BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **240,** 167–172 (1997)
ARTICLE NO. RC977528

Expression and Characterization of a *trpl* Homolog from Rat

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Received September 19, 1997

Mammalian homologues of the Drosophila *trp/trpl*-gene-family code for "Ca^{2+}-store-operated" channels. Here we describe the cloning and expression of a *trp/trpl* homologous gene from rat brain. The clone is named *Rtrp3* because of its high homology to the recently described *Htrp3* (Zhu et al (1996) Cell 85:661-671). Expression of *Rtrp3* in the mammalian COS-1 cell line leads to 100 % increase of capacitative Ca^{2+} entry as compared to the controls. This capacitative calcium entry can be completely blocked with La^{3+} (10 μM). We conclude that *Rtrp3,* a new member of the growing family of *trp/trpl* homologues in mammalians, is involved in "Ca^{2+}-*store-operated*" Ca^{2+} entry into the cell.

In a variety of cells hormonal stimulation leads to breakdown of phosphatidyl-inositol 4,5 bisphosphate to inositol 1,4,5 trisphosphate (IP_3) and diacylglycerol (DAG) followed by a biphasic increase in the intracellular cytosolic free Ca^{2+} concentration. The initial spike due to IP_3-induced release of Ca^{2+} from intracellular stores is followed by a sustained Ca^{2+} plateau caused by Ca^{2+} influx into the cell from the outside. Putney et al. (1, 2) have proposed that depletion of IP_3-sensitive Ca^{2+}-stores activates an influx-pathway for Ca^{2+}. Although the mechanism how the signal from the depleted Ca^{2+} pool is transferred to the plasma membrane has not yet been clarified, this "capacitative calcium entry" hypothesis has been largely accepted.

Most probably this "store operated Ca^{2+} influx" (SOC) has different biophysical properties in different cells. In mast cells (3), RBL cells (4), *Xenopus* oocytes (5) and Jurkat T-cells (6) the so called I_{CRAC} (calcium release activated calcium current) with a single channel conductance <1 pS and a high selectivity for Ca^{2+} over monovalent cations was described. However in other cells currents with different properties than I_{CRAC} exist (7). In pancreatic acinar cells from mouse we recently described a nonselective cation current which is activated by IP_3-induced Ca^{2+} store depletion (8).

For *Drosophila melanogaster* it has been shown that the *trp* and *trpl* gene products function as Ca^{2+} permeable channels in phototransduction (9) and are regulated by store depletion when expressed in SF9 cells (10) or *Xenopus* oocytes (11, 12). Recently *trp* or *trpl* homologues from bovine (*bCCE* (13)) and human (*TRPC1* (14); *TRPC1A* (15); *Htrp-1* (16); *Htrp3* (17)) tissues were cloned and identified as store operated by functional expression.

Here we describe cloning and expression of a *trp/trpl*-like gene isolated from rat brain. Due to its high homology to *Htrp3* we called it *Rtrp3* (R for rat). Functional expression of the *Rtrp3* protein in COS-1 cells resulted in a higher "capacitative calcium entry" (CCE) after store depletion than in the control indicating its participation in store operated Ca^{2+} entry. Interestingly we found a higher La^{3+} sensitivity for Rtrp3 than described for Htrp3 which might be due to differences in the amino acid sequences.

MATERIALS AND METHODS

Database searches. Database searches and sequence similarity analysis were performed using the "Heidelberg Unix Sequence Analysis Resources" (HUSAR) of the "Deutsches Krebsforschungszentrum", which is based on the Wisconsin Sequence Analysis Package from the Genetics Computer Group (GCG). TBLASTN does a Pearson and Lipman search for similarity between a query peptide sequence and any group of nucleotide sequences. TBLASTN translates the nucleotide sequences in all six reading frames before performing the comparison.

Isolation of trp fragments by RT-PCR. Total RNA was purified from rat brain and human brain following the method of Chomczynski & Sacchi (18). Alternatively the "triazol method" was used (Life Technology, Eggenstein, FRG). Total RNA was converted to cDNA by reverse transcriptase (RT). Two primers, 5′-GATACGTTCTTTATGG-3′ and 5′-TTTTGGACTAGGAACTAGAC-3′ (antisense), were designed and predicted to amplify a 320 bp fragment. For polymerase chain reaction (PCR) the following conditions were used: 3 min initial denaturation at 94 °C, 40 cycles : 94 °C for 1 min, 50 °C for 1 min

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Abbreviations: SDS, sodium dodecylsulfate; AA, amino acid.

0006-291X/97 $25.00

and 72 °C for 1 min, followed by a final extension at 72 °C for 10 min. PCR reaction mixture (50 μl) contained 10 ng of cDNA template, 10 pmol of each primer, 0.2 mmol/l of each of the dNTPs, 0.5 units of Taq DNA Polymerase (Life Technology, Eggenstein, FRG), 50 mmol/l KCl, 10 mmol/l Tris-HCl (pH 9.0), 1.5 mmol/l $MgCl_2$ and 0.1 % W1 (Life Technology, Eggenstein, FRG). The resulting PCR fragments were purified by agarose gel electrophoresis, subcloned into pUC18 and sequenced.

Screening of cDNA library. A λZAP II cDNA library of rat adult brain was purchased from Stratagene (Heidelberg, FRG). This library (about 10^6 phages) was screened with ^{32}P random-labelled DNA-probes following standard protocols (19) in Roti-Hybri-Quick-Solution (Roth, Karlsruhe, FRG) at 68 °C and washed with 0.5 × SSC, 0.1 % SDS (20×SSC: 3 M NaCl, 300 mM sodium citrate pH 7) at 45 °C. The pBS plasmids containing a cDNA insert of a positive clone were excised using a helper phage (ExAssist, Stratagene, Heidelberg, FRG) and sequenced using either universal primers or *Rtrp3* specific synthetic oligonucleotides as primers. Other standard nucleic acid and bacteriological manipulations were performed as described (19).

Primer extension libraries. Poly(A)-RNA was prepared from rat brain using the method of Chomczynski & Sacchi (18) or using the QuickPrep Micro mRNA Purification Kit (Pharmacia, Freiburg, FRG). Complementary DNA was synthezised using the ZAP-cDNA kit (Stratagene, Heidelberg, FRG), starting with 5 μg of mRNA and a mixture of the following primers: 5′-TTTTGGACTAGGAACTAGAC-3′, 5′-CCAAAGCTCTCATTTGC-3′, 5′-GTCCTTTACAGTCCTTCC-3′, 5′-GCGCGTGCCATCCTCGTCGT-3′, each at a concentration of 1 μM. A pUC18 library was constructed using standard techniques (18). This library was screened with a PCR fragment corresponding to the extreme 5′-part of the existing *Rtrp3* clone. Inserts of positive clones were sequenced as described.

PCR with degenerated oligonucleotide primers. 4 redundant primers were designed, which are common to *Dtrp, Dtrpl, Ctrp* and which are optimised for rat codon usage: 5′-CAYAARAAYAAYTTYGARAT-3′ (AA 162-167 of *Dtrpl*), 5′-ATHWSNTAYAARCARAARAA RTT-3′ (AA 304-310 of *D*trpl), 5′-CCNTTYATGAARTTYYTSATHCA-3′ (AA 373-379 of *Dtrpl*), 5′-AAYATYAAYTGYATGGA-3′ (AA 71-75 of *Dtrpl*). PCR conditions were as described above except annealing temperature and elongation time which were optimised for each primer pair and varied from 40 °C to 58 °C and 1 min to 3 min respectively. Since the PCR product exhibited no visible or multiple bands (because redundant primers were used) nested PCR's were performed. The whole PCR mixture was subcloned in pUC18, screened with a PCR fragment corresponding to the extreme 5′-part of the existing *Rtrp3* clones. Inserts of positive clones were sequenced as described.

DNA sequencing. DNA sequencing was performed according to the dideoxy chain termination method (20) using the T7 Sequencing kit (Pharmacia, Freiburg, FRG) and [^{35}S]dATP (Amersham, Braunschweig, FRG).

Construction of the full-length clone and subcloning in expression plasmids. Restriction sites for subcloning were introduced by PCR mutagenesis without changing the amino acid sequence. The resulting fragments were ligated together to give the full-length clone. Sequencing was performed to show the correct fusion. This construct was subcloned in the eukaryotic expression vector pcDNA3.1 (Invitrogen, Leek, Netherland) by use of the BamHI and EcoRI cloning sites resulting in pcDNA3_*Rtrp3*.

Cell culture and transfection of COS-1 cells. Cells were cultured in MEM-α-medium (Life Technology, Eggenstein, FRG) supplemented with 10 % (v/v) heat-inactivated fetal calf serum (PAA Laboratories, Cölbe, FRG) and 200 U/ml of penicillin and streptomycin each.

Cells were plated 24 h prior to transfection at a density of 2×10^5 cells per well onto 384 mm^2 glass coverslips placed in 6-well-plates. Cells were then transfected with 200 μl of transfection mixture containing 10 μg pcDNA3_*Rtrp3*, pcDNA3 alone or the "nonsense" construct (same insert as pcDNA3_*Rtrp3* but opposite orientation) and 10 μg Lipofectamine in Optimem with GLUTAmax medium (Life Technology, Eggenstein, FRG). As a control parallel transfection with pEGFP (Clontech, Heidelberg, FRG) was done. Transfection efficiency was about 50-70 % as shown by the fluorescence of the green fluorescence protein (GFP, (21)).

Ca^{2+} imaging. The intracellular Ca^{2+} concentration was measured in individual cells using the fluorescent indicator fura-2 in combination with a microscopic imaging system (T.I.L.L. Photonics, Planegg, Germany) coupled to an inverted microscope (Carl Zeiss, Germany). The use of a Fluar 10× objective (Carl Zeiss, Germany) allowed us to observe up to 200 cells per experiment.

COS-1 cells grown on glass coverslips (see cell culture) were transferred to a solution containing: 140 mM NaCl, 4.7 mM KCl, 1 mM $MgCl_2$, 1,3 mM $CaCl_2$, 10 mM HEPES, 10 mM glucose, pH 7.4 and were loaded with 4 μM of fura-2/AM (Molecular Probes, USA) for 30 min at 37 °C. Coverslips with loaded cells were mounted to a perfusion chamber on the stage of the inverted microscope. For measuring $[Ca^{2+}]$ cells were excited alternately at 345 and 385 nm for 500 - 1000 ms every 10 s, and the resultant emission was collected above 510 nM. Cells were continuously perfused at room temperature (21-24 °C) with solutions containing varying $[Ca^{2+}]$ and different test-compounds as indicated in the figures. Estimates of the absolute $[Ca^{2+}]$ were calculated from the fluorescence ratio (345nm/380nm) using the Grynkiewicz-relation (22) assuming a K_D of 225 nM for the Ca^{2+}-binding of fura-2.

In-vivo calibration was performed for each cell at the end of an experiment. Therefore cells were permeabilised for Ca^{2+} with 10 μM ionomycine and superfused with solutions containing first 10 mM EGTA for determination of R_{min} and subsequently 10 mM Ca^{2+} to determine R_{max}.

RESULTS

Cloning of a Rat Homologue of trp/trpl Genes

The sequences for the *Dtrp* and *Dtrpl* genes from *Drosophila melanogaster* and the *Ctrp* gene from *C.elegans* were used for a homology-search in the genbank database as deduced amino acid sequences. As a result of the TBLASTN search a human sequence (R34716) was found to encode an amino acid sequence that shares similarity with the *Drosophila* trpl protein. This sequence was the result of the human genome project and was only a partial "single-pass" sequence, determined from a cDNA clone isolated from human fetal brain (23).

```
emnew3:HS716100 R34716 yg61a02.r1 Homo sapiens cDNA clone 37199
Identities = 39/68 (57%), Positives = 56/68 (82%), Frame = +3

dtrpl:          645 GLLMFGSYSVINVIVLLNLLIAMMSNSYAMIDEHSDTEWKFARTKLWMSYFEDSATLPPP FNVLPSVK 712
                    G +++G Y+V  V+VLLN+LIAM+++SY  I++ SD EWKFAR+KLW+SYF+D  TLPPP
trans.R34716:    16 GYVLYGIYNVTMVVVLLNMLIAMINSSYQEIEDDSDVEWKFARSKLWLSYFDDGKTLPPP XSLVPSPK 83
```

```
emnew3:HS67337 T67673 yd10h03.r1 Homo sapiens cDNA clone 66773
Identities = 22/53 (41%), Positives = 36/53 (67%), Frame = +3

dtrp:          504 KWHDFDPQLIAEGLFAAANVFSALKLVHLFSINPHLGPLQISLGRMVIDIVKF 556
                   +WH  DPQ +AE  LF A  ++ S  +L ++   +  LG LQIS+G+M+ D+++F
trans. R34716:  34 EWHTEDPQFLAEVLFTATSMLSFTRLAYILPAHESLGTLQISIGKMIEDMIRF 86
```

This human sequence was found to code for a peptide sequence with an identity of 57 % to *Dtrpl* (Gly-16 to Lys-83) and 41 % to *Dtrp* (Glu-34 to Phe-86). Comparision of the deduced sequence of R34716 to a *C. elegans trp* homologue also revealed that it is homologous to the middle part of all known *trp*-type proteins. We thus synthesized two oligonucleotides and amplified a 320 bp fragment of R34716 from human brain RNA. After veryfiing the sequence this fragment was used as a probe to screen a rat adult brain library.

From 10^6 recombinant phages a clone was isolated and shown to contain an insert of about 2 kb. Sequencing revealed that this clone is coding for an open reading frame of about 450 amino acids which is the 3′-end of a rat *trp* homologue.

For cloning the missing 5′-end we combined 2 different strategies, primer extension libraries and PCR with degenerated primers.

For primer extension libraries we prepared poly(A)-RNA from rat brain and synthesized complementary DNA by using a mixture of 3 different oligonucleotide primers. A library was construced using pUC18 as the cloning vector. After screening with a *trp* specific probe a new rat clone was isolated coding for additional 230 AA of the rat *trp* homologue.

While this work was in progress new cDNA sequences of two human homologs of *Dtrp* were published. *TRPC1* (14) and *Htrp1* (16). Both these cDNAs vary only in the amino-terminal part of the deduced amino acid sequences. For PCR with degenerated primers we aligned all known trp and trpl amino acid sequences (*Dtrp, Dtrpl, Ctrp, TRPC1, Htrp1*) and identified regions of homology. Using the rat codon usage table we choose 4 redundant primers. After nested PCR using these primers cDNA fragments coding for a rat trp homologue were found.

Taking together we had the complete coding region for the rat homologue of *Dtrpl*. It is coding for a protein of 828 AA.

Recently the cDNA sequence of a new human homologue of the *Drosophila trpl* was published, named *Htrp3* (17). Our protein and the Htrp3 protein are identical in large parts but vary in the amino-terminal part of the deduced amino acid sequence. The homology of these two proteins is 94 %. As a consequence of the high similarity to the human sequence our protein was named Rtrp3 (fig. 1).

We have also detected the presence of this protein Rtrp3 in rat pancreas.Starting with mRNA and performing RT-PCR with different primer combinations we obtained the corresponding Rtrp3 fragments coding for AA 220-710. These fragments are identical to those derived from rat brain as shown by cloning and sequencing (data not shown).

For expression of Rtrp3 we had to construct a full-length clone. This was done by silent mutagenesis introducing single restriction sites. Following this attempt we were able to get a full length clone. The coding region was subcloned in pcDNA3 to give pcDNA3_*Rtrp3*. This construct was used for expression in COS-1 cells.

Functional Expression of Rtrp in COS-1 Cells

To characterise the role of the cloned *Rtrp* gene in capacitative calcium entry (CCE) we expressed it in the mammalian COS-1 cell-line by the use of the "Lipofectamine" transfection system (Life Technology, Eggenstein, FRG). Parallel transfection experiments with the gene for the fluorescence marker GFP (green-fluorescence-protein, Cubitt et al (24), Chalfie (21)) indicated a transfection rate of 50 - 70 % (data not shown). Because of this high transfection rate we decided to omit any marker-gene in our construct and to evaluate functional expression statistically.

To characterize expression of *Rtrp3* and its influence on CCE we measured cytoplasmic [Ca^{2+}] after store depletion in both control cells and *Rtrp3*-transfected cells by single cell flurometry and the use of the ratiometric Ca^{2+} dye fura-2 method. We depleted Ca^{2+} stores by using the store specific Ca^{2+}-ATPase inhibitor thapsigargin (100 nM) in a nominally Ca^{2+} free solution. This induced an initial $[Ca^{2+}]_i$ spike by Ca^{2+} release from intracellular pools which subsequently declined to the resting level within a few minutes since Ca^{2+} is pumped out of the cell. After the resting level had been reached, Ca^{2+} (1.3 mM) was readded to the perfusion medium and $[Ca^{2+}]_i$ increase due to Ca^{2+} influx was recorded (fig. 2a,b). When measured 16 h after transfection, the increase in $[Ca^{2+}]_i$ was significantly higher in *Rtrp3* transfected cells (fig. 2b) as compared to cells transfected with the empty vector (pcDNA3) (fig. 2a) or to "nonsense"-DNA-transfected cells (fig. 2c) (for information about the controls see "Materials and Methods"). La^{3+} (10 μM), an inhibitor of capacitative Ca^{2+} entry (25), completely abolished Ca^{2+} influx in *Rtrp3*-transfected cells (fig. 2b), in empty-vector- (fig. 2a) and "nonsense"-DNA-transfected cells (not shown) as well as in wild-type COS-1 cells (not shown).

In the experiments shown in fig. 2 two min after

FIG. 1. Comparision of Rtrp3 (lower line) and Htrp3 (upper line) proteins as predicted by the HUSAR program BESTFIT. | means identity; : means similarity; · means gap.

Ca^{2+}-readdition $[Ca^{2+}]_i$ reached 81 ± 15 nM (54 cells) in the vector-transfected controls (a) and 131 ± 35 nM (88 cells) in the *Rtrp3*-transfected cells (b).

Since absolute $[Ca^{2+}]_i$ values were variable from cell passage to cell passage, we normalized all data relative to their respective control (vector pcDNA3) which was measured in the same cell passage. The La^{3+} inhibitable component of vector-transfected cells was defined thereby as 100%. An increase in CCE of 96 ± 10,7 % was found in *Rtrp3*-transfected cells as compared to pcDNA3- and 101±11 % in comparison to "nonsense"-DNA-transfected cells. (16 h after transfection, *Rtrp3:* 4 transfections, 370 cells; pcDNA3: 4 transfections, 420 cells; "nonsense": 3 transfections, 326 cells) (fig. 2c). Capacitative calcium entry was maximal at 16 h after transfection and was downregulated later on (not shown).

DISCUSSION

The present study describes a new member (*Rtrp3*) of a growing group of *trp/trpl* homologous genes of mammalian cells from which the *Htrp1* and the *TRPC1* gene were initially cloned from human tissues (16, 14). Recently different homologous variants of these clones were discovered (17, 15) A new clone from bovine tissue which seems to represent another class of *trp/trpl* genes was also detected and was called *bCCE* (13).

The here described *Rtrp3* is the first clone found in rat. *Rtrp3* is highly homologous to *Htrp3* and less homologous to other members of the family including the *Drosophila trp/trpl* genes (about 40 % identical to *trp* and 57 % to *trpl*).

For several of the above mentioned clones participation in "store-operated Ca^{2+} entry" had been verified (13, 15-17). To examine if *Rtrp3* participates in CCE, intracellular calcium stores of transfected COS-1 cells were depleted by the highly specific endoplasmic reticulum Ca^{2+}-ATPase inhibitor thapsigargin. In *Rtrp3*-transfected cells we found an increase in Ca^{2+} influx double as high as in control cells.

It is difficult to decide if Rtrp3 code for the Ca^{2+} influx-mechanism itself (i.e. a channel protein or a subunit of a channel) or for an activating factor in the cascade between empty Ca^{2+} stores and the Ca^{2+} influx-channel. In our expression system Rtrp3-induced CCE could not be distinguished qualitatively from an endogenous component. Therefore both explanations seem to be possible. Zhu et al. (17) and Zitt et al. (15) conclude from their functional data that TRPC1A and Htrp3 are most probably channel proteins. The electrophysiological data of Zitt (15) demonstrate appearance of a current after expression of the TRPC1A which was not present in the control. These results are supporting the idea that TRPC1A is coding for a channel. Furthermore, if Rtrp3 is a Ca^{2+} channel, we can not unequivocally decide from our data if it is directly activated by the emptiness of the Ca^{2+} stores (as it is postulated for the "capacitative entry hypothesis"). Another possibility would be Ca^{2+} activation of this channel by increase in cytosolic $[Ca^{2+}]$ due to Ca^{2+} release from the Ca^{2+} store.

Despite of the high homology of our Rtrp3 to Htrp3

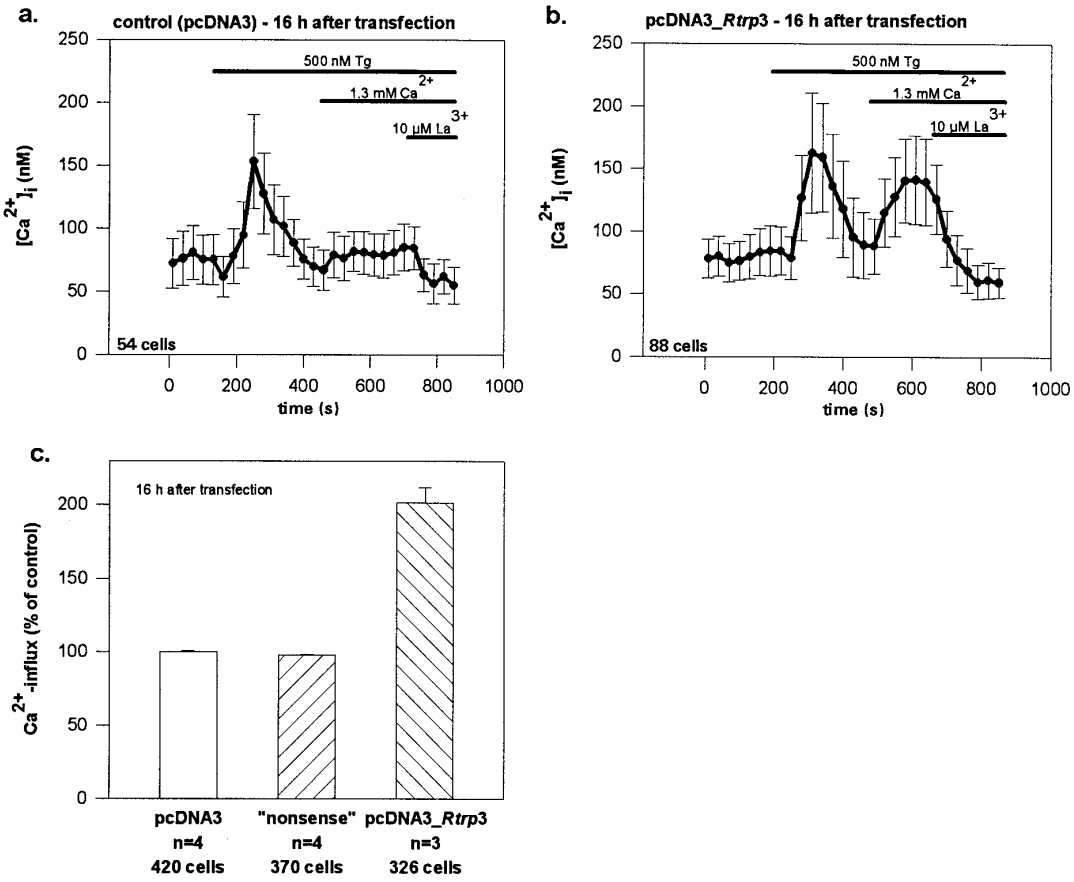

FIG. 2. Capacitative Ca^{2+}-entry in transfected COS-1 cells. Bath solutions were changed as indicated by the bars in *a* and *b*. (a) Four pcDNA3-transfections were performed and CCE was analysed 16 h later. Mean ± SEM values of 54 cells from one of these transfections are shown. (b) Four pcDNA3_*Rtrp3*-transfections were performed and CCE was analysed 16 h later. Mean ± SEM values of 88 cells from one of these transfections are shown. (c) Summarized data on CCE with pcDNA3-, pcDNA3_*nonsense*- and pcDNA3_*Rtrp3*-transfected cells (n = number of transfections). 100 % is defined as the La^{3+}-inhibitable Ca^{2+}-influx after pcDNA3-transfection (see fig. 2a). Ca^{2+}-Influx after pcDNA_*Rtrp3*-transfection is double as high as Ca^{2+}-influx after pcDNA3- or pcDNA3_*nonsense*-transfection. Data are means ± SEM for the given numbers of cells.

it seems that there are differences between the two clones when they are functionally expressed in mammalian cells. Zhu et al. (17) described that La^{3+} was less effective to block the CCE in *Htrp3*-transfected COS-1 cells than in wild type COS-1 cells (250 μM La^{3+} blocked CCE in the wild type by 90 % but in *Htrp3*-transfected cells only about 30% (17)). In contrast we could show that the La^{3+} block of CCE remains unchanged after expression of Rtrp3 in comparison to the block in the wild type cells. Future studies with mutated *Rtrp3* genes may clarify which amino acids are responsible for La^{3+} selectivity. If Rtrp3 is indeed a Ca^{2+} channel protein this approach may also help to find the selectivity filter for La^{3+}.

ACKNOWLEDGMENTS

We are grateful to Ms. Bärbel Kohler for excellent technical assistance. The authors thank the "Deutsche Forschungsgemeinschaft" (SFB 246, Project B11) for support of this work.

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