create a PLC- and IP3-dependent Ca\(^{2+}\) entry path-
way (6). This function of Trp3 was again confirmed by the
promotion of CCh-induced Ca\(^{2+}\) signaling observed in the pres-
ent study (Fig. 1). Fig. 7 illustrates that DEANO (3 \(\mu\)M) as well as
CCCP (300 \(\mu\)M) slightly inhibited CCh-induced Ca\(^{2+}\) signal-
ing in control cells (Fig. 7, A and C) but promoted CCh-induced
Ca\(^{2+}\) entry into T3-9 cells (Fig. 7, B and D). In T3-9 cells,
DEANO (3 \(\mu\)M), authentic NO (100 \(\mu\)M; \(n = 4\); data not shown)
as well as CCCP (300 \(\mu\)M) increased the CCh-induced Ca\(^{2+}\)
entry signals measured in cell populations. Both NO as well as
CCCP reduced the CCh-induced Ca\(^{2+}\) mobilization in Ca\(^{2+}\)-
free solution but augmented the Ca\(^{2+}\) signals induced by sub-
sequent Ca\(^{2+}\) entry. Promotion of CCh-induced Ca\(^{2+}\) entry into
T3-9 cells was associated with a change in kinetics of the Ca\(^{2+}\)
signals. In the presence of DEANO or CCCP Ca\(^{2+}\) entry-in-
duced intracellular Ca\(^{2+}\) levels did not decline but remained
elevated during prolonged rises in extracellular Ca\(^{2+}\). The con-
centration of Ca\(^{2+}\) measured at 200 s after initiation of Ca\(^{2+}\)
entry was 475 ± 14 \(nM\) (\(n = 12\)) in the presence of vehicle
and 758 ± 15 \(nM\) (\(n = 10\)) in the presence of DEANO. Similarly,
CCCP promoted Ca\(^{2+}\) signals in T3-9 cells, resulting in signif-
cantly elevated intracellular Ca\(^{2+}\) levels (968 ± 94 \(nM\), \(n = 6\),
Fig. 7D) at 200 s after initiation of entry. Thus, the Trp3-
derived PLC-regulated Ca\(^{2+}\) entry pathway and CCE were
divergently regulated by NO. Because Trp3 channels were
found previously to exhibit Ca\(^{2+}\)-mediated negative feedback
modulation similar to CCE channels, it was of interest to inves-
tigate the effects of NO on the relation between bulk cytoplasmic
Ca\(^{2+}\) and Trp currents in single adherent HEK293 cells.

**NO Promotes CCh-stimulated Trp3 Currents because of Sup-
pression of Ca\(^{2+}\)-dependent Negative Feedback Regulation**—
Fig. 8 shows time courses of intracellular Ca\(^{2+}\) and membrane
currents measured simultaneously in single T3-9 cells, which
were stimulated with CCh in Ca\(^{2+}\)-free solution and subjected
to subsequent elevation of extracellular Ca\(^{2+}\) in the absence
(Fig. 8A) and presence (Fig. 8B) of DEANO (3 \(\mu\)M). The simul-
taneous recording of cytoplasmic Ca\(^{2+}\) and membrane currents
in voltage-clamped T3-9 cells demonstrated a qualitatively
similar relation between overall cytoplasmic Ca\(^{2+}\) levels and
membrane conductances in the absence and presence of NO.
CCh stimulated a membrane conductance in Ca\(^{2+}\)-free extra-
cellular solution, which was suppressed during re-addition of

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**Fig. 4. Inhibition of TG-induced divalent cation entry by the mitochondrial uncoupler CCCP is reduced in T3-9 cells.** Time courses of [Ca\(^{2+}\)]\(_i\) (A and B) and [Mn\(^{2+}\)]\(_i\) (C and D) in HEK\(^{\mathrm{VT}}\) and T3-9 cells. Mean time courses derived from 6–10 individual experiments are shown. Thin lines denote experiments performed in the absence of CCCP and thick lines represent experiments performed in the presence of CCCP. CCCP (300 \(\mu\)M) was administered 140 s before stimulation with thapsigargin.
extracellular Ca\textsuperscript{2+}. This CCh-stimulated membrane conductance was not detected in vector-transfected control cells (n = 3, data not shown). Two distinct modes of regulation of Trp3 channels were evident. (i) Trp3 channels were activated in response to stimulation of muscarinic receptors, a situation that is characterized by depletion of intracellular Ca\textsuperscript{2+} stores and a transient rise in cytoplasmic Ca\textsuperscript{2+}; and (ii) the channels were suppressed during subsequent elevation of extracellular Ca\textsuperscript{2+}, which caused large rises in cytosolic Ca\textsuperscript{2+} via Ca\textsuperscript{2+} entry. Fig. 9 shows a comparison of intracellular Ca\textsuperscript{2+} levels and current densities obtained in parallel experiments. In the absence of DEANO, Ca\textsuperscript{2+} re-addition was associated with a sub-

Fig. 5. Antimycin A\textsubscript{1} but not oligomycin mimics the effect of DEANO. Time courses of [Ca\textsuperscript{2+}]\textsubscript{i}, A and B in flou-3 loaded single HEK\textsuperscript{VT} cells. Mean time courses derived from 18–44 individual experiments are shown. Thin lines denote control experiments and thick lines represent experiments performed in the presence of A\textsubscript{A}, (2 μM; A) or oligomycin (Oligo; 6 μM; B). A\textsubscript{A} or oligomycin was administered 140 s before stimulation with thapsigargin. C, columns represent percentage inhibition in change in flou-3 fluorescence intensity measured in single HEK\textsuperscript{VT} cells in the presence of DEANO (open), CCCP (black), A\textsubscript{A} (gray), or oligomycin (crossed), treated cells. The compounds were added 140 s prior to stimulation with TG. Mean values ± S.E. are given for n = 18–44. * denotes statistically significant different at p < 0.05 versus controls.

Fig. 6. DEANO and CCCP prevent mitochondrial Ca\textsuperscript{2+} uptake in HEK\textsuperscript{VT} and T3-9 cells. Intramitochondrial Ca\textsuperscript{2+} was monitored in cells loaded with the cell permeant rhod-2 AM dye. Images illustrate rhod-2 fluorescence in single HEK293 cells before and after exposure of cells to CCh in control cells (A and B) or experiments performed in the presence of DEANO (E) or CCCP (H). Corresponding time courses of rhod-2 fluorescence are shown in C, F, and I. Mean time courses derived from 55–60 individual experiments are shown. Note: Images A, D, and G represent rhod-2 fluorescence in single cells at the beginning of the experiment. Images B, E, and H represent rhod-2 fluorescence after the addition of CCh alone (control), CCh plus DEANO or CCh plus CCCP respectively.
stantial and significant reduction in membrane currents. This Ca$^{2+}$ entry-induced suppression of Trp3 currents was less pronounced in the presence of DEANO. By contrast, Ca$^{2+}$ entry-induced increments in cytoplasmic Ca$^{2+}$ were significantly larger in the presence of the NO donor ($\Delta F_{488} = 50 \pm 2$) as compared with controls ($\Delta F_{488} = 36 \pm 1$). Thus, NO affects Ca$^{2+}$-dependent autoregulation of PLC-regulated Trp3 channels in a manner oppositional to that of native CCE channels.

FIG. 7. DEANO and CCCP promote Ca$^{2+}$ entry signals in T3-9 cells. Time courses of [Ca$^{2+}]_i$, in HEKVT (A and C) and T3-9 (B and D) cells. Mean time courses derived from 6–12 individual experiments are shown. Thin lines denote experiments performed in the absence of DEANO or CCCP and thick lines represent experiments performed in the presence of DEANO or CCCP. Either DEANO (3 µM) or CCCP (300 µM) was administered 140 s before stimulation with carbachol (200 µM).

FIG. 8. Simultaneous measurement of intracellular Ca$^{2+}$ and membrane currents in T3-9 cells. Effects of carbachol stimulation and Ca$^{2+}$ readdition under control conditions (A, absence of NO) and in the presence of DEANO (B, 3 µM). Upper panel: Time-courses of single-cell fluo-3 fluorescence at 488 nm and extracellular conditions as indicated. Middle panel: Time courses of membrane currents recorded from the same cell by perforated patch-clamp technique. Membrane potential was voltage clamped at a holding potential of 0 mV and slow voltage ramps (0.08 mV/ms; −100 mV to +60 mV) were applied at a rate of 0.2 Hz. Values in time courses were taken at a potential of −70 mV. Lower panel: Single current traces obtained during voltage-ramp stimulation at the time points indicated.
Role of Trp3 in Cellular Regulation of Ca\textsuperscript{2+} Entry by NO

**Inhibition of CCE Channels by NO**—Nitric oxide is a potent modulator of cellular Ca\textsuperscript{2+} homeostasis because of cGMP-dependent and cGMP-independent mechanisms such as S-nitrosylation of proteins (23). Here we demonstrate that NO, administered as authentic NO in solution or by use of the NO donor DEANO, substantially inhibits endogenous CCE of HEK293 cells. Similar inhibitory effects of NO on CCE have been reported previously for platelets (15) and smooth muscle cells (17), and have been attributed to promotion of uptake of Ca\textsuperscript{2+} into the ER (17). Enhanced refilling of the ER as the basis of the inhibitory action of NO observed here appears unlikely because CCE was activated by exposure of cells to thapsigargin, which effectively prevents refilling of the regulatory pool of the ER. In contrast to promotion of refilling of intracellular Ca\textsuperscript{2+} pools, we observed a slight, distinct release of Ca\textsuperscript{2+} from intracellular storage sites by NO. The NO-sensitive Ca\textsuperscript{2+} store was identified as mitochondria. This effect of NO on intracellular Ca\textsuperscript{2+} handling by mitochondria is likely to be involved in suppression of CCE because Ca\textsuperscript{2+} sequestration by mitochondria has been demonstrated as a determinant of CCE (14). NO-mediated suppression of mitochondrial function as well as respiration has been observed in various tissues (14, 24, 25), and this phenomenon has been attributed to direct, cGMP-independent effects of NO on components of the mitochondrial respiratory chain, in particular on cytochrome oxidase (24, 25). Consistently, inhibition of CCE by NO was found independent of cGMP, as 8-bromo-cGMP failed to mimic NO in terms of Ca\textsuperscript{2+} mobilization or inhibition of CCE.

**Role of Mitochondria**—Our results demonstrate that NO and the mitochondrial uncoupler CCCP exert similar effects on Ca\textsuperscript{2+} signaling in HEK293 cells. Both compounds were found to inhibit CCE at concentrations that mobilize Ca\textsuperscript{2+} from mitochondria and prevent Ca\textsuperscript{2+} uptake into this organelle during IP\textsubscript{3}-induced Ca\textsuperscript{2+} release. A link between impairment of mitochondrial Ca\textsuperscript{2+} sequestration ability and inhibition of CCE has been demonstrated previously for T-lymphocytes (14). Thus, mitochondria may well represent the primary target of NO within the mechanism leading to modulation of Ca\textsuperscript{2+} entry. This role of mitochondria was further confirmed by experiments with the mitochondrial uncoupler antimycin A\textsubscript{3}, which exerted inhibitory effects on CCE in vector-transfected HEK293 cells. Antimycin A\textsubscript{3} suppresses the build-up of mitochondrial H\textsuperscript{+} gradients, resulting in a rapid reduction of mitochondrial membrane potential and impaired Ca\textsuperscript{2+} uptake (21). By contrast, oligomycin, an inhibitor of ATP synthase that fails to rapidly depolarize mitochondria (22), was barely effective as an inhibitor of CCE. Our results strongly suggest a role of mitochondrial membrane potential and Ca\textsuperscript{2+} handling in the observed regulation of CCE.

**Modulation of the NO Sensitivity of CCE Channels by Trp3**—In Trp3-overexpressing cells, the regulatory properties of CCE were strikingly altered, in that CCE lost its sensitivity to NO. This substantial change in cellular regulation of CCE may be explained by either a change in the CCE ion channel complex itself or by a change in the regulatory mechanisms involved. It is tempting to speculate that Trp3 interferes with the formation of CCE channel complexes, which, in wild type HEK293 cells, are composed of as yet unidentified channel proteins. Overexpression of Trp3 protein may alter the stoichiometry of these CCE channels, resulting in heteromultimeric complexes containing Trp3 in addition to the generation of Trp3 homomultimers. A respective model, which is consistent with the observation of three Ca\textsuperscript{2+} entry pathways of different regulatory properties, is depicted in Fig. 10. The ability of Trp3 proteins to form functional heteromultimers has repeatedly been demonstrated (12, 26), and it is reasonable to speculate that the endogenous CCE channel proteins are members of the Trp family. Alternatively, we cannot exclude at present that Trp3 functions as a regulatory protein of CCE channels without...
was surprising because our results suggest that NO as well as
ulation of CCh-stimulated Trp3 channels and CCE channels
Interestingly, Mn\(^2+\) Ca\(^2+\) unique Ca\(^2+\) Trp3-overexpressing cells. Hence, Trp3 expression resulted in a
ular Ca\(^2+\)/H\(11001\)
pathway is insensitive to inhibition by NO or mitochondrial 
press the Trp3-mediated cation conductance because of extra-
and a contribution of Trp3 to the pore-forming complex of CCE 
channel complex,
entry pathways in native HEK293 cells. Channels' sensitivity to 
press - and - signs represent promotion and inhibition re-
vation of tetrameric Ca\(^2+\) entry channel complexes and accounts for the observation that Trp3 overexpression produces two Ca\(^2+\) entry pathways with regulatory properties different from that of the Ca\(^2+\) entry pathways in native HEK293 cells. Channels' sensitivity to store-depletion, NO/mitochondrial uncoupling and PLC-stimulation are given in bars, + and - signs represent promotion and inhibition respectively. Dashed arrow with cross represents insensitivity.
being a component of the pore-forming channel complex itself. Nonetheless, in case that Trp3 is able to contribute to a CCE channel complex, e.g. a tetrameric ion channel as illustrated in the model shown in Fig. 10, it appears likely that Trp3 alters the biophysical and pharmacological properties of CCE. Indeed, a Trp3-induced change in the sensitivity of CCE channels to the pore blocker Gd\(^3+\) has been reported previously (10). Interestingly, Mn\(^2+\) quench experiments revealed a Trp3-induced change in the sensitivity of CCE channels to extracellular Ca\(^2+\). Mn\(^2+\) entry was barely affected by micromolar (10 \(\mu\)M) concentrations of extracellular Ca\(^2+\) in vector-transfected cells but significantly suppressed by 10 \(\mu\)M extracellular Ca\(^2+\) in Trp3-overexpressing cells. Hence, Trp3 expression resulted in a unique Ca\(^2+\) sensitivity of the thapsigargin-stimulated Mn\(^2+\) entry, suggesting a change in the divergent binding properties and a contribution of Trp3 to the pore-forming complex of CCE channels.

Promotion of Phospholipase C-regulated Trp3 Channel Activity by NO—It has been demonstrated repeatedly that expression of Trp3 generates a phospholipase C-dependent Ca\(^2+\) entry pathway. We report here that this Ca\(^2+\) entry pathway is insensitive to inhibition by NO or mitochondrial uncouplers. Interestingly, carbachol-induced Ca\(^2+\) mobilization from intracellular stores was blunted by NO as well as CCCP in T3-9 but not in control cells. The mechanism by which mitochondrial uncoupling interferes with IP\(_3\)-induced Ca\(^2+\) mobilization is unlikely to involve depletion of an endoplasmic reticulum Ca\(^2+\) pool because thapsigargin-induced Ca\(^2+\) release was not affected by either CCCP or NO. Despite suppression of Ca\(^2+\) release, both NO and CCCP failed to inhibit but rather augmented CCh-induced Ca\(^2+\) entry. Oppositional regulation of CCh-stimulated Trp3 channels and CCE channels was surprising because our results suggest that NO as well as CCCP acts by alteration of local Ca\(^2+\) feedback mechanisms, and inhibitory autoregulation by Ca\(^2+\) has been reported for both Trp3 channels as well as CCE channels. Inhibition of Trp3 by cytoplasmic Ca\(^2+\) was again confirmed in the present study by simultaneous measurements of intracellular Ca\(^2+\) and membrane currents. In single voltage-clamped cells, Trp3-mediated cation currents measured during re-addition of extracellular Ca\(^2+\) were promoted, and the Trp3-mediated Ca\(^2+\) entry signal was augmented by NO. Elevation of extracellular Ca\(^2+\) from micromolar to millimolar concentration is known to suppress the Trp3-mediated cation conductance because of extra-
cellular as well as intracellular inhibitory effects of Ca\(^2+\) (12). This Ca\(^2+\)-mediated inhibitory modulation of Trp3 channels was suppressed by NO, a phenomenon that is in clear contrast to the observed inhibitory effects of NO and CCCP on endoge-
ous CCE channels in vector-transfected controls. The diver-
genation of these two Ca\(^2+\) entry channels by NO and CCCP indicates a distinctly different coupling of the endoge-
nous CCE channels and the phospholipase C-sensitive Trp3 channels to mitochondria. We suggest that a specific property of Trp3 enables up-regulation of channel activity in response to impaired mitochondrial Ca\(^2+\) sequestration. It is tempting to speculate that the specific functional interaction of Trp3 proteins with IP\(_3\) receptors that have been reported to communicate tightly with mitochondria (27, 28) may provide the basis of this specific coupling. Alternatively, PLC-regulated Trp3 channels and endogenous CCE channels may be targeted to distinct microdomains of the plasma membrane, which may accommo-
date divergent local changes in cytoplasmic Ca\(^2+\) during mito-
chondrial uncoupling. The molecular property that renders Trp3 channels insensitive to inhibition by NO and mitochon-
drial uncoupling remains to be clarified. Nonetheless, our study clearly demonstrates that overexpression of Trp3 confers insensitivity to inhibition by NO, as a Trp3-specific regulatory property, to endogenous CCE channels.

In summary, our results demonstrate that Trp3 expression determines Ca\(^2+\) entry by generation and modification of two distinct channel types, i.e. phospholipase C-controlled Ca\(^2+\) entry channels and classical CCE channels. These two channel types are oppositively controlled by nitric oxide and mito-
chondria. Our results provide evidence for a close relationship between Trp3 expression and the function of CCE channels. We suggest that Trp3 determines physiologically important prop-
erties of CCE channels such as sensitivity to regulation by NO and mitochondrial function. Thereby, variations in the expres-
sion of Trp3 may provide the basis of diverse properties of CCE channels in different tissues and cell types.

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