Endogenously expressed canonical transient receptor potential (TRPC) homologs were investigated for their role in forming store-operated, 1-oleoyl-2-acetyl-sn-glycerol-stimulated, or carbachol (CCh)-stimulated calcium entry pathways in HEK-293 cells. Measurement of thapsigargin-stimulated Ba\(^{2+}\) entry indicated that the individual suppression of TRPC1, TRPC3, or TRPC7 protein levels, by small interfering RNA (siRNA) techniques, dramatically inhibited (52–68%) store-operated calcium entry (SOCE), whereas suppression of TRPC4 or TRPC6 had no effect. Combined suppression of TRPC1-TRPC3, TRPC1-TRPC7, TRPC3-TRPC7, or TRPC1-TRPC3-TRPC7 gave only slightly more inhibition of SOCE (74–78%) than seen with suppression of TRPC1 alone (68%), suggesting that these three TRPC homologs work in tandem to mediate a large component of SOCE. Evidence from co-immunoprecipitation experiments indicates that a TRPC1-TRPC3-TRPC7 complex, predicted from siRNA results, does exist. The suppression of either TRPC3 or TRPC7, but not TRPC1, induced a high Ba\(^{2+}\) leak flux that was inhibited by 2-APB and SKF96365, suggesting that the influx is via leaky store-operated channels. The high Ba\(^{2+}\) leak flux is eliminated by co-suppression of TRPC1-TRPC3 or TRPC1-TRPC7. For 1-oleoyl-2-acetyl-sn-glycerol-stimulated cells, siRNA data indicate that TRPC1 plays no role in mediating Ba\(^{2+}\) entry, which appears to be mediated by the participation of TRPC3, TRPC4, TRPC6, and TRPC7. CCh-stimulated Ba\(^{2+}\) entry, on the other hand, could be inhibited by suppression of any of the five endogenously expressed TRPC homologs, with the degree of inhibition being consistent with CCh stimulation of both store-operated and receptor-operated channels. In summary, endogenous TRPC1, TRPC3, and TRPC7 participate in forming heteromeric store-operated channels, whereas TRPC3 and TRPC7 can also participate in forming heteromeric receptor-operated channels.

Many G protein-coupled receptors stimulate the release of internal Ca\(^{2+}\) stores and the influx of Ca\(^{2+}\) via multiple Ca\(^{2+}\) channels (1), including store-operated channels (SOCs) (2, 3).
TRPC1-TRPC3-TRPC7 Complex Forms Store-operated Channels

When taken together, recent overexpression (24, 29) and antisense studies (9, 30) suggest that the physiological role of individual TRPC homologs may be more complex than originally thought. The type of native channel formed by a given TRPC protein may depend on which other TRPC homologs are endogenously expressed. What is required to clarify this issue is the identification of all endogenous TRPC homologs expressed in a given cell type, followed by a comprehensive investigation of the contribution of each TRPC homolog to the channel type(s) under investigation. The siRNA approach seemed best suited to evaluate the quantitative contribution of each individual TRPC protein to various channel subtypes and to determine whether co-suppression of multiple TRPC proteins has additive inhibitory effects on store-operated, OAG-stimulated, or CCh-stimulated Ca²⁺ entry pathways. The results from such a comprehensive investigation of endogenous TRPC protein function in HEK-293 cells suggest that TRPC1, TRPC3, and TRPC7 assemble to form native SOCs and that certain TRPC proteins (TRPC3 and TRPC7) can simultaneously participate in forming native store-operated and native OAG-stimulated channels.

EXPERIMENTAL PROCEDURES

Materials—Fura 2 free acid, fura 2-AM, and Pluronic F-127 were purchased from Molecular Probes, Inc. (Eugene, OR). Thapsigargin was purchased from LC Laboratories, G418 from Mediatech, hygromycin from Invitrogen, and puromycin from ICN. HBSS, Dulbecco’s modified Eagle’s medium, and Ca²⁺-free HBSS solutions came from Invitrogen. All other reagents were purchased from Sigma, or their source is acknowledged when the agent is first discussed.

Cultured HEK-293 Cells—HEK-293 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 50 units/ml penicillin, 50 μg/ml streptomycin, and 2 mm glutamine. Cells were grown in an incubator at 37 °C with humidified 5% CO₂ and 95% air.

Ca²⁺ Imaging—[Ca²⁺], concentration and Ba²⁺ entry were measured in cells loaded with the fluorescent indicator fura 2 by previously described methods (42).

siRNA Constructs—Using the complementary oligonucleotides shown in Table I, siRNAs targeting each individual TRPC homolog were made by previously described methods (42). For the co-expression of siRNAs for TRPC1, TRPC3, and TRPC7, siRNAs were expressed in different vectors containing neomycin-, hygromycin-, or puromycin-selectable markers. For mock transfection, the negative control vector that expresses a hairpin siRNA with limited homology to any known sequence in human, mouse, and rat genomes was used. This plasmid that expresses a hairpin siRNA with limited homology to any known sequence in human, mouse, and rat genomes was used. This plasmid was provided by Ambion. Several mock transfections, using different selection markers, were done.

Transfection—HEK-293 cells were grown in 75-cm² flasks to 80% confluence. Cells were transfected with the appropriate siRNA construct(s) by using PerFectin Transfection reagent (Gene Therapy Systems Inc.). 48 h after transfection, G418 (or G418 + hygromycin or G418 + hygromycin + puromycin) was added. Cells surviving after 2 weeks were collected and used in all experiments.

Total RNA Isolation—Total RNA was isolated from HEK-293 cells using the RNeasy Mini Kit (Qiagen Inc.) and treated with DNase I (Invitrogen). The RNA sample was additionally purified by ethanol precipitation, and its concentration was determined by measuring absorbance at 260 nm.

Quantitative Real Time RT-PCR—PCR primers for the TRPC homologs were designed based on published sequences in GenBank™ (Supplemental Table I). First-strand cDNA was prepared from 1 μg of total RNA using SuperScript™ III RNase H⁻ reverse transcriptase (Invitrogen) and 1 μg of oligo(dT). Real time PCR was performed on the ABI Prism 7700 sequence detection system by using SYBR Green PCR core reagents (Applied Biosystems) and cDNA synthesized as described above. PCR was performed using the kit protocol in a 25-μl reaction volume. The integrity of the RT-PCR products was confirmed by melting curve analysis. Melting curves for TRPC1, TRPC3, TRPC4, TRPC6, and TRPC7 showed one specific peak. The amount of PCR products in HEK-293 cells or in cells transformed with siRNA constructs was calculated in reference to the individual calibration curves based on cDNA obtained from control HEK-293 cells.

Western Blotting—HEK-293 cells or cells expressing siRNA were grown on 10-cm dishes under the conditions described above. Cells were lysed in modified radioimmune precipitation buffer (10 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.1% SDS, 1% Nonidet P-40, 1% sodium deoxycholate, 2 mM EDTA, 2 mM Na₂VO₄, 2 mM Na₃P₂O₇, 2 mM NaF). Western blots were performed as described (42). Each Western blot was repeated at least three times using different cell lysates.

Co-immunoprecipitation—HEK-293 cells on 10-cm dishes were incubated for 1 h at 37 °C in HBBS and then lysed in modified radioimmune precipitation buffer. After centrifugation (15,000 rpm for 30 min), the protein concentration of the supernatants was determined, and 2-mg aliquots of total protein were used for each immunoprecipitation reaction. 10 μg of TRPC1 antibody (Santa Cruz Biotechnology) was added to the cell lysate, and the mixture was incubated for 3 h at room temperature with rotation. Then 100 μl of protein A⁺G-Sepharose was added, and the rotation continued for 2 h. The Sepharose was washed four times with ice-cold lysis buffer, and 100 μl of sample buffer (with 5% mercaptoethanol) was added to solubilize proteins. The Sepharose-sample buffer mixture was incubated at room temperature for 30 min and then boiled for 5 min at 95 °C. After centrifugation, the supernatants were applied to a 6% SDS-PAGE (16 × 16 cm), and electrophoresis was run overnight. Proteins were transferred onto an Immobilon membrane and Western blotted with anti-TRPC1 antibodies. The membranes were sequentially stripped and rebotted with anti-TRPC3 antibodies (Alomone Laboratories), anti-TRPC7 antibodies, and in some cases anti-TRPC4 antibodies (Santa Cruz Biotechnology).

RESULTS

Suppression of Individual TRPC Protein Levels by Expression of siRNA—Expression of TRPC1, TRPC3, TRPC4, and TRPC6 mRNA (9) and TRPC7 mRNA (this study) is seen in our HEK-293 cells. Whereas our previous antisense studies suggested that endogenously expressed TRPC3 was involved in mediating SOCE, the efficiency of protein reduction was not sufficient to determine its quantitative contribution toward SOCE (9). To obtain a more efficient suppression of TRPC3 protein levels and to evaluate thoroughly the quantitative contribution of each endogenously expressed TRPC homolog to SOCE, we turned to the methods of siRNA. Constructs were designed to express siRNA targeting a specific site on each endogenously expressed TRPC homolog. A minimum of two

### Table I

| mRNA Accession number | Orientation | Hairpin oligonucleotides sequence (5'-3') (target sequence in boldface type) | Target location |
|-----------------------|------------|-----------------------------------------------------------------|-----------------|
| TRPC1 U31110          | Forward    | GATCCCGGTTTCCCAAATTTTATTTTATTGTTTCAGGGAGAACAACTGTTATTAGCTTCTTTTAA | 906–924         |
| TRPC3 U47050          | Forward    | GATCCCGGTTTCCCAAATTTTATTTTATTGTTTCAGGGAGAACAACTGTTATTAGCTTCTTTTAA | 1505–1523       |
| TRPC4 NM_016179       | Reverse    | GATCCCGTACCCTTTGATTTTGTGTTTCAGGGAGAACAACTGTTATTAGCTTCTTTTAA    | 2005–2023       |
| TRPC6 AJ006276        | Reverse    | GATCCCGGTTTCCCAAATTTTATTTTATTGTTTCAGGGAGAACAACTGTTATTAGCTTCTTTTAA | 670–678         |
| TRPC7 NM_020389       | Reverse    | GATCCCGGTTTCCCAAATTTTATTTTATTGTTTCAGGGAGAACAACTGTTATTAGCTTCTTTTAA | 102–120         |
constructs were designed for each TRPC homolog, so that multiple target sites for each TRPC homolog could be evaluated. Initial experiments determined the most effective construct for suppressing each TRPC homolog, and then that construct was used to establish stable cell lines. This work produced five cell lines, each with a different TRPC homolog suppressed. As in the past, when establishing stable cell lines, all surviving clones were harvested and combined to create a cell population stably expressing the siRNA, in order to avoid the problems associated with selecting individual cell clones from a cell population that shows dramatic clonal variation in SOCE (33).

Real time RT-PCR analysis was utilized to assess both the efficiency of suppression of mRNA levels for the targeted TRPC homolog and to evaluate whether the siRNA construct had any cross-reactivity in terms of suppressing nontargeted TRPC homologs. The data in Fig. 1 show that the efficiency of suppression was high, with the percentage reduction of mRNA levels ranging between 84 and 96%. The specificity was also excellent in that there was no suppression of mRNA for nontargeted TRPC homologs.

Western blots were run to evaluate the degree of suppression of individual TRPC proteins (Fig. 2), and these data show that the reduction of individual TRPC protein levels ranged from 78 to 91%.

Role of Individual TRPC Proteins in Mediating Native SOCE—The role of endogenous TRPC homologs in mediating native SOCE was assessed by monitoring thapsigargin-stimulated Ba^{2+} entry in control HEK-293 cells versus cells expressing siRNA targeting individual TRPC homologs. Ba^{2+} entry was chosen to monitor SOCE, since Ba^{2+} is not pumped by Ca^{2+}-ATPases (34, 35), thereby allowing effects on Ca^{2+} entry pathways to be distinguished from effects on Ca^{2+} pump pathways. The data in Fig. 3 illustrate the effects of suppressing individual TRPC homologs on SOCE. The suppression of TRPC1 protein levels produced a 68% inhibition of SOCE, whereas the suppression of TRPC3 or TRPC7 protein levels produced slightly less, but significant, reductions of SOCE (52 and 58% inhibition, respectively). In contrast, the suppression of TRPC4 or TRPC6 protein levels caused no measurable effect on SOCE in HEK-293 cells. These data suggest that multiple TRPC homologs, but not all TRPC homologs, are involved in mediating SOCE.

Effect of Suppressing Multiple TRPC Homologs on Native SOCE—The combined percentage of inhibition for individually suppressing TRPC1, TRPC3, and TRPC7 totaled ~178%, suggesting that these three TRPC homologs cannot form three independent SOCE pathways. In order to test this prediction, it was necessary to determine whether co-expression of siRNA for two of the involved TRPC homologs or for all three produces additive inhibition of SOCE. Thus, new siRNA constructs targeting these three TRPC homologs were designed to have a different selectable marker (hygromycin or puromycin versus neomycin) than the siRNA construct already stably expressed in the cells. The data in Fig. 4 show the effect of expressing multiple siRNA constructs on the protein levels for TRPC1, TRPC3, and TRPC7. The suppression of TRPC proteins levels are dramatic, ranging between 78 and 91%. The cells expressing multiple siRNA allowed us to assess whether the inhibitory effects of siTRPC1, siTRPC3, and siTRPC7 on SOCE are additive.
The data in Fig. 5 show that the combined suppression of TRPC1 and TRPC3 had only slightly more effect on SOCE than the suppression of TRPC1 alone (74% inhibition versus 68% inhibition). Similar results were obtained with the combined suppression of TRPC1 and TRPC7 (78% inhibition) or the combined suppression of TRPC3 and TRPC7 (76% inhibition). The combined suppression of TRPC1, TRPC3, and TRPC7 also gave only slightly more inhibition (74% inhibition) than was observed with the suppression of TRPC1 alone (68% inhibition). Thus, these data indicate that there cannot be three independent SOCs formed from homomeric assembly of TRPC1, TRPC3, and TRPC7. Instead, the data indicate that all three TRPC proteins (TRPC1, TRPC3, and TRPC7) are required to form one channel subtype that mediates over 75% of the native SOCE in HEK-293 cells.

The hypothesis that TRPC1, TRPC3, and TRPC7 combine to form a single pathway that mediates a major fraction of SOCE is also supported by the observed effects of TRPC protein suppression on Ba\(^{2+}\)/H11001 leak influx. The data in Fig. 6A show that the suppression of TRPC1 has no significant effect on Ba\(^{2+}\) leak influx. However, suppression of either TRPC3 or TRPC7 leads to significantly higher Ba\(^{2+}\) leak flux than that observed in control HEK-293 cells. The leak flux is also low if both TRPC3 and TRPC7 are suppressed. These data suggest that with the suppression of two of the three proposed subunits, either no channel is formed, or a channel is formed that has low basal activity and little response to store depletion.

To determine whether TRPC1, TRPC3, and TRPC7 associate to form a channel complex in HEK-293 cells, cells were lysed in a modified radioimmune precipitation buffer, and immunoprecipitation of TRPC1 was performed. Proteins from the resulting pellet were run on SDS-PAGE and transferred to Immobilon membranes for Western blotting with TRPC1 antibody. The membranes were sequentially stripped and reprobed with antibodies to TRPC3 and then with antibodies to TRPC7. As seen in Fig. 7, TRPC3 and TRPC7 were co-immunoprecipitated together with TRPC1. In some experiments, a reblot of these membranes with anti-TRPC4 antibody showed that TRPC4 did not come down with the TRPC1-TRPC3-TRPC7 complex (data not shown).

However, if one suppresses TRPC1 together with TRPC3, the Ba\(^{2+}\) leak flux is decreased back down to the level observed in control HEK-293 cells (Fig. 6A). Likewise, the combined suppression of TRPC1 and TRPC7 reduces the Ba\(^{2+}\) leak flux back down to levels seen in control HEK-293 cells. The leak flux is also low if both TRPC3 and TRPC7 are suppressed. These data suggest that with the suppression of two of the three proposed subunits, either no channel is formed, or a channel is formed that has low basal activity and little response to store depletion.

**Co-immunoprecipitation of Endogenous TRPC1, TRPC3, and TRPC7 from HEK-293 Cells**—To determine whether TRPC1, TRPC3, and TRPC7 associate to form a channel complex in HEK-293 cells, cells were lysed in a modified radioimmune precipitation buffer, and immunoprecipitation of TRPC1 was performed. Proteins from the resulting pellet were run on SDS-PAGE and transferred to Immobilon membranes for Western blotting with TRPC1 antibody. The membranes were sequentially stripped and reprobed with antibodies to TRPC3 and then with antibodies to TRPC7. As seen in Fig. 7, TRPC3 and TRPC7 were co-immunoprecipitated together with TRPC1. In some experiments, a reblot of these membranes with anti-TRPC4 antibody showed that TRPC4 did not come down with the TRPC1-TRPC3-TRPC7 complex (data not shown).

**Role of TRPC Homologs in Forming Native OAG-stimulated Channels**—It has been proposed that one subtype of ROCs can be regulated by diacylglycerol, in a protein kinase C-independent manner (37). The data in Fig. 8 show that for HEK-293 cells grown in our laboratory, there is a dramatic effect of OAG on Ba\(^{2+}\) entry. An effect can be observed at a dose of 10 \(\mu\)M, and substantially more Ba\(^{2+}\) influx is seen in...
the presence of 100 μM OAG, a common dose used in TRPC overexpression studies.

Although OAG-stimulated channels are thought to be formed by TRPC3/TRPC6/TRPC7 subfamily members, an important question that has not been addressed is whether multiple endogenously expressed subfamily members can combine to form native OAG-stimulated channels. Data in figure 9 illustrate that whereas the suppression of TRPC3 protein levels has a dramatic effect on the OAG-sensitive Ba^{2+} influx (75% inhibition), the suppression of TRPC1 has no measurable effect. However, it was found that suppressing either TRPC6 or TRPC7 produces a dramatic effect (60% inhibition) on OAG-stimulated Ba^{2+} influx. Suppression of TRPC4 protein levels also has a substantial impact (75% inhibition) on the OAG-stimulated Ba^{2+} influx in HEK-293 cells, suggesting that TRPC homologs outside of the TRPC3/TRPC6/TRPC7 subfamily also can participate in forming native OAG-stimulated channels.

Are the Store-operated and OAG-stimulated Channels Truly Independent Channels?—Previous studies have supported the contention that store-operated and OAG-stimulated channels represent two independent calcium entry pathways, based partially on the observation that SOCs, but not OAG-stimulated channels, can be inhibited by 2-APB (38, 39). The data in figure 10A show the effect of various doses of 2-APB on SOCE in our HEK-293 cells, with full inhibition of SOCE occurring at a dose of 100 μM. The data in Fig. 10B show that the OAG-stimulated Ba^{2+} influx is not affected by 100 μM 2-APB.

The next set of experiments investigated whether OAG could stimulate additional Ba^{2+} entry after SOCs have been fully activated. Following activation of SOCE with thapsigargin, the addition of OAG produces a dramatic increase in Ba^{2+} influx (Fig. 11A). Also, after store depletion, if the SOCE is inhibited by the addition of 100 μM 2-APB, the subsequent addition of OAG still results in a dramatic increase in Ba^{2+} influx (Fig. 11B). Furthermore, when Ca^{2+} stores are depleted in siTRPC1 cells, the addition of Ba^{2+} produces only low levels of SOCE, yet the subsequent addition of OAG produces a dramatic stimulation of Ba^{2+} entry. When taken together, these inhibitor data and siRNA data argue that SOCE and OAG-stimulated Ba^{2+} entry occur via two independent pathways in HEK-293 cells.

Role of TRPC Homologs in Forming Native CCh-stimulated Channels—Given the role of various TRPC homologs in mediating store-operated and OAG-stimulated Ba^{2+} entry, it was important to determine the role of these TRPC homologs under more physiological conditions, where a receptor agonist is
likely to activate both SOCs and ROCs. Thus, the effect of suppressing individual TRPC homologs on CCh-stimulated Ba\(^{2+}\) influx was investigated. The data in Fig. 12 show that CCh-stimulated Ba\(^{2+}\) entry is inhibited by the suppression of any of the five endogenously expressed TRPC homologs.

Although suppression of TRPC1 protein levels has no effect on OAG-stimulated Ba\(^{2+}\) entry, it does have a significant inhibitory effect on CCh-stimulated Ba\(^{2+}\) entry. It is likely that this effect is via inhibition of SOCE that is activated in response to Ca\(^{2+}\) store-depletion following the CCh addition. To estimate the fraction of CCh-stimulated Ba\(^{2+}\) entry that occurs via SOCs, the effect of 2-APB on CCh-stimulated Ba\(^{2+}\) entry was determined. The experiments were similar to those in Fig. 10 that 2-APB was added during the linear phase of the Ba\(^{2+}\) influx. With the acute addition of 100 \(\mu\)M 2-APB, 31 \(\pm\) 4.6\% (n = 9) of the CCh-stimulated Ba\(^{2+}\) entry was blocked (data not shown). This is roughly equivalent to the amount of CCh-stimulated Ba\(^{2+}\) entry inhibited by suppressing TRPC1 protein levels.

Suppression of TRPC6 also inhibited ~30\% of the CCh-stimulated Ba\(^{2+}\) entry, whereas suppression of TRPC3, TRPC4, or TRPC7 protein levels inhibited ~60\% of the CCh-stimulated Ba\(^{2+}\) entry.

Although most cell lines express multiple endogenous TRPC homologs, to our knowledge, there has been no comprehensive study where every endogenously expressed TRPC protein was systematically reduced, by antisense or siRNA methods, to evaluate the quantitative contribution of individual TRPC homologs to native calcium entry pathways. Furthermore, there has been no investigation of the effects of simultaneously suppressing multiple TRPC homologs to determine whether two or more endogenous TRPC proteins work in tandem to form native channels. Several years ago, our laboratory began an extensive series of experiments to pursue such a comprehensive investigation in HEK-293 cells.

Whereas there are reported differences in the TRPC expression profiles for HEK-293 cells grown in different laboratories (9, 40, 41), the HEK-293 cells grown in our laboratory express five TRPC homologs (TRPC1, TRPC3, TRPC4, TRPC6, and TRPC7). siRNA techniques were utilized to produce a series of five cell lines, each with a single TRPC protein stably suppressed. These cell lines represent a valuable resource for investigating physiological events regulated downstream of particular calcium entry pathways, thereby eliminating the need to use pharmacological agents for such studies. More immediately, these cell lines allowed a quantitative assessment of the role of individual TRPC homologs in mediating SOCE. The results from the siRNA studies indicate that TRPC1, TRPC3, and TRPC7 all play a significant role in mediating SOCE in HEK-293 cells, since suppression of each individual TRPC homolog inhibits greater than 50\% of the SOCE. Our results, showing TRPC1 involvement in mediating SOCE, are consistent with a number of overexpression studies and with several recent antisense studies (see Introduction) but also significantly extend those observations by showing that endogenously expressed TRPC1 works in a heteromeric complex to mediate native SOCE.

The observation that TRPC3 is involved in mediating SOCE (Fig. 3) differs from the results of most overexpression studies, which suggest that TRPC3 forms ROCs (see Introduction). The most notable exception is the report indicating that TRPC3 proteins form SOCs when overexpressed at low levels but form ROCs when overexpressed at higher levels (24). The data from the siTRPC3 cells support our earlier antisense data, suggesting that TRPC3 proteins are involved in forming SOCs in HEK-293 cells (9), but also represent an important extension of that work because the suppression of 89\% of TRPC3 protein now allows a quantitative assessment of the contribution of TRPC3 to this pathway. The observation that TRPC7 is involved in mediating SOCE also differs from the general hypothesis that members of the TRPC3/TRPC6/TRPC7 subfamily form ROCs and not SOCs. However, this observation is consistent with one early overexpression study suggesting that overexpressed TRPC7 could be store-operated (28) and with a more recent study reporting that overexpressed TRPC7 could form both SOCs and ROCs in HEK-293 cells (29). To our knowledge, the data in Fig. 3 represent the first indication that endogenous TRPC7 is involved in forming native SOCs.

Whereas our previous antisense studies provided an important indication that TRPC3 can participate in mediating SOCE (9), they did not address the central question of whether this protein works alone or in tandem with other proteins to form native SOCs. The observation that the combined suppression of TRPC1, TRPC3, and TRPC7 proteins produced only slightly more inhibition of SOCE than suppression of TRPC1 protein alone (74 \textit{versus} 68\%) suggests that TRPC1, TRPC3, and TRPC7 assemble in a heteromeric complex to form a single complex...
channel type that mediates \( \sim 75\% \) of the native SOCE in HEK-293 cells. This raises the question of which channel mediates the fraction of SOCE not affected by suppression of TRPC proteins. Since recent whole cell patch clamp measurements of store-operated currents in HEK-293 cells revealed the presence of both \( I_{\text{CRAC}} \)-like and \( I_{\text{SOC}} \)-like currents (49), it is possible that the residual fraction of SOCE, after suppression of TRPC homologs, represents \( \text{Ca}^{2+} \) entry via one particular subtype of SOC. Given the lack of evidence that TRPC proteins can form highly \( \text{Ca}^{2+} \)-selective channels, it seems most likely that the residual SOCE after TRPC protein suppression is mediated by an \( I_{\text{CRAC}} \)-like channel.

The contention that SOCE in HEK-293 cells is mediated by a heteromeric TRPC channel is further supported by the effect of TRPC siRNA on \( \text{Ba}^{2+} \) leak flux. Suppression of either TRPC3 or TRPC7 results in a high \( \text{Ba}^{2+} \) leak flux (38% of the maximal activity) that can be inhibited by 2-APB or SKF96365, suggesting that this \( \text{Ba}^{2+} \) influx is mediated by leaky SOCs. The suppression of TRPC1 in cells in which which channel mediates the fraction of SOCE not affected by suppression of TRPC proteins. Since recent whole cell patch clamp measurements of store-operated currents in HEK-293 cells revealed the presence of both \( I_{\text{CRAC}} \)-like and \( I_{\text{SOC}} \)-like currents (49), it is possible that the residual fraction of SOCE, after suppression of TRPC homologs, represents \( \text{Ca}^{2+} \) entry via one particular subtype of SOC. Given the lack of evidence that TRPC proteins can form highly \( \text{Ca}^{2+} \)-selective channels, it seems most likely that the residual SOCE after TRPC protein suppression is mediated by an \( I_{\text{CRAC}} \)-like channel.

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channel type that mediates \( \sim 75\% \) (the higher value is after a rough normalization for incomplete protein suppression) of the native SOCE in HEK-293 cells. This raises the question of which channel mediates the fraction of SOCE not affected by suppression of TRPC proteins. Since recent whole cell patch clamp measurements of store-operated currents in HEK-293 cells revealed the presence of both \( I_{\text{CRAC}} \)-like and \( I_{\text{SOC}} \)-like currents (49), it is possible that the residual fraction of SOCE, after suppression of TRPC homologs, represents \( \text{Ca}^{2+} \) entry via one particular subtype of SOC. Given the lack of evidence that TRPC proteins can form highly \( \text{Ca}^{2+} \)-selective channels, it seems most likely that the residual SOCE after TRPC protein suppression is mediated by an \( I_{\text{CRAC}} \)-like channel.

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TRPC3 (or TRPC7) is already suppressed dramatically reduces the Ba$^{2+}$ leak flux, whereas the suppression of TRPC1 alone has no effect on the Ba$^{2+}$ leak flux (Fig. 6), suggesting that the formation of channels with just TRPC1 and TRPC7 (or just TRPC1 and TRPC3) results in leaky SOCs, a finding that is similar to the observation that suppressing expression of the adaptor protein Homer in submandibular HSG cells results in leaky SOCs (36). These data imply that the right combination of TRPC proteins, and probably the right combination of accessory proteins, must be assembled to form channels that are not constitutively active but are responsive to store depletion. This could help explain why overexpression studies with either TRPC3 or TRPC7 produce constitutively active, rather than store-operated channels (see Introduction). It should be mentioned at this point that our recent study in H19-7 cells also concluded that TRPC1 and TRPC3 combine to mediate SOCE in cultured hippocampal neurons (42). Whereas the data in H19-7 cells indicate that TRPC7 does not play a role in mediating SOCE in H19-7 cells, the potential roles of TRPC5 and TRPC6 remain to be tested in that cell system. Perhaps TRPC6 could help explain why overexpression studies with either TRPC4 or TRPC5 (45). On the other hand, there are two studies that indicate that TRPC1 and TRPC3 can interact directly (46, 47). In the former study, jointly overexpressed TRPC1 and TRPC3 could be immunoprecipitated together. In the latter study, it was observed that the joint overexpression of TRPC1 and TRPC3 in HEK-293 cells produced channels that, in the absence of extracellular Ca$^{2+}$, are constitutively active and can be further stimulated with diacylglycerol. The addition of external Ca$^{2+}$ reduces the constitutive activity to some degree. These channels are slightly sensitive to store depletion, but the store-operated currents are small in comparison with those elicited by OAG (47). The constitutive activity of the TRPC1-TRPC3 channels and their low levels of store-operated currents would be consistent with the high Ba$^{2+}$ leak and low SOCE observed when TRPC7 is suppressed and thereby prevented from participating in the TRPC1-TRPC3-TRPC7 complex proposed for native SOCs. The low level of store-operated channel activity when TRPC1 and TRPC3 are overexpressed together could result from either a lack of TRPC7 expression or a lack of Homer expression. As far as we know, the combination of TRPC1, TRPC3, and TRPC7 has not been expressed, and the interactions have not been investigated; however, future studies are planned to do just that.

Another important finding is that endogenous TRPC3 can simultaneously participate in forming native SOCs and native OAG-stimulated channels. These are considered to be two independent channel types based on their differential sensitivity to 2-APB (38, 39) (Fig. 10), the additive effects of store depletion and OAG addition (Fig. 11), and the different biophysical characteristics of the two channels (48, 49). This is further supported by our finding that TRPC1 mediates SOCE but not OAG-stimulated Ba$^{2+}$ entry, and TRPC4 mediates OAG-stimulated Ba$^{2+}$ entry but not SOCE in HEK-293 cells.

The finding that OAG stimulates a native Ca$^{2+}$ entry pathway differs from some studies in HEK-293 grown in other laboratories (38, 50), an interlaboratory difference that probably results from the high levels of TRPC3 expression as well as the presence of TRPC7 in our HEK-293 cells. The HEK-293 cells grown in our laboratory clearly have a substantial, native OAG-stimulated Ba$^{2+}$ entry pathway and therefore serve as an important model cell for investigating the potential role of endogenous TRPC homologs in forming native OAG-stimulated channels. The initial point to emphasize from the siRNA studies on OAG-stimulated channels (Fig. 9) is that TRPC1 plays no role in mediating OAG-stimulated Ba$^{2+}$ entry, whereas TRPC3 plays a substantial role. Thus, although TRPC1 and TRPC3 appear to combine to mediate SOCE in HEK-293 cells, TRPC3 plays a role in OAG-stimulated Ba$^{2+}$ entry that is independent of TRPC1.

In addition to TRPC3 involvement, TRPC6 and TRPC7 also play a role in mediating OAG-stimulated Ba$^{2+}$ entry (Fig. 9).
in mediating SOCE in HEK-293 cells. It will probably be some time before we understand this degree of subtle variation in the physiological roles of TRPC3/TRPC6/TRPC7 subfamily members.

The data from the siTRPC4 cells also argue for TRPC4 involvement in mediating OAG-stimulated Ba\(^{2+}\) entry (Fig. 9), a finding consistent with our previous antisense studies (30). However, the TRPC4 siRNA data represent an important extension of our earlier studies, in that the reduction of TRPC4 levels by antisense produced only a 42% inhibition of OAG-stimulated cation entry. The more efficient suppression of TRPC4 by siRNA methods (a 88% reduction in protein levels) now demonstrates that TRPC4 is involved in mediating ~75% of the OAG-stimulated Ba\(^{2+}\) entry in HEK-293 cells (Fig. 9), a contribution as significant as that of any individual member of the TRPC3/TRPC6/TRPC7 subfamily. Furthermore, TRPC4, like TRPC3, may participate in forming multiple channel types in HEK-293 cells, since our previous antisense studies suggested that TRPC4 is also involved in forming the ROC important for Ca\(^{2+}\) oscillations in response to low doses of carbachol (30), suggested to be the arachidonic acid-stimulated channel (51). Clearly, the data in Fig. 3 indicate that TRPC4 is not involved in mediating SOCE. Although this finding is in contrast to studies for mouse and bovine TRPC4, which argue strongly that TRPC4 does mediate SOCE in some cell systems (52), it is consistent with our recent report on the role of TRPC homologs in the rat-derived H19-7 hippocampal cells. In that study, it was observed that a dramatic reduction of TRPC4 by siRNA techniques does not alter the level of SOCE in undifferentiated H19-7 cells. Furthermore, SOCE is up-regulated 3.5-fold during differentiation of H19-7 cells at the same time that TRPC4 protein levels are dramatically down-regulated (42).

Given the effects of suppressing various TRPC homologs on store-operated and OAG-stimulated Ba\(^{2+}\) entry, it was important to determine the role of these TRPC homologs in mediating a more physiological response, such as CCh-stimulated entry.
Ba\textsuperscript{2+} entry. The results shown in Fig. 12 are slightly more difficult to interpret than those in Figs. 3 and 9, due to the CCh stimulation of both SOCs and ROCs. Agonist binding to G protein-coupled receptors leads to the production of both inositol 1,4,5-trisphosphate and diacylglycerol, as well as to the production of other signaling molecules known to activate ROCs, such as arachidonic acid. The fact that suppression of TRPC1 has no effect on OAG-stimulated Ba\textsuperscript{2+} entry and that the effect of suppressing TRPC1 on CCh-stimulated Ba\textsuperscript{2+} entry is roughly the same as the effect of adding 100 \mu M 2-APB suggests that \textasciitilde30% of the CCh-stimulated Ba\textsuperscript{2+} entry is via SOCs.

Suppression of TRPC6 levels also affects only one of the two pathways studied, namely the OAG-stimulated pathway. Thus, one could use its effect to say that the OAG-stimulated pathway mediates \textasciitilde30% of the CCh-stimulated Ba\textsuperscript{2+} entry. Suppression of TRPC3 or TRPC7 levels affected both the store-operated and the OAG-stimulated pathways and therefore can produce more inhibition (\textasciitilde60%) of CCh-stimulated Ba\textsuperscript{2+} entry than suppression of TRPC1 levels, which presumably affects only the store-operated pathway. Suppression of TRPC3 or TRPC7 would also be predicted to produce more inhibition (\textasciitilde60%) than suppression of TRPC1 levels, which presumably affects only the OAG-stimulated pathway. Thus, one is left to explain only why suppressing TRPC4 levels gives more inhibition of CCh-stimulated Ba\textsuperscript{2+} entry than the 30% inhibition seen by suppressing TRPC6 levels. As mentioned above, our antisense results suggested that TRPC4 may be involved in forming the arachidonic acid-stimulated pathway as well as the OAG-stimulated pathway, and thus the larger inhibition may result from the combined inhibition of these two pathways.

In summary, a comprehensive siRNA approach to investigating all five TRPC homologs endogenously expressed in HEK-293 cells led to important new information on the physiological roles of TRPC proteins. First, the data demonstrate for the first time that endogenous TRPC7 can mediate native SOCE. Second, TRPC7 joins with TRPC1 and TRPC3 to form a TRPC1-TRPC3-TRPC7 complex that mediates native SOCE. Third, all endogenously expressed TRPC3/TRPC6/TRPC7 subfamily members participate in forming native OAG-stimulated channels, although there is one important distinction between subfamily members, in that TRPC3 and TRPC7 also are involved in mediating SOCE, whereas TRPC6 is not. Finally, endogenous TRPC3 and TRPC7 are both involved in forming two independent types of native calcium channels, one store-operated and the other OAG-stimulated. This is the first demonstration that an individual endogenous TRPC protein can simultaneously form native store-operated and native OAG-stimulated channels in a single cell line, with the type of channel formed being dictated by its interaction with other endogenous TRPC proteins. Thus, the finding that TRPC3 and TRPC7 can participate with TRPC1 to form native SOCs and with TRPC4 and TRPC6 to form native OAG-stimulated channels supports the theory that the physiological role of a particular TRPC protein may be dictated by the expression profile of other TRPC proteins in that particular cell type.

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Note Added in Proof—A recent paper demonstrated that antisense to TRPC1 and antisense to TRPC3 attenuate thapsigargin- and OAG-stimulated Ca\textsuperscript{2+} entry in HSY cells and that TRPC1 and TRPC3 co-immunoprecipitate (53).
TRPC1-TRPC3-TRPC7 Complex Forms Store-operated Channels

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