Calcium controls smooth muscle TRPC gene transcription
via the CaMK/calcineurin-dependent pathways

Sara Morales,1 Amalia Diez,2 Antonio Puyet,2 Pedro J. Camello,1 Cristina Camello-Almaraz,1 Jose M. Bautista,2 and Maria J. Pozo1
1Department of Physiology, University of Extremadura, Cáceres; 2Department of Biochemistry and Molecular Biology IV, University Complutense of Madrid, Madrid, Spain

Submitted 1 March 2006; accepted in final form 30 August 2006

Morales S, Diez A, Puyet A, Camello PJ, Carmello-Almaraz C, Bautista JM, Pozo MJ. Calcium controls smooth muscle TRPC gene transcription via the CaMK/calcineurin-dependent pathways. Am J Physiol Cell Physiol 292: C553–C563, 2007. First published September 6, 2006; doi:10.1152/ajpcell.00096.2006.—Transient receptor potential protein family C (TRPC) has been proposed as a candidate for channels involved in capacitative Ca2+ entry (CCE) mechanisms, but the modulation of their gene expression remains unexplored. In this study we show that guinea pig gallbladder smooth muscle contains mRNA encoding TRPC1, TRPC2, TRPC3, and TRPC4 proteins whose abundance depends on cytosolic Ca2+ level ([Ca2+]i). Thus lowering the levels of cellular calcium with the chelators EGTA and BAPTA AM results in a downregulation of TRPC1–TRPC4 gene and protein expression. In contrast, activation of Ca2+ influx through L-type Ca2+ channels and Ca2+ release from intracellular stores induced an increase in TRPC1–TRPC4 mRNA and protein abundance. Activation of Ca2+/calmodulin-dependent kinases (CaMK) and phosphorylation of cAMP-response element binding protein accounts for the increase in TRPC mRNA transcription in response to L-type channel-mediated Ca2+ influx. In addition to this mechanism, activation of TRPC gene expression by intracellular Ca2+ release also involves calcineurin pathway. According to the proposed role for these channels, activation of CCE induced an increase in TRPC1 and TRPC3 mRNA abundance, which depends on the integrity of the calcineurin and CaMK pathways. These findings show for the first time an essential autoregulatory role of Ca2+ in Ca2+-homeostasis at the level of TRPC gene and protein expression.

transient receptor protein family C channels; cytosolic calcium levels; gallbladder

Calcium (Ca2+) plays a signaling role in many important cellular functions, such as fertilization, embryonic pattern formation, differentiation, proliferation, contraction, secretion, and metabolism (5). Although Ca2+ is a ubiquitous second messenger, cells have found ways to endow distinct Ca2+ signals with specific functions. The versatility of the Ca2+-signaling mechanism in terms of speed, amplitude, and spatiotemporal patterning enables elevations of Ca2+ to specifically regulate many processes of cell activity, including gene expression (23, 26, 44). One potential Ca2+-dependent step in the process of new gene expression is mRNA transcription (6, 17), which also has been shown to be differentially regulated by the pattern of Ca2+ elevation (23). Detection of cytosolic Ca2+ level ([Ca2+]i) increases by specific Ca2+ sensors such as calmodulin (9) or members of the recoverin subfamily of Ca2+-binding proteins (7) transduce the Ca2+ signal into changes in the transcription rate of specific genes through three general mechanisms: activation of phosphorylation/phosphorylation cascades that modify transcription factors, induction of protein-protein interaction between the Ca2+ sensor and transcription factors, or changes in the binding properties of the Ca2+ sensor to specific sites in the DNA (reviewed in Ref. 26).

Among other genes mediating proliferation and cell survival, mRNA transcription of genes encoding ion channels has been shown to be regulated by Ca2+ (1, 13, 30). Experimental manipulations of Ca2+ entry via L-type channels have shown that increased cytosolic Ca2+ enhances L-type Ca2+ currents either upregulating transcriptional expression of these channels (13) or downregulating Kv mRNA expression (1, 30), thereby causing membrane depolarization, which causes further Ca2+ influx through L-type Ca2+ channels. Ca2+ influx through store-operated channels (SOC) is involved in controlling gene expression during the activation of T cells by antigens in a calcineurin/nuclear factor of activated T cells (NFAT)-dependent manner (for review, see Ref. 18). In keeping with this, it has been shown that transcriptional activation may be regulated by store-operated Ca2+ entry in vascular smooth muscle through changes in cAMP-response element binding protein (CREB)-mediated c-fos expression (32).

At present, the best molecular candidates for SOC are the TRP proteins (so called because of their homology with the transient receptor potential protein that underlies phototransduction in Drosophila). The TRP superfamily has been subdivided into multiple subfamilies on the basis of sequence similarity (27). In the case of SOC, much attention has been focused on the canonical TRP (TRPC) subfamily. Despite considerable effort, it is unclear exactly which of the seven TRPC isoforms are the molecular constituents of endogenous SOC. The mechanisms that regulate TRPC gene expression remain one of the important unexplored areas of research in this field. Transcriptional changes of TRPC gene expression in response to organ culture (4, 14) and to injury (4) have been shown recently in arterial smooth muscle, but the signal pathway and mechanisms mediating the plasticity of TRPC gene expression have not been explored yet. In addition, short-term changes in TRPC expression recently were demonstrated in pulmonary arteries. Thus extracellular ATP induces upregulation of TRPC4 expression, which was mediated by phosphorylation of the transcription factor CREB, probably through Ca2+-independent mechanisms (47). The aim of the present work was to explore the hypothesis that [Ca2+]i participates in...
the regulation of TRPC transcription and protein expression. If correct, such a proposal increases the complexity of Ca\(^{2+}\) signaling as a consequence of this apparent positive feedback: Ca\(^{2+}\)-mediated regulation of Ca\(^{2+}\)-permeable TRPC channels.

**MATERIALS AND METHODS**

**Animals.** Gallbladders were isolated from 300- to 450-g male guinea pigs following cervical dislocation and exsanguination and immediately placed in cold Krebs-Henseleit solution (KHS; for composition, see Solutions). All procedures were reviewed and approved by the office of Animal Care Management at the University of Extremadura.

**RT-PCR assay.** Total RNA was isolated from gallbladder smooth muscle (GBSM) using the TRI reagent. Residual genomic DNA was removed by treatment with DNA-free (Ambion). Total RNA was quantified with RIBO green. First-strand cDNA was synthesized from 1 µg of total RNA using random decamers and MMLV reverse transcriptase (Ambion). The cDNA was amplified by PCR using known rat-specific glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers and a set of TRPC isoform-specific primers described by Ong et al. (29) in guinea pig airway smooth muscle. Amplification was carried out using Applied Biosystems AmpliTaq Gold DNA polymerase and a Gene-Amp PCR system 2700. cDNA (2 µl) was added to 23 µl of PCR reaction mixture containing 2.5 µl of 10× PCR buffer, 1/25 µl of 25 mM MgCl\(_2\), 1 µl of 10 mM dNTPs, 0.3 µM of each primer (except for the degenerate primer MTA, which was used at 1 µM) and 0.125 µl of Taq polymerase (5 U/µl). The PCR conditions were as followed: 95°C for 7 min, 45 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min, with final extension of 10 min at 72°C. A negative control reaction (no template) to test for general contamination was included. Under these conditions we never observed unspecific products. Reaction products were separated by electrophoresis on 2% agarose gel stained with ethidium bromide. The obtained RT-PCR products were sequenced either directly by using the same oligonucleotides as primers or after being subcloned into a pGEM-T vector (Promega) by using Applied Biosystems sequencing kits.

**Real-time quantitative RT-PCR.** Relative abundance of TRPC1–TRPC4 mRNA was assessed using the 5’ fluorogenic nucleic acid assay (TaqMan) on an ABI Prism7700 sequence detector system (Applied Biosystems). Primers and probes were designed to the TRPC1–TRPC4 mRNA sequence by Ong et al. (29) in guinea pig airway smooth muscle. Amplification was carried out using Applied Biosystems AmpliTaq Gold DNA polymerase and a Gene-Amp PCR system 2700. cDNA (2 µl) was added to 23 µl of PCR reaction mixture containing 5 µl of 10× PCR buffer, 1/25 µl of 25 mM MgCl\(_2\), 1 µl of 10 mM dNTPs, 0.3 µM of each primer (except for the degenerate primer MTA, which was used at 1 µM) and 0.125 µl of Taq polymerase (5 U/µl). The PCR conditions were as followed: 95°C for 7 min, 45 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min, with final extension of 10 min at 72°C. A negative control reaction (no template) to test for general contamination was included. Under these conditions we never observed unspecific products. Reaction products were separated by electrophoresis on 2% agarose gel stained with ethidium bromide. The obtained RT-PCR products were sequenced either directly by using the same oligonucleotides as primers or after being subcloned into a pGEM-T vector (Promega) by using Applied Biosystems sequencing kits.

**RT-PCR determinations were done in three independent experiments, with each experiment containing tissue from three to four different animals.**

**Western blot analysis for TRPC.** Guinea pig GBSM layers were divided into strips and treated under different experimental conditions. In these protocols, the control and time-course treatments were always performed in strips coming from the same gallbladder. The muscle strips were later ground in a liquid nitrogen-cooled mortar and pestle, homogenized in lysis solution (for composition, see Solutions) using a homogenizer (OMNI International), and then sonicated for 5 s. Lysates were centrifuged at 10,000 g for 15 min at 4°C to remove nuclei and unlysed cells. Protein concentration was measured in the supernatant by using the Bradford method. Protein extracts (25–35 µg) were heat-denaturalized at 95°C for 5 min with DTT, electrophoresed on 7.5% polyacrylamide-SDS gels, and then transferred to a nitrocellulose membrane. For control and treated samples, the same amount of protein was always loaded in the polyacrylamide-SDS gels. Membranes were blocked for 1 h at room temperature using 10% bovine serum albumin (BSA) and incubated overnight at 4°C with an appropriate dilution of the primary antibody (1:500 for GADPH, 1:200 for TRPC1, 1:200 for TRPC3, and 1:100 for tubulin). The secondary antibody was used as load control. After washing, the membranes were incubated for 1 h at room temperature with anti-rabbit IgG-horseradish peroxidase conjugated secondary antibody (1:10,000; Bio-Rad Laboratories, Hercules, CA) or anti-mouse IgG horseradish peroxidase-conjugated secondary antibody (1:10,000; Bio-Rad Laboratories, Hercules, CA). Positive bands were visualized using an LAS-3000 fluorography apparatus (Fuji). Membrane autoradiographs were evaluated using a densitometer (Bio-Rad). Band intensities were normalized to the internal control (GADPH or tubulin) in each experiment. Results were expressed as means + SE. A significance level of P < 0.05 was used to determine statistical significance. The Student’s t test was used for comparison of means between control and experimental groups (Sigma Stat).

**Table 1. Oligonucleotide sequences of primers and probes used for real-time RT-PCR**

<table>
<thead>
<tr>
<th>Gene</th>
<th>GenBank Accession</th>
<th>Primers and Probes</th>
<th>Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRPC1</td>
<td>AY572429</td>
<td>QTRPS-1 (TRP-1 sense)</td>
<td>AAAACGCGACAAAGACTCTGGTATCTT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>QTRPA-1 (TRP-1 antisense)</td>
<td>AAGGGAGGTTCCGTTGAACGAA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>QTRRP-1 (TRP-1 probe)</td>
<td>TGGGAGCGACAAAGACTGACACCTTCC</td>
</tr>
<tr>
<td>TRPC2</td>
<td>AY572483</td>
<td>QTRPS-2 (TRP-2 sense)</td>
<td>CTGTGCTGCTTACTGGTG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>QTRPA-2 (TRP-2 antisense)</td>
<td>GACCCGAGGGCCTCCTATTGGAACG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>QTRRP-2 (TRP-2 probe)</td>
<td>GAGGAGGAAGATGACCATGA</td>
</tr>
<tr>
<td>TRPC3</td>
<td>AY574230</td>
<td>QTRPA-3 (TRP-3 sense)</td>
<td>AACTCTCTCTTATGCACTGTAAGAAGAA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>QTRPP-3 (TRP-3 antisense)</td>
<td>GATGATATTTAAAGCAAATCTGGTAAGCCT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>QTRPP-3 (TRP-3 probe)</td>
<td>TATCACCAGCTTTGGTGATATTTTGAGGTGTG</td>
</tr>
<tr>
<td>TRPC4</td>
<td>AY572431</td>
<td>QTRPS-4 (TRP-4 sense)</td>
<td>GGCATACGATGGGAAAAGCAAGA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>QTRPA-4 (TRP-4 antisense)</td>
<td>AGCCCATTAATGGCAGAAGAAAGAAT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>QTRPP-4 (TRP-4 probe)</td>
<td>TGACATTTTGATGATGATGATACTGGT</td>
</tr>
<tr>
<td>GADPH</td>
<td>AB06340</td>
<td>QGADPH (sense)</td>
<td>TGCTGACATGATGGGAAAAGAAGCC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>QGADPH (antisense)</td>
<td>CAGGCACTACCCAGCATTGGATG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>QGADPH (probe)</td>
<td>TTCCAGGAGGGGAGATCCGGG</td>
</tr>
</tbody>
</table>

**AJP-Cell Physiol • VOL 292 • JANUARY 2007 • www.ajpcell.org**
Expression of TRPC family members in GBSM. TRPC1–TRPC7 expression was evaluated using RT-PCR in GBSM layer. No TRPC5–TRPC7 transcripts were detected in GBSM. Figure 1A shows a 2% agarose gel with ethidium bromide staining of the PCR products for TRPC found in GBSM (n = 5 experiments in 3 animals). All the amplified products were of the predicted sizes: TRPC1, 400 bp; TRPC2, 370 bp; TRPC3, 340 bp; and TRPC4, 370 bp. The cDNA products of TRPC1–TRPC4 were sequenced, and the identities of the amplicons were verified by database homology searches. The partial TRPC sequences found in guinea pig GBSM were deposited at GenBank under the following accession numbers: TRPC1, AY572429; TRPC2, AY574383; TRPC3, AY572430; and TRPC4, AY572431 (Table 1).

Western blot studies using TRPC1, TRPC3, and TRPC4 polyclonal antibodies (Fig. 1B) revealed the expression of a protein for TRPC1 with a molecular mass around 120 kDa. The molecular mass of 120 kDa is in the range previously reported for TRPC1 protein with this commercial antibody in rat (2, 21, 24) but not human tissue (11, 46). Differences in the mobility of the band between humans and other animal species may reflect species differences in the expression of TRPC1 such as glycosylation. For TRPC3 and TRPC4, the molecular masses were 90–95 kDa. These sizes are in agreement with previous reports regarding TRPC3 and TRPC4 (2, 11, 12, 24). We did not perform Western blotting for TRPC2 because a specific antibody is currently not available. To further investigate the expression of TRPC proteins, we stained isolated GBSM cells. Immunofluorescence experiments using anti-TRPC1, anti-TRPC3, and anti-TRPC4 polyclonal antibodies showed that TRPC1, TRPC3, and TRPC4 proteins are present in GBSM (Fig. 1C), which agrees with our PCR and Western blot data, and are mainly located in plasma membrane.

To determine the relative abundance of the four TRPC transcripts expressed in GBSM, we performed real-time quantitative RT-PCR using RNA from freshly isolated GBSM and specific primers and probes designed on gallbladder TRPC1–TRPC4 mRNA sequences. Steady-state transcripts were determined relative to the endogenous control housekeeping gene GAPDH. TRPC1 and TRPC2 mRNA were the most highly expressed, whereas TRPC4 was expressed at the lowest level. TRPC expression relative to GAPDH (arbitrary units) was 0.145 ± 0.020, 0.191 ± 0.025, 0.076 ± 0.015, and 0.0012 ± 0.00059 for TRPC1, TRPC2, TRPC3, and TRPC4, respectively (n = 7).

Intracellular \( \text{Ca}^{2+} \) regulates TRPC gene transcription and protein expression. To test the hypothesis that TRPC gene expression is regulated by \( \text{Ca}^{2+} \) levels, we carried out real-time quantitative RT-PCR on total RNA extracted from GBSM pretreated with the \( \text{Ca}^{2+} \) chelators EGTA and BAPTA AM. We have previously shown that both approaches are effective in lowering \( \text{Ca}^{2+} \) (28). Figure 2A shows that exposure of GBSM to \( \text{Ca}^{2+} \)-free solution containing 1 mM EGTA for 2 h significantly decreased TRPC1, TRPC2, TRPC3, and TRPC4 mRNA abundance relative to control conditions (n = 5, \( P < 0.05 \) for TRPC3 and TRPC4, \( P < 0.001 \) for TRPC1 and TRPC2). A similar pattern was also observed when GBSM was treated with 10 \( \mu \)M BAPTA AM for 30 min.
Fig. 1. Detection of transient receptor protein family C (TRPC) mRNA and TRPC protein expression in gallbladder smooth muscle (GBSM) layer. A: RT-PCR-amplified products displayed in an agarose gel stained with ethidium bromide for the TRPC isoforms detected in GBSM layer: TRPC1 (400 bp), TRPC2 (370 bp), TRPC3 (340 bp), and TRPC4 (370 bp). L, 100-bp DNA ladder marker. The results shown were obtained in 1 of 5 experiments employing cDNA from 3 different animals. B: original Western blots obtained using anti-TRPC1, anti-TRPC3, and anti-TRPC4 antibodies on GBSM in 1 experiment representative of 4 others. Bands around 120, 90, and 95 kDa were obtained for TRPC1, -3 and -4, respectively. C: confocal immunofluorescence images showing TRPC1, TRPC3, and TRPC4 staining in isolated GBSM cells. No immunofluorescence was evident when primary antibodies were omitted and only FITC-labeled secondary antibody was used.

These data suggest that cytosolic Ca\(^{2+}\) ions regulate TRPC gene expression in guinea pig GBSM, but at this stage we cannot rule out the possibility that Ca\(^{2+}\) may just be necessary for gene expression without having a regulatory role in this

(n = 4, P < 0.05, Fig. 2B). In agreement with the observed reduction in mRNA content, Ca\(^{2+}\) chelator conditions also reduced the amount of TRPC proteins in GBSM cells (Fig. 2, C and D).

Fig. 2. Intracellular Ca\(^{2+}\) is necessary for TRPC gene transcription and protein expression. A and B: real-time quantitative RT-PCR was used to quantify the TRPC1, TRPC2, TRPC3, and TRPC4 mRNA in GBSM. The smooth muscle was treated with 1 mM EGTA in Ca\(^{2+}\)-free medium for 2 h (A) and with 10 μM BAPTA AM for 30 min (B) before total RNA was extracted. Values are expressed as 2\(^{-ΔΔCt}\), as described in MATERIALS AND METHODS; TRPC mRNA levels under treatments are expressed as fold increases or decreases with respect to TRPC mRNA levels in control conditions (represented as a dotted line). The smallest amount of cDNA detected yielded a C\(_t\) = 40.2 ± 0.3, being significantly different from the nontemplate and non-reverse transcriptase controls, for which no detectable fluorescence was detected along the 55 PCR cycles analyzed. Data are means ± SE of 4–5 experiments. Statistical analysis was performed using the nonparametric Mann-Whitney test. *P < 0.05, ***P < 0.001. A typical example of Western blot analysis (C) and summarized data (D) of changes in TRPC1, TRPC3, TRPC4, and α-tubulin protein expression in smooth muscle are shown under the same treatment conditions as in A and B. Summary data are expressed as fold increases with respect to control conditions normalized to α-tubulin content. Data are means ± SE of 3–4 experiments. *P < 0.05.
process. To further test these possibilities, we raised intracellular Ca\(^{2+}\) levels by activation of Ca\(^{2+}\) influx through L-type Ca\(^{2+}\) channels by depolarizing smooth muscle with 60 mM KCl. We have previously demonstrated that L-type Ca\(^{2+}\) channels are the only subtype of Ca\(^{2+}\) voltage-sensitive channels activated by 60 mM KCl depolarization, since the specific blocker of L-type Ca\(^{2+}\) channels, nifedipine, almost abolished the Ca\(^{2+}\) influx in response to KCl and to the selective L-type channel opener BAY K 8644 (16). Under KCl challenge for 10 min, mRNA encoding TRPC1–TRPC4 proteins were substantially enhanced (69.9, 60.9, 90.4, and 92.9% increase for TRPC1, TRPC2, TRPC3, and TRPC4, respectively, \(n = 4\); Fig. 3A). These increases in TRPC mRNA levels were still detected when GBSM was exposed to KCl for 60 min (Fig. 3A). Even higher increases in TRPC1–TRPC3 mRNA accumulation were observed when GBSM was challenged for 10 and 60 min with 10 nM CCK, a hormone that releases Ca\(^{2+}\) from the intracellular Ca\(^{2+}\) stores (28) (Fig. 3B). Associated with these elevations in mRNA, KCl and CCK also induced a significant increase in TRPC expression at the level of protein, as shown in Fig. 3, C and D. These findings suggest that upregulation of gallbladder TRPC channel expression is mediated by increases in [Ca\(^{2+}\)]\(_i\), and support an active role of [Ca\(^{2+}\)]\(_i\) in controlling TRPC gene expression. To ascertain whether the accumulation of TRPC mRNA was caused by transcriptional induction, we carried out a control experiment in which the tissue was previously incubated for 30 min with the transcription inhibitor actinomycin D (5 \(\mu\)g/ml) and then challenged with KCl or CCK. The results, shown as insets in Fig. 3, A and B, indicate that no significant changes in the mRNA levels were detected compared with the controls without actinomycin D (\(n = 4–6\), \(P > 0.05\)). Similar results were obtained when smooth muscle layers were exposed to BAPTA (data not shown). This suggests that Ca\(^{2+}\) behaves as a transcriptional inducer (either direct or indirect) of the TRPC gene transcription.

To give some insights into the possible mechanisms by which Ca\(^{2+}\) signal could be transduced into changes in gene activity, and because CaMKs have been suggested to be important signaling molecules in Ca\(^{2+}\)-induced changes in gene transcription (19), we examined the effects of KN-93, an inhibitor of CaMK activity, on KCl/CCK-induced increases in TRPC1–TRPC4 gene expression. As shown in Fig. 4, A and B, pretreatment of smooth muscle layer with 30 \(\mu\)M KN-93 for 30 min was able to abolish the increases in TRPC1–TRPC4 gene expression in response to both KCl and CCK (\(n = 5\)). It should be noted that even a decrease in TRPC1–TRPC4 mRNA abundance relative to control conditions (expressed as the value of 1) was caused by KN-93, suggesting that CaMKs regulate TRPC gene transcription not only in response to

---

**Fig. 3.** Depolarization-induced Ca\(^{2+}\) influx and Ca\(^{2+}\) release from intracellular stores enhance TRPC gene transcription and protein expression. A and B: real-time quantitative RT-PCR was used to quantify the TRPC1, TRPC2, TRPC3, and TRPC4 mRNA in GBSM. The smooth muscle was treated with 60 mM KCl (A) or 10 nM CCK (B) for 10 or 60 min, respectively, before total RNA was extracted. Values are expressed as 2\(^{-\Delta\Delta CT}\), as described in MATERIALS AND METHODS; TRPC mRNA levels under treatments are expressed as fold increases or decreases with respect to TRPC mRNA levels in control conditions (represented as a dotted line). Data are means ± SE of 4–6 experiments. Statistical analysis was performed using the nonparametric Mann-Whitney test. *\(P < 0.05\), **\(P < 0.01\), ***\(P < 0.001\). Insets: effect of the transcriptional inhibitor actinomycin D (Act D; 5 \(\mu\)g/ml) on the TRPC gene expression in response to 60 mM KCl (A) or 10 nM CCK (B) for 10 min after 20 min of incubation in the presence of Act D. The control value corresponds to tissues not treated with Act D. Data are means ± SE of 4 experiments. A typical example of Western blot analysis (C) and summarized data (D) of changes in TRPC1, TRPC3, and TRPC4 protein expression in smooth muscle are shown under the same treatment conditions as in A and B. Summary data are expressed as fold increases respect to control conditions normalized to α-tubulin content. Data are means ± SE of 4–6 experiments. *\(P < 0.05\).
[Ca^{2+}], increases but also at resting [Ca^{2+}], levels. This was confirmed when TRPC mRNA abundance was measured in control smooth muscle layers pretreated with KN-93 (59, 60, 56, and 61% inhibition for TRPC1, TRPC2, TRPC3, and TRPC4, respectively, n = 6, P < 0.01).

CREB has been identified as an important signal transduction element in CaMK-mediated gene transcription (37). Thus we performed immunofluorescence experiments to detect changes in CREB phosphorylation in response to KCl and CCK. As can be observed in Fig. 4C, nuclear phospho-CREB was undetectable in resting cells and was only evident under both treatments, suggesting that the phospho-CREB may be involved in enhancing TRPC gene expression. This phosphorylation was dependent on CaMK activation, since the pretreatment with the CaMK inhibitor KN-93 abolished and reduced KCl- and CCK-induced CREB phosphorylation, respectively (Fig. 4C). Another important molecule in mediating Ca^{2+} release from intracellular stores. Although it is firmly established that NFAT is the calcineurin effector for gene transcription, we studied whether KCl and CCK treatments were associated to the translocation of NFAT to the nucleus. However, our immunofluorescence experiments failed in showing any change in the location of NFAT in control, which may indicate nonspecific binding of the primary antibodies in our preparation. In agreement with this hypothesis, Western blot experiments showed that in addition to the expected band at ~130 kDa, additional, apparently nonspecific, bands at 100, 85, 75, 70, and 60 kDa also were present, indicating lack of specificity of these antibodies.
Capacitative Ca\(^{2+}\) entry modulates expression of TRPC genes. We have previously demonstrated that GBSM exhibits capacitative Ca\(^{2+}\) entry (CCE) in response to Ca\(^{2+}\) release from internal stores (28). Given that TRPC family proteins have been suggested to be molecular counterparts of plasma membrane proteins activated by depletion of Ca\(^{2+}\) stores (3, 10), we conducted real-time quantitative RT-PCR experiments to test a possible change in GBSM TRPC gene expression as the result of CCE activation. We activated CCE by using a protocol previously validated in this cellular model (28). Before RNA isolation, Ca\(^{2+}\) stores were depleted by application of the sarco(endo)plasmic Ca\(^{2+}\)-ATPase (SERCA) pump inhibitor thapsigargin (1 \(\mu\)M) plus 1 \(\mu\)M nitrendipine (to block L-type Ca\(^{2+}\) channels) in a Ca\(^{2+}\)-free medium for 30 min, and then external Ca\(^{2+}\) was reintroduced for 10 or 60 min. TRPC1 and TRPC3 mRNA contents were significantly higher in the CCE-activated group than in the control group (\(P < 0.05\) for both). However, no significant changes were observed in TRPC2 and TRPC4 expression (\(n = 4\), Fig. 5A). These changes are due to transcriptional changes in gene expression, since no changes were detected in smooth muscle layers pretreated with actinomycin D (\(n = 4\), Fig. 5A, inset). In addition, the CCE protocol caused an increase in TRPC1 and TRPC4 proteins at 10 min that did not last for 60 min (Fig. 5, B and C).

The CCE-induced increases in TRPC1 and TRPC3 proteins suggest that these proteins may be subunits of SOC, activated when the stores are depleted. To further explore this, we quantified Ca\(^{2+}\) entry in fura-2-loaded cells following a protocol involving depletion of the pools by a 30-min pretreatment with thapsigargin plus nitrendipine, followed by treatment with two pulses of extracellular Ca\(^{2+}\) separated by a 20-min interval. In control conditions, the [Ca\(^{2+}\)]\(_{i}\), plateaus induced by the Ca\(^{2+}\) pulses, indicative of CCE, were repetitive (0.059 \(\pm\) 0.006 vs. 0.057 \(\pm\) 0.004 \(\Delta F_{340/380}\), \(n = 7\), Fig. 5D, top) in keeping with previously described results (28). However, as shown in Fig. 5D, bottom, when we treated cells with TRPC1 antibody (5 \(\mu\)g/ml) during the interval between the two Ca\(^{2+}\) applications, there was a marked attenuation of CCE (0.056 \(\pm\) 0.005 vs. 0.023 \(\pm\) 0.004 \(\Delta F_{340/380}\), \(n = 12\), \(P < 0.001\)). TRPC1 antibody treatment did not induce any significant change in TRPC3 mRNA abundance (3.6, 3.9, 6.6, and 10.1% increase for TRPC1–TRPC4, \(n = 4\), \(P > 0.05\)), indicating that CCE attenuation would be the result of local effects of TRPC1 antibody on TRPC1 membrane proteins. In fact, this antibody was targeted to a TRPC1-specific peptide that was predicted to be extracellular and located between fifth and sixth membrane-spanning domains. Thus, when we performed immunostaining in nonpermeabilized cells and used this antibody in the same conditions as in the Ca\(^{2+}\) imaging experiments (20-min treatment with the primary antibody), the staining was similar to that obtained in permeabilized cells after overnight treatment with the primary antibody (Figs. 5E and 1B). When we repeated both experiments with the antibody raised against an intracellular epitope of TRPC3, we did not see any specific staining in the immunofluorescence experiments (Fig. 5E). However, in 6 of 15 cells we recorded a reduction in CCE (39% inhibition), for which we do not have any explanation yet. TRPC4 antibody did not cause any change in CCE and was unable to stain nonpermeabilized cells (data not shown).

Similar to the results obtained after the smooth muscle was challenged with CCK, CCE-enhanced TRPC1 and TRPC3 expression was sensitive to the treatment with both KN-93 and cyclopiazonic A, indicating that Ca\(^{2+}\) influx in response to store depletion activates TRPC expression through CaMK- and calcineurin-mediated pathways (Fig. 6A). CCE activation for 10 min also caused phosphorylation of nuclear CREB that was sensitive to KN-93 pretreatment, as shown in Fig. 6B, which suggests that phospho-CREB can be a mediator of the enhanced TRPC expression. Although the inhibition of mRNA transcription by cyclopiazonic A indicates that translocation of NFAT may also mediate the positive regulation induced by CCE, we could not perform NFAT immunostaining because of the nonspecificity shown by the commercially available antibodies, as reported above.

**DISCUSSION**

Identification of TRPC proteins has received increasing attention due to their possible role as molecular counterparts of some plasma membrane Ca\(^{2+}\) channels, including channels activated by depletion of the intracellular Ca\(^{2+}\) stores. In the present study, we have identified the TRPC isoforms present in GBSM cells and their relative abundance. In addition, we sought to explore the regulation of TRPC gene expression, an area of research that remains unknown in this field. Thus we have demonstrated that intracellular Ca\(^{2+}\) levels participate in the modulation of TRPC gene transcription and protein expression. [Ca\(^{2+}\)]\(_{i}\) resting levels are necessary to maintain TRPC gene expression, and elevation of [Ca\(^{2+}\)]\(_{i}\) by different maneuvers, including activation of CCE, is associated with an up-regulation of TRPC gene expression, which indicates that this ion autoregulates its levels by positive feedback mechanisms, adding an extra level of complexity to Ca\(^{2+}\) signaling.

mRNA encoding for TRPC1, TRPC2, TRPC3, and TRPC4 but not for TRPC5, TRPC6, and TRPC7 proteins is present in GBSM. TRPC2 and TRPC1 were expressed in the greatest abundance relative to GAPDH, whereas TRPC4 was expressed at the lowest level. We also presented evidences for expression of TRPC1, TRPC3, and TRPC4 proteins by Western blot and immunofluorescence experiments, although we cannot ensure that these are the only ones expressed, because specific antibodies for the TRPC2 isoform are not available. There are conflicting reports whether these proteins function as channels gated by G protein-coupled receptors or by depletion of Ca\(^{2+}\) stores. This has been attributed, in part, to the use of different techniques such as Ca\(^{2+}\) imaging or electrophysiology (see Ref. 33). Inhibition of TRPC1 with the use of TRPC-specific tools such as a functional anti-TRPC1 antibody (Refs. 4, 45; present study) or antisense DNA targeted to TRPC1-encoding mRNA (40) is associated with a decrease in Ca\(^{2+}\) reentry in response to store depletion in smooth muscle (Ref. 45; Fig. 5D), inhibition of contractions with a pharmacological profile similar to that of store-operated Ca\(^{2+}\) entry (4), and reduction in the amplitude of the nonselective cationic current evoked by store depletion (40). In addition, overexpression of TRPC1 enhanced pulmonary artery contraction evoked by depletion of the stores (21). Together, these results support a role for TRPC1 in the store-operated Ca\(^{2+}\) entry phenomenon in smooth muscle. There is ample evidence that TRPC1 forms heteromeric complexes with TRPC4 and TRPC5 but also some
involving TRPC3 and TRPC2 (see Ref. 3). Our results do not support or rule out an association of the TRPC isoforms present in our preparation to form nonselective cationic SOC but strongly indicate that TRPC1 is a component of the native channels accounting for Ca\(^{2+}\) influx in response to store depletion.

Although the number of studies focused in TRPC proteins and their functions is increasing, reports concerning the mechanisms that regulate TRPC gene expression are still lacking. The findings reported in the present study demonstrate, for the first time, a link between cytosolic Ca\(^{2+}\) and TRPC gene expression at transcriptional and transductional levels. This
hypothesis is based on the observation that lowering the level of cellular Ca\(^{2+}\) with the use of Ca\(^{2+}\) chelators such as EGTA and BAPTA AM leads to a decrease in the expression of all TRPC members found in GBSM, whereas elevations in cellular Ca\(^{2+}\) as a result of Ca\(^{2+}\) influx or store depletion leads to an increase in TRPC gene expression.

It is physiologically relevant that Ca\(^{2+}\) homeostasis-mediated regulation results in changes at the TRPC protein levels, since this open the possibility of the presence of a positive feedback mechanism aimed to enhance Ca\(^{2+}\) signal in response to different stimuli. Another possibility could be that this mechanism participated in prolonging the Ca\(^{2+}\) increase beyond the duration of the stimuli or even that exacerbation of this autoregulation could collaborate in Ca\(^{2+}\) overload and cellular apoptosis. However, this last possibility seems uncertain, since the experimental maneuvers used in this study to increase [Ca\(^{2+}\)]\(_i\) (CCK, KCl, and CCE) do not cause any functional damage to the smooth muscle. It is noteworthy that activation of CCE for 60 min did not cause any increase in TRPC protein expression, although 60-min treatment with KCl and CCK enhanced the expression. This finding could indicate that the TRPC turnover is increased when CCE is specifically stimulated, but it also could reflect that exhaustive and prolonged store depletion with thapsigargin impairs protein synthesis in sarcoplasmic reticulum (42). The requirement of Ca\(^{2+}\) for protein expression is a well-known effect. Ca\(^{2+}\) depletion of endoplasmic reticulum reduces protein synthesis through an active, regulated response (reviewed in Ref. 20). The effect of thapsigargin and Ca\(^{2+}\) depletion in our experiments is in keeping with previous reports of protein synthesis impairment (e.g., Ref. 38, 48). Regarding the turnover of proteins in Ca\(^{2+}\) transport, studies in cardiac ischemia-reperfusion have shown that mRNA for ryanodine receptors, SERCA pumps, phospholamban, and calsequestrin exhibit a significant decrease in response to short protocols (even less than 20 min in duration) (41). In addition, mRNA and protein expression of inositol 1,4,5-trisphosphate type II receptors decrease within 2–3 h in response to Ca\(^{2+}\) depletion (22).

Changes in the [Ca\(^{2+}\)]\(_i\) constitute one of the main routes by which information is transferred from extracellular signals received by animal cells to intracellular sites, including the nucleus (5). This idea is the basis of excitation-transcription coupling (ET coupling), a process by which common signaling pathways that regulate excitation-contraction coupling (EC coupling) also translate into transcriptional gene regulatory events (43). ET coupling represents a potential integrative mechanism whereby short-term regulation of Ca\(^{2+}\) signaling and contraction are transduced into long-term regulation of smooth muscle growth, differentiation, and remodeling. Our study provides novel evidence showing that intracellular signals that regulate short-term contraction also can regulate short-term expression of genes involved in EC coupling, which adds more complexity to the Ca\(^{2+}\) signaling pathway in the control of short- and long-term gene expression.

Two general paradigms in ET coupling in smooth muscle are the dependence on subcellular changes in Ca\(^{2+}\) and the activation of specific transcription factors through different intracellular pathways (43). The underlying Ca\(^{2+}\)-dependent signaling mechanisms involved in TRPC gene transcription include CaMK and calcineurin pathways, since the inhibitors KN-93 and cyclosporine A caused a dramatic reduction in the upregulated TRPC mRNA. The Ca\(^{2+}\)-mediated regulation is specific for the different modes of Ca\(^{2+}\) signaling: Ca\(^{2+}\) influx through L-type Ca\(^{2+}\) channels induces TRPC upregulation by a CaMK-sensitive pathway, whereas intracellular Ca\(^{2+}\) release and store-mediated Ca\(^{2+}\) influx activate TRPC transcription using both CaMK- and calcineurin-dependent pathways. When [Ca\(^{2+}\)]\(_i\) was increased by a standard Ca\(^{2+}\) reentry protocol after store depletion, the TRPC1 and TRPC3 isoforms were upregulated. As demonstrated in the present study, TRPC1 at least seems to be a component of the channels mediating store-operated Ca\(^{2+}\) entry, which shows the presence of a positive feedback mechanism(s) to ensure Ca\(^{2+}\) entry and replenishment of stores.

Among other kinases activated downstream of Ca\(^{2+}\) increases, CaMK have been shown to phosphorylate CREB (8,
vascular smooth muscle, it has been reported that elevation of Ca\(^{2+}\) influx activated by membrane depolarization failed to induce NFAT nuclear translocation, via calcineurin, the transcriptional effector NFAT is provided by Ca\(^{2+}\) influx through SOC (36). In ileal and vascular smooth muscle, it has been reported that elevation of [Ca\(^{2+}\)], by membrane depolarization failed to induce NFAT nuclear accumulation (15, 39). In skeletal myocytes, the ryanodine-sensitive Ca\(^{2+}\) pool is insufficient to maintain NFAT translocation, whereas Ca\(^{2+}\) entering through non-voltage-dependent channels such as TRPC3 is required for NFAT-dependent transactivator function. In addition, expression of TRPC3 is increased in response to activated calcineurin and is limiting to NFAT-dependent transactivation, indicating that TRPC3 channels participate in a positive feedback circuit (35).

As commented in RESULTS, we could not correlate NFAT nuclear translocation with TRPC mRNA upregulation, probably because of nonspecificity of the antibodies used in our tissue.

We do not know whether TRPC-enhanced expression is due to direct effects of the nuclear transcription factors in the TRPC gene promoters or indirectly through changes in intermediate factors as c-fos or c-jun that subsequently upregulate TRPC gene expression. Pulver et al. (32) recently reported that influx of Ca\(^{2+}\), caused by thapsigargin-induced depletion of intracellular stores in vascular smooth muscle, results in transient phosphorylation of CREB, and transcription of c-fos.

In conclusion, we have shown for the first time that cytosolic free Ca\(^{2+}\) controls TRPC expression through activation of CaMK-dependent CREB phosphorylation and calcineurin-dependent pathway. In addition, we have revealed that expression of TRPC proteins involved in CCE operates as a positive feedback mechanism in Ca\(^{2+}\) homeostasis. These findings provide a basis for further studies investigating the peculiar regulation of TRPC gene in mammalian cells.

ACKNOWLEDGMENTS

We thank M. P. Delgado and S. Perez for technical assistance.

GRANTS

This work was supported by the Spanish Ministry of Education and Science grants SAF-2001-0295 and BFU 2004-0637. S. Morales was supported by a Ministry of Education Predoctoral Research Grant.

REFERENCES

11. Dalrymple A, Slater DM, Beech D, Poston L, Tribe RM. Calcium-dependent CREB phosphorylation and calcineurin dependence pathways. In addition, we have revealed that expression of TRPC proteins involved in CCE operates as a positive feedback mechanism in Ca\(^{2+}\) homeostasis. These findings provide a basis for further studies investigating the peculiar regulation of TRPC gene in mammalian cells.

ACKNOWLEDGMENTS

We thank M. P. Delgado and S. Perez for technical assistance.

GRANTS

This work was supported by the Spanish Ministry of Education and Science grants SAF-2001-0295 and BFU 2004-0637. S. Morales was supported by a Ministry of Education Predoctoral Research Grant.