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Short communication

Capacitative Ca^{2+} entries and mRNA expression for TRPC1 and TRPC5 channels in human epidermoid carcinoma A431 cells

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Abstract

In human epidermoid carcinoma A431 cells, capacitative Ca^{2+} entries in response to intracellular Ca^{2+} store depletion with thapsigargin, an endoplasmic reticulum Ca^{2+}-ATPase inhibitor, and uridine 5′-triphosphate, a phospholipase C-linked agonist, were inhibited by trivalent cations such as Gd^{3+} and La^{3+}, and by the store-operated Ca^{2+} channel inhibitor, 2-aminoethoxydiphenyl borate. Of the seven types of canonical transient receptor potential (TRPC) channels as molecular candidates for store-operated Ca^{2+} channels, mRNAs for TRPC1 and TRPC5 were detected in the cells with the reverse transcription-polymerase chain reaction. Western blotting confirmed the protein expressions of TRPC1 and TRPC5 in A431 cells. The present results suggest that TRPC1 and/or TRPC5 channels serve as store-operated Ca^{2+} channels in A431 cells, and may function as regulators for intracellular Ca^{2+} signaling.

Keywords: TRPC1; TRPC5; Capacitative Ca^{2+} entry; Human epidermoid carcinoma A431 cell; Thapsigargin; Uridine 5′-triphosphate

1. Introduction

Intracellular Ca^{2+} is indispensable for the proliferation of various cell types. The cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) is controlled by Ca^{2+} entry pathways in the plasma membrane and Ca^{2+} release from internal Ca^{2+} stores localized in the sarco(endo)plasmic reticulum. There are at least two classes of Ca^{2+}-permeable channels in the plasma membrane: voltage-dependent Ca^{2+} channels (VDCCs) and non-voltage-gated Ca^{2+}-permeable channels, such as store-operated Ca^{2+} channels (SOCs) or receptor-activated cation channels. The latter class of channels are activated by the depletion of intracellular Ca^{2+} stores and evoke capacitative Ca^{2+} entry (Putney et al., 2001).

In previous studies, we found that dihydropyridine Ca^{2+} channel blockers such as amlodipine inhibited the growth of human epidermoid carcinoma A431 cells in vitro (Yoshida et al., 2003; Yoshida et al., 2004), though this cell line lacks dihydropyridine-sensitive VDCCs (Moolenaar et al., 1986). Fluorometric measurements of $[Ca^{2+}]_i$ revealed that amlodipine specifically attenuated the capacitative Ca^{2+} entries elicited by thapsigargin, an endoplasmic reticulum Ca^{2+}-ATPase (Yoshida et al., 2003), and UTP, a phospholipase C (PLC)-linked agonist. Thus, we suggested that the anti-proliferative actions of some dihydropyridine Ca^{2+} channel blockers, such as amlodipine, may be partly mediated by inhibiting capacitative Ca^{2+} entry through SOCs in the plasma membrane of A431 cells (Yoshida et al., 2004).

Although the molecular identity of I_{SOC} channels in A431 cells has not yet been determined, mammalian homologs of the *Drosophila* canonical transient receptor potential (TRPC) channel family have been implicated as molecular candidates for SOCs and receptor-activated cation channels in both excitable and non-excitable tissues (Ma et al., 2000; Vennekens et al., 2002). It is plausible that TRPC channels are expressed in A431 cells and may function as

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SOCs. To explore the possible mechanisms underlying the antiproliferative action of amlodipine, we examined the properties of thapsigargin- and UTP-induced capacitative Ca^{2+} entries by means of single-cell Ca^{2+} imaging and mRNA expression for the seven types of human TRPC (hTRPC1–7) in A431 cells by the reverse transcriptional polymerase chain reaction (RT-PCR).

2. Materials and methods

2.1. Reagents

Thapsigargin was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10 mM. UTP and SKF 96365 (Sigma-Aldrich Co.) were dissolved in distilled water at concentrations of 100 mM and 10 mM, respectively. 2-Aminoethoxydiphenyl borate (2-APB; Tocris Cookson Inc., Ellisville, MO, USA) was dissolved in DMSO (100 mM). Anti-TRPC1 and anti-TRPC5 antibodies were purchased from Alomone Labs Ltd. (Jerusalem, Israel).

2.2. Tumor cell lines

Human epidermoid carcinoma A431 cells, kindly supplied by Professor Katsuzo Nishikawa (The Second Department of Biochemistry, Kanazawa Medical University), were cultured as described previously (Yoshida et al., 2001; Yoshida et al., 2003, 2004). Briefly, the cells were cultured in Dulbecco's modified Eagle medium (DMEM), containing 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 12.7 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 0.12% sodium bicarbonate, 100 U/ml penicillin G and 100 μg/ml streptomycin, at 37 °C in humidified air containing 5% CO_2. Cells were seeded at a density of 3×10^5/plate in 10 cm diameter plastic culture dishes and passaged every 3–4 days.

2.3. Measurement of intracellular free Ca^{2+} concentrations

The microscopic fluorometric measurement of intracellular free Ca^{2+} concentrations ($[Ca^{2+}]_i$) was performed as described previously (Yoshida et al., 2004). Briefly, A431 cells, grown on poly-D-lysine coated glass bottom dishes (MatTek, Ashland, MA, USA), were washed with a HEPES-buffered physiological saline solution (HBSS) (in mM: 130 NaCl, 2.5 KCl, 1.2 $MgCl_2$, 10 HEPES, 10 glucose, and 2 $CaCl_2$, pH adjusted to 7.4 with NaOH) and loaded with 3 μM acetoxymethyl ester of fluo-3 (fluo-3/AM: Dojindo Laboratories, Kumamoto, Japan) containing 0.005% Cremophore EL (Sigma-Aldrich Co.) for 30 min in the dark at room temperature (25±2 °C). Cells were post-incubated in HBSS for 30 min. In the experiment, the cells were washed with Ca^{2+}-free HBSS, and then the dishes were placed on a Nikon inverted microscope (ECLIPSE TE 300, Nikon, Tokyo, Japan) equipped with a Nikon ×40 S-fluor objective. Fluorescence images of the cells were recorded and analyzed with a video image analysis system (ARGUS/HiSCA, Hamamatsu Photonics, Hamamatsu, Japan). The agents in volumes of 20–100 μl were sequentially applied to the cells bathing in 2 ml of recording medium. Image pairs were captured at 10-s intervals. Fluo-3 fluorescence was monitored at an emission wavelength of 527 nm by exciting fluo-3 at 480 nm. Normalized fluorescent intensities of $F480/F480_0$ were used to indicate changes in $[Ca^{2+}]_i$, where $F480_0$ indicates basal fluorescence.

2.4. RNA preparation and RT-PCR

Total RNA was extracted from A431 cells using the ISOGEN reagent (Nippon Gene, Tokyo, Japan). First-strand cDNA was synthesized from the RNA preparations with a ProSTAR™ First-Strand RT-PCR Kit (Stratagene, La Jolla, CA, USA); RNA (10 μg) was reverse transcribed using the oligo(dT) primer (6 ng/μl) in a total volume of 50 μl. To perform PCR amplifications, the following sets of primers were used: TRPC1 (accession no. U31110): forward 5′ CAA GAT TTT GGA AAA TTT CTT G 3′, reverse 5′ TTT GTC TTC ATG ATT TGC TAT 3′ (PCR product, 372 bp); TRPC3 (accession no. Y13758): forward 5′ TGA CTT CCG TTG TGC TCA AAT ATG 3′, reverse 5′ TCT GAA GCC TTC TCC TTC TGC 3′ (PCR product, 315 bp); TRPC4 (accession no. X90697): forward 5′ TCT GCA GAT ATC TCT GGG AAG GAT GC 3′, reverse 5′ AAG CTT TGT TCG AGC AAA TTT CCA TTC 3′ (PCR product, 415 bp); TRPC5 (accession no. AF054568): forward 5′ GTT CCT GTT TCC CAT GCT GT 3′, reverse 5′ AGT GCT TCC GCA ATC AGA GT 3′ (PCR product, 429 bp); TRPC6 (accession no. U49069): forward 5′ GCT CAT CCA AAC TGT CAG CA 3′, reverse 5′ CAG CAT TCC AAA GTC AAG CA 3′ (PCR product, 468 bp); TRPC7 (accession no. AJ272034): forward 5′ GCT GCC TAC TTG TCC CTG TC 3′, reverse 5′ AAA AGG GAG GCC TAT GGA GA 3′ (PCR product, 411 bp); VDCC (accession no. L04569): forward 5′ AGT CCG TCA ACA CCG AAA AC 3′, reverse 5′ CCA GTT GGG CTG GTT GTA GT 3′ (PCR product, 239 bp); VDCC (accession no. U95019): forward 5′ GTA CCT TCC ATG CGA CCA GT 3′, reverse 5′ TCC GCT AAG CTT GAC CTT GT 3′ (PCR product, 227 bp); hGAPDH (accession no. AB062273): forward 5′ CCA CCC ATG GCA AAT TCC ATG GCA 3′, reverse 5′ TCT AGA CGG CAG GTC AGG TCC ACC 3′ (PCR product, 598 bp). All primers were obtained from the Hokkaido System Science Co. Ltd. (Sapporo, Japan). PCR was performed on 1–2 μl cDNA from A431 cells with a HotStarTaq Master Mix Kit (QIAGEN K.K., Tokyo, Japan) in a total volume of 25 μl. Human brain first strand cDNA (Adult male, Stratagene) and cDNA from NCI-H295 human adrenocortical carcinoma cells, prepared using the same procedure for A431 cells, were used as positive control samples. A negative control was included in each PCR reaction whereby cDNA was replaced with ultrapure

water. Each reaction was performed in the presence of 1 μM 5′ and 3′ gene-specific primers. The PCR program consisted of an initial denaturation at 95 °C for 15 min, 35 cycles at 94 °C for 1 min, 60 °C (50 °C for hTRPC1) for 1 min, and 72 °C for 1 min, and a final extension step at 72 °C for 10 min. PCR products were separated by 1.5–2.0% agarose gel electrophoresis and visualized by ethidium bromide staining.

2.5. Western blot analysis

Cultured A431 cells were lysed in 0.5 ml of mammalian cell lysis/extraction reagent (CelLytic™-M; Sigma-Aldrich Co.) containing protease inhibitors (Protease inhibitor cocktail; Sigma-Aldrich Co.), 1 mM phenylmethylsulfonyl fluoride and 1 mM Na_3VO_4 by repeated passage through a 23-gauge needle. The lysate was centrifuged at 12,500×*g* for 15 min at 4 °C to remove insoluble material, and the protein concentration of the supernatant was determined with the Bio-Rad protein assay reagent (Bio-Rad Laboratories, CA, USA). The cell lysate (25 μg protein) was denatured in 5× sample buffer (625 mM Tris–HCl, 10% SDS, 25% glycerol, 0.015% bromophenol blue and 5% 2-mercaptoethanol, pH 6.8) at 95 °C for 5 min, then electrophoretically separated on a 4–20% polyacrylamide gel (PAG Mini "DAIICH" 4/20: DAIICHI Pure Chemical Co., Ltd., Tokyo, Japan), and the protein bands were transferred to a nitrocellulose membrane. The membrane was blocked with phosphate buffered saline (PBS) containing 5% non-fat milk and 0.1% Tween 20, and subsequently probed with polyclonal antibodies to TRPC1 and TRPC5 (1:200; Alomone Labs, Jersalem, Israel) for 40 min at room temperature. Following a wash with PBS containing 0.1% Tween 20, the membrane was incubated with horseradish peroxidase-conjugated anti-rabbit IgG (Santa Cruz Biotechnology, CA, USA) at room temperature for 40 min. Bound horseradish peroxidase was visualized with enhanced chemiluminescence using Western blotting detection reagents (Amersham Biosciences Corp., Piscataway, NJ, USA) and laser densitometry (Molecular Dynamics, Sunnyvale, CA, USA).

3. Results

3.1. Effects of SOC inhibitors on thapsigargin- and UTP-induced Ca^{2+} responses in A431 cells

In the absence of external Ca^{2+}, thapsigargin induced Ca^{2+} release from internal Ca^{2+} stores by inhibiting endoplasmic reticulum Ca^{2+}-ATPase, and induced capacita-

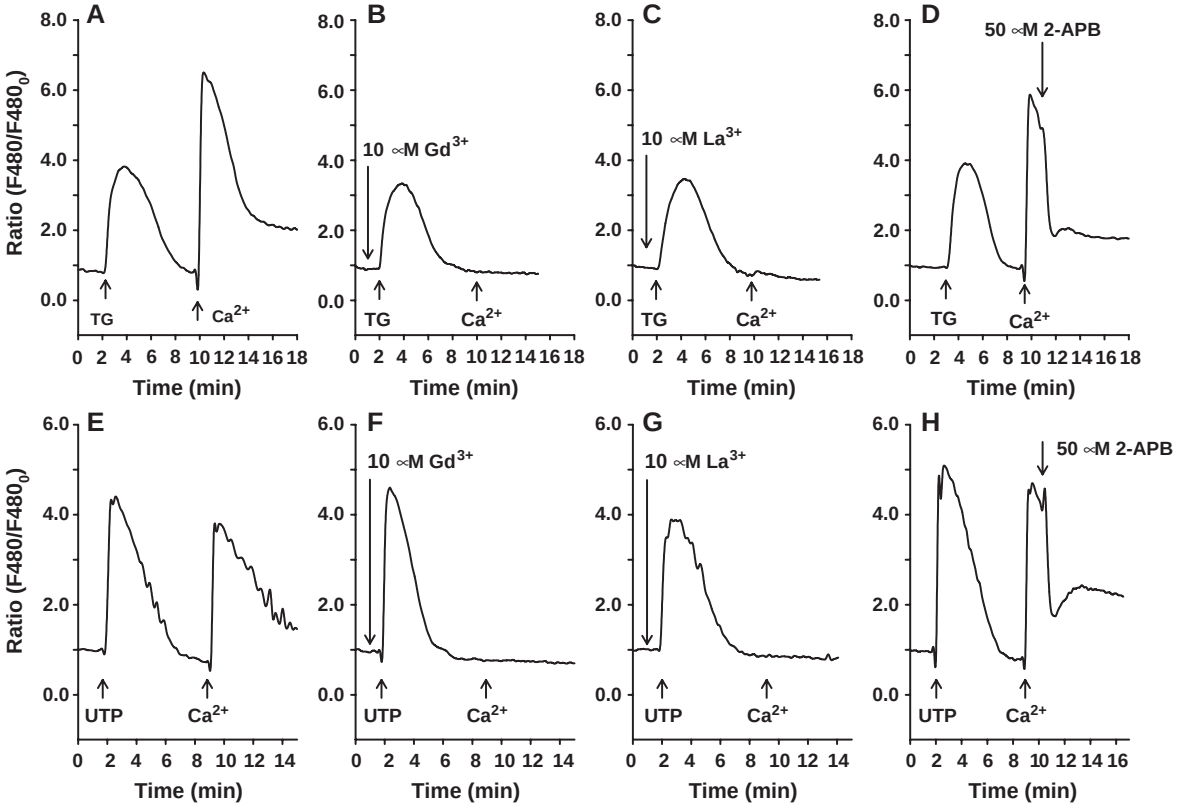

Fig. 1. Effects of trivalent cations and a store-operated Ca^{2+} channel inhibitor on thapsigargin- and UTP-induced Ca^{2+} responses in fluo-3-loaded A431 cells. In the experiment, the medium was changed from HBSS to Ca^{2+}-free HBSS. Test agents were applied as indicated with arrows. After store depletion by thapsigargin (TG, 1 μM, A–D) or UTP (100 μM, E–H), $CaCl_2$ (Ca^{2+}, 2 mM) was added to the medium to initiate capacitative Ca^{2+} entry. Data shown are representative records from at least three experiments. Each trace shown is the mean value of five to seven cells in a field. Ratio (F480/F480$_0$); normalized fluorescent intensity to indicate the changes in $[Ca^{2+}]_i$, where F480$_0$ indicates basal fluorescence.

tive Ca^{2+} entry upon Ca^{2+} addition (Fig. 1A). On the other hand, UTP, a PLC-linked agonist, induced Ca^{2+} release from endoplasmic reticulum in the absence of external Ca^{2+} and then evoked capacitative Ca^{2+} entry upon the addition of external Ca^{2+} (Fig. 1E). Trivalent cations such as Gd^{3+} and La^{3+} (10 μM) completely blocked the capacitative Ca^{2+} entries induced by thapsigargin and UTP, although these cations did not prevent Ca^{2+} release from intracellular Ca^{2+} stores (Fig. 1B, C, F and G). Thapsigargin- and UTP-induced capacitative Ca^{2+} entries into A431 cells were less sensitive to Ni^{2+}, and were inhibited with higher concentrations of Ni^{2+} (>200 μM, data not shown). 2-APB (50 μM), a SOC inhibitor, inhibited capacitative Ca^{2+} entry extremely rapidly when applied after the activation of SOCs with thapsigargin or UTP (Fig. 1D and H). SKF-96365 (25–50 μM), another SOC inhibitor, inhibited the capacitative Ca^{2+} entries induced by both agents, but the potency was modest (data not shown).

3.2. Expression of mRNAs and proteins for TRPC channels

hTRPC1 and hTRPC5 mRNAs were detected in A431 cells, whereas mRNAs for hTRPC1, 3, 4, 5, 6 and 7 were detected in human brain cells as a positive control (Fig. 2A). The human orthologue of the TRPC2 gene, which appears to be a pseudogene, was neither detected in A431 cells nor human brain cells (data not shown). mRNAs for α and β subunits of L-type voltage-dependent Ca^{2+} channels (L-VDCC), marked in human brain cells and NCI-H295 human adrenocortical carcinoma cells (Fig. 2B), were not detected in A431 cells, in accordance with electrophysiological studies revealing the absence of L-VDCC in this cell line (Moolenaar et al., 1986). Western blotting with anti-TRPC1 and TRPC5 antibodies revealed the expression of a protein for TRPC1 and TRPC5 with a molecular weight of around 60 kDa in A431 cells (Fig. 2C, lanes 1 and 3). Bands for TRPC1 and TRPC5 did not appear when antibodies were preincubated with their respective antigens (Fig. 2C, lanes 2 and 4).

4. Discussion

The previous finding that amlodipine, a Ca^{2+} channel blocker, inhibited the growth of A431 cells partly via the attenuation of capacitative Ca^{2+} entry led us to perform experiments to identify the molecular components of SOCs in A431 cells. Mammalian homologs of the *Drosophila* canonical TRPC channel family have been suggested to act as molecular components of SOCs and receptor-activated cation channels in many tissues (Vennekens et al., 2002). In proliferating human pulmonary artery smooth muscle cells, it was shown that TRPC1 and TRPC6 mRNA and protein expressions are upregulated and the amplitude of capacitative Ca^{2+} entry is enhanced (Landsberg and Yuan, 2004). Another study demonstrated that siRNA targeting TRPC4 attenuated ATP-mediated increases in TRPC4 expression, capacitative Ca^{2+} entry amplitude, and ATP-induced proliferation of human pulmonary artery smooth muscle cells (Zhang et al., 2004). Thus,

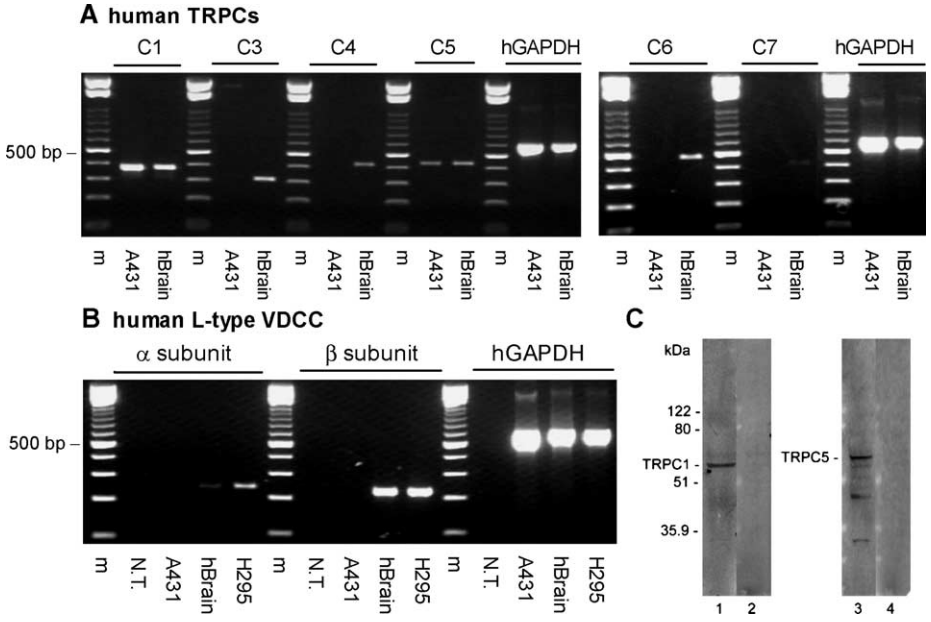

Fig. 2. (A) RT-PCR-amplified products for hTRPC1, hTRPC3–7, and hGAPDH (house keeping gene). hTRPC1 and hTRPC5 mRNAs were detected in A431 cells. All types of hTRPC mRNAs except for that of hTRPC2 were expressed in the human brain (hBrain; Stratagene) as a positive control. (B) RT-PCR-amplified products for α and β subunits of hVDCC. Human brain cDNA (hBrain; Stratagene) and cDNA from human adrenocortical carcinoma cells H295 (H295) were used for positive controls. m; marker, 100 bp ladder. N.T., negative control without template cDNA. (C) Western blots for hTRPC1 and hTRPC5 in A431 cell lysate. The blot was probed with anti-TRPC1 antibody (lane 1), anti-TRPC1 antibody preincubated with the control peptide antigen (lane 2), anti-TRPC5 antibody (lane 3), and anti-TRPC5 antibody preincubated with the control peptide antigen (lane 4).

there is growing evidence to suggest that capacitative Ca^{2+} entry through SOCs is related to cell proliferation in several cell types.

At first, we performed fluorometric Ca^{2+} imaging to elucidate the properties of SOCs in A431 cells in response to store depletion with thapsigargin or UTP. We have already shown that UTP-induced Ca^{2+} responses in A431 cells are mediated via the stimulation of P2 receptors and 1,4,5-triphosphate (IP_3) production by PLC activation, since the responses were inhibited by the putative non-selective P2 receptor antagonist, suramin, and the PLC inhibitor, U73122 (Yoshida et al., 2004). We examined the effects of inorganic inhibitors such as Gd^{3+}, La^{3+}, and Ni^{2+} and organic SOC inhibitors such as 2-APB and SKF-96365 (Clementi and Meldolesi, 1996), on thapsigargin- or UTP-induced Ca^{2+} responses in A431 cells. Both Gd^{3+} and La^{3+}, at concentrations of 10 μM, completely inhibited the capacitative Ca^{2+} entry evoked by thapsigargin or UTP. 2-APB (50 μM), known as an inhibitor of IP_3 receptors and SOC channels (Ma et al., 2000), blocked both agents from inducing capacitative Ca^{2+} entry when applied after the activation of SOCs by Ca^{2+} addition (Fig. 2D and H). Application of 2-APB (>25 μM) itself in Ca^{2+}-free HBSS caused a transient rise in $[Ca^{2+}]_i$ (data not shown), possibly due to its inhibitory action on endoplasmic reticulum Ca^{2+}-ATPase (Bilmen et al., 2002). The above findings clearly indicated the presence of functional SOCs in A431 cells.

To screen human TRPCs (hTRPCs) expressed in A431 cells, we then performed an RT-PCR assay. RT-PCR revealed that mRNAs for hTRPC1 and hTRPC5 are expressed in A431 cells. In Western blot analysis, antibodies to TRPC1 and TRPC5 (Alomone Labs.) detected a protein with an apparent molecular mass of about 60 kDa. This molecular weight is less than the putative molecular masses of 75.9 and 97 kDa for TRPC1 and TRPC5, respectively; however, this band disappeared in both cases following preabsorption with respective specific antigens, suggesting signal specificity. Though the splice variants for human TRPC5 have not been reported, it is possible that A431 cells express a splice variant form of TRPC5. A deletion may occur in the cytoplasmic amino-terminus region encoded by exons 2 and 3, because a PCR-amplified product was undetectable in A431 cells using another set of primers (location of sequence; 879–1785, PCR product; 907 bp) for TRPC5, while a product was detected in the human brain as a positive control (data not shown). It is also possible that the anti-TRPC1 antibody detected a splice variant or truncated form of TRPC1 in A431 cells. Ma et al. (2003), using a mouse polyclonal antibody against the amino-terminus region of hTRPC1, demonstrated that A431 cells express detectable levels of TRPC1 protein. They also suggested that TRPC1 may function as a receptor-operated Ca^{2+} channel through an association with TRPC4 or TRPC5 in the cells after conducting electrophysiological studies (Ma et al., 2003). In this study, we demonstrated the expression of mRNAs and proteins for hTRPC1 and hTRPC5 with functional properties of SOCs in native A431 cells for the first time. Diacylglycerols directly activate hTRPC3 and hTRPC6 (Hofmann et al., 1999) and have no effect on TRPC5 (Venkatachalam et al., 2003). We observed that 1, 2-dioctanoyl-*sn*-glycerol, an analogue of diacylglycerols, did not evoke Ca^{2+} responses in A431 cells (data not shown). This observation appears not to be in conflict with the results from the RT-PCR indicating the mRNA expression of hTRPC5 but not hTRPC3 or hTRPC6 in A431 cells. TRPC1 is widespread and can form heteromultimeric channels with subgroup family members (Vennekens et al., 2002). Indeed, a systematic biochemical study of TRPC interactions in the mammalian brain have shown that TRPC1 forms a heteromer with TRPC4 or TRPC5 (Strübing et al., 2003). It is possible that hTRPC1 is co-expressed with hTRPC5 in heteromultimers and may function as an SOC and/or receptor-activated cation channel in A431 cells. To verify this possibility, gene downregulation studies with antisense oligonucleotides or siRNAs for TRPC1/5 will be needed.

In conclusion, this study revealed that capacitative Ca^{2+} entry in A431 cells in response to store depletion was sensitive to Gd^{3+}, La^{3+} and 2-APB and that A431 cells express mRNAs and proteins for hTRPC1 and C5. These results will be useful to help identify the role of TRPCs in tumor cell proliferation and to clarify mechanism(s) underlying the antiproliferative effect of some dihydropyridine Ca^{2+} channel blockers, such as amlodipine, in A431 cells.

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