Expression of human TRPC genes in the megakaryocytic cell lines MEG01, DAMI and HEL

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Abstract Store-regulated Ca^{2+} entry represents a major mechanism for Ca^{2+} influx in non-excitable cells although many details remain to be evaluated including the identification of cation entry channels. Recently human homologues of the *Drosophila* proteins TRP and TRPL, have been described (TRPC1, TRPC1A, HTRP1) and suggested as candidate cation channels. In this study we sought to examine if the producers of blood platelets, megakaryocytic cells (using the cell lines MEG01, DAMI, HEL), expressed these genes. RNA was prepared from the cell lines and platelets and converted to cDNA. The cDNA was then subjected to 30–35 cycles of PCR using gene specific primers for TRPC1–3. PCR products of the expected sizes were observed for all three TRPC genes in the three cell lines. Direct sequencing confirmed their identity. Additionally for TRPC1, a larger species, and for TRPC2, a smaller species was detected in all three cell lines with sequencing revealing the fragments to contain TRPC sequence, suggesting that they were either products of alternative splicing events or from closely related genes. These results suggest that TRPC genes are expressed in megakaryocytic cell lines and that the TRPC proteins may play a role in mediating cation influx in both megakaryocytes and platelets.

Key words: TRPC; Store-regulated channel; Megakaryocyte; Platelet

1. Introduction

The elucidation of Ca^{2+} influx mechanisms in non-excitable cells represents a major challenge. In platelets Ca^{2+} influx may occur via three main mechanisms. Firstly activation of a receptor operated cation channel (ROC) by agonists such as ADP, may lead to very rapid Ca^{2+} entry with studies using the stopped flow technique measuring millisecond time intervals confirming that ADP induces Ca^{2+} entry without a measurable time delay [1]. However, with most agonists acting via G protein linked receptors or through tyrosine phosphorylation linked mechanisms, Ca^{2+} influx is slower than that induced by an ROC mechanism and is thought to be regulated by the filling state of the intracellular stores (store-regulated or capacitative Ca^{2+} entry [2]). With this mechanism the second messenger inositol 1,4,5-trisphosphate (IP_3) serves to release Ca^{2+} from intracellular stores, which in turn leads to the generation of a (presently) ill-defined signal that opens Ca^{2+} channels in the plasma membrane. Thirdly Ca^{2+} entry may occur as a consequence of the action of second messengers acting directly on the plasma membrane as has been demonstrated with Ca^{2+} and inositol 1,3,4,5-tetrakisphosphate in endothelial cells [3].

There is evidence for multiple types of Ca^{2+} entry channels in non-excitable cells and work towards the identification of members of this family has attracted considerable interest (for a short review see [4]). In *Drosophila* the *trp* and *trpl* genes have been identified to code for cation channels (TRP and TRPL) which can be activated by store depletion and agonists acting at G-protein linked receptors (respectively) [5–7]. The expressed TRP channel shows a higher selectivity for Ca^{2+} than TRPL and has some characteristics similar to the store regulated Ca^{2+} current identified in mast cells [6,8,9]. These studies have led to the search for human homologues of TRP and TRPL proteins that may represent candidate plasma membrane Ca^{2+} channels. Recently, two groups have reported two cDNA stretches coding for proteins termed TRPC1 [10] and Htrp-1 [11] whose primary structure shares approx. 37–40% sequence identity to TRP and TRPL. The cDNAs for both TRPC1 and Htrp-1 are identical in large parts except for variations in the region coding for the amino terminal part of the proteins. Sequences for two transcripts from highly homologous genes (TRPC2 and TRPC3) have also been reported, suggesting other members within this family [10,11a]. A splice variant of the Htrp-1, termed TRPC1A has been functionally expressed in CHO cells and shown to be activated by IP_3 and thapsigargin [12] and Htrp-3 expressed in COS cells also shows capacitative Ca^{2+} entry [11a].

As Ca^{2+} entry forms an essential component of the activation pathway in platelets and a component of Ca^{2+} entry is mediated by store depletion, we sought to examine whether platelets and megakaryocytes contain transcripts from any of the known TRPC genes. For the latter we have used leukaemic cell lines that have phenotypic properties of megakaryocytic precursors namely MEG01, DAMI and HEL cells. We report that all three cells lines express mRNA transcripts derived from TRPC1–3 genes with the possibility of products of alternative splicing events for TRPC1 and TRPC2.

2. Materials and methods

2.1. Cell culture and platelet preparation

The DAMI cell line was obtained from Dr. R. Handin (Boston, MA, USA), HEL and MEG01 were obtained from Dr. S.P. Watson (Oxford, UK) and Dr. G. Périès (Paris, France). The cells were grown in RPMI 1640 plus 10% fetal calf serum. Cells were harvested by centrifugation and washed once in PBS before extraction of RNA. Platelets were isolated from fresh human blood and washed exactly as described previously [13].

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2.2. Preparation of RNA

Total RNA was isolated according to the principle of Chomczynski and Sacchi [14] but using RNAzol reagent (Tel-Test, Inc., Friendswood, USA) according to the manufacturer's instructions. Briefly, cells were lysed in 2 ml guanidine thiocyanate-phenol-chloroform solution. Following centrifugation at 10000×*g*, the RNA-containing aqueous phase was removed and the total RNA precipitated using 1 vol. isopropanol. After one wash in 70% ethanol and resuspension, RNA was directly used for cDNA synthesis.

2.3. cDNA synthesis and PCR amplification (RT-PCR)

6 μg total RNA were used for each reverse transcription reaction using the 'ready to go' T-Primed First strand Kit (Pharmacia, Uppsala, Sweden). cDNA synthesis was carried out at 45°C. Aliquots (1/10 to 1/6 of the reaction mixture) of cDNA were then used directly as template in subsequent PCR reactions which employed AmpliTaq Gold polymerase (Roche Molecular Systems, Inc., Branchburg, USA) and its 10×PCR buffer containing 15 mM $MgCl_2$. Cycling conditions were: 1 min at 94°C, 1 min at 55°C or 59°C, 1 min at 72°C for the number of cycles specified in the Results section. The initial denaturing step was 12 min at 94°C, the final extension was 10 min at 72°C. Oligonucleotide primers used were: for TRPC1, trpc1f and trpc1r; for TRPC2, trpc2f and trpc2r; for TRPC3, trpc3f and trpc3r; the sequence and location of these oligonucleotides are given in Table 1. Oligonucleotide primers for amplification of glyceraldehyde 3-phosphate dehydrogenase (G3PDH) mRNA were obtained from Clontech (Palo Alto, CA, USA) and used according to the manufacturer's instructions.

2.4. Analysis of PCR products

PCR-amplified fragments were visualised by ethidium bromide staining following agarose gel electrophoresis. PCR products were excised from agarose gels and purified using the Geneclean II kit. Where stated sequencing of the purified fragments was carried out by the method of Sanger et al. [15] using a sequenase PCR product sequencing kit (USB, Cleveland, OH, USA).

3. Results

In order to investigate the presence of TRPC transcripts in human megakaryocytic cell lines, an RT-PCR based strategy was employed. Oligonucleotide primers were designed so as to bind specifically to either TRPC1, TRPC2 or TRPC3 cDNA (regions of strong homology were avoided) and to generate PCR products of different sizes.

Total RNA was prepared from the three human megakaryocyte cell lines (DAMI, HEL and MEG01). Following reverse transcription with oligo dT primer, aliquots of each cDNA mixture were subjected to 35 cycles of PCR using gene specific primers (see Table 1). Subsequent agarose gel electrophoresis revealed PCR products of the expected size for each of the three TRPC transcripts (427 bp for TRPC1, 343 bp for TRPC2 and 304 bp for TRPC3; Fig. 1A), suggesting that all three genes are expressed in these cell lines. In identical PCR reactions which contained 100 ng genomic DNA (instead of cDNA) as template, no products were observed (data not shown), excluding the possibility that the bands were derived from chromosomal contamination. Unexpectedly, additional PCR products were observed for both TRPC1 and TRPC2 (and a very faint additional band for TRPC3). When PCR reactions were performed under more stringent conditions (59°C annealing temperature) and for only 30 cycles, a prominent additional band for both TRPC1 and TRPC2 was still present (slightly above TRPC1 and slightly below TRPC2, respectively; Fig. 1B shows typical results obtained with the DAMI cell line, similar results were obtained with MEG01 and HEL), raising the possibility that these bands did not result from PCR artefacts but could instead represent either alternatively processed TRPC transcripts or transcripts from novel homologous genes.

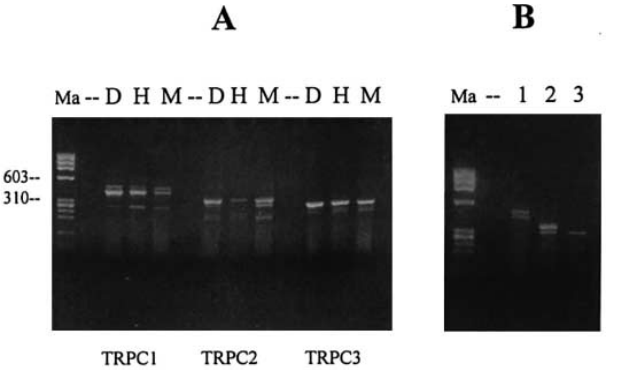

Fig. 1. Detection of PCR products derived from transcripts of TRPC1–3 genes in megakaryocytic cell lines MEG01, DAMI and HEL. Shown are ethidium bromide stained agarose gels. (A) Products after 35 cycles of PCR with primers for TRPC1–3. Ma, size marker (φX174/*Hae*III); M, MEG01; D, DAMI; H, HEL. (B) Products after 30 cycles of PCR with DAMI cDNA using TRPC1–3 primers (labelled 1–3). (–) Negative PCR control (no cDNA).

The PCR products of the expected size were isolated and purified by the Geneclean II kit for further characterization. Direct sequence analysis confirmed their identity as wild-type TRPC1–3 transcripts (data not shown). This demonstrates that all three genes are transcribed in human megakaryocytes and suggests that several store-operated ion channels may be involved in megakaryocyte/platelet Ca^{2+} homeostasis. Furthermore, these findings indicate that the expression of the not yet fully characterised TRPC2 (pseudo) and TRPC3 genes is not restricted to those cell types from which the respective cDNA clones had been derived (fetal liver/spleen and fetal brain, respectively).

In addition, the major two aberrantly sized types of PCR product, presumed to be derived either from the TRPC genes or from homologous genes, were isolated. Direct sequencing revealed that both the larger product formed with the TRPC1

Table 1
Oligonucleotide primers used for PCR of TRPC cDNA

Primer	Sequence	Location
TRPC1f	5′-ACTCTGGTATGAAGGGTTGGA-3′	2766–2786
TRPC1r	5′-GGTATCATTGCTTTGCTGTTC-3′	3172–3192
TRPC2f	5′-GGTATGGAAGAGCACAGCGTG-3′	308–328
TRPC2r	5′-GATAGTCTGGCTTCTTGGTTT-3′	630–650
TRPC3f	5′-CCGTTGTGCTCAAATATGATC-3′	7–27
TRPC3r	5′-AGCCTTCTCCTTCTGCATTTG-3′	290–310

f, forward; r, reverse; location refers to the sequences deposited in GenBank accession numbers: X89066 (TRPC1), X89067 (TRPC2) and X89068 (TRPC3).

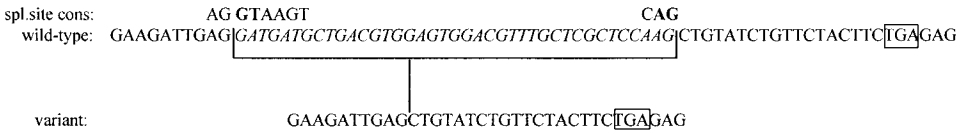

Fig. 2. Sequence comparison of TRPC2 wild-type and variant PCR products derived from DAMI cell cDNA. The additional 39 bp in the wild-type transcripts are shown in italics and the premature stop codon is boxed in both sequences. Since the nucleotides in this region exhibit some similarity to splice sites, consensus sequences are given above for comparison (invariant dinucleotides in bold).

primers and the smaller product formed with the TRPC2 primers contained TRPC1 and TRPC2 sequence (respectively). The smaller TRPC2 fragment lacked 39 bp (nucleotides 461–499 of Genbank sequence accession no. X89067) of the wild-type sequence (Fig. 2) and had most likely been generated via alternative splicing (splice site consensus sequences are shown in Fig. 2 for comparison with the relevant TRPC2 nucleotides). The aberrantly sized TRPC1 PCR product also contained a stretch of wild-type sequence but linked to additional nucleotides of unknown origin (data not shown); it is not known whether this transcript species represents a TRPC1 splice variant or is derived from mRNA of a homologous gene. Both possibilities are currently under investigation.

Although differentiated platelets cannot produce mRNA themselves, transcripts of several genes active at the megakaryocyte stage can be detected in human platelets. In order to establish whether TRPC transcripts were also present, total platelet RNA (representing stored rather than freshly transcribed mRNAs) was isolated and converted into cDNA. PCR reactions employing 40 amplification cycles failed to detect any correctly sized TRPC cDNA (Fig. 3). PCR using the TRPC2 primers did generate two products, however, as these were markedly different from the expected sizes they were not characterised further. From the same RNA preparation, cDNA derived from the housekeeping gene G3PDH, employed as a positive control, was detected (Fig. 3) demonstrating that the RNA quality had been adequate.

4. Discussion

The identification of human genes encoding potential store-operated ion channels [10] will now provide the opportunity to analyse the role of the corresponding proteins in various cell types. The finding that TRPC1A expressed in CHO cells can be activated by thapsigargin or IP_3 is strong evidence that these proteins play an important role in receptor mediated Ca^{2+} entry. As a first step to assess the function of such proteins in cells of the megakaryocyte lineage, we studied the expression of the three known human TRPC genes in megakaryocyte cell lines. RT-PCR analysis revealed that transcripts from all three genes were present in all three cell lines, suggesting that the respective proteins may be involved in signalling events both in megakaryocytes and in platelets.

Northern blot analysis has previously demonstrated high-level expression of TRPC1 in heart, brain, testes and ovary, and lower levels of TRPC1 transcripts in all other human tissues tested with the exception of liver [10,11]. However, homologous TRPC1 mRNA in mouse liver could be detected by RT-PCR analysis, suggesting that low levels of TRPC1 transcripts may also be found in human liver. This is consistent with our finding that TRPC1 mRNA could be amplified with 30 PCR cycles from human hepatoma cell cDNA (data not shown). The presence of TRPC1 mRNA in megakaryocytic cell lines reported here lends further support to the notion that TRPC1 is ubiquitously expressed in human cells, albeit at cell type specific levels. We have shown here that both TRPC2 and 3 are transcribed in cells other than fetal liver/spleen and fetal brain, respectively, from which the original cDNA clones had been isolated. It therefore appears that all three human TRPC genes may be ubiquitously transcribed. Wes et al. [10] have reported that the TRPC2 cDNA sequence contains a stop codon in a position corresponding to residue 690 in TRPC1 (Fig. 2) and may therefore represent an expressed pseudogene.

The relative levels of transcription from the three genes are difficult to assess without the use of sophisticated internal PCR controls. Based on the comparison of band strengths, we suggest that the three genes are expressed at similar levels in these cell lines. With each of the specific primer pairs used, distinct RT-PCR products were observed: an additional band was reproducibly observed with TRPC1 and TRPC2 specific primers (Fig. 1B). DNA sequence analysis of these bands confirmed their origin as derived from the respective TRPC genes. We suggest the TRPC2 derived transcript represents an alternative splicing product which remains in frame and retains the stop codon (Fig. 2). The elucidation of the TRPC1 exon/intron structure will reveal whether the second aberrantly sized transcript species is also derived from aberrant RNA processing. Our detection of these transcript species is consistent with previous findings by Zhu et al. [11] who demonstrated alternative processing of another region of the human TRPC1 gene (omission of the nucleotides encoding amino acids 102–143 of Htrp-1).

In summary, we have demonstrated that transcripts from three currently known human TRPC genes are present in different human megakaryocyte cell lines. This is consistent with a potential role of TRPC proteins in Ca^{2+} homeostasis

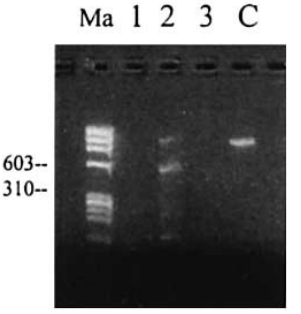

Fig. 3. Analysis of platelet cDNA using TRPC1–3 primers. Analysis was carried out using 40 cycles of PCR. (1–3) PCR with TRPC1–3 primers; Ma, size marker (ϕX174/*Hae*III); C, G3PDH cDNA (positive control).

in megakaryocytes and in blood platelets. That we were unable to detect mRNA derived from TRPC genes in platelets may reflect rapid processing of these transcripts after release of the cells into the circulation. Our findings are consistent with the notion that TRPC1 is ubiquitously expressed albeit at tissue-specific levels. The presence of TRPC2 and TRPC3 transcripts in megakaryocytes suggests that both genes, like TRPC1, may also be expressed in a variety of cell types, although the TRPC2 mRNA contains a premature stop codon and is therefore unlikely to produce a functional protein. Our study also suggests that the megakaryocytic cell lines may represent good model systems to study the role of TRPC proteins in agonist induced Ca^{2+} influx.

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