Suppression of TRPC3 Leads to Disappearance of Store-operated Channels and Formation of a New Type of Store-independent Channels in A431 Cells*

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In non-excitable cells, storage of Ca2+ in endoplasmic reticulum (ER) serves as the central role in maintaining the proper level of intracellular Ca2+ (1–3). Several types of store-operated calcium currents have been characterized in various tissues. Depending on the cell type, these currents display variability in biophysical characteristics and modes of regulation (4–6, 7, 8), suggesting that different proteins may be involved in forming store-operated channels (SOC) in the plasma membrane and/or regulating SOC activity. Several lines of evidence have suggested that STIM1 (9, 10) and Orai1 (11–13) play essential roles in activation of calcium release-activated Ca2+ currents (ICRAC) (4–6).

Recent studies have shown that STIM1 acts as a sensor of the intracellular Ca2+ stores (9, 14) while Orai1 may be directly involved in forming ICRAC channels in the plasma membrane (15–17). Although it is well established that ICRAC is activated by store depletion, ICRAC may only represent one subtype of store-operated channels since the SOC currents with properties distinct from ICRAC have been characterized (7, 8). Despite extensive calcium imaging and electrophysiological studies, the molecular composition of SOC types other than ICRAC remains poorly understood.

The members of mammalian TRP-related family of ion channels have been implicated to both receptor- and store-operated Ca2+ influx (reviewed in Ref. 18). TRPC3 has been shown to form non-selective cation channels that can be activated in a PLC-dependent manner (19). Although most studies suggest that overexpressed TRPC3 forms receptor-operated channels (20–24), several reports provided the evidence for regulation of TRPC3 by depletion of the intracellular calcium stores (25–27). In different cell types, activation of overexpressed TRPC3 channels have been shown to depend on InsP3, DAG, Ca2+, G-proteins, and store depletion. For example in DT40 cells, transiently expressed TRPC3 forms the InsP3-R-dependent and InsP3-R-independent channels (28, 29). These controversial observations may at least in part be reconciled by the fact that TRPC3 forms heterooligomers with the other members of TRPC subfamily (30). Interestingly, recent knockdown studies suggested that native TRPC3 is essential for store-operated calcium entry in HEK293 cells (31, 32).

Our previous studies have shown that A431 carcinoma cell line expresses highly selective ICRAC currents and moderately selective I_{SOC} currents. We found that I_{SOC} currents are at least partly mediated by I_{InsP3} channels, whose activity is controlled by downstream products of PLC (33–38) and could be triggered by passive depletion of intracellular Ca2+ stores with Tg and/or

In non-excitable cells, calcium (Ca2+) release from the inositol 1,4,5-trisphosphate (InsP3)-sensitive intracellular Ca2+ stores is coupled to Ca2+ influx through the plasma membrane Ca2+ channels whose molecular composition is poorly understood. Several members of mammalian TRP-related protein family have been implicated to both receptor- and store-operated Ca2+ influx. Here we investigated the role of the native transient receptor potential 3 (TRPC3) homologue in mediating the store- and receptor-operated calcium entry in A431 cells. We show that suppression of TRPC3 protein levels by small interfering RNA (siRNA) leads to a significant reduction in store-operated calcium influx without affecting the receptor-operated calcium influx. With single-channel analysis, we further demonstrate that reduction of TRPC3 levels results in suppression of specific subtype of store-operated calcium channels and activation of store-independent channels. Our data suggest that TRPC3 is required for the formation of functional store-operated channels in A431 cells.

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‡ The abbreviations used are: PLC, phospholipase C; SOC, store-operated channel; TRPC, canonical transient receptor potential; InsP3, inositol 1,4,5-trisphosphate; PIP2, phosphatidylinositol 4,5-bisphosphate; Tg, thapsigargin; I_{CRAC}, Ca2+ release-activated channel; R, receptor; TPEN, N,N',N'-tetraakis(2-pyridylmethyl)ethylenediamine; siRNA, small interfering RNA; NMDG, N-methyl-D-glucamine.
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BAPTA-AM (39). In the present study, we aimed to determine the role of TRPC3 in mediating the receptor- and store-operated calcium influx in A431 cells. Using calcium imaging and whole cell recordings, we found that suppression of TRPC3 expression with siRNA results in significant reduction in store-operated calcium entry. Because most of the functional studies linking TRP family to receptor-operated and store-operated Ca\(^{2+}\) influx utilized Ca\(^{2+}\) imaging or whole cell current recordings, we further extended our analysis with single channel recordings to obtain more detailed information on functional properties of individual types of channels supporting store-operated Ca\(^{2+}\) influx. We found that suppression of TRPC3 in A431 cells results in selective inhibition of specific subtype of store-operated calcium channels and activation of store-independent channels. Together, these data indicate that TRPC3 is essential for formation of functional SOCs in A431 cells.

EXPERIMENTAL PROCEDURES

Cell Culture—Human carcinoma A431 cells (Cell Culture Collection, Institute of Cytology, St. Petersburg, Russia; ATCC CRL-1555) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 80 µg/ml gentamicin, and 2 mM glutamine. The cells were maintained in 37 °C incubator (5% CO\(_2\)). For patch clamp and Ca\(^{2+}\)-imaging experiments, the cells were plated on glass coverslips and main-

Transfections—Cells were transfected by Effectin Transfection Reagent (Qiagen) with the appropriate siRNA vectors, and transfections were confirmed by sequencing.

RNAi—We designed three different 19-nucleotide siRNA targeting human TRPC3 homolog (sense strands: I, 5’-GATCCC-CATGCCAGATATTGGAATGTCCAGAGACATTACCA-TATCTGGCATTTTGGAAA-3’ (748–766); II, 5’-GATCC-CATGCTAATTATGCTGGGTCAAGAGACCCAG-ACCATAATTAGCATTITTTGGAAA-3’ (1313–1331); III, 5’-GATCCCGGCTTCAGAGATATAGCTTAAGGAGA-CTATATCTCCTCTGAGCCTTTTGGAAA-3’ (2196–2214).

The synthesized double-stranded oligonucleotides were annealed and ligated into pUB/Bsd/H1 RNAi vector containing a cassette for blasticidin selection (40). The oligonucleotide inserts were confirmed by sequencing.

The whole cell recordings were performed with 3–5 MW sylgard-coated, fire-polished glass pipettes. The pipette solution contained (in mM) 145 NMDG aspartate, 10 Cs-EGTA, 10 Cs-HEPES pH 7.3, 1.5 MgCl\(_2\), and either 4.5 CaCl\(_2\) (pCa 7.0) or 0 CaCl\(_2\) (pCa > 9). Extracellular solution contained (in mM) 140 NMDG aspartate, 10 BaCl\(_2\), 10 Cs-HEPES, pH 7.3. The currents were sampled at 5 kHz and filtered digitally at 500 Hz. In all experiments, the holding potential was 0 mV. Periodically (once every 5 s) the holding potential was shifted to −100 mV.
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Our biochemical analysis has shown that A431 cells express several isoforms of TRPC protein family, including TRPC3 (Fig. 1A). This data are in agreement with the unpublished observations of Dr. Schilling. Additional evidence of TRPC3 expression in A431 was obtained using transient transfection of Myc-tagged human TRPC3 cDNA in these cells (Fig. 1B). To assess the role of TRPC3 in mediating agonist- or store-induced currents in A431 cells, we knocked down TRPC3 expression using three different siRNA constructs and tested the protein levels in individual clones by immunoblotting. A clone that showed the highest reduction in TRPC3 expression (siTRPC3) was expanded and used for electrophysiological and imaging analyses (Fig. 2). To determine the role of TRPC3 in mediating agonist-evoked Ca$^{2+}$ entry, we compared the UTP-induced Ca$^{2+}$ entry in control A431 cells and the cells expressing TRPC3 siRNA (siTRPC3). Application of 100 μM UTP to Fura-2-loaded cells maintained in Ca$^{2+}$-free media, resulted in transient elevation of cytosolic Ca$^{2+}$ levels due to calcium release from the intracellular InsP$_3$-sensitive Ca$^{2+}$ stores (Fig. 3A). Subsequent re-addition of 2 mM extracellular Ca$^{2+}$ (for 30 ms) and a 170-ms voltage ramp to +70 mV was applied. The traces recorded before current activation were used as templates for leak subtraction. Whole cell currents were normalized to the cell capacitance. The mean value of cell capacitance was 25 ± 4 pF (n = 45).

The single channel recordings were performed with 8–15 MΩ glass pipettes. The pipette solution contained (in mM): 105 BaCl$_2$, 10 Tris-HCl (pH 7.3). In cell-attached experiments, the bath solution contained 140 mM KCl and 2 mM CaCl$_2$ to nullify the resting membrane potential. In store depletion experiments, 1 mM TPEN, or 1 μM thapsigargin were added to the bath solution containing (in mM): 140 KCl, 5 NaCl, 10 K-Hepes, and 2 EGTA (pH 7.4). In inside-out experiments, the intracellular solution contained (in mM): 140 potassium glutamate, 5 NaCl, 1 MgCl$_2$, 10 K-HEPES pH 7.4, 2 K-EGTA, and 1.13 CaCl$_2$ (pCa 7.0), with or without InsP$_3$. The drugs were applied by bath perfusion. The time required for a complete exchange of solution around the patch pipette was less than 1 s. The single channel recordings were digitized at 5 kHz and filtered at 100 Hz for analysis and presentation. The amplitudes of single-channel currents were determined from the current traces and all-point amplitude histograms. The channel open probabilities (NPo) were determined by using the following equation: NPo = (I)/i, where (I) is a mean channel current; and i is the unitary current amplitude. The (I) was estimated from the time integrals of the currents above the baseline, and i was determined from the current traces and all-point amplitude histograms. The data were collected from the current traces after channel activity reached steady state.

Chemicals—HEPES, EGTA, Tg, NMDG, and TPEN were from Sigma-Aldrich, UTP, and InsP$_3$ were from Calbiochem, Fura-2AM and Pluronic were from Molecular Probes.

RESULTS

Suppression of TRPC3 Expression Reduces Store-operated Calcium Influx in A431 Cells—Our biochemical analysis has shown that A431 cells express several isoforms of TRPC protein family, including TRPC3 (Fig. 1A). This data are in agreement with the unpublished observations of Dr. Schilling. Additional evidence of TRPC3 expression in A431 was obtained using transient transfection of Myc-tagged human TRPC3 cDNA in these cells (Fig. 1B). To assess the role of TRPC3 in mediating agonist- or store-induced currents in A431 cells, we knocked down TRPC3 expression using three different siRNA constructs and tested the protein levels in individual clones by immunoblotting. A clone that showed the highest reduction in TRPC3 expression (siTRPC3) was expanded and used for electrophysiological and imaging analyses (Fig. 2). To determine the role of TRPC3 in mediating agonist-evoked Ca$^{2+}$ entry, we compared the UTP-induced Ca$^{2+}$ entry in control A431 cells and the cells expressing TRPC3 siRNA (siTRPC3). Application of 100 μM UTP to Fura-2-loaded cells maintained in Ca$^{2+}$-free media, resulted in transient elevation of cytosolic Ca$^{2+}$ levels due to calcium release from the intracellular InsP$_3$-sensitive Ca$^{2+}$ stores (Fig. 3A). Subsequent re-addition of 2 mM extracel-
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We next employed the whole cell recordings to investigate the effects of suppression of TRPC3 on cation currents induced by agonists and store depletion. In these experiments, we used 10 mM Ba2+ as a charge carrier. Under these conditions, extracellular application of UTP induced the cation currents that reached the maximum at ~4 min after addition of the agonist (Fig. 4A). Consistent with the calcium imaging experiments, we found that both control and siTRPC3 cells displayed similar UTP-mediated responses (Fig. 4, A and B). Passive store depletion with Tg also induced the cation currents that displayed kinetics of onset similar to the agonist-induced currents (Fig. 4C). In a few cells (2 out of 15 in control group and 3 out of 13 in siTRPC3 group), we only observed the highly selective I_CrAC-like inward calcium currents that were not affected by suppression of TRPC3 (Fig. 4D). We therefore excluded these experiments from the final analysis. In the remaining experiments, the cation currents induced by Tg were reduced on average to 38% in siTRPC3 cells when compared with control (Fig. 4, C and E). Thus, our calcium imaging and whole-cell experiments consistently show that reduction in TRPC3 levels results in selective inhibition of calcium influx mediated by depletion of the intracellular calcium stores. We next used the single channel analysis to explore the effects of TRPC3 suppression on various types of calcium- and cation-selective channels expressed in A431 cells.

TRPC3 Does Not Regulate I_min and I_max Cation Channels—We have previously shown that A431 cells express highly selective I_min calcium channels that could be activated by extracellular UTP, intracellular InsP3, and depletion of intracellular Ca2+ stores (35, 36, 39, see also Fig. 5F). We also found that I_min channels can be directly activated by N-terminal ligand-binding domain of InsP3R (33). To test whether suppression of TRPC3 affects I_min channels in A431 cells, we performed single channel recordings and tested the effects of extracellular application of UTP or TPEN to the cell-attached patches, and intracellular application of InsP3 to the inside-out patches (Fig. 5). We found that similarly to control A431 cells, siTRPC3 cells express I_min channels that can be activated by extracellular agonists (UTP), store depletion and InsP3 (Fig. 5). The biophysical characteristics of single I_min channels monitored in A431 cells with reduced levels of TRPC3 were identical to I_min channels that we previously described in control A431 cells: the single channel conductance measured in the presence of 105 mM extracellular Ba2+ was 1.2 pS (Fig. 5D); the extrapolated reversal potential was higher than +30 mV, indicating high selectiv-

lular Ca2+ induced the second peak of intracellular Ca2+ with practically equal transients in A431 and siTRPC3 cells.

To test whether TRPC3 forms or regulates store-operated channels in A431 cells, we compared Ca2+ entry mediated by passive store depletion in control and siTRPC3 cells. In contrast to the agonist-induced responses, thapsigargin (Tg) or TPEN-induced Ca2+ entry was reduced by respectively 40 and 17% in siTRPC3 cells (Fig. 3, B and C). Analysis of the areas underneath the transient Ca2+ peaks showed that control and siTRPC3 cells released similar amounts of calcium from the intracellular stores in response to Tg treatment. These results suggest that TRPC3 suppression does not affect calcium storage or calcium release from the intracellular stores. To further assess the role of TRPC3 in mediating calcium influx in response to store depletion, we analyzed the effect of gadolinium (Gd3+, 10 μM). At micromolar concentrations, Gd3+ blocks the calcium entry triggered by store depletion (41–43). We found that in the presence of Gd3+, the Tg-induced calcium peaks were reduced by ~10- and ~2-fold in control and siTRPC3 cells, respectively (Fig. 3, B and D). Together, these results suggest that suppression of TRPC3 in A431 cells results in significant reduction of calcium influx mediated by depletion of the intracellular calcium stores.

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FIGURE 4. Whole cell recordings of agonist- and store-operated currents in A431 and siTRPC3 cells. A and B, average UTP-induced currents monitored in A431 (filled circles, n = 8) and siTRPC3 (open circles, n = 6) cells. The point 0 s corresponds to the time of 100 μM UTP application. The current amplitudes were measured at ~80 mV. The pipette solution contained (in mM) 154 NMDG aspartate, 10 Cs-EGTA, 10 Cs-HEPES pH 7.3, 1.5 MgCl2, and 4.5 CaCl2 (pCa 7.0). Extracellular solution contained (in mM) 140 NMDG aspartate, 10 BaCl2,1 0 Cs-HEPES, pH 7.3. B, average current-voltage relationships of currents induced by 100 μM UTP in A431 (black) and siTRPC3 (gray) cells. The current-voltage relationships were measured when the inward currents reached the maximum (at least 250 s after UTP addition). C, average Tg-induced currents monitored in A431 (filled circles, n = 10) and siTRPC3 (open circles, n = 6) cells. The point 0 s corresponds to the time of Tg (1 μM) application. The current amplitudes were measured at ~80 mV. The pipette solution contained (in mM) 145 NMDG aspartate, 10 Cs-EGTA, 10 Cs-HEPES pH 7.3, 1.5 MgCl2 (pCa > 9). D, the current-voltage relationships of I_max currents recorded form A431 (black) and siTRPC3 (gray) cells. The currents were mediated by passive Ca2+ store-depletion induced by bath application of Tg (1 μM). E, current-voltage relationships of currents evoked in A431 (black) and siTRPC3 (gray) cells by passive depletion of calcium stores with 1 μM Tg. To generate the curves, the current-voltage relationships were measured after inward currents reached the maximum (at least 250 s after Tg addition). The experiments that only displayed I_CrAC currents were not included. The data from the same experiments were used to generate C and E.
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FIGURE 5. Single I_{min} channels in siTRPC3 cells. A and B, single I_{min} channels were monitored in cell-attached mode after bath application of 100 μM UTP (A) or 1 mM TPEN (B). The holding potential was −70 mV. Expanded current traces are shown on the bottom. C, activity of single I_{min} channels was induced by application of μM InsP_{3} to the cytosolic side of inside-out patch. The holding potential was −70 mV. Expanded currents traces are shown on the bottom. D, mean current-voltage relationship of I_{min} channels. Each point shows the average amplitude of single-channel currents induced by UTP or TPEN (n > 9). The data are shown as mean ± S.E. The linear fit corresponds to single-channel conductance (g) of 1.2 pS. E, an open-time histogram of I_{min} channel was constructed from 1010 single-channel opening events. The events with time duration of less than 3 ms were discarded. Single exponential fit (solid line) corresponds to a time constant of 8.8 ms. Single-channel currents were monitored at −70 mV and filtered at 500 Hz. F, summary of I_{min} channels occurrence in A431 and siTRPC3 cells. The cells were stimulated with UTP or TPEN (Tg) in cell-attached mode or with InsP_{3}, in inside-out mode. The reversal potential was −70 mV. Expanded currents traces are shown on the bottom. G, activity of single I_{min} channels was induced by application of μM InsP_{3} to the cytosolic side of inside-out patch. The holding potential was −70 mV. Expanded currents traces are shown on the bottom. H, mean current-voltage relationship of I_{min} channels. Each point shows the average amplitude of single-channel currents induced by UTP or TPEN (n > 9). The data are shown as mean ± S.E. The linear fit corresponds to single-channel conductance (g) of 1.2 pS. I, an open-time histogram of I_{min} channel was constructed from 1010 single-channel opening events. The events with time duration of less than 3 ms were discarded. 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The linear fit corresponds to single-channel conductance (g) of 1.2 pS. U, an open-time histogram of I_{min} channel was constructed from 1010 single-channel opening events. The events with time duration of less than 3 ms were discarded. Single exponential fit (solid line) corresponds to a time constant of 8.8 ms. Single-channel currents were monitored at −70 mV and filtered at 500 Hz. V, summary of I_{min} channels occurrence in A431 and siTRPC3 cells. The cells were stimulated with UTP or TPEN (Tg) in cell-attached mode or with InsP_{3}, in inside-out mode. The reversal potential was −70 mV. Expanded currents traces are shown on the bottom. W, activity of single I_{min} channels was induced by application of μM InsP_{3} to the cytosolic side of inside-out patch. The holding potential was −70 mV. Expanded currents traces are shown on the bottom. X, mean current-voltage relationship of I_{min} channels. Each point shows the average amplitude of single-channel currents induced by UTP or TPEN (n > 9). The data are shown as mean ± S.E. The linear fit corresponds to single-channel conductance (g) of 1.2 pS. Y, an open-time histogram of I_{min} channel was constructed from 1010 single-channel opening events. The events with time duration of less than 3 ms were discarded. Single exponential fit (solid line) corresponds to a time constant of 8.8 ms. Single-channel currents were monitored at −70 mV and filtered at 500 Hz. Z, summary of I_{min} channels occurrence in A431 and siTRPC3 cells. The cells were stimulated with UTP or TPEN (Tg) in cell-attached mode or with InsP_{3}, in inside-out mode. The reversal potential was −70 mV. Expanded currents traces are shown on the bottom.

regulation properties were described by us in HEK293 cells and we have named them I_{max} (44). Similarly to the I_{min} channels, I_{max} in A431 cells could be activated by application of InsP_{3} to inside-out patches (Fig. 6H). We found that in siTRPC3 cells, I_{max} could also be evoked by UTP (Fig. 6D) or depletion of intracellular stores with TPEN (Fig. 6E). In addition, in both control and siTRPC3 cells, I_{max} channels had similar conductance and gating characteristics (Fig. 6, G and F), suggesting that TRPC3 is not involved in formation or regulation of I_{max} channels.

Non-selective Channels in A431 and siTRPC3 Cells—Further single-channel analysis indicated that in addition to I_{min} and I_{max} channels, A431 cells express non-selective cation channels. These channels can be activated by UTP (10 out of 87 experiments, Fig. 7A) or store-depletion (13 out of 74 experiments, Fig. 6B) but not by InsP_{3} (n = 119, Fig. 7D) or 10 μM OAG (n = 12, data not shown), indicating receptor-independent mode of their activation. We previously characterized the channels with similar characteristics in HEK293 cells, and named them I_{NS} (44). In A431 cells, I_{NS} channels displayed the following basic properties: the reversal potential near 0 mV; the single-channel conductance of 4.5 pS (Fig. 7J); the lack of voltage dependence of channel open probability (Fig. 7E); and the mean open time −3.4 ms (Fig. 7C). In contrast, no I_{NS} channel activity was observed in siTRPC3 cells treated with Tg or TPEN (n = 58, Fig. 7I). Selective suppression of I_{NS} in siTRPC3 cells suggests that TRPC3 is an essential subunit or regulator of I_{NS} channels. Because the I_{CRAC}, I_{min} and I_{max} currents were similar in control A431 and siTRPC3 cells, suppression I_{NS} channel activity in siTRPC3 cells is the most likely explanation for reduction of Tg-induced Ca^{2+} influx that we observed in our Ca^{2+}-imaging experiments. On the other hand, Ca^{2+}-imaging data suggests that suppression of TRPC3 did not affect the agonist-induced Ca^{2+} entry (Fig. 3A). The later observation indicates that receptor-operated calcium influx is compensated in siTRPC3 cells. Indeed, in 15% of the single-channel experiments, the agonist application to siTRPC3 cells induced the activity of a new type of Ca^{2+}-permeable channels (n = 62, Fig. 7F). In 10 experiments (16%, n = 62) the moderate constitutive activity of these channels was observed and was further enhanced after application of UTP (data not shown). In contrast to I_{NS}, the open probability of these channels was highly voltage-dependent with increased activity observed at the membrane potentials above −50 mV (Fig. 7, E and G). Kinetic characteristics of these channels were also different from the I_{NS}. Analysis of open-time histograms revealed the time constants of 6.6 ms (Fig. 7H), which is 2-fold higher than the time constant of I_{NS}. Similar to I_{NS} in control A431 cells, the channels observed in siTRPC3 displayed the reversal potential near 0 mV and the unitary conductance of −4.5 pS (Fig. 7J). Thus, we called these channels I_{NS-2}, whereas the non-selective channels (I_{NS}) in A431 cells were renamed as I_{NS-1}. Further analysis has shown that activity I_{NS-2} could not be evoked by store depletion with Tg or TPEN (n = 58). Neither constitutive nor UTP-induced I_{NS-2} channel activity could be blocked by Gd^{3+} (10 μM) (n = 22, data not shown). Together these data indicate that in contrast to I_{NS-1}, I_{NS-2} are store-independent cation channels. In addition, activi-
TRPC3 is essential for activity of Ca\(^{2+}\) permeable channels that support store-operated Ca\(^{2+}\) influx. Despite the increasing evidence that TRPC proteins mediate store-operated currents, the role of TRPCs in forming or regulating SOC channels remains controversial. Although some overexpression studies have suggested that recombinant TRPC3 forms receptor-operated channels (20–24), several knockdown studies indicate that TRPC3 is required for SOC activity. For example, the TRPC3 antisense decreased the store-dependent calcium influx in HSY and PS1 cells (45, 46). In addition, TRPC3 antisense and siRNA reduced receptor- and store-operated Ca\(^{2+}\) influx in HEK293 cells (31, 32). In our previous studies we demonstrated that HEK293 cells express four types of agonist- and store-activated Ca\(^{2+}\) channels that display distinct permeability and activation profiles: I\(_{\text{CRAC}}\), I\(_{\text{min}}\), I\(_{\text{max}}\), and I\(_{\text{NS}}\) (44). We also characterized the highly selective I\(_{\text{CRAC}}\) and moderately selective I\(_{\text{SOC}}\) currents in A431 cells, and shown that in these cells, I\(_{\text{SOC}}\) are partly supported by I\(_{\text{NS}}\) (33). In the present study, we investigated the role of native TRPC3 in mediating receptor- and store-operated currents in A431 cells. We generated a stable cell line in which the expression levels of native TRPC3 were significantly reduced (siTRPC3, Fig. 2). We found that in siTRPC3 cells, the UTP-induced Ca\(^{2+}\) influx was normal (Figs. 3A and 4A) suggesting that in these cells, TRPC3 is not directly involved in regulation of receptor-operated cation channels. However, it is also possible that the effect of reduction of TRPC3 on receptor-operated calcium influx was compensated by other TRP homologues. In contrast to the agonist-induced currents, the calcium currents triggered by passive store-depletion were significantly reduced in siTRPC3 cells (Figs. 3 and 4). Thus, in A431 cells, TRPC3 is essential for Ca\(^{2+}\) entry mediated by depletion of intracellular stores. The Tg-induced currents observed in control A431 cells and siTRPC3 cells, displayed different sensitivity to Gd\(^{3+}\) (Fig. 3D) suggesting that reduction of TRPC3 levels affects the composition of functional cation channels in the plasma membrane. In siTRPC3 cells, more than 45% of Ca\(^{2+}\) influx was store-independent and Gd\(^{3+}\)-insensitive, indicating that Ca\(^{2+}\) influx in these cells is supported by constitutive activity of store-independent Ca\(^{2+}\) channels. Thus, we investigated the single cation channels expressed in A431 cells. The cells were stimulated with UTP or TPEN (Tg) in cell-attached mode or with InsP\(_3\) in inside-out mode. Our detailed single-channel analysis has shown that A431 cells express several types of cation channels. Although we

**FIGURE 6. Single I\(_{\text{NS}}\) channels in A431 and siTRPC3 cells.** A and B, single I\(_{\text{max}}\) channels were monitored in cell-attached mode in A431 cells after bath application of 100 \(\mu\)M UTP at holding potential \(-70\) mV (A) or 1 mM TPEN at holding potential \(-50\) mV (B). Expanded current traces are shown on the bottom. C and D, open time histograms of I\(_{\text{max}}\) channel were constructed from 542 single-channel opening events in A431 cells (C) and 628 single-channel opening events in siTRPC3 cells (D). Events with time duration of less than 3 ms were discarded. Double exponential fit (solid line) corresponds to a time constant of \(\tau_{\text{a}}\), 2.5 ms and \(\tau_{\text{c}}\), 21.3 ms for A431 and \(\tau_{\text{a}}\), 2.01 ms and \(\tau_{\text{c}}\), 13.3 ms for siTRPC3 cells. Single-channel currents were monitored at \(-70\) mV and filtered at 500 Hz. D and E, single I\(_{\text{max}}\) channels were monitored in cell-attached mode in siTRPC3 cells after bath application of 100 \(\mu\)M UTP (D) or 1 mM TPEN (E). The holding potential was \(-70\) mV. Expanded current traces are shown on the bottom. G, mean current-voltage relationship of I\(_{\text{max}}\) channels in A431 (filled squares) and siTRPC3 (open circles) cells. Each point represents the average amplitude of single-channel currents induced by UTP or TPEN \(n > 4\). The data are shown as mean \pm S.E. The linear fit corresponds to single-channel conductance \(g\) of 18 pS and reversal potential \(E_{\text{rev}}\) +15 mV. H, summary of I\(_{\text{max}}\) channels occurrence in A431 and siTRPC3 cells. The cells were stimulated with UTP or TPEN (Tg) in cell-attached mode or with InsP\(_3\) in inside-out mode.

**DISCUSSION**

By using a combination of Ca\(^{2+}\) imaging and electrophysiological recordings we found that in A431 cells, the native activity of I\(_{\text{NS-2}}\) in siTRPC3 cells could not be evoked by application of 2.5 \(\mu\)M InsP\(_3\) to the inside-out patches \((n = 51,\) Fig. 7) or 10 \(\mu\)M OAG to the cell-attached or inside-out patches \((n = 10\) and data not shown). Thus, I\(_{\text{NS-2}}\) and I\(_{\text{NS-1}}\) channels display distinct biophysical properties and modes of activation. Our single-channel analysis indicates that in A431 and siTRPC3 cells, agonist-induced Ca\(^{2+}\) influx is mediated by diverse set of ion channels. Considering that the single-channel conductance, selectivity and the observation frequency of I\(_{\text{NS-1}}\) and I\(_{\text{NS-2}}\) are very similar, we conclude that suppression of I\(_{\text{NS-1}}\) is compensated by up-regulation of I\(_{\text{NS-2}}\) channels that contribute to the agonist-induced Ca\(^{2+}\) influx in siTRPC3 cells. Thus, suppression of TRPC3 in A431 cells has not altered the agonist-evoked Ca\(^{2+}\) entry, but affected its regulation which can be elucidated only by single-channel analysis. The different situation was observed with store-dependent Ca\(^{2+}\) influx. Because I\(_{\text{NS-2}}\) channels are store-independent, and do not mediate the store depletion-evoked Ca\(^{2+}\) influx, the decrease of Tg-induced Ca\(^{2+}\) entry in siTRPC3 cells was observed in both Ca\(^{2+}\) imaging and whole cell experiments.
could not analyze the I_{CRAC} channels because their unitary conductance is too small (6), we found that similarly to the HEK293 cells, A431 cells express three distinct types of channels: I_{min}, I_{max}, and I_{NS}. The I_{min} channels displayed a 1.2 pS unitary conductance for divalent cations (35, 36), a 10 pS conductance for monovalent cations (33) and high selectivity for divalent cations (36). In A431 cells, I_{min} could be activated by extracellular UTP, intracellular InsP_{3}, depletion of intracellular Ca^{2+}/H^{1+} stores with Tg, or BAPTA-AM (35–39, see also Fig. 5F). The I_{max} channels displayed a 18 pS unitary conductance for divalent cations and lower selectivity than I_{min} (Fig. 6G). Similar to the HEK293 cells (44), activity of I_{max} channels in A431 cells could be evoked by extracellular UTP or intracellular InsP_{3} (Fig. 6, A and H). Together, these data indicate that in A431 cells, I_{min} and I_{max} channels function in both receptor- and store-operated modes. In addition to I_{min} and I_{max}, we also identified non-selective 4.5 pS INS-1 channels (Fig. 7H). The INS-1 channels could be activated by store-depletion but not by intracellular InsP_{3} (Fig. 7I). Thus, INS-1 function exclusively as store-operated channels. We found that siTRPC3 cells also express I_{min} and I_{max} channels that display the biophysical characteristics and modes of regulation similar to control A431 cells. In addition, the observation frequency of I_{min} and I_{max} channels was not affected by suppression of TRPC3. These findings rule out the possibility that TRPC3 directly acts as a subunit for I_{min} and I_{max} channels.

In contrast to control A431 cells, we could not detect active I_{NS-1} channels in the plasma membrane of siTRPC3 cells. Neither passive store depletion, nor agonist application could activate I_{NS-1} in siTRPC3 cells indicating that TRPC3 is essential for forming functional I_{NS-1} channels. However, in 15% cell-attached experiments we registered a new type of nonselective, Gd^{3+}-insensitive I_{NS-2} channels. INS-2 dis-
played a unitary conductance of 4.5 pS conductance for divalent cations (Fig. 7H) and high voltage-dependence with increased activity at membrane potentials above −50 mV (Fig. 7, E and F). InNS-2 could be activated by UTP but not by InsP₃ or depletion of intracellular calcium stores (Fig. 7I). The application of high resolution single-channel patch clamp analysis in our study revealed that reduction of TRPC3 expression level caused complex changes in functional properties of store- and receptor-operated Ca²⁺ influx channels in A431 cells. These changes could not be clearly identified using low-resolution methods such as Ca²⁺-imaging techniques (Fig. 3) or whole-cell Ca²⁺ current recordings (Fig. 4). In the future, similar single-channel approach can be taken to dissect functional roles of other proteins proposed to serve as subunits of store-operated Ca²⁺ channels in cells.

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