The transient receptor potential, TRP4, cation channel is a novel member of the family of calmodulin binding proteins

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The mammalian gene products, transient receptor potential (trp)1 to trp7, are related to the *Drosophila* TRP and TRP-like ion channels, and are candidate proteins underlying agonist-activated Ca^{2+}-permeable ion channels. Recently, the TRP4 protein has been shown to be part of native store-operated Ca^{2+}-permeable channels. These channels, most likely, are composed of other proteins in addition to TRP4. In the present paper we report the direct interaction of TRP4 and calmodulin (CaM) by: (1) retention of *in vitro* translated TRP4 and of TRP4 protein solubilized from bovine adrenal cortex by CaM–Sepharose in the presence of Ca^{2+}, and (2) TRP4–glutathione S-transferase pull-down experiments. Two domains of TRP4, amino acid residues 688–759 and 786–848, were identified as being able to interact with CaM. The binding of CaM to both domains occurred only in the presence of Ca^{2+} concentrations above 10 μM, with half maximal binding occurring at 16.6 μM (domain 1) and 27.9 μM Ca^{2+} (domain 2). Synthetic peptides, encompassing the two putative CaM binding sites within these domains and covering amino acid residues 694–728 and 829–853, interacted directly with dansyl–CaM with apparent K_d values of 94–189 nM. These results indicate that TRP4/Ca^{2+}-CaM are parts of a signalling complex involved in agonist-induced Ca^{2+} entry.

Key words: agonist-activated calcium entry, calcium, calmodulin, ion channels, transient receptor potential proteins.

INTRODUCTION

Many Ca^{2+}-dependent cellular processes are controlled by the ubiquitous calcium sensor calmodulin (CaM), including the cell cycle, cytoskeletal organization [1] and development of membrane excitability [2]. In addition to the regulation of many intracellular proteins, such as kinases and phosphatases, CaM is an important modulator of various types of ion channels, including cyclic-nucleotide-gated channels, *N*-methyl-D-aspartate ('NMDA') receptors, some potassium channels and voltage-gated Ca^{2+} channels (for a review see [3]).

Additional CaM-regulated channels are the transient receptor potential (TRP)-like and TRP channels involved in phototransduction in the *Drosophila* eye [4–9]. These channels are responsible for the light-activated cationic conductance changes in *Drosophila* photoreceptor cells [10,11]. A variety of TRP-related channels have been identified in mammals, and these mammalian channels have been implied to underly agonist-activated Ca^{2+}-permeable channels [12,13]. So far, the cDNAs of seven mammalian *trp*-related genes have been cloned and characterized as functional channels following heterologous expression in various cell systems. The gating mechanisms for the recombinant mammalian TRP channels are still controversial and may include store-operated and store-independent activation mechanisms. For example, mammalian TRP3 [14,15] and TRP4 [16–19] channels have been shown to be responsible for store-depletion-activated Ca^{2+} entry, but direct activation by diacylglycerol (TRP3) [20] or by G-protein-coupled receptors independently of store depletion (TRP4) [21] has also been demonstrated.

The mechanisms linking Ca^{2+}-store depletion to channel activation are still not understood but might include diffusible messengers [22–24], exocytotic insertion of channels preformed in vesicles [25–27] or, as in the case of the recombinant TRP3 [14,15,28] and TRP1 [29] channels, direct coupling [30] of the InsP_3 receptor of the store to the TRP plasma membrane channel proteins. Recently, it has been shown that the C-terminus of human TRP4 (also known as TRP4α; GeneBank® accession number AF063822), but not human TRP4$_{\Delta785-868}$ (also known as TRP4β; GeneBank® accession number AF063823), physically interacts with the C-terminus of the InsP_3 receptor [31]. Accordingly, TRP4 channel activation might require assembly with the InsP_3 receptor, Na^+/H^+-exchanger regulatory factor ('NHERF') [32] and other regulatory and scaffolding proteins, as has been reported for the *Drosophila* TRP and TRP-like channels. The *Drosophila* channels are organized through a large complex formation, which includes phospholipase C, G-proteins, protein kinase C, the scaffolding protein INAD ('inactivation no after potential') and CaM [11,33,34].

In the present report, we show that mammalian TRP4 binds CaM in a Ca^{2+}-dependent way. CaM binding occurs at two different domains within the cytoplasmic C-terminal region of the TRP4 protein. Interestingly one CaM binding domain localizes to a region which has been identified as the interaction site of TRP3 with the N-terminal portion of the InsP_3 receptor [15], whereas the second CaM binding domain localizes to a region which is absent in TRP4 splice variants, including murine TRP4$_{\Delta781-864}$ (GenBank® accession number U50921), which was used previously in heterologous expression experiments [21].

EXPERIMENTAL

Materials

The murine TRP4 (mTRP) cDNA (GenBank®: U50922) was kindly provided by Dr Feng Qian and Dr L. H. Philipson (Pharmacological and Physiological Sciences, The University of Chicago, Chicago, IL, U.S.A.). The *Xenopus laevis* CaM cDNA

Abbreviations used: TRP, transient receptor potential; mTRP, murine TRP; bTRP, bovine TRP; hTRP, human TRP; CaM, calmodulin; GST, glutathione S-transferase.

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in pTD39 was kindly provided by Professor T. Davis (Department of Biochemistry, University of Washington, Seattle, WA, U.S.A.) [35]. Peptides corresponding to mTRP4, amino acid residues 694–718 RRAADNLRRHHQYQEVMRNLVKRYV (peptide 421), residues 829–853 QRKVNFADIKNFGLFHRRSKQNAA (peptide 422) and residues 708–728 EVMRNLVKRYVAAMIREAKTE (peptide 423), were synthesized and purified at the Institut für Medizinische Biochemie (Universität des Saarlandes, Homburg, Germany). L-[^{35}S]Methionine (> 1000 Ci/mmol), pGEX-2T and CaM–Sepharose were obtained from Amersham Pharmacia Biotech and bovine brain CaM and CaM–agarose was obtained from Calbiochem.

Construction and expression of glutathione S-transferase (GST) TRP4 and TRP5 fusion proteins

To produce C-terminal GST fusion proteins, a *Bam*HI/*Eco*RI, an *Eco*RI and an *Eco*RV cDNA fragment, corresponding to amino acids 1–140, 140–239 and 844–981 of bovine TRP4 (bTRP), was fused in-frame in pGEX4T1-3 vectors. A *Stu*I/*Not*I cDNA fragment of rabbit TRP5, corresponding to amino acid residues 894–974 was fused into pGEX vector. Fusion protein epitopes of TRP4, covering residues 625–698, 688–759, 741–791 and 786–848 (mTRP4) and 538–558 (bTRP4), were constructed by PCR, amplifying cDNAs with the following primer pairs: 5′-CGG GAT CCC AAC TAA TTG CCG ACC ATG-3′, 5′-CGG AAT TCA GCA GCA CGC CGC CCA-3′, 5′-CGG AAT TCA GAG CTT GCT TCC TCT GAG-3′, 5′-CGG GAT CCA GCT TCG GGA CAA TTG GG-3′, 5′-CGG GAT CCC AAG ACA TTT CTA GCT TCC G-3′, 5′- CGG AAT TCG CTG AGA TTC TTTCTC TTG TC-3′, 5′-CGG AAT TCA CCG TCTATG AAA TAA CCC G-3′, 5′-CGG GAT CCA AGA GAA AGA ATC TCA GCC TC-3′, 5′-CGG GAT CCC AGT TGT ACT TCT ATT ATG AA-3′ and 5′-CGG AAT TCT CAC TTT TCA CAT CGT ATG CCT-3′. The amplified cDNA products were digested with *Bam*HI and *Eco*RI and subcloned in-frame in pGEX vectors. The identity of the amplified cDNA fragments was confirmed by sequence analysis. DNA was transformed into *Escherichia coli* BL21, and the expression of GST–TRP4 and GST–TRP5 fusion proteins was carried out as described previously [17].

Fluorometric measurements using dansyl–CaM

Bovine brain CaM (0.5 mg) was dissolved in 250 μl of 20 mM NH_4HCO_3 (pH 7.4)/1 mM $CaCl_2$ and incubated with 1.5 μl of dansyl chloride (10 mg/ml acetone; Molecular Probes, Eugene, OR, U.S.A.) for 2 h at room temperature [36]. Non-reacted dansyl chloride reagent was removed by centrifugation with Vivaspin 5000 concentrators (Vivascience, Lincoln, U.K.), and dansylated CaM was washed three times with 20 mM NH_4HCO_3/HCl (pH 7.4). Measurement of absorbance at 340 nm (molar absorption coefficient, 3400 $M^{-1} \cdot cm^{-1}$) showed incorporation of 1.1 mol dansyl/mol CaM.

Fluorescence emission spectra were obtained at 20 °C using a luminescence spectrometer (LS50; Perkin Elmer) in the presence of 20 mM Hepes (pH 7.4), 200–400 nM dansyl–CaM, various concentrations of peptides and either 1 mM $CaCl_2$ or 2 mM EGTA. The excitation wavelength was 340 nm, the slit width was 5 nm, and emission wavelengths between 400 and 600 nm were measured. The fraction of saturated dansyl–CaM fluorescence (a) was calculated as: $a = (F - F_0)/(F_\infty - F_0)$, where F_0 is the dansyl–CaM fluorescence in the presence of Ca^{2+}, before the addition of peptide, and F_∞ represents the fluorescence intensity at saturated peptide levels. By plotting a as a function of free peptide concentrations the CaM-titration curve was obtained. The dissociation constants for the CaM peptide binding were calculated by curve fitting using SigmaPlot 4.0 software.

Translation of bTRP4 and CaM *in vitro* and retention of bTRP4 by CaM–agarose

To obtain pcDNA3-CaM, the entire protein coding region of *X. laevis* CaM [33], including the consensus sequence for initiation of translation in vertebrates [37], was subcloned into pcDNA3 (Invitrogen) and the resulting vector construct was sequenced on both strands. *In vitro* transcription and translation of CaM (pcDNA3-CaM) and bTRP4 (pCCE, [16]) cDNA, under control of the T7 promotor, were performed in the presence of L-[^{35}S]methionine using the TNT7®Quick translation kit (Promega).

In vitro translated [^{35}S]bTRP4 was isolated by gel filtration using Sephadex G-50 columns (Amersham Pharmacia Biotech) in the presence of 50 mM Tris/HCl (pH 7.4), 150 mM NaCl, 0.1 % (v/v) Triton X100 and 1 mM Ca^{2+}. CaM–agarose (20 μl), equilibrated in the same buffer, was incubated with 40000 c.p.m. of [^{35}S]bTRP4 in a final volume of 250 μl for 12 h at 4 °C. After three washes, the CaM–agarose was resuspended in SDS sample buffer [50 mM Tris/HCl (pH 6.8), 1 % (w/v) SDS, 15 % (v/v) glycerol, 0.005 % (w/v) Bromophenol Blue, 2 % (v/v) 2-mercaptoethanol] and proteins were separated by SDS/PAGE.

Ca^{2+}-dependent TRP4–GSH–Sepharose chromatography with ^{35}S-labelled CaM

Ca^{2+} solutions were prepared in 20 mM Hepes (pH 7.2), 150 mM KCl and the [Ca^{2+}] was adjusted by adding EGTA, *N*-hydroxyethylethylenediaminetriacetic acid ('HEDTA'), nitrilotetra-acetic acid ('NTA') and $CaCl_2$. The free calcium concentrations were calculated using Sliders v2.00 software (http://www.stanford.edu/~cpatton/maxc.html). The *in vitro* translated [^{35}S]CaM was isolated by gel filtration in the presence of the appropriate Ca^{2+}-buffer. Approx. 1.5 μg GST–TRP4 fusion proteins bound to glutathione–Sepharose were equilibrated in Ca^{2+}-buffers and incubated with 40000 c.p.m. of [^{35}S]CaM, in a volume of 300 μl, for 3–12 h at 4 °C. After three washes with the appropriate Ca^{2+}-buffer, the column was resuspended in SDS sample buffer. GST fusion proteins and [^{35}S]CaM were separated by SDS/PAGE. The gels were stained with Coomassie Brilliant Blue, scanned, dried and exposed to phosphorimager (BAS-2500; Fuji) screens for 2 h. The amount of [^{35}S]CaM and Coomassie-Blue stained TRP4 fusion protein present in the same lane was measured densitometrically and analysed using AIDA software (Raytest).

Solubilization of TRP4 protein and CaM chromatography

All steps were performed at 4 °C. Bovine adrenal glands were obtained from the local slaughterhouse and the cortex was homogenized in buffer A [50 mM Tris/HCl (pH 7.4)/0.5 M EDTA containing protease inhibitor mixture: 1 μg/ml antipain, 1 mM benzamidine, 1 mM iodacetamide, 1 μg/ml leupeptine, 1 mM *ortho*-phenanthroline, 1 mM pepstatin A, 1 mM PMSF] using a Polytron tissue homogenizer (Kinematica AG). The homogenate was sedimented by centrifugation at 5000 $\boldsymbol{g}$ for 15 min, the supernatant was filtered through eight layers of gauze and centrifuged at 35000 $\boldsymbol{g}$ for 20 min. The pellet was resuspended in buffer B [20 mM Tris/HCl (pH 7.4), 0.303 M sucrose, protease inhibitor mixture and 0.6 M KCl] using a glass/Teflon homogenizer, and the suspension was stirred for 20 min and filtered through gauze. After centrifugation at

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186000 g for 60 min, the pellet was resuspended in buffer B using a glass/Teflon homogenizer, stirred for 15 min and filtered through gauze. After sedimentation by centrifugation at 72000 g for 45 min, the microsomal membranes were resuspended in 50 mM Tris/HCl (pH 7.4)/10 % sucrose and stored at −80 °C. Protein concentrations were determined using the BCA method (Pierce). Microsomal membranes (110 mg protein) were resuspended using a glass/Teflon homogenizer in 2.5 % (v/v) polyoxyethylene (9) lauryl ether (Lubrol®; Sigma), 5 mM 2-mercaptoethanol, 20 mM Hepes (pH 7.5), 150 mM NaCl and protease inhibitor mixture, and incubated for 1 h with agitation. After centrifugation at 72000 g for 45 min, the pellets were treated four times as above. The combined supernatants, containing the solubilized membrane proteins were adjusted in buffer C [0.1 % (v/v) Lubrol, 20 mM Hepes (pH 7.4), 150 mM NaCl] containing 1 mM $CaCl_2$, and incubated in the presence of 10 ml of CaM–Sepharose for 1 h, which had been pre-equilibrated in buffer C containing 1 mM $CaCl_2$. The column was extensively washed with buffer C containing 1 mM $CaCl_2$, and thereafter CaM binding proteins were eluted with buffer C containing 2 mM EGTA. The eluted fractions were precipitated with 2 vol. of acetone at −20 °C, centrifuged at 12000 g for 15 min, washed with methanol and resuspended in SDS sample buffer. Proteins were separated by SDS/PAGE, and Western-blot analysis was performed with the bTRP4 specific antibody 236 [17].

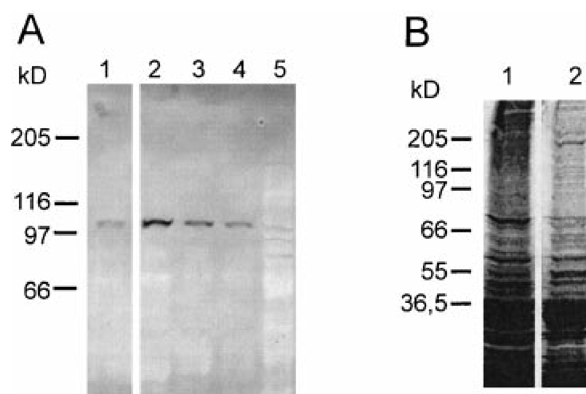

Figure 2 Binding of bovine adrenal gland cortex TRP4 to Ca^{2+}–CaM

Adrenal cortex membrane proteins were solubilized and applied to CaM–Sepharose. After extensive washing, the retained proteins were eluted with 2 mM EGTA. Proteins were separated by SDS/PAGE and the gels were subjected to Western-blot analysis (**A**) or silver staining (**B**). (**A**) Western blot after SDS/PAGE (6.5% gel) and immunoblotting: lane 1, adrenal cortex membrane proteins with affinity-purified TRP4 antibody 236 [17]; lane 5, void volume of the CaM–Sepharose column; lanes 2–4, solubilized proteins eluted from CaM–Sepharose in the presence of EGTA. (**B**) Silver stained gel after SDS/PAGE (17–6.5% gradient gel): lane 1, solubilized membrane proteins; lane 2, eluate from the CaM–Sepharose column.

RESULTS

To demonstrate the direct interaction of TRP4 with CaM, TRP4 cDNA was translated *in vitro* in the presence of [^{35}S]methionine (Figure 1), and the TRP4 protein was incubated with CaM–agarose in the presence of 1 mM Ca^{2+}. After extensive washing with Ca^{2+}-containing buffer, the bound TRP4 protein was eluted by SDS sample buffer. As shown in Figure 1, TRP4 binds to CaM–agarose.

Recently, we have shown that TRP4 is abundantly expressed in the cortex of bovine adrenal gland [17] and, in the present study, we investigated whether native TRP4 and CaM were able to associate. We therefore solubilized membrane proteins from bovine adrenal cortex microsomes and incubated them with CaM–Sepharose in the presence of Ca^{2+}. CaM-binding proteins were eluted with EGTA, separated by SDS/PAGE and subjected to Western-blot analysis using the polyclonal antibody 236 [17] to detect TRP4. As shown in Figure 2(A), lane 1, the antibody 236 recognized the 105 kDa bTRP4 among the solubilized membrane proteins. The bTRP4 protein was completely retained on CaM–Sepharose in the presence of Ca^{2+}. No significant amounts of bTRP4 were detectable in the flow through (Figure 2A, lane 5), whereas, in the presence of 2 mM EGTA, the bTRP4 protein was eluted from the CaM–Sepharose (Figure 2A, lanes 2–4). The Ca^{2+}-dependent retention of TRP4 by CaM–Sepharose demonstrates that TRP4 from native tissue was able to bind to CaM in the same way as other proteins present in the solubilized membrane fraction (Figure 2B, lane 2).

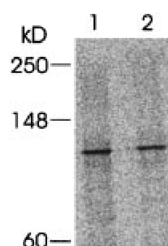

Figure 1 Binding of [^{35}S]bTRP4 to Ca^{2+}–CaM

In vitro translated [^{35}S]bTRP4 protein was incubated with CaM–agarose in the presence of 1 mM Ca^{2+}. After extensive washing, the agarose was resuspended in SDS sample buffer and the proteins were separated by SDS/PAGE (7% gel). The gel was then exposed to a phosphorimager screen. Lane 1, input of *in vitro* translated [^{35}S]bTRP4; lane 2, [^{35}S]TRP4 retained by CaM–agarose. The input represents 100% of [^{35}S]TRP4 protein added to the binding assay; ~ 89% of the input was retained in each of four experiments.

To identify the CaM binding domain in the TRP4 protein, we performed GST pull-down experiments with [^{35}S]CaM. For this purpose, a series of N- and C-terminal GST–TRP4 fusion proteins covering most parts of the cytosolic portions of the protein were expressed in *E. coli* (Figure 3A) , immobilized on glutathione–Sepharose and incubated in the presence of CaM, translated *in vitro*, and Ca^{2+}. After extensive washing, the retained proteins were analysed by SDS/PAGE (Figure 3B, upper panel), and the gel was exposed to a phosphorimager screen (Figure 3B, lower panel). [^{35}S]CaM was retained by two different fusion proteins covering amino acid residues 688–759 and 786–848 of TRP4 (Figure 3B, lower panel), indicating that TRP4 contains two independent CaM binding domains. Both domains were localized within the large cytoplasmic C-terminal portion of TRP4. For the other GST–TRP4 fusion proteins (Figure 3A), as well as for the GST–TRP5 fusion protein encompassing the 81 amino acid residues of the C-terminus (Figure 3B), no association with CaM was detectable. In agreement with the results shown in Figure 2, binding of CaM to either of the TRP4 binding domains depends on the presence of Ca^{2+} (Figure 3C).

The fact that CaM binding to TRP4 occurs only in the presence of Ca^{2+} prompted us to investigate the Ca^{2+} dependency of each of the two CaM binding domains more precisely. Therefore approx. 1.5 μg of mTRP4 (residues 688–759) – and TRP4 (residues 786–848) – GST fusion proteins was bound on to glutathione–Sepharose and this was equilibrated in the presence of various [Ca^{2+}], ranging from 27 nM to 1 mM, and thereafter incubated with [^{35}S]CaM. After extensive washing with the respective Ca^{2+} buffer, GST–TRP4 fusion proteins were analysed

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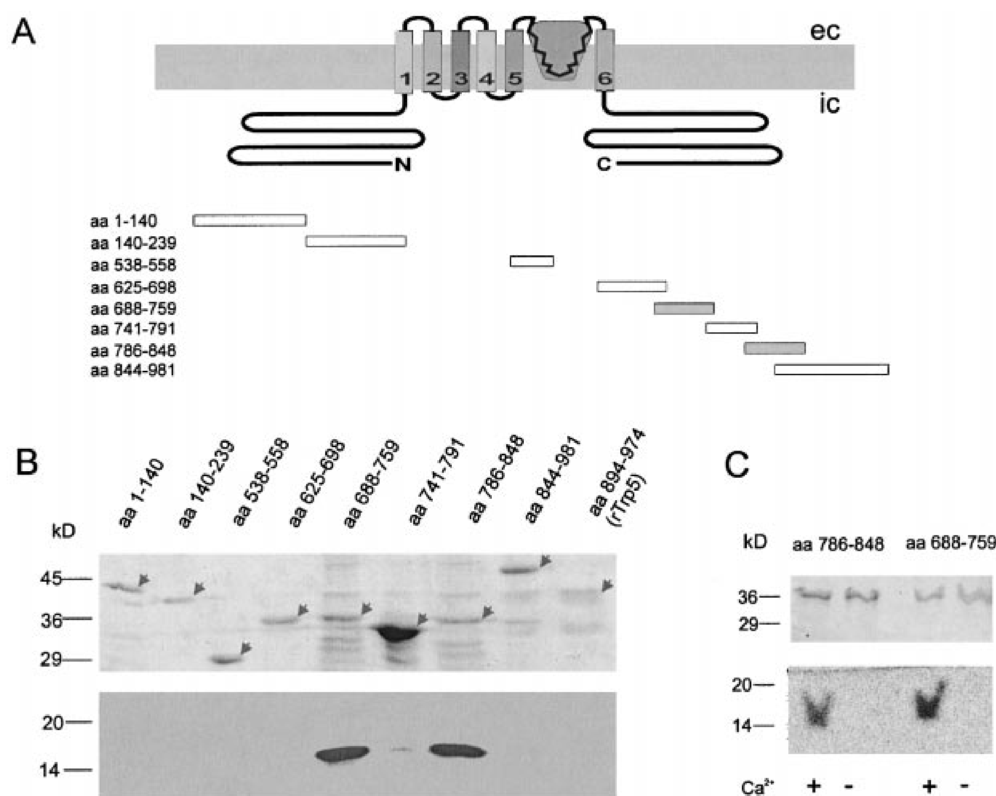

Figure 3 Identification of two CaM binding domains within the TRP4 protein

Transmembrane topology of TRP4, including the six putative transmembrane segments and a predicted pore region between segments 5 and 6. Lower panel: bars represent epitopes of TRP4 fused to the C-terminus of GST, resulting in fusion proteins which were expressed in bacteria. Grey bars indicate fusion proteins which interact with [^{35}S]CaM. Amino acid (aa) numbering is according to the mTRP4 and bTRP4 sequences (GenBank® accession numbers U50922 and X99792 respectively). (**B**) ^{35}S-labelled CaM was incubated with different GST–TRP4 fusion proteins absorbed on to glutathione–Sepharose. After 12 h incubation at 4 °C, the Sepharose beads were washed and the absorbed complexes were resolved by SDS/PAGE (12% gel), stained and autoradiographed. Upper panel: representative result of four experiments showing a Coomassie-Blue stained gel with various amounts of GST fusion proteins retained on glutathione–Sepharose: 1 μg residues (aa) 1–140 (43 kDa), 1 μg residues 140–239 (38 kDa), 1.5 μg residues 538–558 (30 kDa), 1.5 μg residues 625–698 (36 kDa), 1.5 μg residues 688–759 (35 kDa), 6 μg residues 741–791 (32 kDa), 1 μg residues 786–848 (34 kDa), 1.5 μg residues 844–981 (43 kDa) and 1 μg residues 894–974 [rabbit TRP5 (rTRP5), 37 kDa]. Lower panel, corresponding autoradiogram showing [^{35}S]CaM retained by GST fusion proteins, residues (aa) 688–759 and residues 768–848. (**C**) Ca^{2+} dependence of binding of TRP4 residues (aa) 688–759 and 768–848 to CaM. [^{35}S]CaM was incubated with mTRP4 (residues 786–848) – or mTRP4 (residues 688–759) – GST fusion protein bound on to glutathione–Sepharose in the presence of either 1 mM Ca^{2+} or 2 mM EGTA. Upper panel, Coomassie-Blue stained gel of fusion proteins; lower panel, corresponding phosphorimaging signal, demonstrating that [^{35}S]CaM is retained only in the presence of Ca^{2+}.

by SDS/PAGE for the retention of [^{35}S]CaM and the gels were exposed to phosphorimager screens (Figure 4B). Again, binding of CaM to either domain was not observed in the absence of Ca^{2+} or in the presence of $< 10\ \mu$M Ca^{2+}. CaM was retained by both domains at similar [Ca^{2+}], with half-maximal binding of CaM to domain 1 (residues 688–759) at $16.6 \pm 2.3\ \mu$M Ca^{2+} (Figure 4C, left panel), and domain 2 (residues 786–848) at $27.9 \pm 4.5\ \mu$M Ca^{2+} (Figure 4C, right panel).

The TRP4-derived protein fragments which bind CaM consist of 72 (domain 1) and 63 (domain 2) amino acids. Different recognition motifs for CaM interaction have been shown including the IQ motif, which was first recognized for a Ca^{2+}-independent CaM interaction with unconventional myosins [38] with the consensus sequence IQXXXRGXXXR. However, no such consensus sequence could be detected in either of the two TRP4 domains. Other CaM binding domains reported are stretches of 16–35 residues which show a segregation of basic and polar residues on one side and hydrophobic residues on the other side in a α-helical wheel projection [39]. Similar amphiphilic amino acid sequences are present within domain 1 and domain 2 of TRP4 and the corresponding peptides were synthesized, covering the sequences R694–V718 (peptide 421) and E708–E728 (peptide 423) of domain 1 and the sequence Q829–A853 (peptide 422) of domain 2 (Figures 5A and 5B). The interaction between the TRP4 peptides and CaM was studied by fluorescence measurements with danysl–CaM. Without the peptides, the emission peak of dansyl–CaM (200 nM) in the presence of 1 mM Ca^{2+} was between 501–503 nM (Figure 5C, dotted line). The addition of 200 nM peptides 421, 422 or 423 (Figure 5C, solid line) increased the fluorescence intensity of dansyl–CaM at 470 nm by 2.7, 3 and 1.7 times, as an index of CaM–peptide interaction and shifted the emission peak to a lower wavelength. Lower concentrations of the three peptides produced intermediate blue shifts and increased intensity. Fluorescence intensities for peptides 412, 422 and 423 were reduced by the addition of 2 mM EGTA (Figure 5C, broken line). Titration of fixed amounts of CaM with different concentrations of peptides showed a saturation pattern. When the fraction of bound CaM was plotted as a function of free peptide concentrations (Figure 5D), the dissociation constants (K_d) obtained were 94 ± 6 nM, 138 ± 8 nM and 189 ± 7 nM for peptides 421, 422 and 423, respectively (means $\pm$ S.E.M., $n = 3$). Peptide 421, covering R694 to V718, overlaps peptide 423 (E708–E728) by 11 amino acid residues, indicating that these common residues represent the

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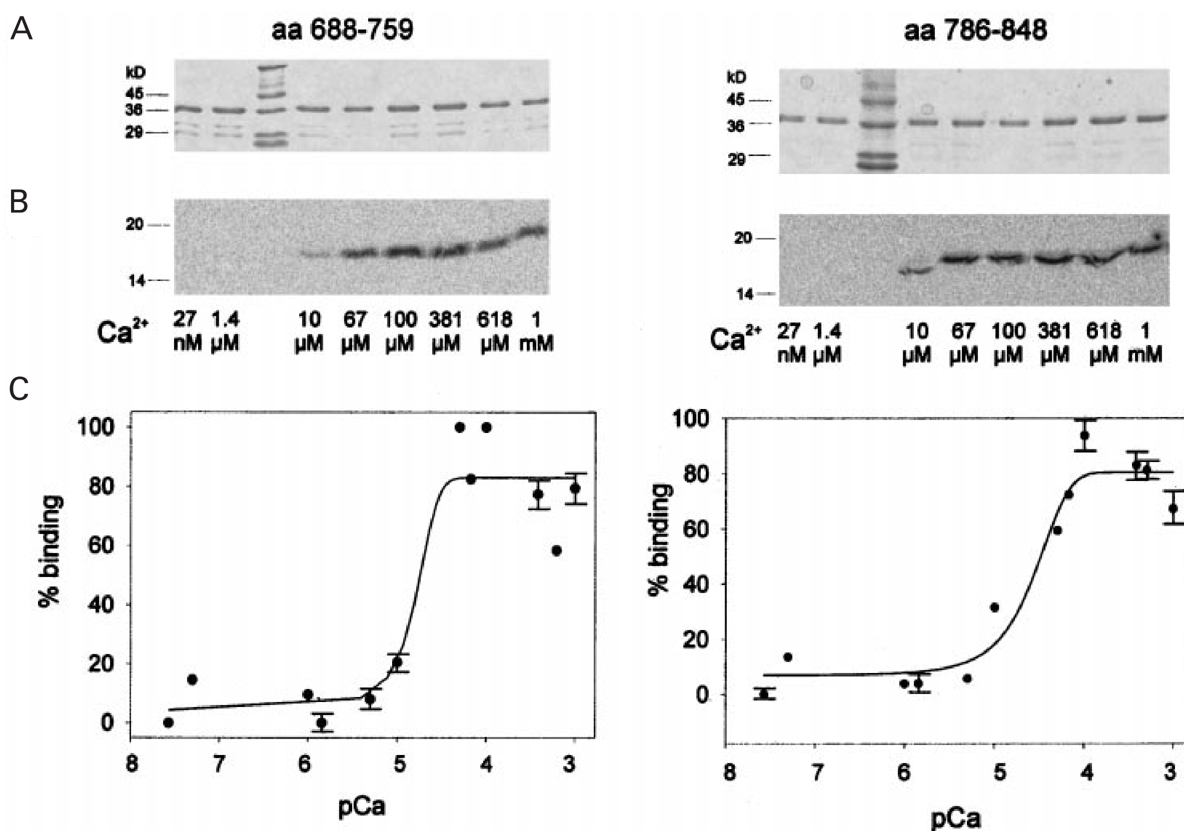

Figure 4 Effect of [Ca^{2+}] on [^{35}S]CaM binding to the CaM binding domains, residues 786–848 and 688–759 of TRP4

(**A**) Representative result showing a Coomassie-Blue stained gel after SDS/PAGE (20–10 % gel) where ~ 1.5 μg of mTRP4 [residues (aa) 688–759] – or mTRP4 (residues 786–848) – GST fusion proteins (right and left panel respectively) were absorbed on to glutathione–Sepharose and incubated with 200 000 c.p.m. of [^{35}S]CaM in the presence of different Ca^{2+} buffers. (**B**) Autoradiogram of retained [^{35}S]CaM from the gel shown in (**A**). (**C**) Binding of [^{35}S]CaM plotted as a function of increasing [Ca^{2+}]. Half maximal binding of CaM to domain 1 and domain 2 occurred at 27.9 ± 4.5 μM and 16.6 ± 2.3 μM respectively (means ± S.E.M). Binding is expressed as a percentage of maximal binding. Values are the means of two to six independent determinations.

core structure of CaM binding site 1 within TRP4, whereas peptide 422 represents CaM binding site 2.

DISCUSSION

The present study identifies TRP4 as a CaM binding protein. TRP4 contains two domains (Figure 6) within its presumptive cytosolic C-terminus, which bind CaM in a Ca^{2+}-dependent manner. The CaM concentration, for example in neuronal cells, is in the order of μM, exceeding the K_d values of domains 1 and 2 for CaM binding (94–189 nM). *In vivo*, occupation of both sites should therefore depend on the cytosolic Ca^{2+} concentration. In the presence of 16.6 μM Ca^{2+} (domain 1) or 27.9 μM Ca^{2+} (domain 2), 50 % of the TRP4 binding sites are occupied by CaM. Cytosolic Ca^{2+} oscillates between 0.1 and 0.2 μM in a resting cell, but could locally be increased due to the release from intracellular stores and/or entry into the cell, and consecutively convert TRP4 into its Ca^{2+}-CaM-bound form.

The first CaM binding domain was identified on the GST–TRP4 (residues 688–759) fusion protein by retention of CaM. Two overlapping peptides within this region, peptides 421 and 423, were predicted to form an amphiphilic α-helix; both peptides bind dansyl–CaM with high affinity and therefore represent the CaM binding domain 1 (residues 694–728) (Figure 6A, upper panel, shaded areas).

The second CaM binding domain was identified by the GST–TRP4 (residues 786–848) fusion protein. A stretch of 20 amino acid residues within this domain and five adjacent C-terminal residues (residues 829–853) (Figure 6A, lower panel, shaded areas) were identified and predicted to form an amphiphilic helical structure. This 25-residue peptide binds dansyl–CaM with high affinity, and apparently represents the CaM binding domain 2. Comparison of this second CaM binding domain with the amino acid sequence of TRP5 [40] reveals some similarity (Figure 6A, lower panel). The C-terminal sequences of TRP1, 3, 6 and 7 are shorter than the TRP4 sequence and end before domain 2 occurs in TRP4.

Recently, it has been shown by co-immunoprecipitation, yeast two-hybrid analysis and GST pull-down experiments that non-corresponding sequences of TRP1, TRP3 and TRP4 physically interact with the InsP_3 receptor [15,29,31]. The N-terminal portion of the InsP_3 receptor interacts with TRP3 and, strikingly, when the amino acid sequences of TRP3 and TRP4 are compared (Figure 6A), the InsP_3 receptor interaction domain of TRP3 (Figure 6A, upper panel, boxed area) aligns with the CaM binding domain 1 (residues 694–728) of TRP4 (Figure 6A, upper panel, shaded area). This finding indicates that TRP3, like TRP4, might bind CaM, and that TRP3-mediated Ca^{2+} entry is not only modulated by the InsP_3 receptor but also by Ca^{2+}-CaM-dependent processes.

In the case of TRP4, it has been shown that the C-terminal portion of human TRP4 (residues 615–977), but not the shorter

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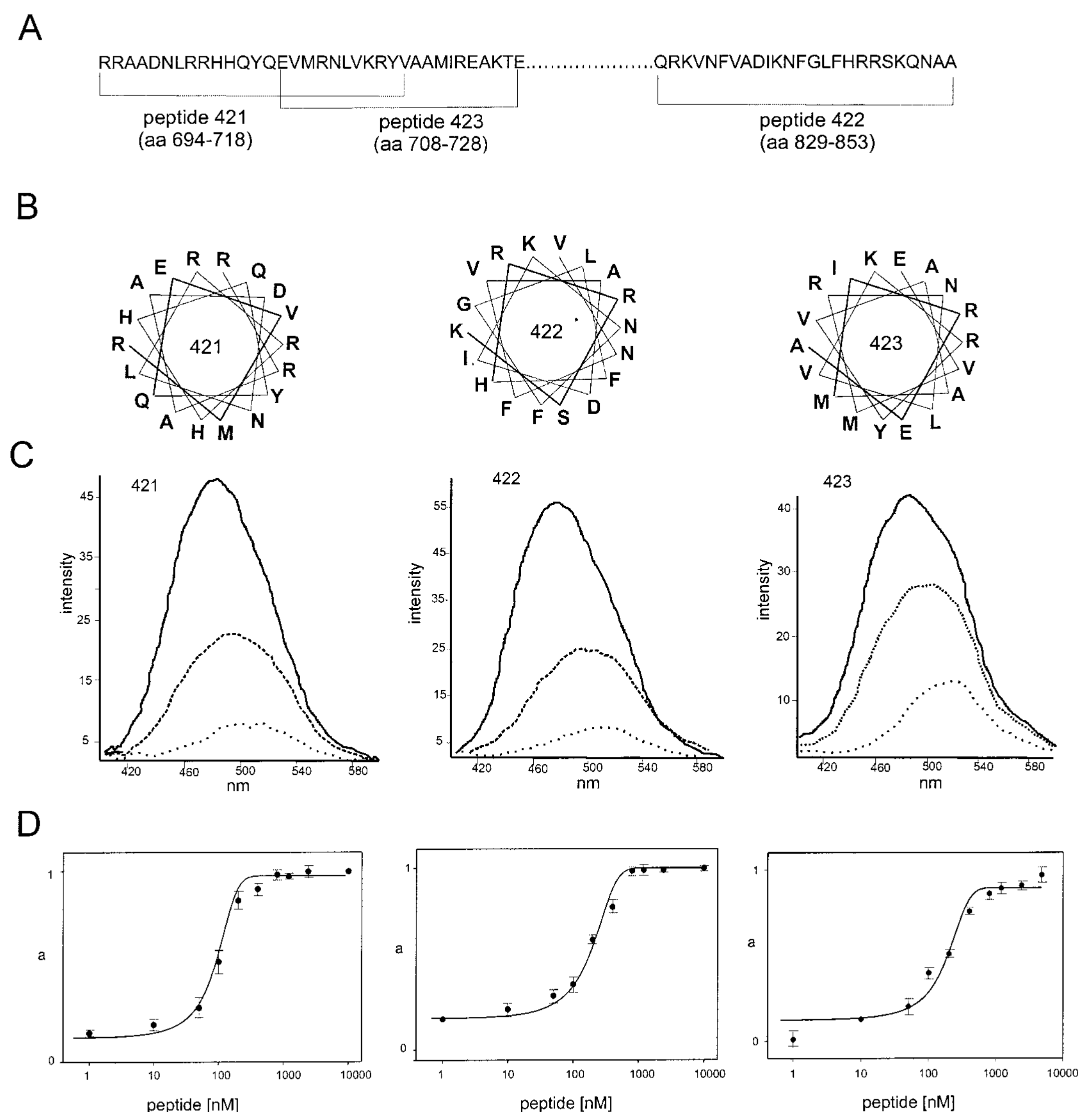

Figure 5 Direct interaction of CaM with TRP4 peptides as measured by the changes in dansyl fluorescence

(**A**) Amino acid sequences of the TRP4 peptides 421, 423 and 422. (**B**) Helical wheel projections of TRP4 peptides 421, 422 and 423. (**C**) Emission spectra of the fluorescence measurements in the presence of 200 nM dansyl–CaM and 1 mM Ca^{2+} (dotted line). The addition of peptide (200 nM) caused a blue shift in the fluorescence peak and increased the total fluorescence intensity (solid line). The addition of 2 mM EGTA completely reversed these fluorescence changes (broken line). (**D**) The interactions between dansyl–CaM and mTRP4 peptides were assayed by examining the fractional increase in dansyl–CaM fluorescence at 470 nm. The fractional degree (a) of dansyl–CaM fluorescence was plotted against the free peptide concentration. The data points were fitted and the apparent K_d values for peptides 421, 422 and 423 were 94, 138 and 189 nM respectively. Data are shown as average ± S.E.M. of three experiments.

$TRP4_{\Delta 785-868}$ version, was able to interact with the C-terminal portion of the InsP_3 receptor [31]. Accordingly, the InsP_3-receptor interaction domain of human TRP (hTRP)4 should be localized within the stretch of 83 residues (G_{785} to S_{868}), which are lacking in the $TRP4_{\Delta 785-868}$ protein. Interestingly, the CaM binding site 2 described in the present work (Figure 6A, lower panel, shaded area) is localized within the same stretch of 83 residues (Figure 6A, lower panel, boxed area).

This second CaM binding site is absent from the $TRP4_{\Delta 785-868}$ splice variant of the human *trp4* gene (Figure 6B), which shares 96 % sequence identity with its mouse counterpart, and from the murine splice variant $TRP4_{\Delta 781-864}$. The tissue-specific expression of $hTRP4_{\Delta 785-868}$ has been demonstrated [31] but, so far, its significance is unknown. Interestingly, expression of a $mTRP4_{\Delta 781-864}$ variant, which additionally lacks the 21 C-terminal amino acid residues [21], including the Na^+/H^+-exchanger regulatory factor interaction domain, leads to the formation of cation channels which are activated independently of Ca^{2+}-store depletion. In contrast, the 'full-length' bTRP4, which shares 97.4 % sequence identity with mTRP4 when expressed in HEK-293 cells, Chinese Hamster ovary cells and rat basophilic leukaemia cells, leads to the formation/augmentation of Ca^{2+} entry channels which are activated by Ca^{2+} store depletion [16–18].

The identification, in the present study, of two CaM binding sites within the TRP4 protein indicates that TRP4/Ca^{2+}-CaM are parts of a signalling complex involved in store-operated Ca^{2+} entry. Future experiments are necessary to analyse the impact of either of the CaM binding domains on TRP4 activity by expressing TRP4 cDNAs in which the CaM binding domains, described here, have been mutated and/or deleted.

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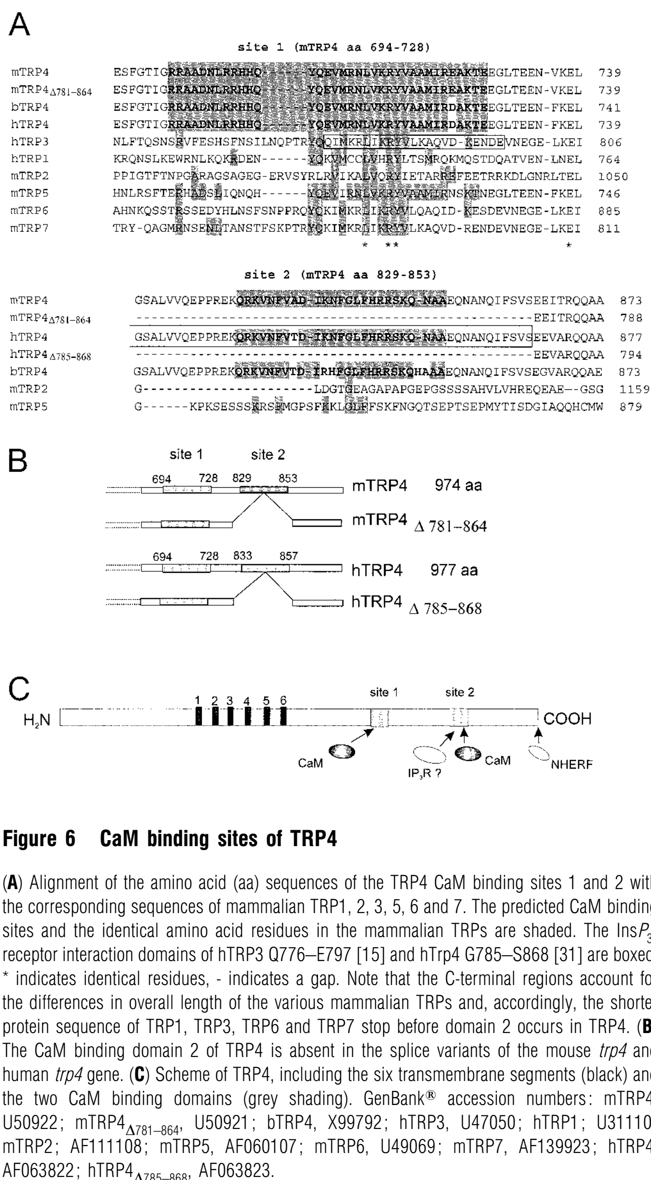

Figure 6 CaM binding sites of TRP4

(**A**) Alignment of the amino acid (aa) sequences of the TRP4 CaM binding sites 1 and 2 with the corresponding sequences of mammalian TRP1, 2, 3, 5, 6 and 7. The predicted CaM binding sites and the identical amino acid residues in the mammalian TRPs are shaded. The InsP_3-receptor interaction domains of hTRP3 Q776–E797 [15] and hTrp4 G785–S868 [31] are boxed. * indicates identical residues, - indicates a gap. Note that the C-terminal regions account for the differences in overall length of the various mammalian TRPs and, accordingly, the shorter protein sequence of TRP1, TRP3, TRP6 and TRP7 stop before domain 2 occurs in TRP4. (**B**) The CaM binding domain 2 of TRP4 is absent in the splice variants of the mouse *trp4* and human *trp4* gene. (**C**) Scheme of TRP4, including the six transmembrane segments (black) and the two CaM binding domains (grey shading). GenBank® accession numbers: mTRP4, U50922; mTRP4$_{\Delta781-864}$, U50921; bTRP4, X99792; hTRP3, U47050; hTRP1; U31110; mTRP2; AF111108; mTRP5, AF060107; mTRP6, U49069; mTRP7, AF139923; hTRP4, AF063822; hTRP4$_{\Delta785-868}$, AF063823.

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