Cloning and Functional Expression of a Human Ca^{2+}-Permeable Cation Channel Activated by Calcium Store Depletion

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Summary

Depletion of intracellular calcium stores generates a signal that activates Ca^{2+}-permeable channels in the plasma membrane. We have identified a human cDNA, *TRPC1A*, from a human fetal brain cDNA library. *TRPC1A* is homologous to the cation channels trp and trpl in Drosophila and is a splice variant of the recently identified cDNA *Htrp-1*. Expression of *TRPC1A* in CHO cells induced nonselective cation currents with similar permeabilities for Na^+, Ca^{2+}, and Cs^+. The currents were activated by intracellular infusion of myo inositol-1,4,5-trisphosphate or thapsigargin. Expression of *TRPC1A* significantly enhanced increases in the intracellular free calcium concentration induced by Ca^{2+} restitution after prolonged depletion. Similar results were obtained in Sf9 cells. We conclude that *TRPC1A* encodes a Ca^{2+}-permeable cation channel activated by depletion of intracellular calcium stores.

Introduction

In most cellular systems, the hormonally induced breakdown of phosphoinositol lipids leads via generation of myo inositol-1,4,5-trisphosphate ($InsP_3$) to a biphasic increase in the intracellular free calcium concentration ($[Ca^{2+}]_i$). The first phase is due to binding of $InsP_3$ to its specific receptor located in the membranes of intracellular calcium stores and the subsequent mobilization of calcium from these stores. The second phase is caused by Ca^{2+} influx through the cell membrane (for review see Putney, 1990; Penner et al., 1993; Fasolato et al., 1994; Clapham, 1995; Berridge, 1995). Evidence has been accumulated that this Ca^{2+} influx is activated by an unknown signal generated by the depletion of $InsP_3$-sensitive calcium stores. Such a store-operated Ca^{2+} influx does not necessarily require activation of hormone receptors, but can be triggered by several substances that deplete intracellular calcium stores independently of $InsP_3$. The prototype of these substances is the inhibitor of endoplasmic calcium ATPases, thapsigargin (Thastrup et al., 1990). Store-operated currents in mast cells and in lymphocytes are highly Ca^{2+} selective. They have been named I_{CRAC} (calcium-release activated Ca^{2+} current) (Hoth and Penner, 1992). Noise analysis of these currents reveals exceedingly small current fluctuations, indicating a conductance of single channels in the fS range (Zweifach and Lewis, 1993). Recent studies have demonstrated other store-operated calcium channels with much higher conductance (Lückhoff and Clapham, 1994). Thus, store-operated Ca^{2+} entry seems to utilize related but functionally different pathways.

In photoreceptor cells of Drosophila melanogaster, light induces a stimulation of phospholipase C with subsequent activation of Ca^{2+}-permeable channels in the plasma membrane (for review see Hardie and Minke, 1993; Zucker, 1996). One of these channels is absent in the transient receptor potential (trp) mutant of Drosophila. The cDNA that is deficient in the trp mutant was cloned (Montell and Rubin, 1989). Expression of *trp* in the Sf9–baculovirus system produced currents with high selectivity for Ca^{2+} when intracellular stores were depleted by thapsigargin (Vaca et al., 1994). Expression of the related Drosophila trp-like cDNA, *trpl* (Phillips et al., 1992), yielded currents with equal permeabilities for Ca^{2+}, Na^+, and Ba^{2+} (Vaca et al., 1994). Although trpl was activated by various heptahelical G protein–coupled receptors (Harteneck et al., 1995; Hu and Schilling, 1995), it was not stimulated by depletion of intracellular calcium stores (Vaca et al., 1994). Thus, structurally related cation channels are not necessarily regulated by the same mechanisms.

While this work was in preparation, two groups independently published the sequences of two human *trp* homologs. The homologs were named *TRPC1* (Wes et al., 1995) and *Htrp-1* (Zhu et al., 1995). They have not yet been functionally expressed and characterized. Here, we report the cloning of a human homolog of *trp*, *TRPC1A*, a splice variant of *Htrp-1*, and its expression in three different expression systems. We provide evidence that *TRPC1A* codes for a Ca^{2+}-permeable store-operated cation channel.

Results

Cloning of a Human Homolog of the Drosophila *trp* and *trpl* Genes

On the assumption that mammalian homologs to *trp* and *trpl* genes of Drosophila may exist, we searched among the expressed sequence tags (ESTs) deposited to the GenBank database, using partial amino acid sequences of trp and trpl proteins as query sequences. A human EST (EST05093) was found coding for a peptide sequence with 50% identity to the trp amino acid sequence (Glu-33 to Asn-80) and 48% identity to trpl (Glu-42 to Asn-89). A 194 bp fragment of EST05093 was amplified from a human fetal brain cDNA library by the polymerase chain reaction (PCR) and used as probe to screen the same cDNA library by plaque hybridization. Three cDNA clones were isolated. Two showed a 5.5 kb insert and displayed an equal restriction enzyme pattern. The third clone carried a 2.1 kb cDNA fragment, a partial sequence of the 5.5 kb cDNA. The 5.5 kb cDNA contained a 2277 bp open reading frame coding for 759 amino acids. While this work was in progress, the cDNA sequences of two human homologs of the Drosophila cation channel trp were published, i.e., *TRPC1* (Wes et al., 1995) and *Htrp-1* (Zhu et al., 1995). Both these cDNAs

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TRPC1A   ------------------------------------------------    0
TRPC1    MCPGIPGPRAEAAVGTTHPFSSPGAWLGSGSGSGPVGAPPPSP       43
Htrp-1   ------------------------------------------------    0
trp      ------------------------------------------------    0
trpl     ------------------------------------------------    0

TRPC1A   --------MMAALYPSTDLSGASSSSLPSSPSSSSPNEVMALK       35
TRPC1    GLPPSWAAMMAALYPSTDLSGASSSSLPSSPSSSSPNEVMALK       86
Htrp-1   --------MMAALYPSTDLSGASSSSLPSSPSSSSPNEVMALK       33
trp      -------------------------MGSNTESDAEKALGSRLD       18
trpl     -- -------------MGRKKKLPTGVSSGVSHASSAPKSVGG       27

TRPC1A   DVREVKEENTLN--EKLFLLACDKGDYYMVKKILEENSSGD-L      75
TRPC1    DVREVKEENTLN--EKLFLLACDKGDYYMVKKILEENSSGD-L      126
Htrp-1   DVREVKEENTLN--EKLFLLACDKGDYYMVKKILEENSSGD-L      74
trp      YDLMMAEEYILSDVEKNFILSCERGDLPGVKKILEEYQGTDKF       61
trpl     CCVPLGLPQPLLLEEKKFLLAVERGDMPNVRRTLQKALRHQHI       70

TRPC1A   NINCVDVLGRNAVTITIENENLDILQLLLDYGCQ---------      109
TRPC1    NINCVDVLGRNAVTITIENENLDILQLLLDYGCQ---------      160
Htrp-1   NINCVDVLGRNAVTITIENENLDILQLLLDYGCQSADALLVAI      116
trp      NINCTDPMNRSALISAIENENFDLMVILLEHNIEVGDALLHAI      104
trpl     NINCMDPLGRRALTLAIDNENLEMVELLVVMGVETKDALLHAI      113

TRPC1A   -------------------------KLMERIQNPEYSTTMDVA      127
TRPC1    -------------------------KLMERIQNPEYSTTMDVA      178
Htrp-1   DSEVVGAVDILLNHRPKRSSRPTIVKLMERIQNPEYSTTMDVA      158
trp      SEEYVEAVEELLQWEETNHKEGQP-YSWEAVDRSKSTFTVDIT      146
trpl     NAEFVEAVELLLLEHEELIYKEGEP-YSWQKVDINTAMFAPDIT      155

TRPC1A   FVILAAHRNNYEILTMLLKQDVSLPKPH ]
TRPC1    FVILAAHRNNYEILTMLLKQDVSLPKPH ] last 604 aa not shown
Htrp-1   FVILAAHRNNYEILTMLLKQDVSLPKPH ]
trp      FLILAAHRNNYEILKILLDRGATLPMPH   last 1101 aa not shown
trpl     FLMLAAHKNNFEILRILLDRGAAVPVPH   last 941 aa not shown
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Figure 1. Sequence Alignment

Alignment of amino-terminal amino acid sequences of TRPC1A, TRPC1 (Wes et al., 1995), and Htrp-1 (Zhu et al., 1995) with the Drosophila trp (Montell and Rubin, 1989) and trpl (Phillips et al., 1992) sequences. Numbers of amino acids are given at the right. Highly conserved amino acid residues (aa) are stippled. Sequences of the last 604 amino acid residues are identical in TRPC1A, TRPC1, and Htrp-1.

are identical in large parts, but vary in the amino-terminal part of the deduced amino acid sequences. The cDNA clone reported here is named *TRPC1A*. Its coding region is identical to that of *Htrp-1* but lacks a 102 bp stretch that encodes the amino acids 109–143 of Htrp-1. The reported sequence of TRPC1 contains 51 additional amino acids at the amino terminus. Figure 1 shows an alignment of the amino acid sequences in the amino-terminal region of TRPC1A, TRPC1, Htrp-1, trp, and trpl.

Alignment of the nucleotide sequences of the 5′-noncoding region of *TRPC1A* and *TRPC1* shows identity from nucleotides −1 to −136 of *TRPC1A* (data not shown). At nucleotide −137 of *TRPC1A*, corresponding to nucleotide +17 of *TRPC1*, identity interrupts. The sequence of *TRPC1* upstream to this position is GGA ATTCC. This is the linker sequence used for construction of the cDNA library exploited by Wes et al. (1995) (Stratagene, personal communication). Thus, it is likely that the extension of the amino terminus of TRPC1 protein, as compared with Htrp-1 and TRPC1A, is derived from a cloning artifact during preparation of the cDNA library. The correct start codon for *TRPC1* may be identical to the start codon in *Htrp-1* and *TRPC1A*. We used the same cDNA library as Wes et al. (1995) and found that the clone containing the *TRPC1A* cDNA showed a similar artifact in the 3′-noncoding region of *TRPC1A*. The base pair identity of the 3′-noncoding region of *TRPC1A* and *TRPC1* ends at position 1362 after the stop codon. At this position starts the linker sequence, followed by a sequence unrelated to *TRPC1*.

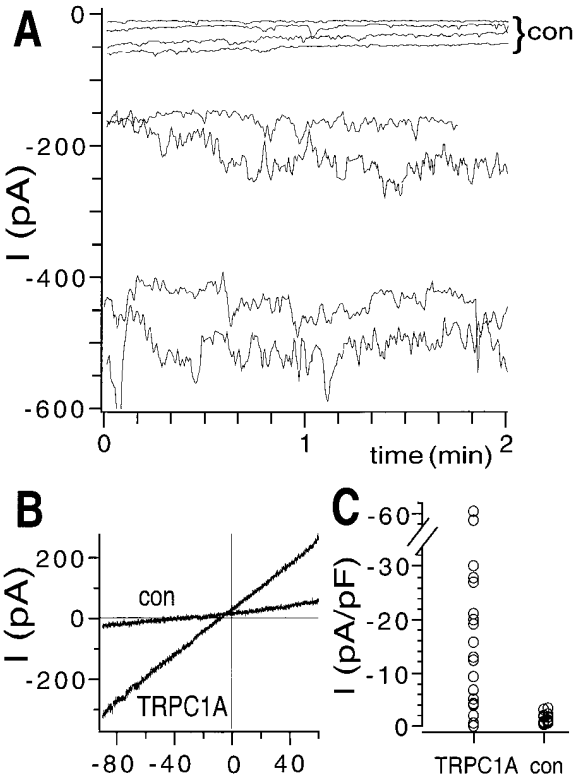

Figure 2. Induction of Currents in CHO Cells by Transient Expression of *TRPC1A*

(A) Inward currents in *TRPC1A*-expressing cells and control cells (con), exposed to Ca^{2+}-free bath solution for 10–30 min. The holding potential was −70 mV. The traces are from four consecutive experiments with cells from each group. The pipette solution was supplemented with 10 μM $InsP_3$.

(B) Current–voltage relation of whole-cell currents in a *TRPC1A*-expressing cell and a control cell (con), as obtained during voltage ramps from −90 to +60 mV.

(C) Summary of all experiments performed in control (con) (n = 15) and *TRPC1A*-injected (n = 22) cells. Currents were measured at −70 mV, 90 s after obtaining the whole-cell configuration, and are expressed in relation to the membrane capacity of each cell (7–22 pF).

Biophysical Properties of TRPC1A Currents in CHO Cells

For transient expression of *TRPC1A*, we microinjected eukaryotic expression plasmids carrying *TRPC1A* cDNA into nuclei of Chinese hamster ovary (CHO) cells. Two days after injection, these cells were tested for the presence of cation currents. When the cells were kept in a Ca^{2+}-free bath solution, they exhibited inward currents (Figure 2A) at negative holding potentials with a linear current–voltage relation (Figure 2B). These currents were significantly ($p = 0.003$, Mann–Whitney rank sum test) larger than the currents in control cells injected with plasmid without insert (Figures 2A and 2C). Control cells produced currents smaller than 4.0 pA/pF (n = 15). Currents in 16 out of 22 *TRPC1A*-injected cells exceeded this value (median 9.4 pA/pF).

To define the permeability of the TRPC1A-related conductance to various ions, we measured whole-cell currents during voltage ramps (Figure 3A) at various extra-

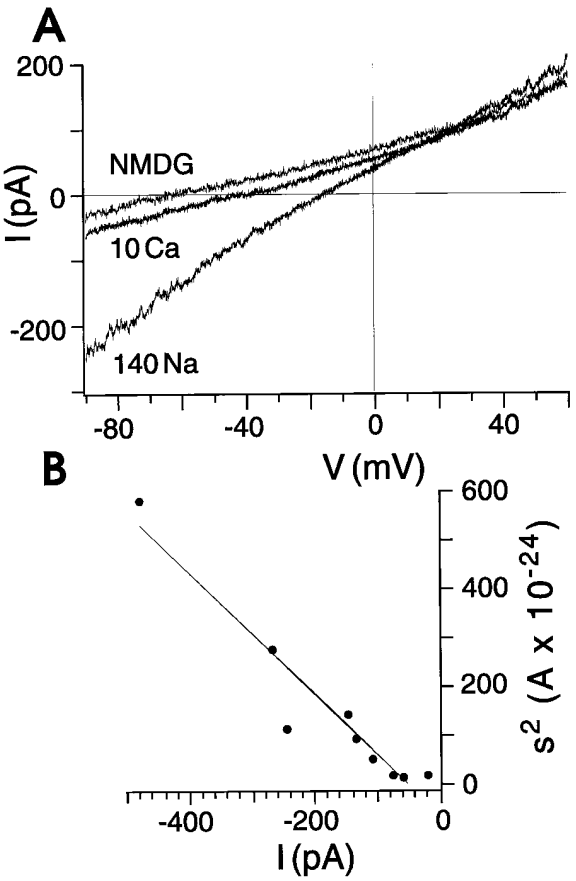

Figure 3. Biophysical Properties of TRPC1A Currents

(A) Current–voltage relation of TRPC1A currents in the presence of various cations. In one *TRPC1A*-expressing cell, voltage ramps from −90 to +60 mV were applied over 450 ms. One ramp was obtained in Ca^{2+}-free solution (140 mM NaCl), one in a solution with 10 mM $CaCl_2$, 0 mM Na^+ and one in a solution containing NMDG as main cation.

(B) Noise analysis of TRPC1A currents. The current amplitudes in several cells at −70 mV are plotted against the variance (s^2) of the amplitude. The rightmost point derives from a control cell. From the regression line, it is predicted (Neher and Stevens, 1977) that the current through single channels has an amplitude of 1.1 pA.

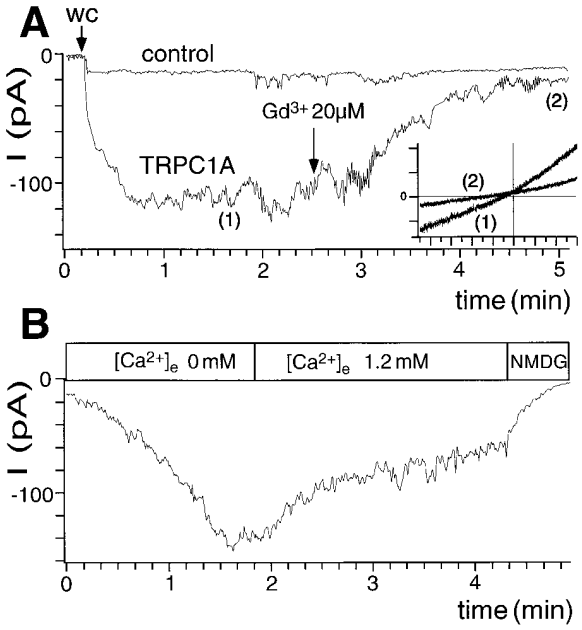

Figure 4. Regulation of TRPC1A Currents

(A) Time course of inward current (at −70 mV) in a CHO cell stably expressing *TRPC1A* and in a control cell during infusion of $InsP_3$ via the patch pipette (10 μM). The whole-cell configuration was obtained at time 15 s (wc, arrow). Gd^{3+} (20 μM) was added to the bath of the TRPC1A cell at time 2.5 min (arrow). The Gd^{3+} concentration was increased to 100 μM at time 4.25 min. The insert shows voltage ramps from −90 to +60 mV (range of ordinate −150 to 220 pA) obtained from the TRPC1A cell prior to (1) and after (2) addition of Gd^{3+}.

(B) Inhibition of TRPC1A current by external Ca^{2+}. A CHO cell stably expressing *TRPC1A* was infused with $InsP_3$. At the time indicated by the bar, the Ca^{2+} concentration in the bath was changed from 0 to 1.2 mM (standard bath solution without EGTA). The bath was changed to Na^+-free NMDG solution at time 4.25 min.

cellular concentrations of Na^+ and Ca^{2+}. Raising the Ca^{2+} concentration in the bath from 0 to 1.2 or 10 mM left the reversal potential (E_{rev}) of whole-cell currents within ±5 mV (n = 3). Substitution of all external Na^+ with 10 mM Ca^{2+} induced a shift of E_{rev} to the left by 19–31 mV (n = 4). A further shift to the left was produced by omitting Ca^{2+} as well (Figure 3A). Thus, TRPC1A supports Na^+ as well as Ca^{2+} currents. Since the control experiments (see Figure 2) were performed in the absence of extracellular Ca^{2+}, we had to exclude that the currents carried by Ca^{2+} might represent an TRPC1A-independent, store-operated Ca^{2+}-selective entry pathway native to CHO cells, such as I_{CRAC} in mast cells (Hoth and Penner, 1992). Therefore, we added Ca^{2+} (10 mM) to control cells kept in a Ca^{2+}-free bath for 10 min and infused with $InsP_3$ (10 μM) via the pipette. No Ca^{2+}-selective currents were observed under these conditions (n = 4; data not shown).

Currents in *TRPC1A*-injected cells were blocked by Gd^{3+} (20 μM), and the Gd^{3+}-sensitive part of the currents reversed close to 0 mV (data not shown for *TRPC1A*-injected cells, but see Figure 4). Taken together, these data characterize TRPC1A currents as cation currents with roughly equal permeability for Na^+, Cs^+, and Ca^{2+} that carry mostly Na^+ but also Ca^{2+} under physiologic ion conditions.

As a test whether TRPC1A currents behave as typical currents through ion channels and to estimate the conductance of these channels, we performed noise analysis of the currents (Neher and Stevens, 1977). The relation between the currents and their variances was roughly linear (Figure 3B). The slope of this relation indicates a single channel amplitude of 1.1 pA at −70 mV. Assuming a linear amplitude–voltage relation, the predicted single channel conductance is 16 pS.

Regulation of TRPC1A Currents in CHO Cells

The regulation of TRPC1A currents was studied in CHO cells stably expressing *TRPC1A*. Cells were kept in Ca^{2+}-free solution and intracellularly infused with a solution containing EGTA (10 mM) and either $InsP_3$ (10 μM) or

thapsigargin (3 μM). Mock-transfected control cells kept under these conditions did not exhibit currents at −70 mV with an either Gd^{3+}-sensitive or Na^+ removal–sensitive part exceeding 40 pA or 4 pA/pF (n = 20). When CHO cells expressing *TRPC1A* were infused with $InsP_3$, they showed substantially higher currents in 9 out of 15 cells (median of all experiments 6.2 pA, range 1.2 to 18.4 pA/pF, p = 0.011). In most cells (n = 7), these currents were not present right from the beginning of the current recording but developed over 10 s to 5 min (Figures 4A and 4B). The currents were inhibited by Gd^{3+} (20–100 μM, Figure 4A). The inhibition was quantitatively the same with both concentrations of Gd^{3+}, but it took place over 1 min with the lower concentration (see Figure 4A) and within 10 s with the higher concentration (data not shown). Substitution of external Na^+ with N-methyl-D-glucamine (NMDG) inhibited the inward component of the currents (Figure 4B). Raising the extracellular Ca^{2+} concentration to 1.2 mM induced a slow decrease in $InsP_3$-induced currents (Figure 4B). The mean inhibition was 32% ± 9.4% (mean ± SEM, n = 6) after 2 min. This inhibition affected both, the inward as well as the outward component of the currents, with no noticeable shift in the reversal potential.

Intracellular infusion of thapsigargin (3 μM) induced currents in 6 out of 9 cells (median of all experiments 9.0 pA/pF; range 1.2 to 32 pA/pF, p = 0.001). In contrast, currents exceeding the range of control cells were observed only in 1 out of 6 *TRPC1A*-expressing cells when kept in the whole-cell configuration for 5 min with neither $InsP_3$ nor thapsigargin in the pipette solution. Thus, currents in *TRPC1A*-expressing cells but not in control cells were induced by $InsP_3$ as well as thapsigargin. These findings characterize TRPC1A as a store-operated channel.

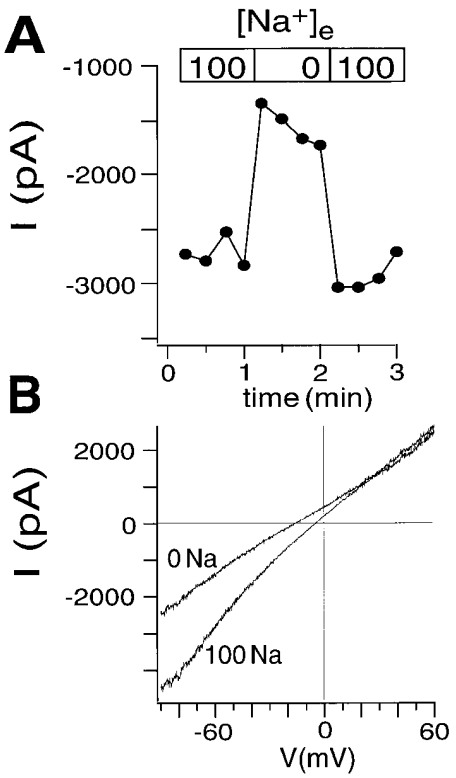

Figure 5. TRPC1A Currents in Insect Sf9 Cells

(A) Currents (at −60 mV) in an Sf9 cell infected with recombinant baculovirus carrying *TRPC1A*. Extracellular Na^+ was completely removed in exchange for NMDG during the time indicated by the bar. The holding potential was 0 mV; a ramp from −90 to +60 mV was applied every 15 s. Measurements were started 1 min after obtaining the whole-cell configuration.

(B) Currents in the same cell during one ramp in the presence and one in the absence of extracellular Na^+.

TRPC1A Currents in Sf9 Cells

TRPC1A was also expressed in insect Sf9 cells by infection with recombinant baculoviruses carrying *TRPC1A* cDNA. Current messurements were performed 35–45 hr after infection, in Ca^{2+}-free bath solution with $InsP_3$ (10 μM) in the pipette solution. These cells exhibited NMDG-sensitive cation currents at −60 mV (Figures 5A and 5B) that exceeded the range of those in control cells (infected with baculovirus carrying histamine H_1 receptor cDNA; n = 11) in 5 out of 17 experiments. Since infected cells showed large leak currents (Figure 5B), no further biophysical analysis of these currents was attempted.

Enhanced Calcium Entry after Expression of *TRPC1A* in CHO Cells, Sf9 Cells, and Xenopus Oocytes

The expression of store-operated Ca^{2+}-permeable cation channels should enhance the increase in $[Ca^{2+}]_i$ in response to resubstitution of Ca^{2+} after Ca^{2+} depletion. This was tested in three different cell types expressing *TRPC1A*, i.e., CHO cells, Sf9 cells, and Xenopus laevis oocytes. CHO cells were transfected with either the plasmid pcDNA3, resulting in control clones, or with pcDNA3 carrying the *TRPC1A* cDNA. Cells stably expressing *TRPC1A* were selected by addition of geneticin. To select cells with highest expression of *TRPC1A*, we subcloned the stably expressing cells by several rounds of dilution. In all subclones, the basal level of $[Ca^{2+}]_i$, as measured with fura-2 (Grynkiewicz et al., 1985) in the presence of 1 mM external Ca^{2+}, was not different from $[Ca^{2+}]_i$ in control cells. Then, different subclones were tested for calcium influx induced by depletion of intracellular calcium stores. Cells were exposed to Ca^{2+}-free solution containing EGTA (2 mM) and thapsigargin (5 μM) for 30 min prior to readdition of Ca^{2+} (8 mM) to the bath. Figure 6 shows the results from two representative subclones of CHO cells stably expressing *TRPC1A*. Subclone 1 (SC1, Figure 6) exhibited a slow increase in $[Ca^{2+}]_i$, larger than but kinetically similar to that in control cells. In contrast, subclone 2 (SC2, Figure 6) displayed a fast increase in $[Ca^{2+}]_i$, resulting in peak $[Ca^{2+}]_i$ values three times higher than control. However, $[Ca^{2+}]_i$ in this clone gradually returned to control levels within 1 min. In spite of kinetic differences, peak levels of $[Ca^{2+}]_i$ in both subclones expressing *TRPC1A* were significantly larger than those in control cells (p < 0.001, n = 13 experiments, Wilcoxon test for paired data).

Similar results were obtained with Sf9 cells (Figure 7). Calcium store depletion was performed by keeping the cells in Ca^{2+}-free buffer (4.5 mM EGTA) containing thapsigargin (0.2 μM) for 10 min. In cells expressing *TRPC1A*,

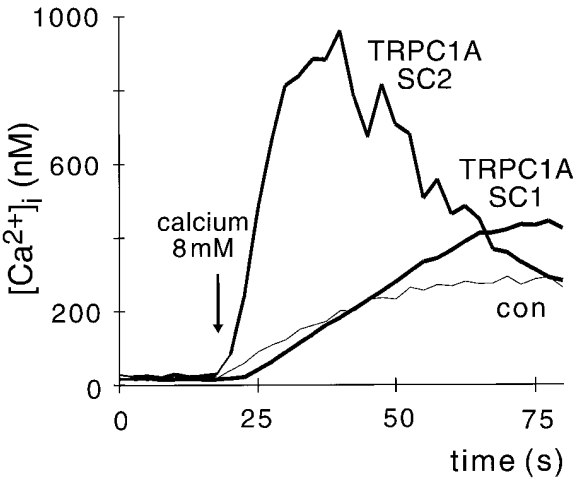

Figure 6. Effects of TRPC1A on $[Ca^{2+}]_i$ in CHO Cells

Cells were kept in a Ca^{2+}-free buffer containing thapsigargin before extracellular Ca^{2+} was resubstituted by addition of $CaCl_2$ (arrow). Comparison of two different subclones (SC1 and SC2) of CHO cells stably expressing *TRPC1A* with mock-transfected CHO cells (con). Each trace represents the mean of 12 (SC1 and SC2) or 18 (con) cells obtained during one experiment.

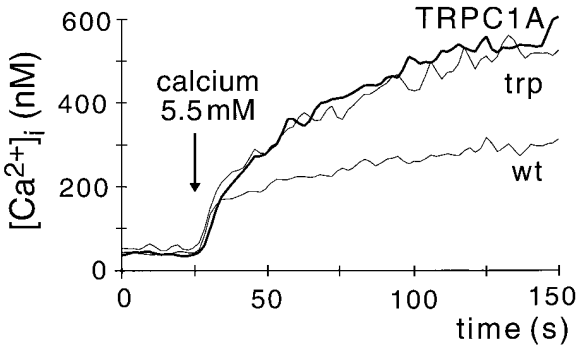

Figure 7. Effects of TRPC1A on $[Ca^{2+}]_i$ in Sf9 Cells

Cells were kept in a Ca^{2+}-free buffer containing thapsigargin before extracellular Ca^{2+} was resubstituted by addition of $CaCl_2$ (arrow). Comparison of Sf9 cells infected with wild-type baculoviruses (wt) with cells infected with recombinant baculoviruses containing the cDNAs from either *TRPC1A* or Drosophila *trp* (representative tracings from one experiment).

Ca^{2+} restitution induced an increase 1.56 ± 0.11 (n = 12 measurements in nine independent infections) of that in controls (cells infected with wild-type virus, $p < 0.001$). In comparison, this increase was 1.46 ± 0.14 (n = 12) of control in cells expressing trp ($p < 0.001$).

Effects of TRPC1A on $[Ca^{2+}]_i$ in Xenopus oocytes were assessed from Ca^{2+}-dependent Cl^- currents with the protocol of Petersen et al. (1995). Oocytes were kept in Ca^{2+}-free solution containing thapsigargin (1 μM) for 3–5 hr before readdition of Ca^{2+}. The resulting Ca^{2+}-dependent Cl^- currents were significantly larger in oocytes injected with *TRPC1A* mRNA (~5 ng, 24–48 hr before measurements) than in oocytes injected with serotonin 5-HT_{2A} receptor mRNA as control (data not shown). The Cl^- currents were 1.02 ± 0.07 μA (TRPC1A, n = 36) versus 0.77 ± 0.06 μA (control, n = 34) in one batch of cells ($p = 0.014$, t test) and 2.65 ± 0.25 μA (n = 24) versus 1.92 ± 0.17 μA (n = 24) in another batch ($p = 0.04$).

Discussion

Whereas previous studies reported only sequence data of *trp* homologs, we were able to express *TRPC1A* functionally. Two days after intranuclear injection of *TRPC1A* cDNA, CHO cells exhibited nonselective cation currents that could be carried by Na^+, Cs^+, and Ca^{2+}. Noise analysis provided evidence that the currents passed through channels with a single channel conductance of about 16 pS. In parallel, we studied the regulation of TRPC1A currents in stably expressing CHO cells. The advantage of these cells was that they were available in large numbers, although the observed currents were smaller in amplitude and did not appear quite as consistently as in the injected cells. Currents were induced by intracellular infusion of either $InsP_3$ or thapsigargin. The findings with thapsigargin make it unlikely that TRPC1A is directly activated by $InsP_3$ or by a metabolite of $InsP_3$. Furthermore, activation of the currents cannot be attributed to an increase in $[Ca^{2+}]_i$, since we used a high concentration of EGTA in the pipette and since addition of extracellular Ca^{2+} decreased the currents (see Figure 4B). The common feature of the effects of $InsP_3$ and thapsigargin is the depletion of intracellular calcium stores (Thastrup et al., 1990). Thus, our experiments provide evidence that TRPC1A is a store-operated cation channel.

However, it is notoriously difficult to decide whether the expressed product of a cDNA constitutes an ion channel or a channel subunit. The amino acid sequence of TRPC1A predicts a protein with at least six transmembrane spanning regions (see discussion in Zhu et al., 1995). Furthermore, the present study shows that expression of *TRPC1A* induced cation currents carried by Na^+ in the mammalian CHO cells as well as in the insect Sf9 cells. Unfortunately, the currents in Sf9 cells could not be better characterized. Thus, we could not address the question whether the currents in both systems are identical with respect to their biophysical properties, which would argue against the idea that TRPC1A requires other subunits to form a functional cation channel.

Our conclusion that expression of *TRPC1A* confers a store-operated Ca^{2+} entry pathway is supported by our measurements of increases in $[Ca^{2+}]_i$ in response to restitution of Ca^{2+} after Ca^{2+} depletion. This was done in three different expression systems: CHO cells, Sf9 cells, and Xenopus oocytes. Expression of *TRPC1A* produced results similar to those of expression of the store-operated channel trp in either Sf9 cells (Vaca et al., 1994) or in Xenopus oocytes (Petersen et al., 1995). In two subclones of stably expressing CHO cells, different kinetics of the increase in $[Ca^{2+}]_i$ were observed. Interestingly, fast and strong initial rises in $[Ca^{2+}]_i$ were followed by a marked decline, whereas only cells with a slow initial response reached a higher plateau than control. We suggest that the initial response rate reflects the amount of *TRPC1A* expression. We interpret the subsequent decline of $[Ca^{2+}]_i$ as a Ca^{2+}-induced inhibition of

Ca^{2+} entry, in line with the observation that Ca^{2+} inhibits currents through TRPC1A. Ca^{2+}-induced inhibition is a general characteristic of store-operated Ca^{2+} entry (Berridge, 1995).

The amino acid sequence of TRPC1A is about 38% identical to the sequences of trp and trpl. Functionally, TRPC1A shares properties of both its insect homologs. In terms of regulation, it resembles trp because it is activated by depletion of intracellular calcium stores. However, with respect to ion selectivity, it is closer to trpl that is also a fairly nonselective cation channel, although the estimated single channel conductance of TRPC1A (16 pS) is almost one order of magnitude below that of trpl (110 pS), as assessed from a similar noise analysis as in the present study and confirmed in single-channel studies (Obukhov et al., submitted).

In conclusion, expression of *TRPC1A* increased Ca^{2+} depletion–induced Ca^{2+} entry in three different expression systems. TRPC1A currents were activated by thapsigargin and by $InsP_3$. Thus, TRPC1A is capable of mediating capacitative Ca^{2+} entry (Putney, 1990; Berridge, 1995). Widespread expression of *Htrp-1* and/or its splice variants has been reported (Zhu et al., 1995). Therefore, TRPC1A is likely to play a general role in the calcium homeostasis of the organism. On the other hand, ion selectivity and single channel conductance of TRPC1A are quite different from those of other mammalian store-operated Ca^{2+} entry pathways (Hoth and Penner, 1992; Zweifach and Lewis, 1993; Lückhoff and Clapham, 1994) as well as from those of their insect homologs trp and trpl (Vaca et al., 1994). Therefore, TRPC1A cannot be the only pathway for capacitative Ca^{2+} entry, but may be the first characterized mammalian member of a family of genes coding for Ca^{2+}-permeable channels responsible for refilling of intracellular calcium stores.

Experimental Procedures

Database Searches

Database searches and sequence similarity analyses were performed using the Heidelberg Unix Sequence Analysis Resources (HUSAR) of the Deutsches Krebsforschungszentrum (DKFZ), which is based on the Wisconsin Sequence Analysis Package from the Genetics Computer Group (GCG). The TFASTA program, which is performing a Lipman and Pearson search, was used to look for related amino acid sequences to different parts of trp and trpl proteins of Drosophila in the database of expressed sequence tags. Before starting the search, TFASTA translates the DNA sequences of the database in all six frames into amino acid sequences.

Isolation of cDNA Clones

The specific oligodesoxynucleotides 5′-TCGCCGAACGAGGTGA TGG-3′ (corresponding to nucleotides 476–494 of *TRPC1A* cDNA) and 5′-GTTATGGTAACAGCATTTCTCC-3′ (corresponding to nucleotides 658–669 of *TRPC1A* cDNA) were used to amplify a 194 bp fragment of the expressed sequence tag (EST05093) by PCR. PCRs were performed by using 0.4 μM of each primer, 50 mM KCl, 1.25 mM $MgCl_2$, 10 mM Tris–HCl (pH 8.3), and 1.25 U of Taq polymerase (Perkin–Elmer, Überlingen, Federal Republic of Germany). As template, we used 1×10^7 plaque forming units (pfu) of the λZAP human fetal brain cDNA library (Stratagene, La Jolla, CA). The resulting amplificate was subcloned and used as a probe for subsequent screening of the human fetal brain cDNA library. We plated 1×10^6 pfu as described by the manufacturer. Filter lifts were performed using reinforced nitrocellulose membrane (Schleicher und Schuell, Dassel, Federal Republic of Germany). Hybridization was carried out in a shaking air incubator at 60°C overnight in 6× SET (1× SET: 150 mM NaCl, 1 mM EDTA, 2.5 mM Tris–HCl [pH 7.4]) containing 1% sodium dodecyl sulfate (SDS) overnight. The filters were washed at 60°C with 1× SET, 0.1% SDS. Sequences of the isolated cDNAs were determined by the dideoxynucleotide termination method.

Subcloning of *TRPC1A* into Various Expression Plasmids

The entire 5.5 kb insert carrying the *TRPC1A* cDNA was cloned into the eukaryotic expression plasmid pcDNA3 (Invitrogen, Leek, Netherlands) by use of the KpnI and XbaI sites on the multiple cloning sites of pBluescript SK(−) and pcDNA3. The 2451 bp Bsp120I–SspI cDNA fragment containing the entire 2277 bp coding region of *TRPC1A* was cloned to the baculovirus transfer vector pVL1392 (Invitrogen) using the NotI and SmaI sites of the vector. The SmaI cDNA fragment missing the first 175 bp of the 5′-noncoding region of the cDNA was cloned into the plasmid pSP64T modified by Krieg and Melton (1984) using the BglII site of the plasmid after producing blunt ends.

Cell Culture and Generation of Stable Cell Lines

CHO-K1 cells were cultured in Ham's F12 medium supplemented with 200 U/ml penicillin, 200 μg/ml streptomycin, and 4 mM glutamine. For stable expression of *TRPC1A*, CHO cells were transfected with either pcDNA3, resulting in control clones, or with pcDNA3 carrying the 5.5 kb of *TRPC1A* cDNA. Cells stably expressing *TRPC1A* were selected with 500 μg/ml geniticin (GIBCO BRL, Eggenstein, Federal Republic of Germany).

Expression of *TRPC1A* in the Sf9–Baculovirus System

Monolayer fall armyworm cells (Sf9 from ATCC, Rockville, MD) were propagated in TMN-FH medium (Sigma, Deisenhofen, Federal Republic of Germany) supplemented with 10% (v/v) fetal calf serum. When cells were grown in suspension, lipid concentrate (1:100, Life Technologies, Karlsruhe, Federal Republic of Germany) was added. cDNAs encoding the guinea pig histamine H_1 receptor, the Drosophila trp channel, and the TRPC1A protein were subcloned to baculovirus transfer plasmid (pVL1392 from Invitrogen). Recombinant viruses were generated by cotransfection of Sf9 cells with the cDNA constructs and BaculoGOLD baculovirus DNA (Dianova, Hamburg, Federal Republic of Germany) by lipofection. The subsequent amplification procedure were performed according to standard techniques (O'Reilly et al., 1992). Cells were infected at a multiplicity of infection of 5 to 7.5.

Microinjection of Expression Plasmids

CHO cells (subclone CHO-K1) were seeded at a density of about 10^3 cells per mm^2 on coverslips imprinted with squares for localization of injected cells. Intranuclear microinjection with the eukaryotic expression plasmid pcDNA3 (control) or with plasmids carrying the 5.5 kb *TRPC1A* cDNA insert was performed with a manual injection system (Eppendorf, Hamburg, Federal Republic of Germany). The injection solution contained 0.3 μg/μl plasmid in water. Approximately 10–20 fl were injected with commercially available microcapillaries (Femtotips, Eppendorf) with an outlet diameter of 0.5 μm. The pressure was 20–40 hPa, and the injection time was 0.3 s. After injection, the cells were kept in culture medium for 40–48 hr.

In Vitro Transcription and Microinjection of Xenopus Oocytes

The cDNAs of *TRPC1A* and the 5-HT_{2A} receptor were subcloned to the plasmide pSP64T modified by Krieg and Melton (1984). We linearized 1 μg of each construct by the restriction endonuclease XbaI and used this as template to produce capped RNA by in vitro transcription with the mMESSAGE mMACHINE SP6 Kit (Ambion, Austin, TX). RNA was diluted by 1:10 with diethyl pyrocarbonate (DEPC)-treated water, and 50 nl was injected per oocyte.

Electrophysiology

Currents in CHO and Sf9 cells were measured with the patch-clamp technique in the whole-cell mode (Hamill et al., 1981). For CHO cells, the intracellular solution contained 140 mM CsCl, 2 mM $MgCl_2$, 10 mM EGTA, 0.3 mM ATP, 0.03 mM GTP, 10 mM HEPES (pH 7.2). The standard bath solution contained 140 mM NaCl, 1.2 mM $MgCl_2$, 1.2 mM $CaCl_2$, 10 mM EGTA, 10 mM glucose, 11.5 mM HEPES (pH 7.4).

The solution labeled "10 Ca" in Figure 3A contained 125 mM NMDG, 10 mM $CaCl_2$ instead of NaCl. The "NMDG" solution in Figure 3A contained 140 mM NMDG instead of NaCl. For noise analysis, currents were filtered at 1 kHz. Mean and variance were calculated from continuous traces of 200 or 400 ms. Pipettes were coated with Sylgard in experiments used for noise analysis. For measurements in Sf9 cells, the pipette solution contained 120 mM CsCl, 1 mM $MgCl_2$, 10 mM EGTA, 30 mM glucose, 30 mM mannitol, 10 mM PIPES (pH 6.5). The bath solution contained 100 mM NaCl, 1 mM $MgCl_2$, 1 mM EGTA, 70 mM sucrose, 50 mM mannitol, 0.02 mM $InsP_3$, 10 mM PIPES (pH 6.5). The access resistance was less than 10 MΩ. Series resistance compensation was set to 50%–70%. Ca^{2+}-dependent chloride currents in Xenopus oocytes were measured in two-electrode voltage clamp at −60 mV two days after injection. The electrodes were filled with 3 M KCl. The extracellular solution contained 115 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 10 mM HEPES (pH 7.4). To deplete intracellular calcium stores, oocytes were incubated for 3 hr in the extracellular solution supplemented with 1 μM thapsigargin and 1 mM EGTA. Chloride currents were induced by addition of 2 mM $CaCl_2$. All experiments were performed at room temperature (19°C–23°C).

Measurement of $[Ca^{2+}]_i$ in CHO Cells

Measurement of $[Ca^{2+}]_i$ in single CHO cells loaded with fura-2/acetoxymethylester (fura-2) (MoBiTec, Göttingen, Federal Republic of Germany) was performed as described (Dippel et al., 1996) with a digital imaging system (T. I. L. L. Photonics, München, Federal Republic of Germany). CHO cells were loaded with 2 μM fura-2 for 60 min at 37°C in a buffer consisting of 138 mM NaCl, 6 mM KCl, 0.1 mM $CaCl_2$, 1 mM $MgSO_4$, 1 mM Na_2HPO_4, 5 mM $NaHCO_3$, 5.5 mM glucose, 20 mM HEPES (pH 7.4), substituted with 0.1% (w/v) BSA. Cells were washed twice with 2 ml of a buffer consisting of 138 mM NaCl, 6 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5.5 mM glucose, 20 mM HEPES (pH 7.4). Before starting the measurements, cells were overlayed for 30 min with 300 μl buffer containing 138 mM NaCl, 6 mM KCl, 1 mM $MgCl_2$, 2 mM EGTA, 5.5 mM glucose, 0.005 mM thapsigargin, 20 mM HEPES (pH 7.4). For each single cell measured, F_{max} and F_{min} were determined by subsequent addition of the wash buffer described above containing 10 μM ionomycin plus 10 mM $CaCl_2$ and the same buffer containing 10 mM EGTA, respectively. Images were digitized and analyzed by the software Fucal 5.14 (T. I. L. L. Photonics). Ratio images were generated in 2.5 s intervals. For background compensation, illumination of an area containing no cells was subtracted. For each cell, $[Ca^{2+}]_i$ was averaged from pixels within manually outlined areas. The significance of the results was determined using Student's t tests.

Measurement of $[Ca^{2+}]_i$ in Sf9 Cells

$[Ca^{2+}]_i$ in suspensions of Sf9 cells was measured with fura-2 as described by Hu et al. (1994) in a Perkin–Elmer luminescence spectrometer LS 50B. Sf9 cells were grown in spinner cultures and loaded with fura-2 by incubation in fura-2 (2 μM in the presence of 10 mM Ca^{2+}) 38–46 hr after infection. For details see Harteneck et al. (1995). Cells were incubated in buffer containing 4.5 mM EGTA and 200 nM thapsigargin for 10 min before starting the measurements.

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GenBank Accession Number

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