Specific detection of the endogenous transient receptor potential (TRP)-1 protein in liver and airway smooth muscle cells using immunoprecipitation and Western-blot analysis

Hwei Ling ONG^{*1}, Jinglong CHEN^{*1}, Tim CHATAWAY^{*}, Helen BRERETON^{*}, Lei ZHANG^{*}, Tamara DOWNS[†], Leonidas TSIOKAS[†] and Greg BARRITT^{*2}

^{*}Department of Medical Biochemistry, School of Medicine, Faculty of Health Sciences, Flinders University, GPO Box 2100, Adelaide, South Australia 5001, Australia, and

[†]Department of Cell Biology, University of Oklahoma Health Sciences Centre, 941 S.L. Young Boulevard, Oklahoma City, OK 73112, U.S.A.

Although there are numerous reports of the presence of mRNA encoding the transient receptor potential (TRP)-1 protein in animal cells and of the detection of the heterologously expressed TRP-1 protein by Western-blot analysis, it has proved difficult to unequivocally detect endogenous TRP-1 proteins. A combination of immunoprecipitation and Western-blot techniques, employing a polyclonal antibody and a monoclonal antibody respectively, was developed. Using this technique, a band of approx. 80 kDa was detected in extracts of H4-IIE rat liver hepatoma cell line and guinea-pig airway smooth muscle (ASM) cells transfected with human TRPC-1 cDNA. In extracts of untransfected H4-IIE cells, ASM cells, rat brain and guinea-pig brain, a band of approx. 92 kDa was detected. Reverse transcriptase PCR experiments detected cDNA encoding both the α - and β -isoforms of TRP-1 in H4-IIE cells. Treatment of protein extracts with peptide N-glycosidase F indicated that the 92 kDa band represents an N-glycosylated protein. Western blots conducted with a commercial

polyclonal anti-(TRP-1) antibody (Alm) detected a band of 120 kDa in extracts of H4-IIE cells and guinea-pig ASM cells. A combination of immunoprecipitation and Western-blotting techniques with the Alm antibody did not detect any bands at 92 kDa or 120 kDa in extracts of H4-IIE and ASM cells. It is concluded that (a) the 92-kDa band detected in untransfected H4-IIE and ASM cells corresponds to the N-glycosylated β -isoform of endogenous TRP-1, (b) the combined immunoprecipitation and Western-blot approach, employing two different antibodies, provides a reliable and specific procedure for detecting endogenous TRP-1 proteins, and (c) that caution is required in developing and utilizing anti-(TRP-1) antibodies.

Key words: intracellular messenger-activated non-selective cation channels, store-operated Ca^{2+} channels, transient transfection.

INTRODUCTION

Store-operated Ca^{2+} channels and intracellular messenger-activated non-selective cation channels are required in the processes by which hormones and other extracellular signals induce changes in the cytoplasmic free Ca^{2+} concentration in non-excitable animal cells [1,2]. The structures and functions of many of these plasma membrane cation channels are not yet well defined. Homologues of the *Drosophila melanogaster* transient receptor potential (TRP) protein, the TRP proteins, are thought to play an important, but as yet not clearly defined, role as store-operated Ca^{2+} channels and/or intracellular messenger-activated non-selective cation channels [3–6].

Four isoforms (splice variants) (α , β , γ and δ) of TRP-1 mRNA have been observed [7]. Two of these, γ and δ , do not appear to be translated and are considered to be non-functional [7]. The predicted sizes of the α - (full length) and β -isoforms are 91.2 and 87.6 kDa respectively [7–9]. Evidence has been obtained to indicate that TRP-1 mRNA and/or the TRP-1 protein is expressed in brain [8,10,11], *Xenopus laevis* oocytes [12,13], liver cells [14], airway smooth muscle (ASM) cells and in a number of other non-excitable cell types [7,15–17]. The results of studies [9,12,14,18–21] in which the TRP-1 protein has been heterologously over-expressed suggest that this protein may be a

store-operated Ca^{2+} channel and/or an intracellular messenger-activated non-selective cation channel. Evidence that suggests that TRP-1 is a store-operated Ca^{2+} channel has been provided by the results of anti-sense DNA and RNA experiments [12,15].

Although it has been relatively easy to detect mRNA encoding TRP-1 in animal cells [7–17], it has proved difficult to unequivocally detect the endogenous TRP-1 protein [8]. Several anti-(TRP-1) antibodies have been developed and used in Western-blot analysis to detect heterologously expressed and endogenous TRP-1 proteins [8,11,12,19,22–24]. However, in Western-blot analysis employing a given anti-(TRP-1) antibody, it has often proven difficult to clearly establish that the antibody recognizes the heterologously expressed TRP-1 protein and, in extracts of untransfected cells, reveals a band of the expected molecular mass corresponding to endogenous TRP-1.

We have developed three anti-(TRP-1) antibodies. The first is a monoclonal antibody, raised against the cytoplasmic region of the N-terminus of TRP-1. The second is a rabbit polyclonal anti-(TRP-1) antibody, raised against 18 amino acid residues at the C-terminus of *Xenopus laevis* TRP-1. This antibody has been shown to recognize the β -isoform of TRP-1 [human (h)TRPC-1], when this is heterologously expressed in *Xenopus* oocytes, and the endogenous *Xenopus* oocyte TRP-1 protein [12]. The third is a rabbit polyclonal anti-(TRP-1) antibody, raised against a

Abbreviations used: ASM, airway smooth muscle; DMEM, Dulbecco's modified Eagle's medium; NP40, Nonidet P40; PNGase F, peptide N-glycosidase F; RT-PCR, reverse-transcriptase PCR; TRP, transient receptor potential; hTRPC-1, human TRPC-1; TRPL, TRP-like.

¹ These authors contributed equally to the work.

² To whom correspondence should be addressed (e-mail Greg.Barritt@flinders.edu.au).

peptide composed of 14 amino acid residues in the pore region of guinea-pig TRP-1. We also tested a commercially available rabbit polyclonal anti-(TRP-1) antibody, which was raised against a peptide comprised of 15 amino acid residues in the pore region of human TRP-1. The aim of the present experiments was to detect endogenous TRP-1 proteins in liver cells and ASM cells using these four antibodies [14]. We report here the use of an approach that combines immunoprecipitation and Western blotting, and employs two anti-(TRP-1) antibodies. This procedure can detect endogenous TRP-1 in rat and guinea-pig brain, ASM cells and in the H4-IIE rat liver hepatoma cell line. This approach may provide a reliable and specific procedure for detecting the endogenous TRP-1 protein in other cell types, for determining the subcellular location of endogenous TRP-1 protein in cultured cells and tissues by subcellular fractionation, and in the investigation of the functions of TRP-1.

MATERIALS AND METHODS

Materials

Two affinity-purified rabbit polyclonal anti-(TRP-1) antibodies (1.12 AP and 13.5 AP) were raised against the peptide CRDLLG-FRTSKYAMFYPRN (1.12 AP) and the peptide CQLYDKGY-TPKEQKD (13.5 AP). A mouse monoclonal anti-(TRP-1) antibody [1F1; obtainable from L. T. (Leonidas-Tsiokas@ouhsc.edu)] was raised against the cytoplasmic region of the N-terminus of human TRP-1 (L. Tsiokas and T. Downs, unpublished work). A commercial anti-(TRP-1) antibody (designated Alm) (raised against the peptide QLYDKGYTSKEQ-KDC) was obtained from Alomone Labs (Jerusalem, Israel). One affinity-purified rabbit polyclonal anti-TRPL antibody (where TRPL is TRP-like protein) was raised against the peptide CDSNFDIHVVLDLDEK corresponding to the 14 amino acids at the C-terminus of *Drosophila* TRPL protein, with an additional cysteine residue on the N-terminus to facilitate coupling to diphtheria toxoid. cDNA encoding human TRPC-1 containing a FLAG tag at the N-terminus (hTRPC-1) was kindly provided by Dr Craig Montell (Department of Biological Chemistry, The John Hopkins University School of Medicine, Baltimore, U.S.A.) [12,25]. Fugene-6 transfection reagent, Nonidet P40 (NP40) and peptide N-glycosidase F (PNGase F) were from Roche Molecular Biochemicals. Goat anti-rabbit and goat anti-mouse IgG peroxidase conjugated antibodies, leupeptin, pepstatin A and PMSF were from Sigma-Aldrich. Enhanced chemiluminescence (ECL[®]) Western-blotting detection reagents, Hyperfilm and Protein G-Sepharose beads were from Amersham Pharmacia Biotech. Dulbecco's modified Eagle's medium (DMEM), foetal calf serum, pEGFP-N1 vector, Opti-MEM and LIPOFECTAMINE[™] 2000 transfection reagent were from Invitrogen. All other chemicals and materials were of the highest grade commercially available.

Cells, cell culture and transient transfection of H4-IIE and ASM cells

The culture of H4-IIE cells (A.T.C.C. CRL 1548) [26], isolation and primary culture of guinea-pig ASM cells [27] and isolation of rat hepatocytes [28] were performed as described previously.

H4-IIE cells were transiently transfected with hTRPC-1 cDNA using Fugene-6 transfection reagent according to the manufacturer's instructions [14]. Cells (2×10^5) were suspended in 0.5 ml of serum- and antibiotic-free DMEM, containing 3 μ g of hTRPC-1 cDNA and 6 μ l of Fugene-6. The mixture was then plated on to a 35-mm diameter dish and incubated for 6 h. Antibiotic-free

DMEM (1.5 ml) was then added, and the cells were cultured for a further 18–22 h before whole-cell protein extraction.

ASM cells were transiently transfected with hTRPC-1 cDNA using LIPOFECTAMINE[™] 2000 transfection reagent, according to the manufacturer's instructions. Cells (1×10^5) were plated on to a 35-mm diameter dish and cultured until 90% confluent. Prior to transfection, cells were washed twice in serum- and antibiotic-free DMEM. Cells were transfected by incubating the cells with 0.3 μ g of pEGFP-N1 vector (marker), 3 μ g of hTRPC-1 cDNA and 9 μ l of LIPOFECTAMINE[™] 2000 in 1 ml of antibiotic-free DMEM containing 400 μ l of Opti-MEM and 200 μ l of foetal calf serum for 6 h. After 6 h, the above transfection mix was removed, and the cells were cultured for a further 24 h in DMEM supplemented with penicillin-G (5000 units/ml), streptomycin (5 mg/ml) and 10% (v/v) foetal calf serum.

Preparation of cell extracts and subcellular fractionation

Whole-cell protein extracts were prepared from H4-IIE and ASM cells as described previously [14]. Rat hepatocyte and rat liver subcellular fractions were prepared as described previously [28]. Crude membrane and cytosolic protein fractions were obtained from rat brain, guinea-pig brain, H4-IIE cells and airway smooth muscle cells using the method adapted from Sambrook et al. [29]. The extraction buffer comprised 50 mM Tris (pH 7.5), 1 mM EDTA, 10 μ M leupeptin, 10 μ M pepstatin, 100 μ M PMSF, 1 mM β -mercaptoethanol and water to make up a final volume of 10 ml [29]. For brain tissue, 1 g of tissue was minced and homogenized with 10 ml of extraction buffer in a glass-glass homogenizer on ice. For cells, 1×10^7 cells were suspended in 2 ml of extraction buffer for 15 min on ice, and the cells were homogenized using an UltraTarax for 30 s on ice and then sonicated for another 30 s on ice.

The homogenized tissue or cell suspension was then centrifuged at 750 *g* for 10 min at 4 °C. After centrifugation, the cloudy supernatant was re-centrifuged at 50000 *g* for 30 min at 4 °C. At the end of centrifugation, the clear supernatant was the cytosolic protein fraction. The remaining pellet was the crude membrane fraction and was dissolved in 500 μ l of extraction buffer containing 1% Triton X-100. Protein concentration of both the cytosolic and membrane fractions was estimated using the modified Lowry method [30].

Western-blot analysis

The protein extracts were subjected to SDS/PAGE (9% gel) with the Laemmli discontinuous buffer system [29], and the resolved proteins were electrotransferred for 2 h to nitrocellulose membranes (Hybond C+) using the HorizBlot semi-dry blotter (Atto Corporation, Tokyo, Japan) [12,14]. The membranes were blocked for 1 h at 22 °C with TBS-T [20 mM Tris/HCl (pH 8.0), 500 mM NaCl and 0.1% (v/v) Tween-20] containing 5% (w/v) skimmed milk powder. The membranes were then incubated with a polyclonal or monoclonal anti-(TRP-1) antibody (1 μ g/ml in TBS-T with 5% skimmed milk powder and 0.02% NaN_3) overnight at 4 °C with rocking, followed by 5 washes (5 min each) in TBS-T. For peptide blocking, a polyclonal anti-(TRP-1) antibody was incubated for 2 h at 22 °C with the peptide (1 μ g/ml in TBS-T) against which the antibody was raised. The antibody-peptide mixture was then centrifuged at 10000 *g* for 10 min at 4 °C, and the resulting supernatant was incubated with the membranes as described above. Membranes were further incubated with secondary antibody (anti-rabbit or -mouse IgG-

peroxidase conjugate, 1:2000 dilution in TBS-T) for 2 h at 22 °C, followed by 5 washes in TBS-T. Bound peroxidase was detected using ECL[®] Western-blotting detection reagents, according to the manufacturer's instructions, followed by exposure of the membrane to Hyperfilm.

Immunoprecipitation

The method used was modified from that described by Harlow and Lane [31]. Where the commercial antibody was used for immunoprecipitation, the method was employed as described elsewhere [22,23]. H4-IIE cells and ASM cells were harvested by trypsinization. Hepatocytes were freshly isolated from rat liver by perfusion with collagenase, whereas brains were freshly isolated from rat or guinea-pig. The cells or tissues were washed three times in cold PBS. The cells (1×10^7 – 2×10^7), hepatocytes (100–400 mg) or brain tissues (200 mg) were suspended in 1 ml of cold RIPA buffer [1% NP40, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 10 mM Tris/HCl (pH 7.5), 1 μ M leupeptin, 1 μ M pepstatin A, 200 μ M PMSF and 1 mM EDTA] for 45 min at 4 °C with end-over-end mixing [31]. The suspension was centrifuged at 10000 *g* at 4 °C for 20 min. Supernatant was carefully removed and quantified using the modified Lowry method [30].

Protein extract (0.5–5.0 mg) was incubated with 1.5 μ g of polyclonal anti-(TRP-1) antibody at 4 °C overnight, with rocking. Protein G-Sepharose beads (30 μ l) were washed three times in cold RIPA buffer and incubated with the above mixture at 4 °C for 2 h with rocking [31]. The beads were collected by centrifugation at 10000 *g* for 5 s at 22 °C and washed twice with RIPA buffer (10 min each wash) at 4 °C. Following one quick rinse in cold PBS, the supernatant was completely aspirated using a 25-gauge needle. Sample buffer [20 μ l; 62.5 mM Tris/HCl, 2% SDS, 10% (v/v) glycerol, 100 mM dithiothreitol and 0.01% Bromophenol Blue, pH 6.8] [29] was added to 30 μ l of Protein G-Sepharose beads and heated in boiling water for 2 min. The samples were then analysed by Western blotting using the monoclonal anti-(TRP-1) antibody, 1F1 (2 μ g/ml) as the primary antibody and goat anti-mouse IgG-peroxidase conjugated secondary antibody (1:1000 dilution).

Enzymic deglycosylation

Following immunoprecipitation of H4-IIE cell extracts with 1.12 AP, the proteins were eluted from Protein G-Sepharose beads with 10 μ l of elution buffer (100 mM Tris/HCl and 1% SDS, pH 7.4). The eluate was heated in boiling water for 3 min, cooled to 22 °C, and then diluted 1 in 10 with incubation buffer (100 mM Tris/HCl and 0.6% NP40, pH 7.4) [32]. The samples were treated with PNGase F (30 units/ml) at 37 °C for the times indicated, before being subjected to SDS/PAGE.

Reverse-transcriptase (RT)-PCR for the detection of mRNA encoding α - and β -isoforms of TRP-1

The isolation of mRNA from H4-IIE cells, preparation of cDNA and RT-PCR were conducted as described previously [14]. The PCR primers employed were sense 5'-GGG AGA AAT GCT GTT ACC AT-3' and antisense 5'-GTT GAG TAT TCC GGA TTC TG-3', which were designed to amplify across the previously reported splicing region in the N-terminus of the TRP-1 protein [8]. The conditions employed for PCR were 30 s at 94 °C, 30 s at 55 °C and 1 min at 72 °C for 40 cycles. The expected sizes for the PCR products corresponding to the α - and β -isoforms were 233 bp and 133 bp respectively.

RESULTS

Four anti-(TRP-1) antibodies were used in the present study. These were the 1.12 AP polyclonal antibody (raised against the RDLLGFRTSKYAMFYPRN sequence in the C-terminal region of human, mouse and *Xenopus laevis* TRP-1), the 1F1 monoclonal antibody (raised against the cytoplasmic region of the N-terminus of hTRP-1) and the 13.5 AP polyclonal antibody (raised against the QLYDKGYTPKEQKD sequence in the S5–S6 pore-forming region of guinea-pig TRP-1); and the polyclonal antibody, designated Alm, (raised against the QLYDKGYTSKEQKDC sequence in the S5–S6 pore-forming region of human, mouse and rat TRP-1). In Western-blot analysis, the 1.12 AP antibody (Figure 1A) and the 1F1 antibody (Figure 1B)

Figure 1 Western-blot analysis of extracts of H4-IIE and ASM cells in which the hTRPC-1 protein is heterologously expressed

The anti-(TRP-1) antibodies employed were 1.12 AP (A) and 1F1 (B). (A) Lanes 1 and 5, extracts of H4-IIE cells transiently transfected with cDNA encoding hTRPC-1. Lane 2, extracts of untransfected H4-IIE cells. Lanes 3 and 6, extracts of ASM cells transiently transfected with cDNA encoding hTRPC-1. Lane 4, extracts of untransfected ASM cells. Lanes 5 and 6 were incubated with 1.12 AP pre-treated with the peptide against which the antibody was raised. The amount of extract applied to each lane was 10 μ g. (B) Lanes 1 and 2, extracts of H4-IIE cells transiently transfected with cDNA encoding hTRPC-1 and untransfected H4-IIE cells respectively (30 μ g per lane). Lanes 3 and 4, extracts of ASM cells transiently transfected with cDNA encoding hTRPC-1 and untransfected ASM cells respectively (15 μ g per lane). The results shown are representative of those obtained in one of 3–5 experiments, which each gave similar results.

Figure 2 Detection of the heterologously expressed hTRPC-1 protein in extracts of H4-IIE and ASM cells using a combined immunoprecipitation and Western-blot protocol

Lane 1, extracts of H4-IIE cells (H4-IIE). Lanes 2 and 3, extracts of ASM cells (ASM). Immunoprecipitation (IP) was conducted with the 1.12 AP (lanes 1 and 2), or the 13.5 AP (lane 3) anti-(TRP-1) antibody. The Western blot (WB) employed the 1F1 anti-(TRP-1) antibody. The amounts of protein used in the immunoprecipitation step were 0.2 mg for H4-IIE cells and 0.5 mg for ASM cells. The results shown are representative of those obtained in one of five experiments, which each gave similar results. IgG_H and IgG_L correspond to the heavy and light chains of IgG respectively.

each detected a band with a molecular mass of approx. 80 kDa in extracts of H4-IIE cells and in ASM cells heterologously expressing the FLAG-tagged β -isoform of TRP-1 (hTRPC-1) (lanes 1 and 3 respectively, Figures 1A and 1B). No band with an apparent size of 80–90 kDa was observed in untransfected cells (lanes 2 and 4, Figures 1A and 1B). The appearance of the 80-kDa band detected by 1.12 AP antibody was blocked by pre-treatment of the antibody with the peptide against which the antibody was raised (lanes 5 and 6, Figure 1A). It was noted that the apparent molecular mass of the expressed hTRPC-1 protein (approx. 80 kDa) is smaller than the predicted size of FLAG-tagged hTRPC-1 (88.6 kDa) [9]. In similar experiments conducted using the 13.5 AP antibody, a band of 80 kDa was not detected either in ASM cells expressing hTRPC-1 or in H4-IIE cells expressing hTRPC-1 (results not shown).

In order to detect endogenous TRP-1 proteins in H4-IIE cells and in ASM cells, a combined immunoprecipitation and Western-blotting protocol was employed. This was tested first with cells heterologously expressing hTRPC-1. Using the 1.12 AP polyclonal antibody for immunoprecipitation and the 1F1 monoclonal antibody for Western-blot analysis, a band of 80 kDa was detected in H4-IIE cells and in ASM cells expressing hTRPC-1 (Figure 2, lanes 1 and 2 respectively). Similar results were obtained for ASM cells expressing hTRPC-1 using the 13.5 AP antibody in place of the 1.12 AP antibody for the immunoprecipitation step (Figure 2, lane 3). However, the intensity of the 80-kDa band was less with 13.5 AP (Figure 2, lane 3 compared with lane 2). The 80-kDa band was not detected when the immunoprecipitation step was conducted with pre-immune serum, with an anti-TRPL antibody or with no antibody present (results not shown). An 80-kDa band was not detected in H4-IIE cells expressing hTRPC-1 using the 13.5 AP antibody in

immunoprecipitation (results not shown). This is most likely to be due to differences in the amounts of the hTRPC-1 protein expressed in ASM and H4-IIE cells, since the method of transfection of H4-IIE cells differed from that employed for ASM cells.

When the combined immunoprecipitation (1.12 AP)–Western blot (1F1) technique was applied to search for endogenous TRP-1, a band with an apparent size of 92 kDa was detected in H4-IIE cells (Figure 3A, lane 1). This was tentatively identified as the endogenous TRP-1 protein. No 92-kDa band was observed when an anti-TRPL antibody, pre-immune serum or no antibody was employed in place of the 1.12 AP antibody in the immunoprecipitation step (Figure 3A, lanes 2, 3 and 4 respectively). A band with an apparent molecular mass of 92 kDa and with an intensity comparable with that observed in H4-IIE cells was also observed in rat and guinea-pig brain and in guinea-pig ASM cells (Figure 3B, lanes 1–3 and 7, compared with H4-IIE cells in lane 6). After subcellular fractionation of rat brain, a 92-kDa band was detected in a crude membrane fraction, but not in the cytosolic fraction (Figure 3B, lane 7 compared with lane 8). The intensity of the band obtained with ASM cell extracts depended on the amount of cell extract employed (Figure 3B, lanes 2 and 3). Formation of the 92-kDa band using extracts of ASM cells and brain was not detected when the immunoprecipitation was conducted with pre-immune serum or with anti-TRPL antibody, or when no antibody was included in the immunoprecipitation (results not shown). The combined immunoprecipitation–Western-blot technique did not detect a 92-kDa band in extracts of freshly isolated hepatocytes or in an liver plasma membrane fraction (Figure 3B, lanes 4 and 5).

Results (not shown) similar to those shown in Figure 2, lane 1 (heterologously expressed hTRPC-1), and Figure 3A, lane 1 (putative endogenous TRP-1) were obtained when the order of the antibodies employed in the combined protocol was reversed (i.e. the 1F1 antibody was used for immunoprecipitation and the 1.12 AP antibody for Western blotting). However, the intensity of the detected 92-kDa bands that corresponded to putative TRP-1 was less than that observed when the 1.12 AP antibody was used for immunoprecipitation.

A 92-kDa band was also detected in extracts of guinea-pig brain and guinea-pig ASM cells using the 13.5 AP antibody in place of the 1.12 AP antibody for the immunoprecipitation in the combined protocol (Figure 3C). The intensity of the 92-kDa band increased with increasing amounts of brain extract (Figure 3C, lanes 2 and 1) and of ASM cell extract (Figure 3C, lanes 4 and 3). However, the 92-kDa band was not detected in extracts of H4-IIE cells using 13.5 AP in the combined protocol (results not shown).

As noted above, the apparent size of the heterologously expressed hTRPC-1 protein (the FLAG-tagged β -isoform of TRP-1) was approx. 80 kDa (compared with 80 kDa reported in [9,12]), whereas the size of the band of putative endogenous TRP-1 was approx. 92 kDa (compared with 92 kDa reported in [8]). This comparison is shown more clearly in Figure 3(D). The two most likely explanations for the difference between the size of the putative endogenous TRP-1 and the heterologously expressed TRPC-1 proteins are that the 92-kDa band corresponds either to the α -isoform of TRP-1 (predicted size of the non-glycosylated α -isoform is 91.2 kDa) or, since several studies have shown that TRP-1 proteins are N-glycosylated [33–35], the glycosylated form of the β -isoform of TRP-1 (predicted size of the non-glycosylated FLAG-tagged β -isoform is 88.6 kDa).

The nature of the isoforms of TRP-1 present in H4-IIE cells was investigated using RT-PCR and primers specific for cDNA encoding each of the isoforms. Bands corresponding to the expected sizes of each of the α - and β -isoforms were observed

Figure 3 Detection of a 92-kDa protein, putative endogenous TRP-1, in extracts of H4-IIE cells, ASM cells and brain using a combined immunoprecipitation and Western-blot protocol

(A) Each lane contained H4-IIE cell extracts (3 mg of protein for the immunoprecipitation). Immunoprecipitation (IP) was conducted with the 1.12 AP anti-(TRP-1) antibody (lane 1), an anti-TRPL antibody (lane 2), pre-immune serum (lane 3) or without any antibody (lane 4). Western-blot analysis (WB) was conducted with the 1F1 antibody. (B) The extracts and the amount of protein used in immunoprecipitation were: guinea-pig brain (0.5 mg, lane 1), ASM cells (3.0 mg and 0.5 mg for lanes 2 and 3 respectively), rat hepatocytes (4 mg, lane 4), rat liver plasma membrane fraction (PM; 3 mg, lane 5), H4-IIE cells (3 mg, lane 6), rat brain crude membrane (CM; 5 mg, lane 7) and rat brain cytosol (Cyt; 5 mg, lane 8). Immunoprecipitation was conducted with the 1.12 AP anti-(TRP-1) antibody and Western blotting with the 1F1 antibody. (C) The extracts employed and the amount of protein used in immunoprecipitation were: guinea-pig brain (3 mg, lane 1; 0.5 mg, lane 2) and guinea-pig ASM cells (3 mg, lane 3; 0.5 mg, lane 4). Immunoprecipitation was conducted with the 13.5 AP anti-(TRP-1) antibody and Western blotting using the 1F1 antibody. (D) Extracts of H4-IIE cells transfected with cDNA encoding hTRPC-1 (+; 0.2 mg, lane 1) and untransfected H4-IIE cells (−; 3 mg, lane 2). Immunoprecipitation was conducted with the 1.12 AP anti-(TRP-1) antibody and Western-blot analysis with the 1F1 anti-(TRP-1) antibody. The results shown are representative of those obtained in one of 5–10 experiments, which each gave similar results. IgG_H and IgG_L correspond to the heavy and light chains of IgG respectively.

(results not shown). The possibility that the 92-kDa band represents a glycosylated form of TRP-1 was tested by treating the immunoprecipitated proteins (obtained using 1.12 AP) from H4-IIE cell extracts with PNGase F (30 units/ml for 2 h or 18 h at 37 °C) in order to remove any N-glycosyl moieties. This treatment abolished the 92-kDa band, but yielded a new band of approx. 87 kDa. In control experiments, conducted under the same experimental conditions, treatment with PNGase F caused the predicted decreases in the apparent molecular masses of human transferrin and human α_1 -acid glycoprotein (results not shown). These results indicate that the 92-kDa band represents a glycosylated protein.

The Alm polyclonal anti-(TRP-1) antibody has been employed in some studies of the TRP-1 protein [22,23]. However, using this

antibody, no band of 80 kDa (the observed molecular mass for hTRPC-1) could be detected in Western-blot analysis of extracts of *Xenopus* oocytes heterologously expressing hTRPC-1 (Figure 4A, lane 2) in contrast with the ability of 1.12 AP to detect hTRPC-1 in these extracts (Figure 4A, lane 1). In Western blots employing the Alm anti-(TRP-1) antibody, no band with an apparent size of 92 kDa was observed in a crude membrane fraction from H4-IIE cells (which might be expected to be enriched in endogenous TRP-1) (Figure 4A, lane 3), and no band with a size of 80–95 kDa (the range of reported sizes for the TRP-1 protein) [8,11,12,19,22–24] was observed in a rat liver plasma membrane preparation, or in crude membrane fractions of mouse liver, rat hepatocytes, or mouse or rat brain (Figure 4A, lanes 4–8). The predominant band observed in most of these

Figure 4 Western-blot analysis of cell and tissue extracts using the Alm polyclonal anti-(TRP-1) antibody

(A) The extracts employed were: *Xenopus* oocytes in which hTRPC-1 is heterologously expressed (10 μ g per lane, lanes 1 and 2), crude membrane (40 μ g) from H4-IIE cells (lane 3), mouse liver (lane 4), rat hepatocytes (lane 5), mouse brain (lane 6), rat brain (lane 7) and plasma membrane from rat liver (40 μ g) (lane 8). For lane 1, the extract was treated with 1.12 AP antibody, whereas extracts in lanes 2–8 were treated with the Alm antibody. (B) The extracts employed were: ASM cell cytosolic extract (40 μ g per lane, lanes 2 and 4), and an ASM cell crude membrane fraction (40 μ g per lane, lanes 1 and 3). For lanes 3 and 4, the extracts were treated with antibody that had been pre-treated with peptide against which the antibody was raised before being used in the Western-blot analysis. The results shown are representative of those obtained in one of three experiments, which each gave similar results.

crude membrane fractions had an apparent size of 120 kDa (Figure 4A). A number of other bands were also observed, including one at 60 kDa. In ASM cells, the 120-kDa band was observed in a crude membrane fraction, but not in a cytosolic fraction (Figure 4B, lanes 1 and 2). A 60-kDa band was also detected in both crude membrane and cytosolic fractions of ASM cells (Figure 4B, lanes 1 and 2). Appearance of the 120-kDa band in the membrane fraction was abolished by pre-treatment of the Alm anti-(TRP-1) antibody with the blocking peptide (Figure 4B, lane 3 compared with lane 1). However, appearance of the 60-kDa band was not abolished by this treatment (Figure 4B, lanes 3 and 4 compared with lanes 1 and 2).

Attempts were made using the Alm anti-(TRP-1) polyclonal antibody to immunoprecipitate endogenous TRP-1 from an extract of H4-IIE cells and to detect any immunoprecipitated TRP-1 [also using the Alm anti-(TRP-1) antibody] on a Western blot (compared with the protocol employed in [22,23]). In the resulting Western blot, no band with an apparent molecular mass of 92 kDa, which might correspond to putative endogenous TRP-1, was detected using this procedure. Also no band with an apparent molecular mass of 120 kDa was detected. Considerable staining of the gel in the region corresponding to 20–70 kDa was observed (results not shown). The majority of these bands are likely to be due to binding of the secondary antibody (anti-rabbit IgG-peroxidase conjugate) used in the Western blot to the rabbit immunoglobulins of the Alm antibody [11].

DISCUSSION

The results of the present study provide evidence that indicates that endogenous TRP-1 in H4-IIE cells and ASM cells can be detected as a band with an apparent size of 92 kDa using a combined procedure involving immunoprecipitation with the 1.12 AP polyclonal anti-(TRP-1) antibody and Western-blot

analysis with the 1F1 monoclonal anti-(TRP-1) antibody. Neither antibody alone was able to detect the endogenous TRP-1 protein in a Western blot without a prior immunoprecipitation step. The following observations are considered to provide reasonable evidence that the observed 92-kDa band is the endogenous TRP-1 protein. (a) Both the 1.12 AP and 1F1 antibodies detect the expressed β -isoform of TRP-1 (hTRPC-1) in Western blots and using the combined protocol. (b) In Western blots, formation of the 80-kDa band (attributed to heterologously expressed hTRPC-1) was abolished by pre-treatment of the 1.12 AP anti-(TRP-1) antibody with the immunizing peptide. (c) In the combined protocol, the 92-kDa band was not detected when 1.12 AP employed in the immunoprecipitation was replaced by a polyclonal anti-TRPL antibody or with pre-immune sera, or when the 1.12 AP used for immunoprecipitation was omitted altogether. (d) The size of the 92-kDa band is similar to the predicted size of the α -isoform of TRP-1 protein [7,8]. (e) In extracts of each of H4-IIE cells, ASM cells and brain, the same size band (92 kDa) was observed.

The apparent molecular mass of the endogenous TRP-1 protein in H4-IIE cells and ASM cells was found to be 92 kDa. The results of RT-PCR experiments showed that mRNA encoding both the α - and β -isoforms of TRP-1 is present in H4-IIE cells. The predicted sizes of the non-glycosylated forms of these isoforms are 91.2 kDa and 87.6 kDa respectively [7–9]. It is expected that two bands of endogenous TRP-1 protein, corresponding to the α - and β -isoforms, would be detected in extracts of H4-IIE cells. However, only one band with an apparent molecular mass of 92 kDa was observed. Moreover, the results conducted using PNGase F indicate that the observed 92-kDa protein is N-glycosylated. A most likely explanation for these results is that the β -isoform of TRP-1 is expressed in much larger amounts than the α -isoform, the β -isoform is glycosylated, and the observed 92-kDa band corresponds to the N-glycosylated β -isoform of TRP-1. However, it cannot be excluded

that the 92-kDa band may correspond to a mixture of the α - and β -isoforms of TRP-1 [7,33–37].

When used in the combined immunoprecipitation and Western-blot procedure, the 13.5 AP anti-(TRP-1) antibody detected endogenous TRP-1 in ASM cells, but not in H4-IIE cells. This is most likely due to the fact that the 13.5 AP antibody was raised against a peptide sequence in the pore-forming region of guinea-pig TRP-1. The equivalent peptide sequence in the human and rat TRP-1 proteins is identical except for the substitution of a proline residue (guinea-pig) for a cysteine residue. Since this is unlikely to be a conservative amino-acid substitution, the conformation of human and rat TRP-1 at this region may differ from that of guinea-pig TRP-1.

The failure of anti-(TRP-1) antibodies 1.12 AP, 13.5 AP and 1F1 to recognize the endogenous TRP-1 protein in Western blots (in the absence of prior immunoprecipitation) is likely due to a relatively low specificity and affinity of each of the antibodies for TRP-1. This may especially be the case under conditions of the Western blot where the TRP-1 protein is denatured, since the antibodies may have a lower affinity for the denatured proteins. In the combined protocol, which improved the detection of TRP-1, the immunoprecipitation step allows partial purification of the TRP-1 protein and the removal of a number of proteins that would otherwise cross-react with the 1F1 antibody used in the second step. The use of a polyclonal antibody (instead of a monoclonal antibody) in the immunoprecipitation step is likely to improve the effectiveness of the procedure, because a polyclonal antibody recognizes multiple epitopes on the target antigen resulting in the formation of large multivalent immune complexes [38]. This would be expected to enhance the avidity of the interactions and increases the efficiency of immunoprecipitation. The use of a monoclonal antibody in the second (Western blot) step may help to overcome a potential disadvantage of using a polyclonal antibody in the first (immunoprecipitation) step. A polyclonal antibody could cross-react with epitopes on other proteins, resulting in higher backgrounds and possible misidentification of antigens [38].

It has been reported that the Alm anti-(TRP-1) antibody can detect the endogenous TRP-1 protein in human platelets [22,23]. However, the present results suggest that this antibody, or at least the batch used in these experiments, does not recognize heterologously expressed hTRPC-1 or endogenous TRP-1 in H4-IIE and guinea-pig ASM cell extracts. In contrast, in parallel experiments conducted using the combined protocol with the 1.12 AP and 1F1 antibodies, it was possible to detect endogenous TRP-1, thus providing evidence that the TRP-1 protein is present in these extracts. The possibility that the 60- and 120-kDa proteins detected by the Alm anti-(TRP-1) antibody is a form of TRP-1 (e.g. a TRP-1 fragment or dimer) cannot be completely excluded. However, it is considered more likely that the 120-kDa protein is another membrane protein that is recognized by Alm. Although results obtained in different laboratories might be explained on the basis of differences in the properties of the cell types employed, the expression of different TRP-1 isoforms, differences between batches of the Alm antibody, and variations in the conditions employed for immunoprecipitation and Western blot, the present study suggests that considerable caution should be exercised in the interpretation of the results obtained with any anti-(TRP-1) antibodies.

No endogenous TRP-1 was detected in freshly isolated rat hepatocytes using the combined immunoprecipitation and Western-blot protocol. However, in previous studies, mRNA encoding TRP-1 was detected in these cells [14]. Moreover, in experiments employing immunofluorescence, evidence has been obtained which indicates that the endogenous TRP-1 protein can be

detected in hepatocytes in sections of rat liver using the 1F1 monoclonal anti-(TRP-1) antibody (L. Tsiokas and T. Downs, unpublished work), and in H4-IIE cells and in freshly isolated rat hepatocytes using either the 1F1 monoclonal anti-(TRP-1) antibody or the 1.12 AP polyclonal anti-(TRP-1) antibody (J. Chen, H. Brereton, T. K. Chataway and G. J. Barritt, unpublished work). The failure to detect the TRP-1 protein in freshly isolated hepatocytes in the present series of experiments may be due to a much lower expression of TRP-1 in rat hepatocytes compared with that in H4-IIE cells, the possibility that the TRP-1 protein comprises a much smaller proportion of the total protein present in hepatocytes compared with the relative amount present in H4-IIE cells, or to a higher level of proteolytic enzyme activity in hepatocytes. However, the procedure for preparation of hepatocyte extracts included a battery of proteolytic enzyme inhibitors that would be expected to block proteolysis, suggesting that it is unlikely that endogenous TRP-1 in hepatocytes has been degraded by proteolysis. Store-operated and intracellular messenger-activated Ca^{2+} channels may be expressed at very low copy number [39,40]. Therefore the most likely explanation for the failure to detect endogenous TRP-1 in rat hepatocytes may be a very low level of expression of TRP-1 in these cells. These considerations underline the importance of establishing specificity, sensitivity and reliability in Western-blot procedures used to detect endogenous TRP-1. They also reflect some of the difficulties associated with the detection of store-operated Ca^{2+} channel proteins and intracellular messenger-activated non-selective cation channel proteins in animal cells.

We gratefully acknowledge Mrs Diana Kassos for preparation of the typescript. This work was supported by grants from the Australian Research Council (G.B.) and the Polycystic Kidney Disease Foundation (L.T.). H.L.O. and J.C. are supported by the International Postgraduate Student Research Scholarship (Commonwealth of Australia Government) and by the Flinders University Research Scholarship (Flinders University).

REFERENCES

- Barritt, G. J. (1999) Receptor-activated Ca^{2+} inflow in animal cells: a variety of pathways tailored to meet different intracellular Ca^{2+} signalling requirements. *Biochem. J.* **337**, 153–169.
- Berridge, M. J., Lipp, P. and Bootman, M. D. (2000) The versatility and universality of calcium signalling. *Nat. Rev. Mol. Cell Biol.* **1**, 11–21.
- Clapham, D. E. (1995) Calcium signalling. *Cell* (Cambridge, Mass.) **80**, 259–268.
- Hofmann, T., Schaefer, M., Schultz, G. and Gudermand, T. (2000) Transient receptor potential channels as molecular substrates of receptor-mediated cation entry. *J. Mol. Med.* **78**, 14–25.
- Montell, C. (1997) New light on TRP and TRPL. *Mol. Pharmacol.* **52**, 755–763.
- Zhu, X. and Birnbaumer, L. (1998) Calcium channels formed by mammalian Trp homologues. *News Physiol. Sci.* **13**, 211–217.
- Sakura, H. and Ashcroft, F. M. (1997) Identification of four trp1 gene variants murine pancreatic β -cells. *Diabetologia* **40**, 528–532.
- Wang, W. C., O'Connell, B., Dykeman, R., Sakai, T., Delporte, C., Swaim, W., Zhu, X., Birnbaumer, L. and Ambudkar, I. S. (1999) Cloning of Trp1B isoform from rat brain: immunodetection and localization of the endogenous Trp1 protein. *Am. J. Physiol.* **276**, C969–C979.
- Sinkins, W. G., Estacion, M. and Schilling, W. P. (1998) Functional expression of TrpC1: a human homologue of the *Drosophila* Trp channel. *Biochem. J.* **331**, 331–339.
- Mizuno, N., Kitayama, S., Saishin, Y., Shimada, S., Morita, K., Mitsuhashi, C., Kurihara, H. and Dohi, T. (1999) Molecular cloning and characterization of rat trp homologues from brain. *Mol. Brain Res.* **64**, 41–51.
- Strubing, C., Krapivinsky, G., Krapivinsky, L. and Clapham, D. E. (2001) TRPC1 and TRPC5 form a novel cation channels in mammalian brain. *Neuron* **29**, 645–655.
- Brereton, H. M., Harland, M. L., Auld, A. M. and Barritt, G. J. (2000) Evidence that the TRP-1 protein is unlikely to account for store-operated Ca^{2+} inflow in *Xenopus laevis* oocytes. *Mol. Cell. Biochem.* **214**, 63–74.
- Bobanovic, L. K., Maine, M., Petersen, C. C. H., Bennett, D. L., Berridge, M. J., Lipp, P., Ripley, S. J. and Bootman, M. D. (1999) Molecular cloning and immunolocalization of a novel vertebrate trp homologue from *Xenopus*. *Biochem. J.* **340**, 593–599.

- 14 Brereton, H. M., Chen, J., Rychkov, G., Harland, M. L. and Barritt, G. J. (2001) Maitotoxin activates an endogenous non-selective cation channel and is an effective initiator of the activation of the heterologously expressed hTRPC-1 (transient receptor potential) non-selective cation channel in H4-IIIE cells. *Biochim. Biophys. Acta* **1540**, 107–126
- 15 Brough, G. H., Wu, S., Cioffi, D., Moore, T. M., Ming, L., Dean, N. and Stevens, T. (2001) Contribution of endogenously expressed Trp1 to a Ca^{2+} -selective, store-operated Ca^{2+} entry pathway. *FASEB J.* **15**, 1727–1738
- 16 den Dekker, E., Molin, D. G. M., Breikers, G., van Oerle, R., Akkerman, J.-W. N., van Eys, G. J. J. M. and Heemskerk, J. W. M. (2001) Expression of transient receptor potential mRNA isoforms and Ca^{2+} influx in differentiating human stem cells and platelets. *Biochim. Biophys. Acta* **1539**, 243–255
- 17 Kohler, R., Brakemeier, S., Kuhn, M., Degenhardt, C., Buhr, H., Pries, A. and Hoyer, J. (2001) Expression of ryanodine receptor type 3 and TRP channels in endothelial cells: comparison of *in situ* and cultured human endothelial cells. *Cardiovasc. Res.* **51**, 160–168
- 18 Wu, X., Babnigg, G. and Villereal, M. L. (2000) Functional significance of human trp1 and trp3 in store-operated Ca^{2+} entry in HEK-293 cells. *Am. J. Physiol.* **278**, C526–C536
- 19 Xu, S.-Z. and Beech, D. J. (2001) TrpC1 is a membrane-spanning subunit of store-operated Ca^{2+} channels in native vascular smooth muscle cells. *Circ. Res.* **88**, 84–87
- 20 Zhu, X., Jiang, M. S., Peyton, M., Boulay, G., Hurst, R., Stefani, E. and Birnbaumer, L. (1996) *trp*, a novel mammalian gene family essential for agonist-activated capacitative Ca^{2+} entry. *Cell (Cambridge, Mass.)* **85**, 661–671
- 21 Zitt, C., Zobel, A., Obukhov, A. G., Harteneck, C., Kalkbrenner, F., Luckhoff, A. and Schultz, G. (1996) Cloning and functional expression of a human Ca^{2+} -permeable cation channel activated by calcium store depletion. *Neuron* **16**, 1189–1196
- 22 Rosado, J. A. and Sage, S. O. (2000) Coupling between inositol 1,4,5-trisphosphate receptors and human transient receptor potential channel 1 when intracellular stores are depleted. *Biochem. J.* **350**, 631–635
- 23 Rosado, J. A. and Sage, S. O. (2001) Activation of store-mediated calcium entry by secretion-like coupling between the inositol 1,4,5-trisphosphate receptor type II and human transient receptor potential (hTrp1) channels in human platelets. *Biochem. J.* **356**, 191–198
- 24 Tsiokas, L., Arnould, T., Zhu, C., Kim, E., Walz, G. and Sukhatme, V. P. (1999) Specific association of the gene product of *PKD2* with the TRPC1 channel. *Proc. Natl. Acad. Sci. U.S.A.* **96**, 3934–3939
- 25 Wes, P. D., Chevesich, J., Jeromin, A., Rosenberg, C., Stetten, S. and Montell, C. (1995) TRPC1, a human homolog of a *Drosophila* store-operated channel. *Proc. Natl. Acad. Sci. U.S.A.* **92**, 9652–9656
- 26 Auld, A., Chen, J., Brereton, H. M., Wang, Y.-J., Gregory, R. B. and Barritt, G. J. (2000) Store-operated Ca^{2+} inflow in Reuber hepatoma cells is inhibited by voltage-operated Ca^{2+} antagonists and, in contrast to freshly isolated hepatocytes, does not require a pertussis toxin-sensitive trimeric GTP-binding protein. *Biochim. Biophys. Acta* **1497**, 11–26
- 27 Inui, T., James, A. F., Fujitani, Y., Takimoto, M., Okada, T., Yamamura, T. and Urade, Y. (1994) ET_A and ET_B receptors on single smooth muscle cells cooperate in mediating guinea pig tracheal contraction. *Am. J. Physiol.* **266**, L113–L124
- 28 Wang, Y.-J., Gregory, R. and Barritt, G. J. (2000) Regulation of F-actin and endoplasmic reticulum organization by the trimeric G-protein G_{12} in rat hepatocytes. *J. Biol. Chem.* **275**, 22229–22237
- 29 Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
- 30 Peterson, G. L. (1977) A simplification of the protein assay method of Lowry et al. which is more generally applicable. *Anal. Biochem.* **83**, 346–356
- 31 Harlow, E. and Lane, D. (1988) *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
- 32 Tarentino, A. L., Gomez, C. M. and Plummer, Jr, T. H. (1985) Deglycosylation of asparagine-linked glycans by peptide: N-glycosidase F. *Biochemistry* **24**, 4665–4671
- 33 Birnbaumer, L., Zhu, X., Jiang, M. S., Boulay, G., Peyton, M., Vannier, B., Brown, D., Platani, D., Sadeghi, H., Stefani, E. and Birnbaumer, M. (1996) On the molecular basis and regulation of cellular capacitative calcium entry: roles for Trp proteins. *Proc. Natl. Acad. Sci. U.S.A.* **93**, 15195–15202
- 34 Boulay, G., Zhu, X., Peyton, M., Jiang, M. S., Hurst, R., Stefani, E. and Birnbaumer, L. (1997) Cloning and expression of a novel mammalian homolog of the *Drosophila* Transient Receptor Potential (*trp*) involved in calcium entry secondary to activation of receptors coupled by the G_q class of G protein. *J. Biol. Chem.* **272**, 29672–29680
- 35 Zhu, X., Jiang, M. S. and Birnbaumer, L. (1998) Receptor-activated Ca^{2+} influx via human Trp3 stably expressed in human embryonic kidney (HEK)293 cells. *J. Biol. Chem.* **273**, 133–142
- 36 Treuheit, M. J., Costello, C. E. and Halsall, H. B. (1992) Analysis of the five glycosylation sites of human α_1 -acid glycoprotein. *Biochem. J.* **283**, 105–112
- 37 Spik, G., Bayard, B., Fournet, B., Strecker, G., Bouquelet, S. and Montreuil, J. (1975) Studies on glycoconjugates. LXIV. Complete structure of two carbohydrate units of human serotransferrin. *FEBS Lett.* **50**, 296–299
- 38 Bonifacio, J. S., Dell'Angelica, E. C. and Springer, T. A. (1999). *Immunoprecipitation*. In *Current Protocols in Molecular Biology*, pp. 10.16.1–10.16.29, John Wiley and Sons, New York
- 39 Zweifach, A. and Lewis, R. S. (1996) Calcium-dependent potentiation of store-operated calcium channels in T lymphocytes. *J. Gen. Physiol.* **107**, 597–610
- 40 Rychkov, G., Brereton, H. M., Harland, M. L. and Barritt, G. J. (2001) Plasma membrane Ca^{2+} release-activated Ca^{2+} channels with a high selectivity for Ca^{2+} identified by patch-clamp recording in rat liver cells. *Hepatology* **33**, 938–947

Received 10 January 2002; accepted 22 March 2002