Expression profile of the transient receptor potential (TRP) family in neutrophil granulocytes: evidence for currents through long TRP channel 2 induced by ADP-ribose and NAD

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An early key event in the activation of neutrophil granulocytes is Ca^{2+} influx. Members of the transient receptor potential (TRP) channel family may be held responsible for this. The aim of the present study is to analyse the expression pattern of TRP mRNA and identify characteristic currents unambiguously attributable to particular TRP channels. mRNA was extracted from human neutrophils, isolated by gradient centrifugation and also by magnetically labelled CD15 antibodies. The presence of mRNA was demonstrated using reverse transcriptase–PCR in neutrophils (controlled to be CD5-negative) as well as in human leukaemic cell line 60 (HL-60) cells, for the following TRP species: the long TRPC2 (LTRPC2), the vanilloid receptor 1, the vanilloid receptor-like protein 1 and epithelial Ca^{2+} channels 1 and 2. TRPC6 was specific for neutrophils, whereas only in HL-60 cells were TRPC1, TRPC2, TRPC3, melastatin 1 and melastatin-related 1 found. Patch-clamp measurements in neutrophils revealed non-selective cation currents evoked by intracellular ADP-ribose and by NAD^+. Both these modes of activation have been found to be characteristic of LTRPC2. Furthermore, single-channel activity was resolved in neutrophils and it was indistinguishable from that in LTRPC2-transfected HEK-293 cells. The results provide evidence that LTRPC2 in neutrophil granulocytes forms an entry pathway for Na^+ and Ca^{2+}, which is regulated by ADP-ribose and the redox state.

Key words: Ca^{2+} influx, patch-clamp, reverse transcriptase–PCR.

INTRODUCTION

One of the early characteristic cellular reactions of neutrophil granulocytes in response to chemoattractants is an increase in the 'free' intracellular cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) [1]. The increase in $[Ca^{2+}]_i$ may be the signal for further reactions or cellular responses such as migration [2,3], synthesis and release of prostaglandins and leukotrienes [4], phagosome–lysosome fusion process [5], exocytosis [6] and respiratory burst [7]. Mechanisms relevant for increases in $[Ca^{2+}]_i$ include release of Ca^{2+} from intracellular stores as well as a Ca^{2+} influx through the plasma membrane. The molecular basis of Ca^{2+} influx in neutrophil granulocytes is not well understood [8]. The same holds true for many other electrically non-excitable cells where the molecular mechanisms of receptor-mediated Ca^{2+} influx await elucidation in detail [9]. Nevertheless, a key role may be attributed [7] to a family of genes homologous with the *Drosophila* gene defective in the transient receptor potential (TRP) mutant [10]. For most of the mammalian members of this TRP family, it has been demonstrated that they code for Ca^{2+}-permeable cation channels, or at least for subunits of such channels [11,12]. Biophysical properties of the channels and even more so their mode of activation, however, are strikingly different within the family, as revealed in experiments utilizing heterologous overexpression [13]. The aim of the present study was to determine, using reverse transcriptase (RT)–PCR, the TRP channels expressed in neutrophil granulocytes. Yet, it has to be conceded that RT–PCR signals are insufficient evidence for the presence of the corresponding proteins and of intact channels. Since heterologous overexpression of some of the TRP family members has resulted in characteristic ion currents, evidence for their functional expression can be obtained in neutrophil granulocytes by demonstrating currents with the same regulatory and biophysical properties as in transfected cells. Using RT–PCR and patch-clamp experiments, we provide evidence for a prominent role for the long TRP channel 2 (LTRPC2) as an Na^+ and Ca^{2+} entry pathway in neutrophil granulocytes.

MATERIALS AND METHODS

Isolation of human neutrophil granulocytes and mRNA extraction

Neutrophils were isolated from anti-coagulated venous blood of healthy volunteers. After dextran sedimentation (Macrodex, Pharmalink, Sweden) and hypo-osmotic lysis of erythrocytes, the cell suspension was layered over Ficoll-Paque Plus (Amersham Pharmacia Biotech, Uppsala, Sweden). After centrifugation for 20 min at 800 ***g***, the interphase (containing lymphocytes, monocytes and platelets) was harvested to serve as a CD5-positive control in RT–PCR, and the sediment containing the neutrophils was subjected to separation with magnetically labelled antibodies as follows. Cells were washed with PBS/EDTA and resuspended in magnetic antibody cell separation (MACS) solution (137 mM NaCl/2.7 mM KCl/10 mM Na_2HPO_4/1.8 mM KH_2PO_4/2 mM EDTA/0.5 % BSA; pH 7.4) to obtain a cell

Abbreviations used: ADPR, ADP-ribose; BVF, bivalent-ion-free; $[Ca^{2+}]_i$, intracellular cytosolic Ca^{2+} concentration; cADPR, cyclic ADPR; ECaC, epithelial Ca^{2+} channel; fMLP, *N*-formylmethionyl-leucylphenylalanine; HL-60, human leukaemic cell line 60; IL-8R, interleukin-8 receptor; MACS, magnetic antibody cell separation; MLSN1, melastatin 1; MTR1, melastatin-related 1; NMDG, *N*-methyl-D-glucamine; OAG, 1-oleoyl-2-acetyl-*sn*-glycerol; RT, reverse transcriptase; SOCE, store-operated Ca^{2+} entry; TRP, transient receptor potential; TRPC, TRPC channel; LTRPC2, long TRP channel 2: VR1, vanilloid receptor 1.

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density of 1.25×10^8 cells/ml. Cells were incubated for 10 min at 8 °C with a monoclonal antibody against human CD15 (20 μl of antibody solution/1×10^7 cells), conjugated with colloidal 'super-paramagnetic' microbeads (Miltenyi, Bergisch Gladbach, Germany). Cells were washed with MACS solution, centrifuged [300 ***g*** at room temperature (21–23 °C) for 10 min] and resuspended in 500 μl of MACS solution. The magnetically tagged cells were applied on to an equilibrated separation column, which was placed in a Mini-MACS unit. The column was washed four times with MACS solution to remove unlabelled cells. After removal of the column from the magnetic field, neutrophils retained magnetically were eluted by flushing the column once with 5 ml of MACS solution. Approx. $(2.5–5) \times 10^7$ cells were isolated from 70 ml of blood. Characterization of leucocytic subtypes in cell suspensions was performed with an automated haematology analyser (CELL-DYN 4000; Abbott GmbH, Wiesbaden, Germany). The cells were centrifuged and quickly lysed by the addition of TRIzol® reagent (Life Technologies, Rockville, MD, U.S.A.). Total RNA was isolated according to the manufacturer's instructions. Approx. 10–20 μg of total RNA was isolated from $(2.5–5) \times 10^7$ granulocytes. The entire amount of total RNA obtained from each isolation was utilized for mRNA extraction using Oligotex (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The mRNA was eluted from Oligotex with 25 μl of elution buffer.

Culture and differentiation of human leukaemic cell line 60 (HL-60) cells

The promyelocytic HL-60 was obtained from the 'Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH' (Braunschweig, Germany). The cells were grown in VLE–RPMI 1640 (Biochrom, Berlin, Germany), supplemented with 10 % (v/v) heat-inactivated foetal calf serum (Biochrom) and 2 mM L-glutamine in humidified air with 5 % CO_2 at 37 °C. For the induction of granulocytic differentiation, cells were seeded at a density of 1×10^6 cells/ml in a medium containing 400 μM dibutyryl cAMP and cultured for 2 or 3 days. The number of viable cells was determined by Trypan Blue exclusion. RNA was isolated from $(1–2) \times 10^7$ differentiated HL-60 cells using the same procedure and chemicals as described above for neutrophils.

RT–PCR

Oligo(dT)-primed first-strand cDNA synthesis was performed with Moloney-murine-leukaemia virus RT (Superscript II; Life Technologies) using 11 μl of neutrophil mRNA or 500 ng of HL-60 mRNA as template in a total volume of 20 μl. Reaction conditions were as described by the manufacturer. For PCR, the cDNAs were diluted 1 : 2 with sterile water. For negative controls without reverse transcription, the mRNA was diluted to give the same mRNA concentration as in the cDNA solutions. These control reactions, with mRNA as template, were performed to exclude genomic DNA contamination. The reaction solution (50 μl) contained 0.4 μM primer (each for reverse and forward primers), 2 mM $MgCl_2$, 0.2 mM dNTP (each) and 1.25 units of *Taq* polymerase (Ampli Taq Gold, PerkinElmer, Roche Molecular Systems, Branchburry, NJ, U.S.A.) in 1 × PCR buffer II supplied by the manufacturer. As template, 0.1 fmol of PCR fragment in pCR2.1, 1 μl of mRNA solution or 1 μl of cDNA solution was used. The cycling conditions were: 95 °C for 10 min, followed by 35 cycles, each consisting of denaturation at 95 °C for 1.5 min, annealing at 63 °C for 2 min, and extension at 72 °C for 2 min, and a final extension at 72 °C for 10 min. PCR products were analysed on 2 % (w/v) agarose gels and visualized by staining with ethidium bromide.

Culture and transfection of HEK-293 cells

HEK-293 cells were obtained from A.T.C.C. (Rockville, MD, U.S.A.). Cells were cultured in Dulbecco's modified Eagle's medium (Biochrom), supplemented with 10 % foetal calf serum (Biochrom), 1 mM sodium pyruvate (Sigma) and 4 mM L-glutamine (Biochrom). Cells were plated on to 0.1 μg/μl poly-lysine (Sigma)-coated glass coverslips for 24 h and then transiently transfected with the full-length clone of LTRPC2 as described previously [14]. Electrophysiological studies were performed 24–48 h after transfection in cells visibly positive for enhanced green fluorescent protein.

Neutrophil isolation for patch-clamp experiments

Anti-coagulated blood was mixed with hydroxyethyl starch (Plasmasteril; FreseniusAG, Bad Homburg, Germany) and allowed to sediment for 30 min at room temperature. The remaining erythrocytes were removed by hypo-osmotic lysis. The cells were resuspended in PBS supplemented with 5 % (v/v) autologous serum and layered over Percoll (Pharmacia, Uppsala, Sweden) with a density of 1.082 g/ml. After centrifugation at 600 ***g*** for 20 min, the sediment containing the neutrophils was washed twice in PBS supplemented with 5 % autologous serum and finally resuspended in a buffer containing 136 mM NaCl, 2 mM $CaCl_2$, 2.8 mM KCl, 20 mM Hepes and 5 mM glucose (pH 7.4) and supplemented again with 5 % autologous serum.

Electrophysiology

HL-60 cells and human granulocytes were studied with the patch-clamp technique in the whole-cell mode, using an EPC 9 amplifier equipped with a personal computer with Pulse 8.5 and X Chart software (HEKA Electronik, Lambrecht, Germany). Pipettes were made of borosilicate glass; when single channels were to be analysed in the whole-cell or inside-out configuration, the tips of pipettes were coated with dental wax (Moyco, Philadelphia, PA, U.S.A.) to reduce thermic noise.

For whole-cell experiments, the standard extracellular bath solution contained (in mM) 140 NaCl, 1.2 $MgCl_2$, 1.2 $CaCl_2$, 5 KCl and 10 Hepes; the pH was adjusted to 7.4 using KOH. For Na^+-free solutions, Na^+ was replaced by 150 mM *N*-methyl-D-glucamine (NMDG) and the titration was performed with HCl. The bivalent-ion-free (BVF; also known as 'DVF') solution contained (in mM) 150 NaCl, 10 glucose, 10 EDTA and 10 Hepes (pH 7.4). The pipette solution contained (in mM) 145 caesium glutamate, 8 NaCl, 2 $MgCl_2$, 10 caesium-EGTA, 10 Hepes; pH 7.2 (CsOH). In some experiments, the pipette solution additionally contained 0.3 mM ADP-ribose (ADPR), 1 mM NAD^+ or 2–7 mM MgATP. Cells were held at a potential of − 60 mV. Current–voltage (*I*–*V*) relationships were obtained from voltage ramps from − 90 to + 60 mV, applied over 400 ms.

For inside-out patches, the bath solution was the same as the pipette solution in whole-cell experiments, supplemented with 0.3 mM ADPR. The pipette solution was the standard bath of the whole-cell experiments. Results from single-channel recordings (in the inside-out or whole-cell configuration) were sampled at 25 kHz and filtered at 1.5 kHz.

Unless otherwise indicated, all chemicals were purchased from Sigma. The experiments were performed at room temperature. Results are expressed as means ± S.D.

Table 1 Sequences of oligonucleotide primers used for RT–PCR expression profiling

Gene	Accession no.	Orientation	Sequence	Location	Product size (bp)
TRPC1	Z73903	Forward	5′-ATGTATACAACCAGCTCTATCTTG-3′	1841–1864	525
		Reverse	5′-AGTCTTTGGTGAGGGAATGATG-3′	2365–2344	
TRPC2	X89067	Forward	5′-TCTGGACCATGTTCGGTATG-3′	294–313	565
		Reverse	5′-GCTACCTCGCTTTGCAGTC-3′	858–840	
TRPC3	U47050	Forward	5′-CTGCAAATGAGAGCTTTGGC-3′	1835–1854	388
		Reverse	5′-AACTTCCATTCTACATCACTGTC-3′	2222–2200	
TRPC4	AF175406	Forward	5′-ATTCATATACTGCCTTGTGTTG-3′	1791–1812	329
		Reverse	5′-GGTCAGCAATCAGTTGGTAAG-3′	2119–2099	
TRPC5	AF054568	Forward	5′-ACTTCTATTATGAAACCAGAGC-3′	2534–2555	289
		Reverse	5′-GCATGATCGGCAATAAGCTG-3′	2822–2803	
TRPC6	AF080394	Forward	5′-AAGACATCTTCAAGTTCATGGTC-3′	1912–1934	322
		Reverse	5′-TCAGCGTCATCCTCAATTTCC-3′	2233–2213	
LTRPC2 (TRPM2)	AB001535	Forward	5′-GTGGACTGGCTGTTCCGAG-3′	3332–3350	511
		Reverse	5′-GGTAGTTCTCCTTCAGGTAG-3′	3842–3823	
MLSN1 (TRPM1)	AF071787	Forward	5′-CTCATGAGTTTCGGAGTAGC-3′	3039–3058	342
		Reverse	5′-CCACACCTGGTTGGATATTG-3′	3380–3361	
MTR1 (TRPM5)	AF177473	Forward	5′-CAAGATCATCGTGGTAGAGC-3′	2592–2611	398
		Reverse	5′-TCCAGAACATGTCTGCGTTG-3′	2989–2970	
VR1 (TRPV1)	AA321554 (EST)	Forward	5′-CGGTCAAGCAGAGTTTCAGG-3′	14–33	216
		Reverse	5′-AGTGTTGACAGTGCTGTCTG-3′	229–210	
VRL1 (TRPV2)	AF129112	Forward	5′-ACACAGGCATCTACAGTGTC-3′	1591–1610	478
		Reverse	5′-CACCACCAATAGCCATTCTC-3′	2068–2049	
ECaC1/ECaC2	AA078617 (EST)	Forward	5′-CTGTTGCTGTCCTACGACAG-3′	2–21	199
		Reverse	5′-CGATCTCTGTGAGGTCATAG-3′	200–181	
ECaC1 (TRPV5)	AJ271207	Forward	5′-GGGGACCGCTGGTTCCTGCGG-3′	1876–1896	312
		Reverse	5′-TCAAAAATGGTAGACCTCCTCTCC-3′	2190–2167	
ECaC2 (TRPV6)	NM_018646	Forward	5′-GGGGACCGCTGGTTCCTGCGG-3′	2092–2112	301
		Reverse	5′-AGATCTGATATTCCCAGCTCT-3′	2392–2372	
IL-8R	L19591	Forward	5′-GGCTTACCATCCAAACAATTC-3′	647–667	537
		Reverse	5′-ATCGATGGTTTTCAGAGGTTG-3′	1183–1163	
CD5	X04391	Forward	5′-ACACACCTCAGAGCTCAATC-3′	368–387	473
		Reverse	5′-GGGCTTGATTTTCCTGAAGC-3′	840–821	

RESULTS

Selection of primers and establishment of PCR conditions

To examine the expression profile of members of the TRP gene family, we performed RT–PCR analysis with the primer pairs listed in Table 1. To test the specificity of these primers, each of them was used to amplify the expected DNA fragments from various human cDNA sources. These DNA fragments were inserted into the pCR2.1 vector by TA cloning and their expected sequences were confirmed by sequencing. To test further the specificity of the primers, we performed control PCRs with full-length cDNA of one specific member (TRPC1, TRPC3 or LTRPC2) as the exclusive template with all primer pairs. Amplificates were obtained only for the reaction with gene-specific primers. Unknown products were not observed in any of the cases.

To test further the sensitivity of our PCR conditions, we used either full-length cDNAs or DNA fragments cloned into pCR2.1 as the template with the respective specific primer pairs. Low amounts of template DNA (0.1 fmol) resulted in equally strong signals of the amplificates after separation on agarose gels and staining with ethidium bromide (Figure 1A). Therefore the established PCR conditions were both specific and sensitive.

Expression pattern of TRP family members in human granulocytes

Several cell-separation methods were combined to obtain highly pure samples of human granulocytes. A cell suspension with $> 96\,\%$ granulocytes and $< 4\,\%$ other leucocytes was obtained after dextran sedimentation, osmotic lysis of the remaining erythrocytes and 2-fold centrifugation through Ficoll cushions. Contamination of this cell suspension by monocytes and macrophages was $< 0.1\,\%$. Additional purity was achieved with MACS using magnetically labelled CD15-specific antibodies [15]. The CD15 antigen is expressed on neutrophils and eosinophils as well as on monocytes and macrophages, but not on basophils and lymphocytes [16]. MACS yielded cell suspensions with $> 98.5\,\%$ neutrophils, $< 0.9\,\%$ lymphocytes and traces of other contaminating cells (monocytes $< 0.01\,\%$, eosinophils $< 0.6\,\%$ and basophils $< 0.01\,\%$). Immediately after the preparation of the cells, mRNAs were extracted and a first-strand cDNA synthesis was performed.

Along with the TRP-specific PCRs, a CD5-specific reaction was performed as a control. CD5 is a transmembrane protein expressed on the surface of T lymphocytes and a subset of B lymphocytes, but not in granulocytes [17]. Results of the RT–PCR experiments were discarded if the CD5 reaction was not clearly negative. A reaction with gene-specific primers for the interleukin-8 receptor (IL-8R), a protein highly expressed in neutrophils, was set up in each experiment as a positive control. Furthermore, in control experiments performed with each cell preparation in which mRNA instead of cDNA was used as the template, no PCR products were obtained. Therefore amplificates in PCRs with TRP-specific primers could be clearly attributed to mRNA transcripts expressed in neutrophils, whereas contamination with genomic DNA or with mRNA expressed in T or B lymphocytes was excluded.

The expression profile for members of the TRP family, as found in these extensively controlled experiments, is shown in

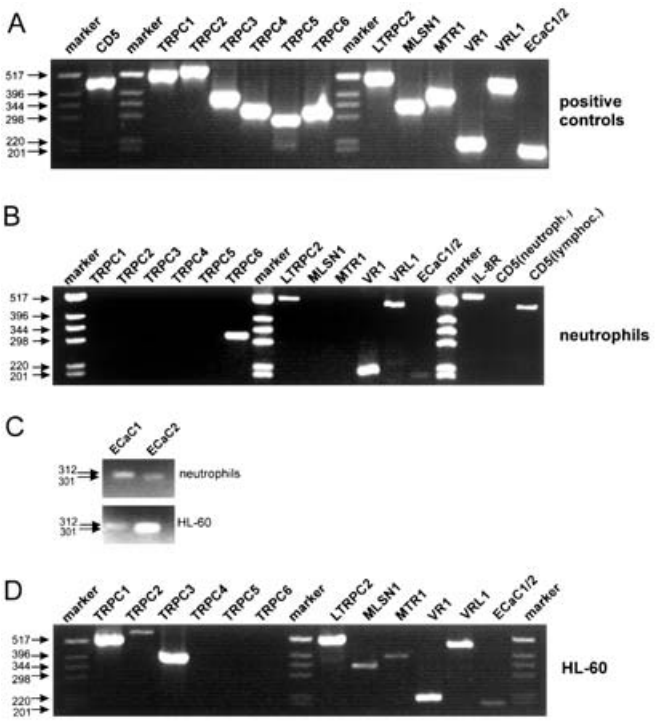

Figure 1 Expression of TRP family members in isolated human neutrophils and HL-60 cells

(**A**) Sensitivity of the established PCR conditions: either full-length cDNA or cDNA fragments of the indicated members of the TRP family were used as template (0.1 fmol of template/reaction) with the respective primer pairs for amplification. Agarose gels were loaded with 15 μl of PCR products/lane and stained with ethidium bromide. The molecular masses of marker bands are given on the left. (**B**) Representative results of RT–PCR analysis with mRNA from one isolation and cDNA preparation of neutrophils with each of the indicated primers are given. The three right lanes show control experiments: IL-8R-specific primers were taken for a PCR using cDNA from isolated granulocytes as template; specific primers targeting the cell marker CD5 were taken for amplification using cDNA from isolated neutrophils [CD5 (neutroph)] or cDNA from isolated lymphocytes [CD5 (lymphoc)] as template. (**C**) Representative results of RT–PCR analysis of primer pairs which discriminate between ECaC1 and ECaC2 are given. The primer sequences were obtained from Hoenderop et al. [33]. (**D**) Representative results of RT–PCR analysis with mRNA of differentiated HL-60 cells are given. VRL1, VR-like protein 1.

Figure 1(B), which represents a characteristic result from RT–PCRs with one preparation of neutrophils. Intensively stained bands of PCR products of the expected sizes were consistently obtained (Table 2) for TRPC6 (in 5 out of 6 preparations), LTRPC2 (in 5 out of 7 preparations), vanilloid receptor 1 (VR1) and VR-like protein 1 (in all the 6 preparations), and for the positive control IL-8R (in 5 out of 6 preparations). Signals were also observed for Ca^{2+} transporter 1/epithelial Ca^{2+} channel (ECaC) (in 4 out of 5 preparations). Since experiments with the primer pair ECaC1/ECaC2 cannot discriminate between ECaC1 and ECaC2 (also named Ca^{2+} transporter 2 and 1 respectively), we repeated the analysis with primer pairs specific for each of these two TRP family members (Figure 1C) and found positive signals for both of them (in all the 4 preparations). In only 2 out of 7 preparations were TRPC3-specific DNA fragments amplified. We did not observe signals for TRPC1 and TRPC2 (in any of the 6 preparations), TRPC4 (in any of the 5 preparations), TRPC5 (in any of the 6 preparations), MLSN1 (in any of the 7 preparations), MTR1 (in any of the 6 preparations) and CD5 (in any of the 7 preparations).

Expression pattern of TRP family members in HL-60 cells

Differentiated HL-60 cells were used as a cell culture model for neutrophil granulocytes. Differentiation of HL-60 cells with dibutyryl cAMP for 2 or 3 days enhanced their response to the chemoattractant peptide *N*-formylmethionyl-leucylphenylalanine (fMLP) in experiments measuring $[Ca^{2+}]_i$ with fura 2, whereas no significant increase in $[Ca^{2+}]_i$ was observed when undifferentiated cells were exposed to fMLP (results not shown). Furthermore, the signal for the fMLP receptor transcript, as detected in RT–PCR, was much stronger in differentiated than in undifferentiated HL-60 cells (results not shown). To verify whether this difference indicates enhanced expression of fMLP receptor mRNA rather than variable efficiencies of the cDNA preparations, the signals for the actin transcript were compared in each preparation. Differentiation did not seem to affect the actin mRNA level in any experiment.

In all preparations of cDNA from differentiated HL-60 cells, detectable amplificates for TRPC1, TRPC2 and TRPC3 as well as for LTRPC2, VR1, VR-like protein 1 and ECaC1/ECaC2 were obtained (Figure 1D). In contrast with experiments with isolated neutrophils, TRPC6 was not identified in any preparation from HL-60 cells. Inconsistent results were obtained for melastatin 1 (MLSN1) and melastatin-related 1 (MTR1). Most of the preparations were positive for MTR1 (in 6 out of 10 preparations), whereas MLSN1 was demonstrated occasionally (in 2 out of 10 preparations). A summary of the RT–PCR experiments is given in Table 2.

Currents through LTRPC2

Among the TRP family members for which we had consistently obtained RT–PCR signals in neutrophil granulocytes, only LTRPC2 produced characteristic and unique currents when it was heterologously expressed in various cells. Therefore we looked for functional evidence of protein expression by measuring LTRPC2-associated currents. LTRPC2 is a non-selective cation channel activated by ADPR [18] and by NAD^+ [19]. Accordingly, we performed patch-clamp experiments in HL-60 cells and in neutrophil granulocytes. ADPR (0.3 mM) was present in the pipette fluid and was allowed to diffuse into the cell after obtaining the whole-cell configuration. These experiments revealed results strikingly similar to those recently obtained in HEK-293 cells transfected with LTRPC2 [14]. In HL-60 cells, currents developed

Table 2 Summary of the expression profiles of members of the TRP gene family in human granulocytes and HL-60 cells

Expression is considered positive and indicated by a + sign if PCR products were found in 70–100 % of the experiments ($n = 5$–7 cell preparations). (+) indicates expression in a smaller fraction of experiments (see the text for details).

	TRPC1	TRPC2	TRPC3	TRPC4	TRPC5	TRPC6	LTRPC2	MLSN1	MTR1	VR1	VRL1	ECaC1	ECaC2
Neutrophils			(+)			+	+			+	+	+	+
HL-60 cells	+	+	+				+	(+)	(+)	+	+	+	+

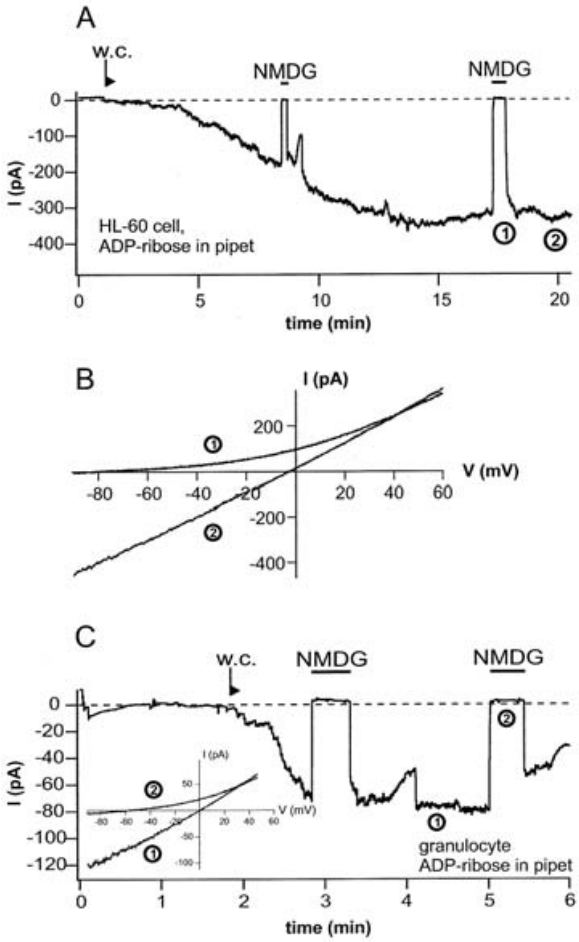

Figure 2 Currents induced by ADPR in HL-60 cells and human neutrophil granulocytes

(**A**) Original current recording from an HL-60 cell patched with 0.3 mM ADPR present in the pipette solution is shown. The whole-cell (w.c.) configuration was obtained at the time indicated. The normal bath medium was replaced by a solution with NMDG as the main cation during the time period indicated. This is depicted by a bar. The holding potential was − 60 mV. (**B**) *I–V* relationships of ADPR-induced currents (same experiment as in **A**) were derived from voltage ramps applied at the time points indicated by numbers in (**A**). Similar results were obtained in eight other experiments. (**C**) Original current recording from a neutrophil granulocyte patched with 0.3 mM ADPR in the pipette is shown. The experimental conditions and procedures were the same as for the HL-60 cells (**A**). The inset shows the *I–V* relationships in normal and NMDG bath, as obtained by voltage ramps. Similar results were obtained in five other experiments.

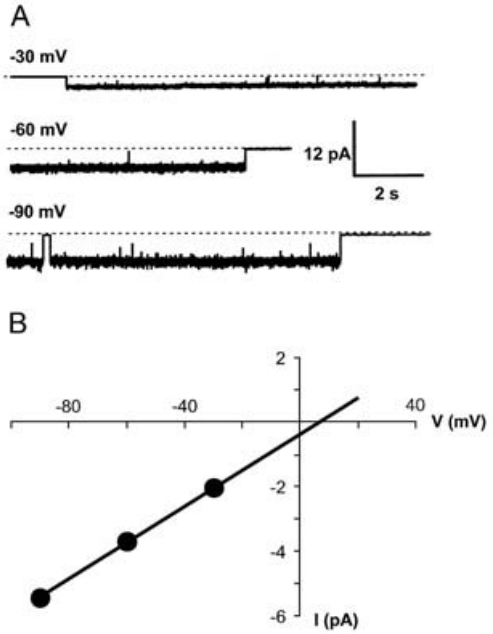

Figure 3 Channel activity in an inside-out patch from an HEK-293 cell transfected with LTRPC2

(**A**) Recordings from one patch at three indicated holding potentials are shown. The bath medium contained 0.3 mM ADPR. The broken line indicates the closed level. Similar results were obtained in two other experiments. (**B**) *I–V* relationships of single-channel events in the experiment depicted in (**A**) are presented. The slope indicates a single-channel conductance of 56 pS. The curve does not cross the ordinate at zero, probably due to the fact that no correction of the liquid junction potential was performed.

gradually during infusion of ADPR into the cytosol (Figure 2A). The reversal potential of the currents was close to 0 mV (Figure 2B), consistent with non-selective cation currents. When the extracellular cations were replaced by the large impermeant cation NMDG, currents were eliminated in the inward direction, but not in the outward direction (Figures 2A and 2B). The mean current at the standard holding potential of − 60 mV was increased from -0.7 ± 0.5 pA/pF at the beginning of the ADPR infusion to -20.8 ± 18.8 pA/pF ($n = 9$) 6–12 min later when the currents had reached a plateau. No such currents were observed when ADPR was omitted from the pipette ($n = 5$).

In neutrophil granulocytes infused with ADPR (Figure 2C), currents were consistently observed. These also developed gradually, although at a higher rate than in HL-60 cells, but they were of smaller amplitudes, as expected in cells of small size [mean capacitance 2.8 ± 0.8 pF versus 8.9 ± 2.2 pF in HL-60 cells in the ADPR experiments ($n = 6$ and 9 respectively)]. The current density increased with ADPR from -4.7 ± 4.8 to -33.0 ± 16.6 pA/pF ($n = 6$) but did not increase in the absence of ADPR in the pipette solution ($n = 3$) or when ADPR was substituted by ADP-glucose ($n = 2$). Again, the reversal potential close to 0 mV (Figure 2C) indicated currents that are non-selective within cations; currents were blocked by NMDG in the inward direction (Figure 2C).

Properties of single channels found in HEK-293 cells after transfection with LTRPC2 were recently reported [18]. We reproduced these findings in inside-out experiments in LTRPC2-transfected HEK-293 cells (Figure 3). The channels were characterized by long open times extending over several seconds with little flickering and a unitary single-channel conductance of 55.4 ± 7.6 pS ($n = 3$ cells). When in whole-cell experiments on neutrophil granulocytes the noise level was minimized by coating the pipette tip with wax, we were able to resolve single-channel events evoked by ADPR at holding potentials between − 30 and − 120 mV ($n = 11$ cells). This was best accomplished in the experiments when the total ADPR-induced current was small. Figure 4(A) shows the successive long-lasting opening of channels during the infusion of ADPR into the cell. Opening of five channels with equal current amplitudes (Figures 4B and 4C) was identified in this experiment. The opening times were in the range of several seconds with little flickering (a detailed analysis of the mean open time was not considered necessary). The single-channel conductance (Figure 4D) was determined to be 55.1 ± 13.5 pS (mean of data obtained from four cells).

NAD^+ has been reported as an alternative stimulus of LTRPC2. When NAD^+ was infused into neutrophil granulocytes

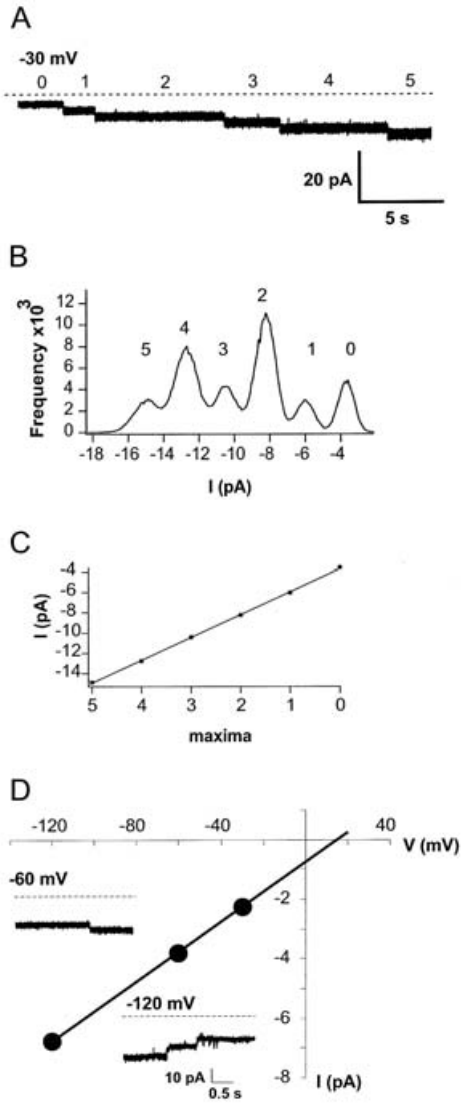

Figure 4 Resolution of single-channel events in a whole-cell recording of a neutrophil granulocyte during the infusion of ADPR (0.3 mM in the pipette solution)

(**A**) Original recording starting from the time when the whole-cell configuration was obtained. The broken line indicates the zero current level. The holding potential was − 30 mV. Numbers indicate the actual number of open channels in the patch. Similar results were obtained in two other whole-cell experiments in which the opening of single channels was resolved at the same holding potential. (**B**) A histogram of the current amplitude of all recorded data points in (**A**) is shown. The ordinate shows the frequency at each current level of the abscissa (bin width = 0.08 pA). Numbers correspond to those in (**A**) and indicate the number of open channels. (**C**) Current values of each maximum in the histogram (**B**) are depicted. (**D**) *I*–*V* relationships of single-channel events in the experiments of (**A**)–(**C**) are presented. The slope indicates a conductance of 50 pS. Characteristic events at holding potentials of − 60 and − 120 mV are shown as insets. Broken lines indicate zero current and the calibration bars indicate 10 pA and 0.5 s respectively.

(Figure 5A), currents developed over time, which were again blocked by NMDG, and had a reversal potential close to 0 mV. The mean current density was -9.5 ± 4.3 pA/pF ($n = 9$ cells). When single-channel events were resolved during NAD^+ infusion (Figures 5B and 5C), these had the same long open times as during infusion of ADPR and exhibited a single-channel conductance of 62.0 ± 4.2 pS ($n = 4$). Thus these channels were indistinguishable from those stimulated by ADPR in neutrophil granulocytes and in LTRPC2-transfected HEK-293 cells.

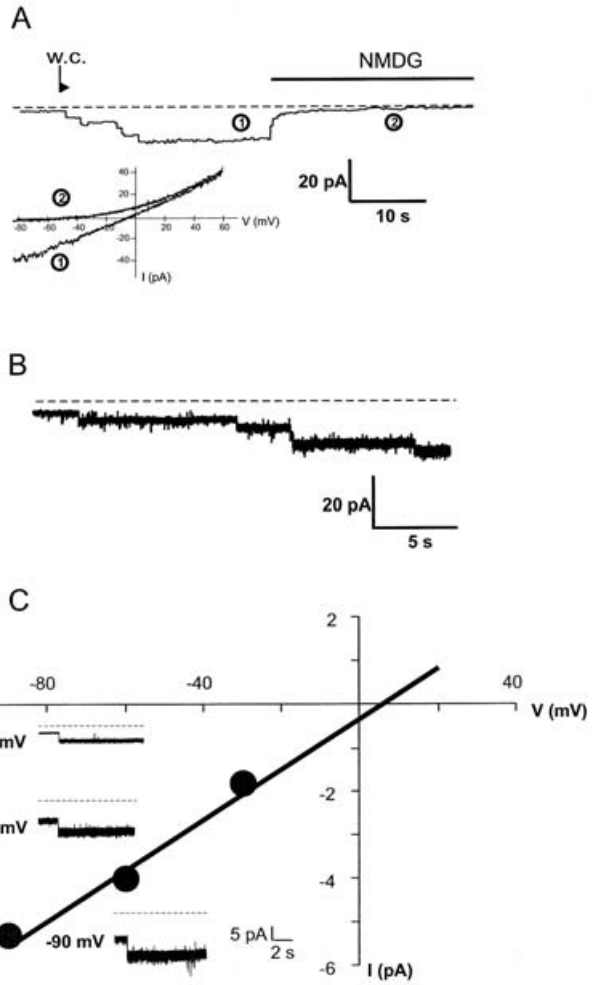

Figure 5 Whole-cell currents and single-channel events in whole-cell recordings of neutrophil granulocytes during infusion of NAD (1 mM in the pipette solution)

(**A**) The continuous current recordings is presented. The whole cell (w.c.) configuration was obtained at the time indicated. The normal bath medium was replaced by a solution with NMDG as the main cation during the time period indicated with a bar. The holding potential was − 60 mV. The inset shows the *I*–*V* relationships obtained by ramps at the time points indicated with numbers. Similar results were obtained in eight other experiments. (**B**) Resolution of single-channel events in another experiment is shown. The recording starts 5 s after obtaining the whole-cell configuration. The holding potential was − 30 mV. Similar results were obtained in three other experiments. (**C**) *I*–*V* relationships for single-channel events are shown. The slope indicates a single-channel conductance of 61 pS. Insets show characteristic events at the indicated holding potential, with the broken lines indicating zero current and the calibration bars being 5 pA and 2 s respectively.

Magnesium-nucleotide-regulated metal ion currents in neutrophil granulocytes

LTRPC7 is also a member of the TRP family. It is ubiquitously expressed in human cells including HL-60 cells and leucocytes; it is responsible for the magnesium-nucleotide-regulated metal ion currents [20]. To demonstrate such currents in neutrophil granulocytes, we followed the procedure of Nadler et al. [20]. During whole-cell current recordings, the bath was replaced by BVF solution. Immediately after exposure to this medium, cells displayed NMDG-blockable cation currents (Figures 6A and 6B) that promptly receded after restitution of a normal bath (results not shown). The mean current density was -10.6 ± 7.4 pA/pF ($n = 10$). The induction of these currents by BVF medium was

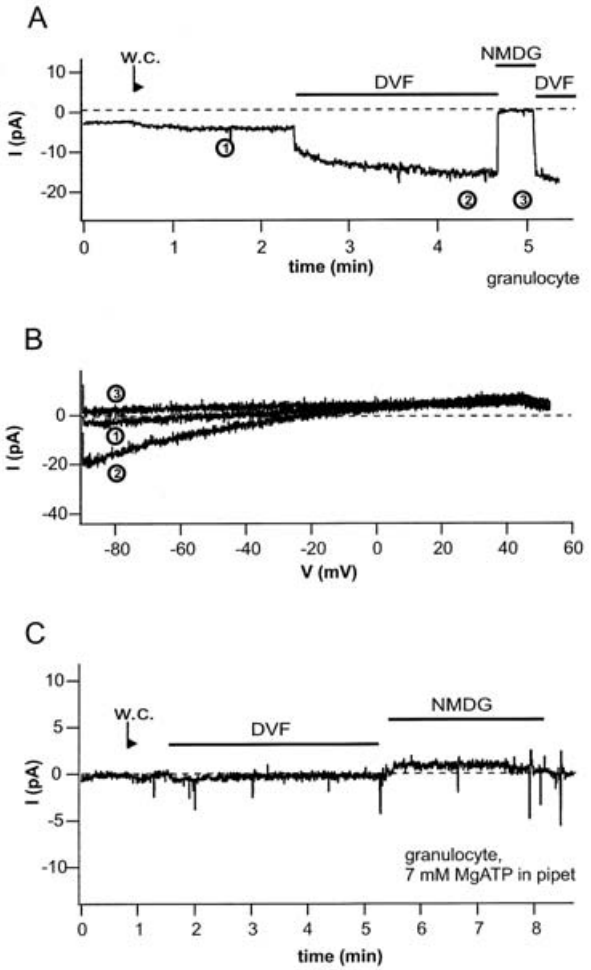

Figure 6 Currents in neutrophil granulocytes induced by the removal of bivalent cations

(**A**) Original current recordings are indicated. The normal bath solution was replaced by a BVF solution and by an NMDG solution during the time periods indicated by bars. (**B**) The corresponding *I–V* relationship is shown. The time points at which ramps were recorded are indicated by numbers corresponding to those in (**A**). (**C**) Original recording from a neutrophil granulocyte is shown. Experimental conditions were the same as in (**A**) with the difference that the pipette solution contained 7 mM MgATP. The holding potential was 60 mV in all these experiments. Similar results were obtained in four other experiments.

prevented (Figure 6D) when the pipette solution was enriched with 7 mM Mg-ATP ($n = 5$).

Characteristic stimuli of other TRP family members without affecting the currents in neutrophil granulocytes

Capsaicin is a well-known stimulus of VR1 (also known as capsaicin receptor) [21]. In spite of the mRNA expression of VR1, application of capsaicin (1–100 μM) failed to induce currents in neutrophil granulocytes. TRPC6 has been described to be activated by diacylglycerol or its analogue 1-oleoyl-2-acetyl-*sn*-glycerol (OAG) [22]. However, we did not obtain evidence that OAG (0.1 mM for 5 min) induces currents in neutrophil granulocytes. Small current fluctuations noticed in the presence of OAG were also observed when the solvent alone (DMSO or ethanol) was applied in control experiments.

DISCUSSION

The present study examined the expression pattern of the mRNAs of TRP channels in neutrophil granulocytes by means of RT–PCR. Furthermore, we have demonstrated non-selective cation currents and single-channel activity induced by ADPR and NAD^+. This is functional evidence for the expression of LTRPC2, which may constitute an important entry pathway for Na^+ and Ca^{2+} in neutrophil granulocytes.

LTRPC2 has been found to be expressed widely in various tissues and forms channels that allow influx of Na^+ and Ca^{2+}, with little selectivity between univalent and bivalent cations. The channels can be activated by intracellular ADPR, possibly by binding to a Nudix box motif in the C-terminal cytosolic tail of the protein [18]. Furthermore, cation influx in LTRPC2-transfected cells could be induced either by intracellular NAD^+ [19,23] or by the application of H_2O_2 [14,19] to the bath medium. H_2O_2 application is an experimental model of oxidative stress. Since the levels of NAD^+ are increased during oxidative stress, H_2O_2 and NAD^+ may represent a common mechanism of LTRPC2 activation [19]. At any rate, both of them may be relevant during oxidative bursts in neutrophil granulocytes when superoxide anions are released, leading to the formation of H_2O_2 and exerting extreme oxidative stress on the granulocytes in an autocrine and paracrine way.

H_2O_2 induced currents in LTRPC2-transfected HEK-293 cells [14] after a delay of several minutes. The currents slowly increased with time and were much smaller than those induced by ADPR. Since neutrophil granulocytes are not stable enough in patch-clamp experiments to allow resolution of currents and channel activity when these develop in a slow and delayed way, we resorted to the rapidly acting NAD^+ as an alternative stimulus of LTRPC2, in addition to ADPR. In neutrophil granulocytes, both stimuli, ADPR and NAD^+, evoked cation currents and channel activity that were indistinguishable from currents and channel activity in HEK-293 cells transfected with LTRPC2 [14,18,19,23]. Especially, the unique current activation by ADPR as well as the characteristic biophysical properties of the channels provides strong evidence for a functional expression of LTRPC2 in neutrophil granulocytes.

ADPR has only recently attracted attention as an intracellular messenger mediating Ca^{2+} influx. More importance has been attributed previously to its related cyclic form, namely cyclic ADPR (cADPR). cADPR induces release of Ca^{2+} from intracellular ryanodine-sensitive calcium stores [24]. Moreover, cADPR has been known to induce Ca^{2+} influx in neutrophil granulocytes [25]. It is formed in neutrophil granulocytes by the transmembrane glycoprotein CD38, which acts as an ADP-ribosyl cyclase, converting NAD^+ into cADPR. Simultaneously, CD38 is an NAD^+ and cADPR hydrolase, thereby forming ADPR as the main (97 %) product of its enzymic activity [26]. CD38 is essential for the function of neutrophil granulocytes, since in CD38 knock-out mice, the chemotactic response of neutrophils to the chemoattractant oligopeptide fMLP is severely impaired, along with diminished Ca^{2+} mobilization and Ca^{2+} influx. The mechanisms by which cADPR induces Ca^{2+} influx have not been elucidated. Our study raises the possibility that cADPR is first hydrolysed to ADPR and then gates LTRPC2.

A well-known principle to explain how Ca^{2+} entry is elicited in electrically non-excitable cells is the store-dependent activation of plasmalemmal Ca^{2+} channels, initiated by the depletion of intracellular calcium stores. Evidence for such a store-operated Ca^{2+} entry (SOCE) has also been found in neutrophil granulocytes [7,27]. TRP channels that have been identified to play a role in SOCE include TRPC1, TRPC3, TRPC4, TRPC5 and ECaC2

(reviewed in [13]). In the light of the expression pattern in neutrophil granulocytes, ECaC2 may be responsible for SOCE in these cells. However, the conditions and requirements that govern the formation of TRP channels to store-operated channels have not been defined. On the other hand, no characteristic properties of store-operated currents or channels have been reported that would make them unambiguously attributable to any given TRP family member. Antisense experiments have demonstrated the contribution of various TRPs to SOCE pathways in several cell types (see e.g. [28,29]). However, neutrophil granulocytes are, probably, unsuitable for any method which uses antisense oligonucleotides. Taken together, the present experiments could not answer the question of whether TRP channels contribute to SOCE in neutrophil granulocytes.

The sequence of LTRPC7 has been reported recently [20], along with information on its ubiquitous expression in human cells including HL-60 cells and leucocytes (although no differentiation of leucocytic subtypes was considered). In view of this evidence, we did not study LTRPC7 expression with RT–PCR but obtained functional evidence for its expression by demonstrating magnesium-nucleotide-regulated metal ion currents in neutrophil granulocytes. Since these currents can be observed only under highly unphysiological conditions, even though LTRPC7 is considered essential for cell survival [20], it is uncertain whether LTRPC7 takes part in signalling pathways during the activation of granulocytes. Even more so, the relevance of the other TRP family members for which mRNA pattern was demonstrated remains questionable, either because their associated currents and mode of activation have not yet been defined or a reported stimulus, as in the case of capsaicin for VR1, failed to induce measurable cellular responses in neutrophil granulocytes. It should be recollected that RT–PCR signals do not necessarily indicate significant protein expression and therefore may have little functional relevance, necessitating additional evidence such as the demonstration of characteristic currents before the role of a given TRP family member can be considered further.

The only member of the subfamily of 'short' TRP channels [10] (which include TRPC1–TRPC7) for which we consistently found mRNA in neutrophil granulocytes was TRPC6. On the other hand, TRPC6 was absent from HL-60 cells. As long as HL-60 cells are considered to be a cell culture model for handling Ca^{2+} influx in neutrophil granulocytes, the discordant expression pattern seems to deny a major role for short TRPCs in neutrophil granulocytes. TRPC6 is closely related to TRPC3; both share one particular mode of activation, namely, they are stimulated by diacylglycerol and by OAG, independently of a protein kinase C. OAG led to gating of TRPC3 and TRPC6 channels in single-channel recordings in excised inside-out patches [22,30] and in $[Ca^{2+}]_i$ measurements with fura 2 [22]. It did not augment whole-cell currents in the present experiments. In general, OAG effects were reported only rarely in whole-cell current measurements [31,32]. Thus we cannot present evidence in favour of or against TRPC6 currents. It appears that LTRPC2 has a more prominent role than TRPC6 in the induction of non-selective cation currents in neutrophil granulocytes.

In conclusion, the present study provides a comprehensive analysis of the endogenous expression of TRP channels in primary human neutrophils. Na^+ and Ca^{2+} currents have been identified which can be attributed to LTRPC2 on the basis of their characteristic regulation and the properties of associated channels. We suggest that LTRPC2 forms an important cation entry pathway in these cells that is regulated by ADPR and the redox state of the cell.

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