Role of Endogenous TRPC6 Channels in Ca$^{2+}$ Signal Generation in A7r5 Smooth Muscle Cells*

The ubiquitously expressed canonical transient receptor potential (TRPC) ion channels are considered important in Ca$^{2+}$ signal generation, but their mechanisms of activation and roles remain elusive. Whereas most studies have examined overexpressed TRPC channels, we used molecular, biochemical, and electrophysiological approaches to assess the expression and function of endogenous TRPC channels in A7r5 smooth muscle cells. Real time PCR and Western analyses reveal TRPC6 as the only member of the diacylglycerol-responsive TRPC3/6/7 subfamily of channels expressed at significant levels in A7r5 cells. TRPC1, TRPC4, and TRPC5 were also abundant. An outwardly rectifying, nonselective cation current significantly levels in A7r5 cells. TRPC1, TRPC4, and TRPC5 were also abundant. An outwardly rectifying, nonselective cation current was activated by phospholipase C-coupled vasopressin receptor activation or by the diacylglycerol analogue, oleoyl-2-acetyl-sn-glycerol (OAG). Introduction of TRPC6 small interfering RNA sequences into A7r5 cells by electroporation led to 90% reduction of TRPC6 transcript and 80% reduction of TRPC6 protein without any detectable compensatory changes in the expression of other TRPC channels. The OAG-activated nonselective cation current was similarly reduced by TRPC6 RNA interference. Intracellular Ca$^{2+}$ measurements using fura-2 revealed that thapsigargin-induced Ca$^{2+}$ entry was unaffected by TRPC6 knockdown, whereas vasopressin-induced Ca$^{2+}$ entry was suppressed by more than 50%. In contrast, OAG-induced Ca$^{2+}$ transients were unaffected by TRPC6 knockdown. Nevertheless, OAG-induced Ca$^{2+}$ entry bore the hallmarks of TRPC6 function; it was inhibited by protein kinase C and blocked by the Src kinase inhibitor, 4-amino-5-(4-chlorophenyl)-7-((butyl)pyrazolo[3,4-d]pyrimidine (PP2). Importantly, OAG-induced Ca$^{2+}$ entry was blocked by the potent L-type Ca$^{2+}$ channel inhibitor, nimodipine. Thus, TRPC6 activation probably results primarily in Na$^{+}$ ion entry and depolarization, leading to activation of L-type channels as the mediators of Ca$^{2+}$ entry. Calculations reveal that even 90% reduction of TRPC6 channels would allow depolarization sufficient to activate L-type channels. This tight coupling between TRPC6 and L-type channels is probably important in mediating smooth muscle cell membrane potential and muscle contraction.

Receptor-induced Ca$^{2+}$ signals are crucial to the function of all cells (1) and involve both release of Ca$^{2+}$ from stores and entry of Ca$^{2+}$ through plasma membrane channels (1–3). Although identification of the latter has proven elusive, members of the canonical transient receptor potential (TRPC) channel family have been leading contenders (2–5). The TRPC channels all appear to be activated in response to phospholipase C (PLC)-coupled receptors (2–8). Within the TRPC family, there are two structurally divided subgroups: TRPC3, TRPC6, and TRPC7 channels (TRPC3/6/7) and TRPC1, TRPC4, and TRPC5 (TRPC1/4/5). One functional characteristic distinguishing these two subgroups is the ability of diacylglycerol (DAG) to activate TRPC3/6/7 channels but not TRPC1/4/5 channels (2, 6–11). DAG also activates TRPC2 channels; however, this channel is not expressed in higher mammals and is restricted mostly to the vomeronasal organ (12). As a product of receptor-induced PLC activation, DAG is an obvious mediator of TRPC channel activation. However, its role in the activation of endogenously expressed TRPC3/6/7 channels is uncertain (7, 8, 13–15). The majority of studies revealing the action of DAG on TRPC channels have been undertaken using overexpression systems (2, 7, 8, 14, 15). Such expression may differ from endogenous TRPC expression. For example, considering that TRPC channels probably function as tetramers (4, 5), overexpression may result in a predominance of homotetrameric structures, whereas endogenous expression may reflect heteromers between TRPC channel subtypes, resulting in quite different properties (2, 4, 5). In addition, since TRPC channels may function within organized signaling domains (2, 16), endogenously expressed TRPC channels may be assembled along with adaptor and/or regulatory proteins, the association of which may be quite different when overexpressed (2, 11, 16–18). Hence, there is a need to examine the activation and function of endogenously expressed TRPC channels.

Whereas TRPC channels are ubiquitously expressed among cell types, there are surprisingly few reports describing the functional role of native TRPC channels. For example, TRPC3 channels are highly expressed in pontine neurons and mediate a nonselective cation current in response to the neurotrophin receptor, TrkB (19). TRPC2 channels appear to mediate the primary electrical response to pheromones in the vomeronasal organ of most mammals except higher primates (12). The TRPC6 channel is highly expressed in a number of different smooth muscle cell types (20–22), and there have been studies indicating that it plays a role in receptor-induced Ca$^{2+}$ signaling in smooth muscle (20–23). In primary portal vein myocytes, current closely corresponding to overexpressed TRPC6 channels was reduced by treatment with TRPC6 antisense oligonucleotides (20). Down-regulation of TRPC6 by antisense sequences in pulmonary vascular smooth muscle cells resulted in reduction of store-operated Ca$^{2+}$ entry (21). Using the clonal A7r5 aortic-derived smooth muscle cell line, Jung et al. (22) described a TRPC6-
like current activated by the permeant diacylglycerol analogue, oleoyl-2-acetyl-sn-glycerol (OAG). In this case, the current was enhanced by PLC-coupled receptor activation but was not modified by Ca\(^{2+}\) store depletion (22). In recent studies using TRPC6 knockout mice, a phenotype of increased arterial blood pressure, augmented arterial tone, and enhanced agonist- and DAG-induced current in smooth muscle was observed (6–8, 24). Whereas such knockout would be expected to prevent rather than augment the Ca\(^{2+}\)-mediated responses, there appeared to be an overcompensatory increase in expression of the closely related TRPC3 channel (6–8, 24).

The primary role of Ca\(^{2+}\) entry in controlling smooth muscle contraction and the possible role of TRPC channels in these responses prompted us to examine the presence and possible function of TRPC channels using A7r5 cells. A careful examination of the expression of TRPC channels at both transcript and protein levels reveals that, of the closely related TRPC3/6/7 subfamily members, only TRPC6 channels are detected. This provides a system in which the function of endogenously expressed TRPC6 channels can be examined in relative isolation from the other subfamily members, TRPC3 and TRPC7. Using an RNAi knockdown approach, we have identified TRPC6 channel function in A7r5 cells and reveal that the channel may be more important as a mediator of Na\(^{+}\) ion entry resulting in the activation of voltage-dependent Ca\(^{2+}\) entry channels.

**EXPERIMENTAL PROCEDURES**

**Cells and RNAi**—Rat aortic smooth muscle A7r5 cells, passage 10–25, were cultured in Dulbecco’s modified Eagle’s medium supplemented with fetal bovine serum (10%), penicillin, and streptomycin as described previously (17, 25). For TRPC6 knockdown studies, A7r5 cells were mixed with 200 nM TRPC6 siRNA (GCAGCAUCAUCAUG-CAAGAUUUA or GGAUUAUGCUUUGAUGUGUU) or a control sequence (GCAACUAACUUCGUUAGAAUCGUUA), each with its complementary sequence. We used Stealth (Invitrogen) siRNA sequences developed to eliminate nonspecific stress responses of the PKR/interferon pathway induced by siRNA. Cells together with siRNA sequences were electroporated using the Gene Pulser II electroporation system (Bio-Rad) at 350 V, 960 microfarads, and infinite resistance. After a 3-h recovery period in serum-free Opti-MEM, 10% fetal bovine serum was added, and cells were incubated for 48 h on coverslips before fura-2 imaging of electrophysiology measurements was undertaken.

**Real Time PCR**—Real-time quantitative PCR was performed in a Sequence Detector System (ABI Prism 7900 Sequence Detection System and software; PerkinElmer Life Sciences). Amplification was performed in a final volume of 25 µL, containing 30 ng of cDNA from the reverse transcribed reaction, primer mixture (0.3 µM each of sense and antisense primers), and 12.5 µL 2× SYBR Green Master Mix (Applied Biosystems). The oligonucleotide primers shown in TABLE ONE were designed using Primer Express 2.0 software (Applied Biosystems). The standard amplification program included 40 cycles of two steps, each comprising heating to 95 °C and heating to 60 °C. Fluorescent product was detected at the last step of each cycle. In order to verify the purity of the products, a melting curve was produced after each run. To determine the relative quantitation of gene expression, the comparative threshold cycle method (ΔΔCT) was used (26). To control for variation in RNA quantity and quality, we used 18 S ribosome RNA as an internal control to calculate a relative Ct for the target molecules (TRPC channels) of interest. Subtracting the Ct of the housekeeping gene from the Ct of the gene of interest yields the ΔCt. A serially diluted positive control sample (rat brain) was analyzed by linear regression to determine the sensitivity and linearity of the assay. The final mRNA levels were normalized according to their Ct values from the standard curves.

**Western Blot Procedures**—To detect TRPC expression, A7r5 cells were lysed in chilled Nonidet P-40 buffer (1% (v/v) Nonidet P-40, 150 mM NaCl, 50 mM Tris-HCl, pH 8.0, containing 100 µM phenylmethyl-sulfonyl fluoride and Sigma protease inhibitor mixture I) followed by an incubation period of 30 min at 4 °C. Following centrifugation (18,000 × g, 20 min at 4 °C), the protein content of the supernatants was quantified using Bio-Rad DC protein assay kits. For rat brain, tissue was homogenized in Nonidet P-40 buffer in exactly the same manner as above for A7r5 cells. For both A7r5 and rat brain, protein extracts (15 µg/lane of each) were resolved on 6% SDS-polyacrylamide gels (27) and electroblotted onto Bio-Rad nitrocellulose membranes (Bio-Rad) (28). After transfer, the nitrocellulose membranes were blocked (1 h, room temperature) in Tris-buffered saline-Tween 20 (TBST; 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Tween 20) containing membrane blocking agent (5%) (Amersham Biosciences) and subsequently incubated overnight at 4 °C with rabbit anti-TRPC1, -TRPC4, -TRPC5, or -TRPC6 antibodies, with rabbit anti-TRPC3 antibody, or with goat anti-TRPC7. Membranes were then washed two times (7 min) in TBST and incubated with secondary antibody (30 min; goat anti-rabbit or donkey anti-goat IgG conjugated to horseradish peroxidase diluted 1:2500 in TBST). Subsequently, membranes were washed three times (5 min) in TBST followed by a single wash (5 min) in Tris-buffered saline (TBS; 150 mM NaCl, 10 mM Tris-HCl, pH 8.0). Peroxidase activity was visualized using the ECL kit as according to the manufacturer’s instructions (Amersham Biosciences).

**Intracellular Ca\(^{2+}\) Measurements**—Cells grown on coverslips were placed in external solution (107 mM NaCl, 7.2 mM KCl, 1.2 mM MgCl\(_2\), 11.5 mM glucose, 20 mM Hepes-NaOH, pH 7.2; this solution is “cation-free,” free of sulfate and phosphate anions) and loaded with fura-2/acetoxymethyl ester (2 µM) for 30 min at 20 °C. Cells were washed, and dye was allowed to de-esterify for a minimum of 30 min at 20 °C. Approximately 95% of the dye was confined to the cytoplasm as determined by the signal remaining after saponin permeabilization (29). Cells on coverslips were placed in external solution in the absence or presence of 3 mM CaCl\(_2\), as described earlier (10, 30). Ca\(^{2+}\) measurements were made using an InCyt dual wavelength fluorescence imaging system (Intracellular Imaging Inc.). Fluorescence emission at 505 nm was monitored with excitation at 340 and 380 nm; intracellular Ca\(^{2+}\) measurements are shown as 340/380 nm ratios obtained from groups (6–12) of single cells. The details of these Ca\(^{2+}\) measurements were described previously (31). Resting Ca\(^{2+}\) levels in A7r5 cells were 100–200 nM (32). All measurements shown are representative of a minimum of three and in most cases a larger number of independent experiments.

**Whole Cell Patch Clamp Measurements**—For patch clamp experiments, A7r5 cells grown on glass coverslips were transferred to the recording chamber and kept in a standard external solution (145 mM NaCl, 2.8 mM KCl, 10 mM CsCl, 2 mM MgCl\(_2\), 10 mM glucose, 0.2 mM EGTA, 10 mM HEPES-NaOH, pH 7.40. Free Ca\(^{2+}\) was adjusted to 50 µM using WEBMAXC software). Patch clamp experiments were conducted in the tight seal whole cell configuration. High resolution current recordings were acquired with a computer-based patch clamp amplifier system (EPC-10, HEKA, Lambrecht, Germany). Patch pipettes had resistances between 2 and 4 MΩ after filling with the standard pipette solution (145 mM cesium glutamate, 8 mM NaCl, 2 mM ATP, 0.3 mM GTP, 10 mM EGTA, adjusted with CsOH to pH 7.2). Free Mg\(^{2+}\) was adjusted to 1 mM, and free Ca\(^{2+}\) was adjusted to 100 mM with MgCl\(_2\) and CaCl\(_2\) using WEBMAXC software. Immediately after establishment of the whole cell configuration, voltage ramps of 50-ms duration spanning
Endogenous TRPC6 Function

| Target | GenBank™ accession number | Orientation | Sequence (5′–3′) | Predicted size | Nucleotide location |
|--------|--------------------------|-------------|-----------------|----------------|-------------------|
| TRPC1  | AF061266                 | Forward     | CGACACCTTTCACCTGTCCA | 64             | 1716–1780         |
|        |                          | Reverse     | GCGCFAAGGGAGAAGATGTACCGA | 190           | 1804–1949         |
| TRPC2  | NM017011                 | Forward     | GTCACATTCTCTCTTGACCAT    | 74             | 531–605           |
|        |                          | Reverse     | CACGATCTCGATCTCTCTCT    | 74             | 531–605           |
| TRPC3  | AB022331                 | Forward     | CTTGCAGATATATCTGGAAGAAAT | 67             | 1514–1581         |
|        |                          | Reverse     | CACGAGGCACTATATAGAATAGAACCTTT | 102           | 2038–2140         |
| TRPC4  | NM_080396                | Forward     | AGTGTACAGAAGACGCGAAGAAC | 79             | 758–837           |
|        |                          | Reverse     | AGTGTACAGAAGACGCGAAGAAC | 79             | 758–837           |
| TRPC5  | AY064411                 | Forward     | CACGAGGCACTATATAGAATAGAACCTTT | 113           | 2665–2778         |
|        |                          | Reverse     | CACGAGGCACTATATAGAATAGAACCTTT | 113           | 2665–2778         |

the voltage range of −100 to +100 mV were delivered from a holding potential of 0 mV at a rate of 0.5 Hz. All voltages were corrected for a liquid junction potential of 10 mV between external and internal solutions. Voltages were filtered at 2.3 kHz and sampled at 20-μs intervals. Capacitive currents and series resistance were determined and corrected using the automatic capacitance and series resistance compensation of the EPC-10. For analysis, four ramps were averaged after current inactivation and used for leak subtraction. The low resolution temporal development of inward currents (at −80 mV) and outward current (80 mV) was leak-corrected with an individual ramp current record after current inactivation by measuring the current amplitude at −80 and +80 mV. Estimates of leak development in untreated cells measured over 300 s revealed a linear decrease in leak current of no more than 1 pA per 30 s. OAG and VP were added to the standard external solution as indicated.

Materials and Miscellaneous Procedures—Thapsigargin, PP2, GF-109203X, and OAG were from EMD Biosciences (San Diego, CA). Fura-2(acetoxy)methyl ester was from Molecular Probes, Inc. (Eugene, OR). Vasopressin was from Sigma. Rabbit anti-TRPC1, -TRPC4, -TRPC5, or -TRPC6 antibodies were from Alomone Labs (Israel), and rabbit anti-TRPC7 was a generous gift from Dr. Randen Patterson (Penn State University). Goat anti-TRPC1 was from Santa Cruz. Goat anti-rabbit and donkey anti-goat IgG conjugated to horseradish peroxidase were from Jackson Laboratories. The A7r5 cell line was from ATCC (Manassas, VA).

RESULTS AND DISCUSSION

TRPC Channel Profile—TRPC channels have been postulated to serve widely among cell types as mediators of Ca2+ entry in response to PLC-coupled receptors (2, 4). Receptor-mediated Ca2+ entry signals play a prominent role in smooth muscle, and we sought to assess the role of TRPC channels using the well studied A7r5 smooth muscle cell line (17, 32). Our focus has been on the widely expressed TRPC3/6/7 group of channels known to be activated by the PLC product, DAG (2, 9, 29, 30, 33). Crucial to assessment of the function to these channels was an accurate quantitative analysis of the expression pattern for TRPC channels.

levels of expression of TRPC message using real time PCR (Fig. 1A). Our analyses reveal that TRPC1 and TRPC6 are the predominant TRPC channel mRNAs observed in A7r5 cells. However, in contrast to the earlier Northern analysis (22), we detected significant levels of TRPC4 and TRPC7 message. TRPC3 and TRPC5 mRNA levels were barely detectable, whereas no TRPC2 mRNA was detected. The latter is surprising since TRPC2 is only reported expressed in vomeronasal organ (14), erythroid precursor cells (34), and sperm (35). Western analysis of TRPC protein levels in A7r5 cells is shown in Fig. 1B. The reliability of TRPC antibodies has been inconsistent among TRPC subtypes, and we confirmed their effectiveness by their identification of TRPC channels in rat brain extracts in which high levels of most TRPC channels are present (Fig. 1B). In A7r5 cells, TRPC1 and TRPC6 were expressed at levels equivalent to those observed in rat brain, consistent with the real time PCR analysis. Certainly, both proteins were prominent in A7r5 cells. TRPC4 and TRPC5 protein levels were also quite prominent, a result not predicted from their relative mRNA levels. Whereas the size
of TRPC5 appeared distinct from the doublet recognized in brain, a large decrease in the intensity of this band occurred after TRPC5 RNAi, confirming its authenticity (not shown). Significantly, no TRPC3 or TRPC7 protein could be detected despite the presence of prominent bands for each channel in rat brain, indicating the effectiveness of the antibodies used. This does not mean that the TRPC3 and TRPC7 proteins are absent, but their expression level is beneath our minimum detection limit. Although the relative levels of different TRPC channels are difficult to ascertain from Western analyses, it is clear that significant disparities exist between levels of protein and mRNA expression. Such differences are probably explained by the relative stability and turnover of both message and protein. Despite the quantitative uncertainty of Western analysis, assessment of TRPC protein levels rather than mRNA levels is obviously a more reliable indicator of the presence and relative levels of endogenous TRPC channels. In a paper published after completion of this work, Monier et al. (36) revealed significant variations in the expression of TRPC channels in different strains of A7r5 cells. In certain strains, significant levels of TRPC3 message could be detected, although no information on protein levels was given. Considering that A7r5 cells are derived from rat aortic smooth muscle, which does not express TRPC3 (8), it is possible that certain strains have undergone alteration during lengthy passaging.

Vasopressin and OAG-induced Currents in A7r5 Cells—From these data, TRPC6 is the only detectable member of the TRPC3/6/7 subgroup of TRPC channels expressed in A7r5 cells. Hence, A7r5 cells offer a useful system to study the DAG responsiveness of endogenous TRPC6 channels. Recent studies have revealed that the permeant DAG analogue, OAG, can stimulate an inward current in smooth muscle cells (20, 23). Whereas it is established that exogenously expressed TRPC6 channels are responsive to OAG (9, 10), it remains unclear whether endogenously expressed TRPC6 channels respond similarly (13, 14). We compared PLC-coupled VP receptor-mediated and OAG-induced channel activities in A7r5 cells using the whole cell patch clamp configuration, replacing K\(^+\) ions and the reversal potential of -80 mV (Fig. 2A). The time course for VP-induced current at -80 mV (Fig. 2A) revealed inward current detectable within a few seconds of VP addition and rising to a maximum within ~30 s and declining thereafter. The current/voltage dependence (Fig. 2B) of this VP-induced current revealed a pattern of outward rectification and a reversal potential of 0 mV. A similar inward current was activated in response to application of 100 μM OAG (Fig. 2C). However, the I/V relationship for OAG-induced current (see Fig. 4) was subtly different, exhibiting double rectifying properties similar to the properties of TRPC6 channels expressed in a number of different systems (5). We sought to undertake a more rigorous determination of the identity of the channel activity mediating this current by gene silencing approaches.

Knockdown of TRPC6 Using siRNA—In order to determine the role of TRPC6 in OAG- and VP-induced channel activity, an electroporation protocol was utilized to introduce selected stealth siRNA sequences (see “Experimental Procedures”). As shown in Fig. 3, introduction of double-stranded RNA sequences corresponding to nucleotides starting at 1574 or 1609 resulted in substantial reduction in TRPC6 expression. Real time PCR revealed an approximate 90% reduction of TRPC6 mRNA levels within 15 h as compared with a control sequence (Fig. 3A). Based on a time course of protein expression by Western analysis, we determined that maximal down-regulation of the protein was at 72 h after electroporation of 200 nM siRNA. At this time, densitometric analysis of Western blots revealed a 70–90% decrease in TRPC6 protein expression (Fig. 3B). Important was to examine the changes in the expression levels of other TRPC family members. Compensation by related subfamily members can severely complicate analysis of a phenotype, using either gene knockout in whole animals (6) or gene silencing techniques (37). In particular, in recent studies with TRPC6 knockout mice, there appears to be a compensatory expression of TRPC3 channels, which may account for increased smooth muscle cell tone in the knockout animals (6–8, 24). Thus, examination of the expression levels of other TRPC3/6/7 channel members was of particular interest. As shown in Fig. 3A, the levels of message for TRPC1, TRPC3, TRPC5, and TRPC7 were virtually unaffected in the same cells in which TRPC6 knockdown was highly effective. At the protein level, there were no detectable changes in the expression of these same TRPC channel members (Fig. 3C). It is important to qualify this interpretation for TRPC3 and TRPC7, which are expressed at low levels. Whereas we can make no assertion about any changes in expression of TRPC3 and TRPC7 based on protein determinations that were below detection limits, the message for these channels was detectable and revealed no change. Thus, the ability to
decrease TRPC6 expression in this system without significant compensatory changes in other TRPC channels provides an important advantage in analyzing the role of endogenous TRPC6 channels.

**Effect of TRPC6 Knockdown on OAG-induced Current**—Whole cell recordings of A7r5 cells revealed a substantial decrease in the OAG-induced current after treatment with siRNA for TRPC6. As shown in Fig. 4A, the development of peak inward current (measured at −80 mV) was substantially reduced in cells treated with the 1609 siRNA as compared with cells treated with control siRNA. The current-voltage relationship shown in Fig. 4B reveals that both inward and outward current are reduced. The outward current (measured at 80 mV) was reduced on average by more than 80% (Fig. 4C), which is consistent with the reduction in protein observed with siRNA (Fig. 3). Whereas the OAG-induced peak was greatly diminished, a small residual current developed more slowly. Since the other OAG-inducible TRPC channels, TRPC3 and TRPC7, were not detectable even after TRPC6 knockdown (Fig. 3), it is unlikely that this current is related to increased expression of other TRPC channels. Instead, this slower activating residual current might reflect a distinct TRPC6 heteromer resulting from the decreased proportion of TRPC6 versus other TRPC channels. Alternatively, it may reflect other channels perhaps sensitive to OAG through a PKC-dependent process.

**Effects of TRPC6 Knockdown on Store-operated and Receptor-induced Ca²⁺ Entry**—Since TRPC channels have been widely implicated in mediating store-operated and receptor-dependent Ca²⁺ entry (1–5), the role of TRPC6 in mediating Ca²⁺ entry in A7r5 cells was important to ascertain. We initially examined the effects of RNAi for TRPC6 on store-operated Ca²⁺ entry induced by emptying stores with the SERCA pump blocker, thapsigargin. Blockade of the SERCA pump results in passive release of Ca²⁺ from stores, and upon the addition of Ca²⁺ to the outside medium, a substantial entry of Ca²⁺ is observed. As shown in Fig. 5, A–C, there was no significant effect of TRPC6 knockdown on either the amount of Ca²⁺ released by thapsigargin or on the store-operated channel-mediated Ca²⁺ entry (p > 0.05). This is a significant result, indicating that the TRPC6 channel is playing little role in purely store-operated Ca²⁺ entry. We have stressed recently that the complete emptying of stores effected by thapsigargin, while important for observing purely store-operated Ca²⁺ entry, is not a condition encountered physiologically (2, 11, 14, 17, 18, 38). Thus, we also examined the effects of TRPC6 knockdown on Ca²⁺ signals induced by activation of the
store emptying

TRPC channels have been implicated in mediating store-operated Ca\(^{2+}\) entry. Whereas each of the seven mammalian homologues has been shown to contribute to Ca\(^{2+}\) entry in response to OAG addition (6). This increased Ca\(^{2+}\) entry was observed, representing a combination of receptor-induced and store-operated Ca\(^{2+}\) entry in most, but not all cells, as shown in Fig. 5. These spontaneous spikes of Ca\(^{2+}\) entry have been observed previously in A7r5 cells and are mediated by L-type Ca\(^{2+}\) channels, since they are blocked by nimodipine or verapamil (39, 40). Since TRPC6 knockdown results in a significant loss of VP-induced Ca\(^{2+}\) entry, we may infer that the receptor-induced Ca\(^{2+}\) entry is partly, but not wholly, dependent on TRPC6 channels. As shown in Fig. 1, TRPC expression in A7r5 cells is certainly not restricted to TRPC6 alone; hence, the residual receptor-induced Ca\(^{2+}\) entry in cells after TRPC6 RNAi treatment may reflect the operation of other TRPC channels. An alternative interpretation is that the Ca\(^{2+}\) entry response induced by VP comprises discrete store-dependent and store-independent components. Whereas each of the seven mammalian TRPC channels have been implicated in mediating store-operated Ca\(^{2+}\) entry, in most cases, this is controversial with data both in favor and against such a role (reviewed in Refs. 1–3 and 13). The data shown in Fig. 5A provide good evidence against TRPC6 functioning in response to store emptying per se. Earlier, we reported that the closely related homologue, TRPC3, while activated by PLC-coupled receptors, was unaffected by stores (29, 30). In those studies, we utilized transient and stable overexpressing systems to examine TRPC3 function, and we speculated that, under such high expression conditions, TRPC3 channels may be “uncoupled” from stores while still activated by the PLC product, DAG (2, 11). The current results indicate that endogenously expressed TRPC6 channels also are not functioning as store-operated channels.

Effects of TRPC6 Knockdown on OAG-induced Ca\(^{2+}\) Entry — An obvious further experiment was to examine the actions of TRPC6 knockdown on any Ca\(^{2+}\) entry that was mediated by the direct TRPC6 activator, OAG. Indeed, the A7r5 cells showed a substantial increase in Ca\(^{2+}\) entry in response to OAG addition (Fig. 6A). This increased Ca\(^{2+}\) entry was all due to entry, since there was no OAG-induced Ca\(^{2+}\) entry in the absence of extracellular Ca\(^{2+}\) (data not shown). Surprisingly, however, there was little change in this OAG-induced Ca\(^{2+}\) entry in A7r5 cells treated with siRNA for TRPC6 (Fig. 6B) as opposed to cells treated with control siRNA (Fig. 6A). We undertook a total of six individual experiments, each involving multiple separate traces (6–12 cells in each trace) of control RNAi-treated cells or cells treated with either or both of the 1609 or 1574 TRPC6 siRNA sequences. From 11 TRPC6 siRNA traces, only one showed a significant decrease in OAG-induced Ca\(^{2+}\) entry from control-treated cells, one showed a slight decrease, and the remaining nine traces revealed no significant change. Based on the substantial TRPC6-mediated current activity (Fig. 4), this was an unexpected result.

Is OAG-induced Ca\(^{2+}\) Entry Mediated by TRPC6 Channels? — We sought to independently assess whether the OAG-induced Ca\(^{2+}\) entry was actually mediated by TRPC6. Since many actions of DAG are PKC-mediated, an obvious possibility was that OAG induced an effect through PKC. Therefore, we assessed any PKC role by utilizing the aminohexyl bisindolylmaleimide, GF109203X, recognized as a potent inhibitor of multiple PKC subtypes (10, 41). Compared with control (Fig. 7A), there was no inhibition by GF109203X on OAG-induced Ca\(^{2+}\) entry; indeed, there was a slight enhancement at longer times (Fig. 7B). In fact, this slight longer term increase in OAG-induced entry was consistent with the action of PKC to inhibit TRPC channels (10). PKC-induced inhibition of the closely related TRPC3 channel (10) was a
exposure to 100 A7r5 cells maintained under optimal growth conditions were loaded with fura-2.

channels.

induced Ca\(^{2+}\) previously determined that OAG-induced activation of TRPC3 was abol-

levels (10, 42). Using stably TRPC3-transfected HEK 293 cells, we pre-

pronounced PKC-mediated phosphorylation of targets at nanomolar

ment of A7r5 cells with the powerful PKC activator, PMA, which causes

powerful effect and provided a functional "hallmark" in the identifica-

tion of TRPC channels. We sought to assess the actions of prior treat-

ment before they inactivate and how reducing the number of TRPC6

whether TRPC6 channels would be able to mediate membrane depolar-

consistent with other studies (22). We approached the question of

L-type channels in A7r5 cells is in the

membrane potential of cultured A7r5 cells measured in several studies

has been well characterized in this cell line (39, 40, 46). The resting

V

fig. 5 shows the results obtained with TRPC6 knockdown in HEK 293 cells.

OAG-induced Ca\(^{2+}\) entry is strongly inhibited by prior incubation with

10 \(\mu\)M PP2, consistent with its action on TRPC6 channels. Overall,

based on observations in Fig. 7 that Ca\(^{2+}\) entry is activated by OAG

independently of PKC, is blocked by PKC inhibition, and is inhibited by

Src PTK inhibition, there is strong circumstantial evidence that OAG is

inducing its action through TRPC6 channel activation.

Reconciliation: TRPC6 Channels Mediate L-type Channel Activation

The paradox emerging from the TRPC6 knockdown and TRPC6 func-
tional studies appears to have a simple and physiologically important

resolution. The experiment shown in Fig. 8 reveals that the OAG-in-
duced entry of Ca\(^{2+}\) in A7r5 cells is largely blocked by the dihydropyri-
dine L-type channel blocker, nimodipine. Compared with control

OAG-induced Ca\(^{2+}\) entry (Fig. 8A), the addition of 1 \(\mu\)M nimodipine 1

min prior to the addition of Ca\(^{2+}\) resulted in a greatly attenuated Ca\(^{2+}\)

entry response to OAG (Fig. 8B). Similar inhibition was observed with

the nondihydropyridine L-type channel blocker, verapamil, at 10 \(\mu\)M

(data not shown). This indicates that the entry of Ca\(^{2+}\) ions in response
to OAG is mostly carried by L-type Ca\(^{2+}\) channels. This is in contrast to

the electrophysiological measurements (Figs. 2 and 4), which were

undertaken in the presence of nimodipine. TRPC channels, including

TRPC6, are considered to be largely nonselective cation channels, the

predominant inward current passing through them being attributed to

Na\(^+\) ions (4, 5, 45). A7r5 cells, like primary smooth muscle cells, are

clearly established to express L-type Ca\(^{2+}\) channels, and their function

has been well characterized in this cell line (39, 40, 46). The resting

membrane potential of cultured A7r5 cells measured in several studies

is in the \(-40\) to \(-50\)-mV range (39, 46). The threshold for opening of

L-type channels in A7r5 cells is in the \(-10\) to \(-20\)-mV range, and peak

Ca\(^{2+}\) current through L-type channels is observed at approximately

+10 mV (39, 46). A relatively small current mediated by TRPC6 channels
could result in effective L-type channel opening. It is clear that

TRPC6 channels in A7r5 cells inactivate slowly (\(\tau\) \~ 50 s in Fig. 2),

consistent with other studies (22). We approached the question of

whether TRPC6 channels would be able to mediate membrane depolar-

ization before they inactivate and how reducing the number of TRPC6

channels by 90% would change this. To estimate how fast the cell would

be depolarized due to TRPC6 activation, we used the classical capaci-

tance discharge equation,

\[
V = V_{exp}\left(-\frac{t}{R_mC_m}\right) \quad \text{(Eq. 1)}
\]
in which \( V \) represents the membrane potential, \( V_r \) is the resting membrane potential, \( t \) is the time after maximum activation of TRPC6, and \( R_m \) is the membrane resistance (\( V_i/R_m \) after maximal activation of TRPC6 channels. We used the value of \(-15 \text{ mV}\) as the threshold (\( V_t \)) for L-type channels in A7r5 cells (39, 46). Based on our studies and those of others (5), the TRPC6 channel has minimal voltage gating and a reversal potential of \( 0 \text{ mV} \). To simplify calculations, we made the assumption that TRPC6 has a linear voltage dependence between \(-80 \) and \( 0 \text{ mV} \). We also assumed that no other channels become activated as a result of TRPC6 channel opening (either due to the Ca\(^{2+}\) influx or membrane depolarization to \( V_r \)) and hence that the membrane resistance \( R_m \) stays constant during the depolarization. Based on this, the time to reach \( V_t \) is given by the following equation.

\[
t = -\frac{V_m}{I_m} C_m \ln \left( \frac{V_i}{V_r} \right)
\]

(Eq. 2)

Using the value for resting potential of \(-50 \text{ mV}\) and our experimental data for \( I_m/C_m \) of \(-0.5 \text{ pA/picofarads} \) at \(-80 \text{ mV} \), then \( t \) the time to reach threshold potential, would be \(192\) ms. If we were to have reduced the total number of TRPC6 channels by \(90\)%, then the time to reach threshold would be \(192\) s. This means that even with a substantial reduction in TRPC6 channels, the time to reach a potential at which L-type channels are opening would still be very rapid, and a change in L-type channel activation would escape detection in our Ca\(^{2+}\) imaging experiments.

One question arising from the data in Figs. 5 and 6 is the apparent discrepancy between the effects of RNAi for TRPC6 on the function of VP-induced as opposed to OAG-induced Ca\(^{2+}\) entry. Our experiments revealed that the entry of Ca\(^{2+}\) in response to VP was largely unaffected by L-type blockers (data not shown). This is explained by our own observations and those of others (39, 45, 47) that VP causes inhibition of L-type channels. In response to VP, the TRPC6 channel appears to be carrying a larger amount of Ca\(^{2+}\) (Fig. 5, D and E) than it does when activated by OAG in the presence of nimodipine (Fig. 8B). It is also clear that the \( I/V \) curve for VP-induced current (Fig. 2B) is distinct from the purely OAG-activated current (Fig. 4B), which more closely resembles the \( I/V \) curve for TRPC6 (5). One explanation for these differences is the existence of different populations of TRPC6 channels responding to OAG and vasopressin. In a previous study (48), heteromers between TRPC1, TRPC5 (or TRPC4), and TRPC6 were demonstrated in embryonic rat brain and in overexpression studies in HEK293 cells. These heteromers were activated by PLC-coupled receptors but not by OAG. Given the predominant expression of the same spectrum of TRPC proteins in A7r5 cells, the presence of such heteromers would be expected. Thus, whereas OAG may operate solely through TRPC6 homomers, the action of VP may also include the OAG-insensitive TRPC heteromers. This may explain the difference in the amount of Ca\(^{2+}\) entry carried by TRPC6 in response to VP as opposed to OAG. Our preliminary studies revealing TRPC6 immunoprecipitation with TRPC1 (data not shown) provide some support for this concept. Thus, whereas TRPC6 channels are a predominant species in A7r5 cells, interactions with other TRPC subtypes may result in multiple populations of channels with distinct activation properties.

Overall, we have combined targeted RNAi with a rigorous assessment of both message and protein, to provide new information on the presence and function of endogenously expressed TRPC6 channels in A7r5 cells. The results indicate that endogenous TRPC6 channels are not involved in store-operated Ca\(^{2+}\) entry but do contribute to the entry of Ca\(^{2+}\) following PLC-coupled receptor activation. Knockdown experiments reveal that an OAG-activated nonselective cation current is almost completely attributable to TRPC6. A corresponding TRPC6-mediated OAG-dependent entry of Ca\(^{2+}\) is not significantly altered by TRPC6 knockdown, and this entry is mostly through L-type channels. The TRPC6 channel is hence acting as a mediator between PLC-generated DAG and the activation of Ca\(^{2+}\) entry through L-type channels. A scheme depicting this signaling process is shown in Fig. 9. The effectiveness of TRPC6 is sufficient that, even after substantial reduction of channel protein by RNAi, it still allows L-type channel activation. The function of TRPC channels mediating depolarization and activation of L-type channels has also been indicated in other studies. Thus, in cerebral arteries, TRPC6 antisense treatment reduced pressure-induced depolarization and arterial constriction, suggesting that TRPC6 channels are activated as a result of pressure and may play an important role in the control of myogenic tone (49). Very recently, the TRPC3 channel, which is also expressed in cerebral arteries, was shown to mediate purinergic receptor-induced depolarization and contraction (50). Thus, members of the TRPC3/6/7 subfamily of nonselective cation channels may play an important role in the control of smooth muscle cell membrane potential to effect control over voltage-operated Ca\(^{2+}\) entry and muscle contraction.

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