Diabetologia (1997) 40: 528–532

Diabetologia
© Springer-Verlag 1997

Identification of four *trp*1 gene variants murine pancreatic beta-cells

H. Sakura, F. M. Ashcroft

University Laboratory of Physiology, Oxford, UK

Summary Insulin secretion is stimulated by glucose, hormones and neurotransmitters. Both activation of a non-selective cation current and activation of a Ca^{2+} current in response to depletion of intracellular Ca^{2+} stores have been suggested to play a role in this stimulation. The properties of these currents resemble those reported for the *Drosophila* genes *trp* and *trpl*. Using the reverse transcription polymerase chain reaction and Northern blot analysis we found that of the six mammalian *trp*-related genes (*trp*1–6), only *trp*1 was expressed at high levels in the mouse insulinoma cell line MIN6. We cloned the murine homologue of human *trp*1 from MIN6 cells and identified four variants (α, β, γ and δ), generated by alternative splicing near the N-terminus of the protein. In vitro translation showed that only the α and β splice variants are efficiently expressed. The β variant is the dominant form in MIN6 cells (and probably in mouse pancreatic islets), whereas the α variant is the major type in the mouse brain. The β variant showed 99 % identity to the human homologue at the amino acid level. [Diabetologia (1997) 40: 528–532]

Keywords *trp*1, Ca-release activated channel, pancreatic beta cell, MIN6 cell, insulin secretion.

Insulin secretion from the pancreatic beta cell is stimulated by glucose, hormones and neurotransmitters [1]. Glucose is the primary physiological stimulus for insulin secretion. It acts by closing ATP-sensitive potassium (K-ATP) channels in the beta-cell plasma membrane. This produces a membrane depolarisation which opens voltage-dependent Ca^{2+} channels and the ensuing Ca^{2+} influx elevates $[Ca^{2+}]_i$ and so initiates insulin secretion. The inhibition of K-ATP channels cannot by itself explain the depolarization induced by glucose. Rather, it is necessary to postulate that there is a resting inward current with a more positive equilibrium potential, which dominates the resting potential when K-ATP channels close. This background inward current has not been identified but the positive reversal potential suggests that it must be primarily carried by Na^+ and/or Ca^{2+} ions.

A number of hormones which potentiate insulin secretion are also believed to depolarise the beta-cell by activating an inward Na^+-current. Among these are acetylcholine (ACh [2–5]) and the pituitary adenylate cyclase activating polypeptide (PACAP [6]). Although acetylcholine elevates inositol 1,4,5-triphosphate (IP_3) and mediates Ca^{2+} release from intracellular stores, current evidence suggests that the Na^+-current is not activated by Ca^{2+} and is independent of Ca^{2+}-store depletion [5]. Depletion of beta-cell intracellular Ca^{2+} stores by exposure to low extracellular Ca^{2+}, however, does activate a small non-selective inward current [7, 8]. This current has been called I_{CRAC} (for Ca^{2+}-release-activated current). Depletion of intracellular Ca^{2+} stores by

Received: 4 December 1996 and in revised form: 3 February 1997

Corresponding author: Professor F. M. Ashcroft, University Laboratory of Physiology, Parks Road, Oxford OX1 3PT, UK

Abbreviations: RT-PCR, Reverse transcriptase polymerase chain reaction; I_{CRAC}, Ca^{2+} release-activated current; K-ATP channel, ATP-sensitive potassium channel; *trp*, transient receptor potential; MIN, mouse insulinoma cells; HEK, human embryonic kidney cells; SDS, sodium dodecyl sulphate; VDCC, voltage-dependent calcium channel.

thapsigargin modifies the pattern of beta-cell electrical activity and $[Ca^{2+}]_i$ oscillations induced by glucose [8, 9].

The channel (or channels) responsible for the beta-cell background Na^+-current, the Na^+-current activated by hormones, and I_{CRAC} have not been identified at the molecular level. The properties of these currents are, however, reminiscent of those produced by expression of the *trp* and *trpl* genes. The *Drosophila* gene product *trp* (transient receptor potential) encodes a putative plasma membrane Ca^{2+} channel. Expression of *trp* in heterologous systems enhances Ca^{2+} entry in response to depletion of intracellular Ca^{2+} stores with thapsigargin [10, 11]. A related gene product, *trpl* (*trp*-like), encodes a constitutively active non-selective cation channel which is unaffected by store depletion [12]. Recently, mammalian homologues of *trp* and *trpl* have been identified [13–16] and the existence of six *trp*-related genes in the mouse genome (*Mtrp*1–6) has been reported by RT-PCR [17]. In this paper, therefore, we have examined the expression of the *Mtrp* genes in the mouse insulinoma cell line MIN6, which constitutes a pure beta-cell population.

Materials and methods

Isolation of trp fragments by RT-PCR. Poly(A)$^+$ RNA was purified from MIN6 cells (mouse insulinoma cells) and HEK293 cells (human embryonic kidney cells) using a FastTrack 2.0 Kit (Invitrogen, De Schelp, The Netherlands) and converted to cDNA using reverse transcriptase (RT). Two redundant primers, 5′-TNGGNCCN(C/T)TNCA(A/G)AT(A/T/C)TC-3′ (sense) and 5′-CGNGC(A/G)AA(C/T)TTCCA(C/T)TC-3′ (antisense) were designed, which are common to all six *trp* genes and predicted to amplify 367–424 bp fragments. For amplification of *trp* 4 and *trp* 5, we also used 5′-TCTGCAGATATCTCTGGGAAGGATGC-3′ (sense) and 5′-AAGCTTTGTTCGAGCAAATTTCCATTC-3′ (antisense) primers. The PCR conditions consisted of 35 cycles of 94 °C for 1 min, 50 °C for 1 min and 72 °C for 1 min. Each 10 μl PCR reaction contained 100 ng of cDNA template, 10 pmol of each PCR primer, 0.2 mmol/l of each of the dNTPs, 0.25 units of Taq DNA polymerase (Promega, Madison, Wis., USA), 50 mmol/l KCl, 10 mmol/l Tris-HCl (pH 9.0), 0.1 % Triton X-100 and 1.5 mmol/l $MgCl_2$. Since the PCR product exhibited multiple bands (because redundant primers were used: data not shown) we cut out the bands of 350–450 bp, which will contain all fragments amplified from *Mtrp*1–6. These PCR fragments were purified, subcloned into pT7Blue T-Vector (Novagen, Madison, Wis, USA) and sequenced.

Screening of cDNA library. A MIN6 cDNA library was constructed as described elsewhere [18] using a ZAP Express cDNA synthesis kit (Stratagene, La Jolla, Calif., USA). This library (5×10^5 phages) was screened under low stringency conditions (hybridised with 30 % formamide, 5 × Denhardt's, 5 × SSPE (0 · 75 mol/l NaCl, 50 mmol/l Na_2PO_4, pH 7 · 4) 100 μg/ml denatured salmon sperm DNA at 37 °C and washed with 0.5 × SSC (75 mmol/l NaCl, 7 · 5 mmol/l Na-citrate, pH 7 · 0), 0.1 % sodium dodecylsulphate (SDS) at room temperature). PBK-CMV plasmids containing a cDNA insert of a positive clone were excised using a helper phage (ExAssist, Stratagene, La Jolla, Calif., USA) and sequenced.

Northern blot analysis. The full length murine *trp*1 was hybridised with 2 μg of MIN6 poly(A)$^+$ RNA, and with a Multiple Tissue Northern Blot (Clontech, Palo Alto, Calif., USA) containing 2 μg of poly(A)$^+$ RNA from various mouse tissues according to the manufacturer's protocol. This was carried out under low (as described for library screening) or high (hybridised with 50 % formamide, 10 × Denhardt's, 5 × SSPE, 100 μg/ml denatured salmon sperm DNA and 2 % SDS at 42 °C and washed with 0.1 % × SSC, 0.1 % SDS at room temperature) stringency conditions. Autoradiography was carried out for 2 days with intensifying screens. After washing out of the *trp* probe, filters were re-hybridised with β-actin as a control.

Identification of splicing variants. Total RNA was isolated using TRI-Reagent (Molecular Research Centre, Oxford, UK) from mouse brain, kidney, and pancreatic islets and converted to cDNAs. Primer pairs, 5′-TGTACCCGAGCACGGACCTC-3′ (sense) and 5′-GCAGAACACAGTGTGCATTC-3′ (antisense), were end-labelled using [^{32}P] γ-ATP and PCR was performed as above. Amplified products were subjected to electrophoresis using a 5 % polyacrylamide gel and then to autoradiography.

In vitro translation. The cDNAs encoding each of the *Mtrp*1 variants were cloned into the pBF vector. In vitro translation was performed using a TNT Coupled Reticulocyte Lysate System (Promega Madison, Wis, USA). Plasmid DNA (0.5 μg) was translated (reaction volume, 25 μl) using SP6 RNA polymerase for 2 h according to the manufacturer's protocol. We loaded 2 μl of reaction product onto an 8.5 % SDS-polyacrylamide gel. After electrophoresis, the gel was dried and subjected to autoradiography for 2 h.

Nomenclature. We have used the nomenclature of Zhu et al. [17]. Accordingly, the partial sequence obtained from rat brain by Petersen et al. [13] is *Mtrp*4 and the human sequences which appear in references [15] and [16] are *Htrp*1. The full length sequence of *Mtrp*1 is deposited in Gene Bank (Accession No. U73 625).

Results

In order to obtain DNA fragments for Northern blotting and cDNA library screening, we performed RT-PCR with MIN6 cell and HEK293 cell cDNAs as a template and primers which are designed to amplify all six known *trp* genes. Six PCR products were analysed from each cell type. For MIN6 cells, five of these corresponded to *trp*1 and one to *trp*6, whereas two *trp*1, three *trp*3 and one *trp*6 products were obtained from HEK293 cells. Although *trp*1, 3, 6 and 2 (which is possibly a pseudogene) show considerable sequence identity, *trp*4 and *trp*5 show rather less homology. We therefore carried out RT-PCR with separate primers for *trp*4 and *trp*5 and as a result we isolated six *trp*4 PCR products, but no *trp*5, from each cell type.

We next screened a MIN6 cDNA library (5×10^5 phages) with a mixed probe consisting of the *trp*1,3,4

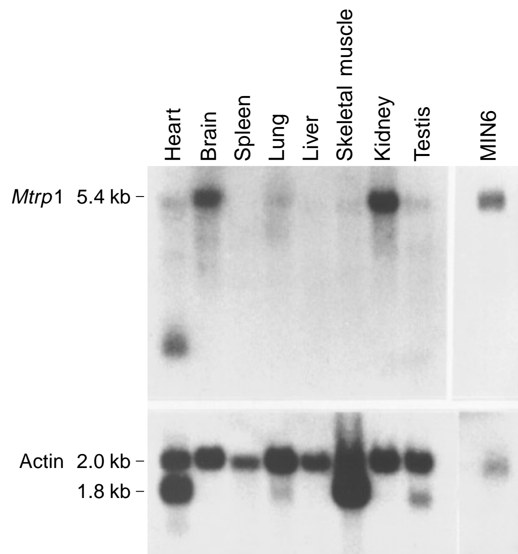
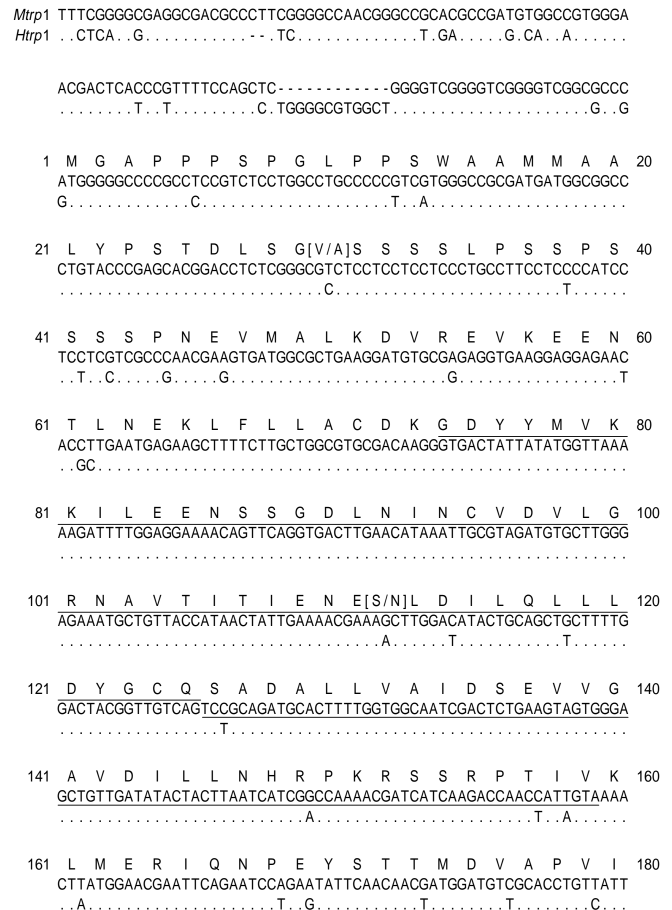


Fig. 1. Northern blot analysis of *Mtrp*1 expression. Adult mouse multiple tissue blot and MIN6 total mRNA (2 μg/lane). *Upper*: Hybridized with *Mtrp*1. The signal migrated at 5.4 kb on each RNA blot. In heart, a 1.4 kb band was also detected. *Lower*: Each filter was rehybridized with an actin probe

and 6 fragments we obtained by RT-PCR. Using low stringency hybridisation conditions, we isolated 9 positive clones. Partial sequencing showed that all clones contained the mouse *trp*1 gene (*Mtrp*1). To determine the tissue distribution of *Mtrp*1 expression, we performed Northern blotting under high stringency conditions. As shown in Figure 1, in most tissues a single band of 5.4 kb was detected. The expression level of *Mtrp*1 was very high in MIN6 cells, brain and kidney, less strong in heart, lung, skeletal muscle, testis and liver, and not detectable in spleen. An additional signal at 1.4 kb was detected in heart. We also performed Northern blot analysis with *Mtrp*3, 4 and 6 as probes, but expression of these genes could not be detected in MIN6 cells, even under low stringency conditions (data not shown).

Since the full length sequence of *Mtrp*1 has not been reported, we determined the nucleotide and the deduced amino acid sequences of four *trp*1 clones, which on the basis of their insert size were expected to contain the full length coding region. There was 91.1% homology between the mouse and human *trp*1 at the nucleotide level and only two amino acids were different (30 [V/A] and 112 [S/N]). As shown in Figure 2A, an additional in-frame methionine codon was found 16 amino acids upstream of the methionine corresponding to the putative initiation codon of human *trp*1. In the case of human *trp*1, this additional methionine is replaced with valine (ATG-GTG). To determine the translation initiation site of murine *trp*1 we carried out in vitro translation. The translated product from the ApaI restriction enzyme site, which is located immediately downstream of the first methionine codon of murine *trp*1, was shorter



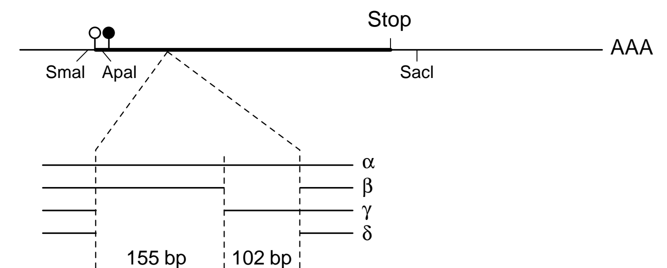


Fig. 2A, B. Nucleotide and deduced amino acid sequences of the *Mtrp*1 splicing region. **A**. Alignment of the *Mtrp*1 gene with the *Htrp*1 gene. Hyphens indicate gaps in the alignment and dots represent identical nucleotides in mouse and man. The deduced amino acid is given above the nucleotide sequence. Differences in amino acids (aa) between mouse and man are shown as [mouse aa/human aa]. The nucleotide sequences which are deleted in the splice variants are shown by single underlining and overlining. The α variant has no deletion. The deletions which occur in the β and γ variants are indicated by underlining and overlining, respectively. The deletions in the δ variant are marked by both underlining and overlining the sequence. **B**. The schema shows the putative initiation site, the restriction enzyme sites and the four splicing variants. The closed circle corresponds to the location of the putative initiation methionine of the human *trp*1. The open circle indicates the location of the first methionine in the murine *trp*1, and is 16 amino acids upstream of that of human *trp*1 (represented by the closed circle)

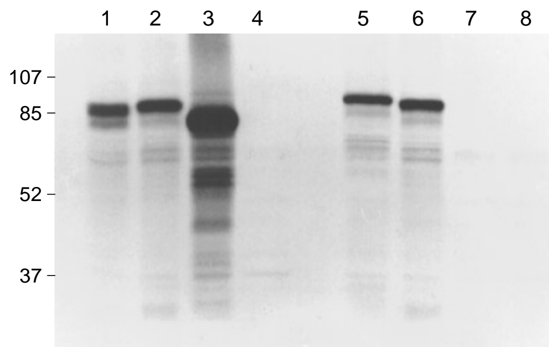


Fig. 3. In vitro translation. Plasmids containing the following inserts were translated using an in vitro reticulocyte system. Lane 1, Full length cDNA (β variant). Lane 2, SmaI-SacI fragment, Lane 3, ApaI-SacI fragment. Lane 4, No DNA, Lane 5–8, α, β, γ and δ variants (SmaI-SacI fragment). The molecular weight (kDa) is shown on the left

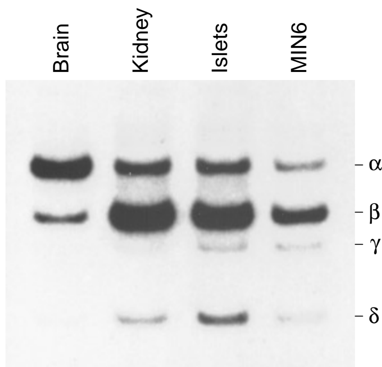


Fig. 4. Four variants of *Mtrp*1 were detected by PCR.The sizes of PCR products; α, β, γ, and δ are 586, 484, 431 and 329 bp, respectively

than that obtained using the full-length clone or the SmaI restriction enzyme site (Figs. 2B and 3). Therefore, the methionine located 16 amino acids upstream of the putative initiation codon of human *trp*1 may be considered to be the main translation initiation codon of murine *trp*1. Thus, the translated protein contains 809 amino acids, in contrast to *Htrp*1 which is 793 amino acids long [14].

During determination of the nucleotide sequence, we found that two clones had the same deletion in the N-terminal region, at a position close to that at which alternative splicing of the human *trp*1 gene is known to occur [16]. We therefore amplified this region using RT-PCR. As shown in Figure 4 four different bands were detected, which we refer to on the basis of their size as α, β, γ and δ variants. Sequencing of each of these PCR products showed that all four variants were produced by alternative splicing, as shown in Figure 2A and B. As the same primers were used in each case, the density of the bands gives an indication of the level of expression. The α type is the major variant in brain, whereas the β variant is dominant in kidney, pancreatic islets and MIN6 cells (Fig. 4). In case of the γ and δ variants, a frameshift is introduced, because the deletion of 155 bp cannot be divided by 3 (Fig. 2B). This suggests that these variants are either not expressed, or that an alternative methionine may be used as initiation codon. As shown in Figure 3, only α and β variants were efficiently translated. Therefore, the γ and δ variants are considered to be non-functional.

Discussion

Among the six types of *Mtrp* genes which have been reported [17], we found that only *Mtrp*1 was expressed at high levels in the insulinoma cell line MIN6. Although *Mtrp* 4 and 6 were also identified by PCR the results of Northern blot analysis and screening of cDNA libraries indicated that the expression level of these genes is very low in MIN6 cells.

The expression pattern of *trp*1 in mouse tissues differs from that reported in man. In the mouse, a strong *trp*1 mRNA signal of 5.4 kb was detected in kidney and brain with a less strong signal in heart and testis, whereas in adult man the highest levels of expression are in brain, heart, testis and ovary with only low levels in pancreas and kidney [14, 15]. *Htrp*1 is, however, expressed at high levels in fetal kidney. Furthermore, an additional 1.4 kb band was detected in mouse heart; it is possible that this 1.4 kb signal comes from a gene related to *Mtrp*1.

We identified four splicing variants of *Mtrp*1. Of these, only the α and β variants have been identified for human *trp*1 (*Htrp*1, [7, 8]): the γ and δ variants have not been reported. The RT-PCR results reported in this study suggest that the γ and δ variants are only a minor component of *Mtrp*1 in all tissues examined. Furthermore, in vitro translation studies indicate that these proteins are unlikely to be expressed (although it is possible that expression may be different in tissues other than rabbit reticulocytes). The *Mtrp*1 β variant was the dominant variant in MIN6 cells. The results of RT-PCR suggest this may also be the case for pancreatic islets.

Hydrophobicity analysis suggests that, like other members of the *trp* family, the *Mtrp*1 protein has six putative transmembrane domains and a pore loop between domains 5 and 6. This predicted topology is similar to that of each of the four repeats of the voltage-dependent calcium channel (VDCC). Unlike the calcium channel, however, there are no charged amino acids in the fourth transmembrane domain of *Mtrp*1 (which in VDCC is believed to act as the voltage sensor). This suggests that the *Mtrp*1 channel will be voltage-independent.

Functional studies have suggested that the *Htrp*1 β variant encodes a non-selective cation channel which is permeable to both Na^+ and Ca^{2+} [16]. This

channel also appears to be activated by depletion of intracellular Ca^{2+} stores [16]. The dominant expression of the *Mtrp*1 β variant in MIN6 cells suggests that this gene may encode a non-selective cation channel in pancreatic beta-cells. This may correspond to the non-selective I_{CRAC} current reported in beta-cells by Worley et al. [7]. Whether it may also underlie the Na^{+}-entry that is activated by acetylcholine stimulation of muscarinic m3 receptors [2–5] remains to be determined. It is worth noting, however, that capacitative Ca^{2+} entry in *Xenopus* oocytes may be enhanced by G-proteins [13], and that m3 receptors are believed to mediate their effects via G-protein activation. Since the *Htrp*1 β-variant encodes a non-selective cation channel, it is also possible that the beta-cell background inward current, which is responsible for depolarizing the beta-cell when K-ATP channels close, may be encoded by the *Mtrp*1 gene. A detailed comparison of the properties of both currents will be necessary to determine if this idea is correct.

Acknowledgements. We thank the Wellcome Trust and the British Diabetic Association for support.

References

1. Ashcroft FM, Rorsman P (1989) Electrophysiology of the pancreatic β-cell. Prog Biophys Molec Biol 54: 87–143
2. Gilon P, Henquin JC (1993) Activation of muscarinic receptors increases the concentration of free Na^{+} in mouse pancreatic B-cells. FEBS Lett 315: 353–356
3. Bertram R, Smolen P, Sherman A et al. (1995) A role for calcium release-activated current (CRAC) in cholinergic modulation of electrical activity in pancreatic B-cells. Biophys J 68: 2323–2332
4. Henquin JC, Garcia MC, Bozem M, Hermans MP, Nenquin M (1988) Muscarinic control of pancreatic B-cell function involves sodium-dependent depolarization and calcium influx. Endocrinol 122: 2134–2142
5. Miura Y, Gilon P, Henquin JC (1996) Muscarinic stimulation increases Na^{+} entry in pancreatic B-cells by a mechanism other than the emptying of intracellular Ca^{2+} pools. Biochem Biophys Res Commu 224: 67–74
6. Leech CA, Holz GG, Habener JF (1995) Pituitary adenylate cyclase activating polypeptide induces the voltage-independent activation of inward membrane currents and elevation of intracellular calcium in HIT T15 insulinoma cells. Endocrinol 136: 1530–1536
7. Worley III JF, McIntyre MS, Spencer B, Dukes ID (1994) Depletion of intracellular Ca^{2+}-stores activates a maitotoxin-sensitive nonselective cationic current in beta-cells. J Biol Chem 269: 32055–32058
8. Worley III JF, McIntyre MS, Spencer B, Mertz RJ, Roe MW, Dukes ID (1994) Endoplasmic reticulum calcium store regulates membrane potential in mouse islet B-cells. J Biol Chem 269: 14359–14362
9. Leech CA, Holtz IV GG, Habener JF (1994) Voltage-independent calcium channels mediate slow oscillations of cytosolic calcium that are glucose dependent in pancreatic B-cells. Endocrinol 135: 365–372
10. Berridge MJ (1996) Capacitative calcium entry. Biochem J 312: 1–11
11. Friel DD (1996) Trp: its role in phototransduction and store-operated Ca^{2+}. Cell 85: 617–619
12. Vaca L, Sinkins WG, Hu Y, Kunze DL, Schilling WP (1994) Activation of recombinant trp by thapsigargin in Sf9 insect cells. Am J Physiol 267:C1501–C1505
13. Petersen CCH, Berridge MJ, Borgese MF, Bennett DL (1995) Putative capacitative calcium entry channels: expression of *Drosophila trp* and evidence for the existence of vertebrate homologues. Biochem J 311: 41–44
14. Zhu X, Chu PBM, Peyton M, Birnbaumer L (1995) Molecular cloning of a widely expressed human homologue for the *Drosophila trp* gene. FEBS Lett 373: 193–198
15. Wes PD, Chevesich J, Jeromin A, Rosenberg C, Stetten G, Montell C (1995) TRPC1, a human homolog of a *Drosophila* store-operated channel. Proc Natl Acad Sci USA 92: 9652–9656
16. Zitt C, Zobel A, Obukhov AG et al. (1996) Cloning and functional expression of a human Ca^{2+}-permeable cation channel activated by calcium store depletion. Neuron 16: 1189–1196
17. Zhu X, Jiang M, Peyton M, Boulay G, Hurst R, Stefani E, Birnbaumer L (1996) *trp*, a novel mammalian gene family essential for agonist-activated capacitative Ca^{2+} entry. Cell 85: 661–671
18. Sakura H, Ämmälä C, Smith PA, Gribble FM, Ashcroft FM (1995) Cloning and functional expression of the cDNA encoding a novel ATP-sensitive potassium channel expressed in pancreatic β-cells, brain, heart and skeletal muscle. FEBS Letts 377: 338–344