Integrative Binding Sites within Intracellular Termini of TRPV1 Receptor

Lenka Grycova¹*, Blanka Holendova¹, Ladislav Bumba², Jan Bily¹, Michaela Jirku¹, Zdenek Lansky¹, Jan Teisinger¹*

¹Institute of Physiology, v.v.i., Academy of Sciences of the Czech Republic, Prague, Czech Republic, ²Institute of Microbiology, v.v.i., Academy of Sciences of the Czech Republic, Prague, Czech Republic

* E-mail: teisingr@biomed.cas.cz (JT); l.grycova@biomed.cas.cz (LG)

Abstract

TRPV1 is a nonselective cation channel that integrates wide range of painful stimuli. It has been shown that its activity could be modulated by intracellular ligands PIP2 or calmodulin (CaM). The detailed localization and description of PIP2 interaction sites remain unclear. Here, we used synthesized peptides and purified fusion proteins of intracellular regions of TRPV1 expressed in E.coli in combination with fluorescence anisotropy and surface plasmon resonance measurements to characterize the PIP2 binding to TRPV1. We characterized one PIP2 binding site in TRPV1 N-terminal region, residues F189-V221, and two independent PIP2 binding sites in C-terminus: residues K688-K718 and L777-S820. Moreover we show that two regions, namely F189-V221 and L777-S820, overlap with previously localized CaM binding sites. For all the interactions the equilibrium dissociation constants were estimated. As the structural data regarding C-terminus of TRPV1 are lacking, restraint-based molecular modeling combined with ligand docking was performed providing us with structural insight to the TRPV1/PIP2 binding. Our experimental results are in excellent agreement with our in silico predictions.

Introduction

The vanilloid receptor (TRPV1) is one of the best characterized members of the TRPV subfamily. This nonselective cation channel serves as a polymodal receptor for various potentially harmful signals. Activation is caused by diverse stimuli, such as noxious heat (>43°C), low pH (<5.4) and chemicals such as capsaicin, its analogs and a wide range of other agonists (e. g. resiniferatoxin, anandamide [1,2,3,4,5]). It is assumed that the TRPV1 channel has six transmembrane domains with a pore domain between the fifth and the sixth segment and as has been recently confirmed by electron microscopy, forms a tetrameric structure with a central localized pore [6]. Both its C- and N-termi are located intracellularly and have been shown to be involved in the regulation of the channel activity [4,7].

A number of studies have demonstrated that the cytoplasmic regions of TRP channels bind agonists and regulatory molecules such as ATP, calmodulin (CaM) and phosphatidylinositol-4, 5-bisphosphate (PIP2) [8,9,10,11,12,13,14,15]. PIP2 is a minor phosphoinositide has been suggested [12]. However the exact role of PIP2 as a TRPV1 activity modulator remains elusive. Whether PIP2 acts directly on TRPV1 [22] or intervenes via the accessory membrane protein PIRT [23] has been discussed. Moreover the exact molecular mechanism of PIP2-dependent regulation of TRPV1 is still unclear, as well as whether PIP2 works as an activator [24,25], inhibitor [9] or causes bi-directional modulation [26,27]. To date several possible PIP2-interacting regions have been proposed within the cytosolic termini of the TRPV1 channel [9,10,22].

We show that there are three different regions on the cytoplasmic domains of TRPV1 interacting with PIP2. We found the precise location of the binding sites within the C- and N-termini and we estimated the corresponding binding affinities. Using the combination of biophysical and bioinformatical methods we identified the key residues involved in PIP2 binding in the proximal and distal regions of the C-tail of TRPV1. We showed that regions on C-terminus L777-S820 and N-terminus F189-V221 overlap with the CaM binding sites and the third PIP2 binding site K688-K718 occupies the TRP domain on C-terminus, a highly conserved sequence among the members of the TRP ion channel family. We found that the presence of PIP2 prevents the interaction of the TRPV1-CT distal region with CaM, which could play an important role in the regulation of TRPV1.
Results

PIP2 Binds to the TRPV1 C-tail Distal Region (712–838)

In this report we studied part of the sequence on the C-tail of TRPV1 (amino acids 712–838; henceforth denoted as TRPV1-CT) containing the distal putative PIP2 interaction region. [9] The region was expressed as a fusion protein in E. coli with thioredoxin at the N-terminus and a 6× His tag located at both termini. Proteins (wild type construct and its site directed mutants) were purified using a two-step purification protocol. All expressed proteins were soluble, and expression yields were sufficient to perform spectroscopic and biochemical studies. We were unable to remove the thioredoxin, as the TRPV1-CT alone has a strong tendency to aggregate. The binding of PIP2 to the TRPV1-CT wild-type was investigated using a steady-state fluorescence anisotropy binding assay. A fluorescent PIP2 analogue, PIP2-Bodipy, was titrated with an increasing amount of TRPV1-CT and the steady state anisotropy was measured (Fig. 1A). We observed the binding of PIP2-Bodipy to TRPV1-CT in a 1:1 ratio and we estimated the equilibrium dissociation constant of the complex formation to be 3.4±0.9 μM. In a control experiment, an increasing amount of thioredoxin was titrated to the PIP2 analogue, PIP2-Bodipy. We observed no thioredoxin-PIP2 binding (Fig. 1B).

To provide confirmation of the results from this technique, we used SPR as a different method for the interactions assessment (Fig. 2). Expressed and purified fusion proteins were washed over the liposome-covered chip and equilibrium dissociation constants were estimated (Tab. 1). The dissociation constant for WT TRPV1-CT was 3.0±0.4 μM, which is in a good agreement with the value estimated by steady state fluorescence anisotropy measurement. PIP2 typically interacts with domains containing a cluster of basic amino acid residues. Thus, in order to identify the residues important for TRPV1-CT binding to PIP2, a set of point mutations was performed on the wild-type (WT) DNA, namely single substitutions R778A, R781A, double substitutions K770A/R785A, R771A/R781A, R771A/R778A and triple substitutions K770A/R778A/R785A and K770A/R781A/R785A. The binding of TRPV1-CT point mutants to PIP2 containing liposomes was investigated using SPR measurements (Fig. 2A, B). The most striking effect was the total loss of binding affinity observed for the single mutant R778A, double mutant R771A/R778A and triple mutant K770A/R787A/R785A (Fig. 2C) respectively. Moreover, the K770A/R785A and R771A/R781A mutations decreased the binding affinity, with estimated K_D values of 5.8±0.8 μM and 33.5±11.7 μM respectively, compared to the value estimated for the WT TRPV1-CT fusion protein (3.0±0.4 μM as determined by SPR and 3.5±0.9 μM as determined by anisotropy measurement) (Tab. 1).

Effect of Liposome Composition on PIP2 Binding to TRPV1-CT Distal Region

As the TRPV1-CT distal sequence contains several basic amino acids which may interact with anionic lipids, we tested the influence of liposome composition on PIP2 - TRPV1 fusion protein interaction by ELISA. The set of liposomes of different composition was prepared and tested whether there is any dependence of TRPV1 binding on the percent composition of PIP2 in the liposomes. Our results showed the modest selectivity for PIP2 over other used lipids (Figure S1). The control SPR experiments with liposomes containing only phosphatidylcholine(PC) and not containing PIP2 were done and just a weak interaction was detected. (Fig. 3) To check and prove that the binding of TRPV1-CT to PIP2 is (highly) specific and that this protein construct does not bind the phosphatidylcholine molecules, we performed a control experiment using fluorescence anisotropy method with 16:0/6:0 NBD PC (1-palmitoyl-2-{6-[7-nitro-2,1,3-benzoxadiazol-4-ylamino]hexanoyl}-sn-glycero-3-phosphocholine) and we observed no interaction. (Fig. 1C).

CaM Binding Site Overlaps with PIP2-Binding Site in TRPV1-CT Distal Region (777–820)

It has been shown that PIP2 and the CaM-Ca^{2+} complex can bind to unstructured clusters of basic amino acids with high affinity [28,29]. To investigate whether the binding sites for PIP2 and CaM in TRPV1-CT distal region overlap, we used a synthetic peptide containing only the TRPV1-CT CaM binding site (L777-S820; here denoted as pTRPV1-CTd) [8,11]. We tested the PIP2 binding to the pTRPV1-CTd peptide using steady-state fluorescence anisotropy measurements. Increasing amounts of TRPV1-CT peptides were titrated into a cuvette containing fluorescently labeled PIP2 (Fig. 4). We observed the binding of PIP2 to this peptide and estimated the equilibrium dissociation constant of the pTRPV1-CTd - PIP2 complex to be 1.86±0.46 μM (Tab. 2).

To confirm that this PIP2 binding site colocalizes precisely with the CaM binding site, we used SPR and tested whether the TRPV1-CT/CaM-Ca^{2+} complex is able to bind PIP2. A protein complex suitable for SPR analysis was prepared and purified (Figure S2). Once liposomes were made and loaded onto the chip, 5 μM TRPV1-CT/CaM-Ca^{2+} complex was washed over the chip. No binding was observed with the protein complex, confirming the colocalization of the PIP2 and CaM binding sites (Fig. 5A). After the regeneration step, the isolated TRPV1-CT fusion protein (712–838) was washed over the chip and binding of approx. 1000 RU was observed. In contrast, further CaM-Ca^{2+} washing over the chip resulted in no change in RU (Fig. 5B). These data might imply that CaM and PIP2 bind to the same or overlapping binding sites within the TRPV1-CT distal region.

PIP2 Interacts with TRPV1-CT Proximal Region (688–718)

The TRPV1 – CT proximal region has been proposed to be an important regulatory site [7], moreover it has recently been suggested that PIP2 can interact directly with the proximal region of TRPV1 [22]. To test the PIP2 binding to the proximal part of the TRPV1-CT, we used the fluorescence anisotropy measurement with synthetic peptides (pTRPV1-CTp) corresponding to the wild type (K688-K718) and its point mutants. The mutations were suggested according to the fact that there are eight basic residues in the pTRPV1-CTp sequence that could bind PIP2 electrostatically: K688, K694, K698, R701, K710, K714, R717 and K718. The equilibrium dissociation constant for the wild type peptide was estimated to be 0.81±0.171 μM (Table 3). The K694A/K698A/K710A triple mutant seemed to completely lose its binding affinity to PIP2 - Bodipy (Fig. 6). Alanine substitutions of the additional candidate residues in the highly conserved QRA region Q700A/R701A significantly attenuated its binding affinity to PIP2-Bodipy (Fig. 6). Indeed these data show that TRPV1-CT proximal region directly binds PIP2 with a high affinity and suggested basic residues play crucial role in the binding.

PIP2 Binds to the CaM Binding Site Present in TRPV1-NT Ankyrin Repeat Domain (189–221)

Recently, one more CaM binding region of TRPV1 was reported. A TRPV1-NT ankyrin binding domain was suggested to interact with CaM and was described in detail using size exclusion chromatography [15]. The CaM binding regions has been commonly considered as a mutual binding sites for CaM and
PIP2. As the PIP2 and CaM interaction regions share key structural features such as interspersed basic and hydrophobic amino acid residues [12], we wondered whether these CaM binding regions of TRPV1 also interact with PIP2. Thus, we designed a peptide that corresponded to this putative binding site – the ankyrin binding domain peptide, pTRPV1-NT (F189-V221). We tested the binding of this peptide to PIP2 by fluorescence anisotropy assay. We observed the binding of PIP2 and estimated the equilibrium dissociation constant \( K_D \) (3.48±0.93 μM) was determined by fitting the data to the Equation 2 as described in Material and Methods. The steady-state fluorescence anisotropy measurement confirmed that the region denoted as CaM interaction site pTRPV1-NT (F189-V221) binds PIP2 with high affinity.

Molecular Modeling and Ligand Docking
To gain structural insight into these experimentally obtained results, a homology model of TRPV1 C-terminus was created using a restraint-based comparative modeling approach. (Fig. 7A) The stereochemical quality was checked and 94% of the residues are in the most favored regions of the Ramachandran plot and has an acceptable geometry. The z-score of the protein is -1.1. This value is within the range of scores typically found for proteins of similar size belonging to one of these groups. In order to be able to perform a structural comparison between template structure and homology model molecule the RMSD value was assessed. To provide the RMSD values of alpha carbons the superimposing of both 3D structures was done using SPDV tool. The RMSD value was calculated to be 1.42 Å. The model of PIP2 molecule which is lacking two aliphatic chains was subsequently docked as PIP2. During the docking procedure the whole molecule of TRPV1 – CT (V746 to K838) was examined and several interaction sites were suggested (Fig. 7B). The 80% of conformations occupy experimentally identified region. Thus in order to gain the detailed insight to the complex forming, the second step of docking procedure was performed. These results provide direct visualization of the interactions between the ligand and nearby PIP2.
atoms in the receptor. In silico binding results are in a good agreement with our experimental work. Our model confirms the crucial interactions between the positively charged residues R778, R781, R785 (Fig. 7C) and PIP2. This is in line with our experimental results, which suggest that R778 plays a pivotal role in this interaction. In our model, the PIP2 molecule also occupies the region previously identified as the binding site for CaM. This region forms an alpha helical structure, as has been predicted before [8].

**Discussion**

PIP2 regulates a wide range of cellular functions and the activity of many ion channels including TRP superfamily members [19,29]. Various domains like PH domains that recognize particular phosphoinositides have been suggested [9,10,22,23]. This study identifies two clusters of basic amino acid residues in the carboxy–terminal and one in the amino–terminal cytoplasmic regions that interact with PIP2. It has been known for a long time that PIP2 typically interacts with the Pleckstrin homology domain (PH) [19]. The PH domain contains a cluster of basic amino acid residues and is known to bind phosphoinositides. Similarly the region of interaction between CaM and its cellular targets often possesses a basic helix consisting of approximately 20 amino acids [8,13,28,30]. It has also been shown that the CaM and PIP2 binding regions could overlap in the one of the members of canonical TRP channels subfamily, TRPC6 and moreover that these two ligands could compete for the mutual binding sites. Initially these overlapping binding sites were described in the MARCKS protein family [31].

Our molecular model of the PIP2 interacting with TRPV1 C-terminal distal region suggested that phosphate head groups of PIP2 form polar interactions with positively charged arginines R778, R781, R785. PIP2 thus occupies the CaM binding groove containing R771, R778, R781, R785 as we had described previously [8]. R771A significantly inhibits the CaM binding to the TRPV1-CT fusion protein, nonetheless this construct preserves its binding ability to PIP2. The next single substitution of R778A inhibited the PIP2 binding, significantly increased the dissociation constant but did not prevent CaM binding [8]. These various effects are in a good agreement with the previously described phosphoinositide/CaM interactions with TRPC6. [12]

The PH domain typically contains at least several basic residues, which participate in the binding of phosphoinositides, by formation of salt bridges between its positively charged amino acid residues and the phosphate groups of PIP2 [19]. Hence a mutagenesis screening of these residues (K770, R771, R778, R781 and R785) was carried out, combined with SPR measurements. This set of experiments revealed the key role of the R778 and R781 residues in the binding of PIP2. Moreover, further combinations of alanine substitutions revealed that the TRPV1-CT distal region participates in PIP2 binding through a cluster of basic residues: the double and triple substitutions of R771A/R778A, K770A/R778A/R785A avoided PIP2 binding totally and the K770A/R785A and R771A/R781A mutations suppressed this interaction partially. The TRPV1–CT proximal region K688-K718 motif has been suggested to be an important regulatory site of TRPV1 [7]. We have thus designed the peptides of this region and tested whether

**Table 1. Analysis of the effects of charge-neutralizing mutations within the TRPV1-CT distal region (712–838) fusion protein on equilibrium dissociation constants determined by surface plasmon resonance experiments.**

| Protein                  | K_d [µM] |
|-------------------------|----------|
| WT                      | 3.0±0.4  |
| K770A/R781A/R785A       | 3.6±0.5  |
| K770A/R785A             | 5.8±0.8  |
| K770A/R778A/R785A       | >150     |
| R771A/R781A             | 33.5±11.7|
| R771A/R778A             | >150     |
| R785A                   | >150     |
| R781A                   | 9.1±3.8  |

The presented values are average ± SD from at least 3 independent measurements.

doi:10.1371/journal.pone.0048437.t001

**Figure 2. Surface plasmon resonance (SPR) analysis of interactions between TRPV1-CT and PIP2-enriched liposomes.** Kinetic binding measurements of TRPV1-CT (A) and the TRPV1-CT-K770A/R778A/R785A triple mutant (B) to the sensor chip coated with PC/PIP2 (80:20) liposomes. The proteins at indicated concentrations were injected in parallel over the lipid vesicles and the flow rate was maintained at 30 µl/min for both association and dissociation phases of the sensograms. (C) SPR equilibrium binding of the TRPV1-CT, TRPV1-CT-K770A/R778A/R785A, and TRPV1-CT-R778A proteins to the sensor chip coated with PC/PIP2 (80:20) liposomes. The proteins were injected at 25 µl/min at different concentrations and washed over the lipid surface and Req values were deduced from steady state (equilibrium) SPR response. The solid lines represent binding isotherms determined by nonlinear least-squares analysis of the isotherm using an equation Req = Rmax/(1+Kd/P0), where Req stands for SPR response value near equilibrium, Rmax is the maximum response and P0 is the protein concentration. Values represent the mean ± S.D from four independent experiments.

doi:10.1371/journal.pone.0048437.g002
its charge neutralization preserves wild-type binding to PIP2. R701 was proposed to be involved in PIP2 dependent activation [7]. Site directed mutation of this arginine reduces binding affinity to PIP2. This is in agreement with previous measurements as the same behavior has been detected in other TRP family members [10]. Moreover we tested other conserved cationic residues within this region that could interact with negatively charged phosphoinositides. The triple substitution at positions K694A/K698A/K710A had the most pronounced effect, completely preventing PIP2 from binding to this region.

It has been previously reported that CaM binds to the ankyrin repeat domain in the N-terminus of TRPV1 [15]. It has also been suggested that PIP2 could be involved in the modulation of TRPV1 functionality via the same region [15]. As the ankyrin repeats sequence shares key structural features with the common PIP2 interaction sites, we tested this region as a possible overlapping binding site for CaM and PIP2. In order to demonstrate the interaction of ankyrin repeats with PIP2, fluorescence anisotropy measurements with synthetized peptide (pTRPV1-NT) were done and the equilibrium dissociation constant was estimated. The resulting $K_{D}$ had an almost identical value to the pTRPV1-CTd peptide $K_{D}$, representing the C-terminal CaM binding site. Lishko at al. showed that CaM interacts with the ankyrin repeats domains of TRPV1-NT and is involved in channel tachyphylaxis as well as PIP2, but the direct interaction of TRPV1-NT with PIP2 was not confirmed [15]. Here we provide direct evidence that the synthetized peptide (pTRPV1-NT) of the part of the ankyrin domains (F189-V221) binds fluorescently labeled PIP2.

Although a number of studies regarding the physiological significance of the regulation of TRPV1 receptor via PIP2 has been published, its precise molecular mechanism remains unsolved. Here, we have identified multiple PIP2 binding sites

**Table 2.** Equilibrium dissociation constants ($K_D$) and their standard deviations of synthetic peptides (pTRPV1) of three different regions on the cytoplasmic tails binding to Bodipy® FL CS, C6-PtdIns(4,5)P2 estimated by fluorescence anisotropy measurement.

| Peptide     | $K_D$ [µM] |
|-------------|------------|
| pTRPV1-CTp  | 0.326±/−0.06 |
| pTRPV1-CTd  | 1.88±/−0.46 |
| pTRPV1-NT   | 1.90±/−0.40 |

doi:10.1371/journal.pone.0048437.t002

**Figure 3.** SPR kinetic binding of TRPV1-CT to PIP2-enriched liposomes (A) and to liposomes made from phosphatidyl choline (PC) (B). Both PIP2-enriched (PIP2/PC 80:20) and PC liposomes were immobilized to the sensor chip at the same density (~1000 RU), and the TRPV1-CT protein at indicated concentrations was injected in parallel over the lipid vesicles at flow rate of 30 µl/min. doi:10.1371/journal.pone.0048437.g003

**Figure 4.** PIP2 recognizes three independent binding sites within the TRPV1 receptor. Steady-state fluorescence anisotropy measurements of interaction between fluorescently labeled phosphatidylinositol-4, 5-bisphosphate (PIP2-Bodipy) and synthetic peptides corresponding to cytoplasmic tails either at the N-terminal region F189-V221 of TRPV1 (pTRPV1– NT), C terminal proximal region K688-K718 of TRPV1 (pTRPV1–CTp), or C-terminal distal region L777-S820 of TRPV1 (pTRPV1–CTd), respectively. PIP2-Bodipy (10 nM) was titrated with indicated concentrations of the peptides and the bound fraction ($F_b$) of PIP2-Bodipy was calculated according to Equation 1 as described in Material and Methods. The solid lines represent binding isotherms determined by nonlinear least-squares analysis of the isotherms using an Equation 2 as described in Material and Methods. Values represent the mean ± SD from at least three independent experiments. doi:10.1371/journal.pone.0048437.g004
within the cytosolic tails of the TRPV1 channel using a combination of biochemical, biophysical and bioinformatical tools. We demonstrated that regions F189-V221 within the N-terminus and K688-K718 and L777-S820 within the C-terminus are involved in PIP2 binding. Interestingly, the F189-V221 and L777-S820 regions overlap with the CaM binding sites suggesting that CaM and PIP2 are competing for the same binding site, which might have implications for regulation of the channel function. The N-terminal F189-V221 occupies the multiple ligands binding site within the ankyrin repeat domain and another binding site lies within the C-terminal CaM binding region. TRPV1 is the first member in the vanilloid subfamily where two regions containing overlapping binding sites for PIP2 and CaM have been identified. Previously, this kind of interaction was shown in the TRPC subfamily [12]. We also revealed a number of amino acid residues within these regions impairing TRPV1-PIP2 interaction. Despite the predicted role of multiple positively charged amino acids in PIP2 binding, we found R778A to have the key role in the interaction. This single mutation leads to a total loss of PIP2 binding.

Figure 5. Both PIP2 and calmodulin (CaM) share the binding site within the C-terminal distal region of TRPV1. (A) SPR kinetic binding of TRPV1–CT and the complex of TRPV1–CT with calmodulin (TRPV1/CaM complex) to the sensor chip coated with PC/PIP2 (80:20) liposomes. TRPV1–CT and the TRPV1–CT/CaM complex (both at 10 μM concentration) were injected in parallel over the lipid vesicles and the flow rate was maintained at 30 μl/min for both association and dissociation phase. (B) A typical SPR kinetic binding of TRPV1–CT to the PIP2-enriched liposomes followed by independent injection of CaM. TRPV1–CT (2 μM) was injected over the sensor chip coated with PC/PIP2 (80:20) liposomes, left to dissociate and then calmodulin was injected onto the identical surface at 10 μM concentration. The flow rate was maintained at 30 μl/min during whole experiment. Black and white strips represent association and dissociation phase of the sensogram, respectively.

doi:10.1371/journal.pone.0048437.g005
of binding affinity of the distal C-terminal region. Our findings provide a characterization of PIP2 interaction sites and indicate interconnection between the PIP2 and CaM binding to the TRPV1, whose physiological significance may need further investigation.

**Materials and Methods**

**Molecular Biology**

Part of the sequence of the rat C-tail of TRPV1 (amino acids 712–838) (TRPV1-CT) (NCBI Reference Sequence: NP_114188.1) was subcloned into the pET-32b expression vector (Stratagene). DNA ligation was verified by DNA sequencing. Point mutations of several amino acid residues for Alanine were performed, namely R771A, R778A as well as the double substitutions K770A/R785A, R771A/R878A and triple substitutions K770A/R778A/R785A, K770A/R771A/R785A. Mutagenesis PCRs were performed using PfuUltra High-fidelity DNA Polymerase (Stratagene). All mutations were confirmed by DNA sequencing.

**Fusion Protein Expression and Purification**

TRPV1-CT was expressed as a fusion protein with thioredoxin tag in Rosetta *Escherichia coli* cells. Protein expression was induced by isopropyl-1-thio-D-galactopyranoside (Roth) for 12 h at 20°C. Cells were disrupted by sonication and the protein was purified using Chelating Sepharose Fast Flow (Amersham Pharmacia Biotech) according to the standard manufacturer’s protocol. Protein samples were concentrated using spin columns (Millipore). The subsequent purification step was a gel permeation chromatography on a Superdex 200 column (Amersham Pharmacia Biotech). Protein concentration was determined by a spectrophotometer (model 6800, Beckman). Protein purity was verified by using 15% SDS-polyacrylamide gel electrophoresis (PAGE).

**Calmodulin (CaM)/TRPV1 – CT Complex Preparation**

Mouse CaM was expressed from the pET3a vector in BL21 *Escherichia coli* cells. Protein expression and purification were done according to the protocol described in our previous study [32]. Binding the recombinant TRPV1 fragment (sequence 712–838) to CaM was done in the presence of CaCl2. The protein mixture, with a 1:1 molar ratio of TRPV1 to CaM, was incubated for 1 h at room temperature (RT). An additional purification step was subsequently done to separate the unbound fraction, gel permeation chromatography in a Superdex 75 column (Amersham Pharmacia Biotech).

**Steady State Fluorescence Anisotropy Binding Assay**

The fusion protein of TRPV1-CT (712–838) was prepared, the series of synthetic peptides (pTRPV1) of three different regions of the cytoplasmic tails of the TRPV1 channel (NCBI Reference Sequence: NP_114188.1) and its mutations were obtained from GenScript USA Incorporated, New Jersey, namely: TRPV1 – CT proximal region K688-K718 (pTRPV1–CTp) or its Q700A/R701A (pTRPV1–CTp-Q700A/R701A) and K694A/K698A/K710A (pTRPV1–CTp-K694A/K698A/K710A) mutant variant, respectively. PIP2-Bodipy (10 nM) was titrated with indicated concentrations of the peptides and the bound fraction (Fb) of PIP2 Bodipy was calculated according to Equation 1 as described in Material and Methods. Values represent the mean ± SD at least three independent experiments.

**Table 3. Summary of equilibrium dissociation constants (Kd) and their standard deviations estimated by fluorescence anisotropy measurement of TRPV1-CT proximal region (688–718) wild-type (peptide pTRPV1-CTp) and its mutants.**

| Peptide        | Kd (μM)       |
|----------------|---------------|
| pTRPV1-CTp     | 0.328 +/- 0.06|
| pTRPV1–CTp K694A/K698A/K710A | >150          |
| pTRPV1-CTp Q700A/R701A | 0.814 +/- 0.17|

**Figure 6. PIP2 binds to the C-terminal proximal region of TRPV1.** Steady-state fluorescence anisotropy measurement of interaction between fluorescently labeled phosphatidyl inositol-4, 5-bisphosphate (PIP2-Bodipy) and synthetic peptide corresponding to the cytoplasmic tail at the C-terminal proximal region K688-K718 of TRPV1 (pTRPV1-CTp) or its Q700A/R701A (pTRPV1–CTp-Q700A/R701A) and K694A/K698A/K710A (pTRPV1–CTp-K694A/K698A/K710A) mutant variant, respectively. PIP2-Bodipy (10 nM) was titrated with indicated concentrations of the peptides and the bound fraction (Fb) of PIP2 Bodipy was calculated according to Equation 1 as described in Material and Methods. Values represent the mean ± SD from at least three independent experiments.

PLOS ONE | www.plosone.org 7 October 2012 | Volume 7 | Issue 10 | e48437
and emission wavelengths were set to 500 nm and 512 nm and for NBD – PC 460 nm and 534 nm respectively. At each peptide concentration, steady-state fluorescence anisotropy was recorded.

The fraction of TRPV1s regions or their mutants bound to fluorescent probe was determined from the anisotropy changes using Eq. (1) [33], where $F_B$ is the fraction bound, $r_{\text{min}}$ and $r_{\text{max}}$, and $r_{\text{obs}}$ are the observed anisotropy, minimum anisotropy, and maximum anisotropy, respectively.
are the anisotropies of the free and bound PIP2-Bodipy with TRPV1-CT, pTRPV1 regions or its mutants, 
\( r_{\text{obs}} \) is the observed anisotropy, and \( Q \) is the ratio of the intensities of the free and bound protein (\( f_{\text{max}}/f_{\text{min}} \)). All experiments were carried out in at least triplicate.

\[
F_B = \frac{(r_{\text{obs}} - r_{\text{min}})}{[(r_{\text{max}} - r_{\text{obs}})Q + (r_{\text{obs}} - r_{\text{min}})]}
\] (1)

**Dissociation Constant Assessment**

The \( F_B \) was plotted against the TRPV1 peptides and fusion proteins concentration respectively and fitted using Eq. (2) [33] to determine the equilibrium dissociation constant (\( K_D \)). Non-linear data fitting was performed using the package SigmaPlot 2000 (6.1) SPSS Inc. \( P_1 \) is the concentration of PIP2-Bodipy and \( P_2 \) is the concentration of TRPV1 fusion protein or TRPPV1 synthetic peptides.

\[
F_B = \frac{K_D + [P_1] + [P_2] - \sqrt{(K_D + [P_1] + [P_2])^2 - 4[P_1][P_2]}}{2[P_1]}
\] (2)

**Liposome Preparation**

The lipids L-\( \alpha \)-phosphatidylinositol-4,5-bisphosphate (PIP2), 1,2-dimyristoyl-sn-glycero-3- Phosphatidylserine (PS), 1,2-dimyristoyl-sn-glycero-3-phosphocholine (PC) and 1,2-dimyristoyl-sn-glycero-3- Phosphatidylethanolamine (PE) were obtained from Avanti Polar Lipids, Inc. A stock solution of each lipid was prepared in chloroform, except for PIP2 stock solution, which was prepared in a chloroform:methanol (2:1) mixture. Liposomes of the following compositions were prepared: PC, PC/PIP2 97/3%, PC/PIP2 90/10%, PC/PIP2/PS 70/10/20%, PC/PIP2/PS/PE 40/10/20/30%, PC/PIP2/PE 70/10/20%, PC/PIP2/PE 77/3/ 20%, PC/PE/PS 50/30/20% by mixing appropriate volumes of the stock solutions. After being dried under an N\(_2\) stream, lipid films were hydrated with 1\% HBSS buffer containing 8 \( \mu \)M oligonucleotide 5'-TATTTCTGATGTCCACCCCC-3', modified at the 3' end with cholesterol (Generi-Biotech, Hradec Kralove, Czech Republic)- followed by extrusion 20 times through a polycarbonate membrane with a 100-nm pore diameter (Avestin Europe, Mannheim, Germany). Finally 8 \( \mu \)M complementary oligonucleotide Biotin conjugate 5'-TGGAATTCAGAAATACCCCC-3' (Generi-Biotech, Hradec Kralove, Czech Republic) was added to the liposome mixture. A 20-min incubation was followed by centrifugation and washing steps to remove the unbound oligonucleotide-Biotin conjugate.

**Elisa**

96-well micro titer plates were used. Each well was coated with 100 \( \mu \)l of (20 \( \mu \)g/ml) C-tail of TRPV1 (amino acids 712–838) in coating buffer (1\times HBSS buffer) or standard (PIP Grip -Avanti Phospholipids) and incubated for 8 h at 4°C. The coating solution was shaken out of the wells and the wells were blocked with 100 \( \mu \)l 1% BSA per well and incubated O/N at 4°C. The next day all eight types of liposomes were added and 2-fold dilutions up to 1/64 \( \times \) were performed. Liposomes were incubated for 1 hour at room temperature. The unbound liposomes were disposed off and the wells were washed with 1\% HBSS buffer. Streptavidin/ Horseradish peroxidase conjugate at 1:3000 dilution was subsequently added and incubated at room temperature for 1 hour followed by the next 1\times HBSS washing step and the addition of substrate solution (\( \text{H}_2\text{O}_2\), o-phenylenediamine (OPD), citrate buffer). The substrate was freshly prepared by adding 40 mg/100 ml of OPD in citrate buffer, and 2 \( \mu \)l of 30% \( \text{H}_2\text{O}_2\) was added to each ml of OPD solution. Thereafter, the plates were further incubated at room temperature in the dark for exactly 15 minutes. The reaction was stopped by adding 50 \( \mu \)l of \( \text{H}_2\text{SO}_4\) into each well. Absorbance was read at 490 nm using a micro plate reader.

**Surface Plasmon Resonance**

All SPR measurements were performed at 25°C using a liposome-coated NLC chip in aProteOn XPR36 Protein Interaction Array System (Bio-rad, Hercules, CA, USA). Liposomes (100 nm in diameter) were made from 1,2-dimyristoyl-sn- glycero-3-phosphocholine (PC) and L-\( \alpha \)-phosphatidylinositol-4,5- bisphosphate (PIP2) (Avanti Lipids, Alabaster, AL, USA) at a molar...
Molecular Modeling and Ligand Docking

A comparative homology model of the three-dimensional structure of the cytoplasmic region (from V746 to K839) of the rat C-terminus TRPV1 was generated using Modeller 9.9 software. [34] As there is no known solved structure of any of the C-terminal regions of the TRPV1 channel subfamily, we used the crystal structure of fragile histidine triad protein (FHT1, PDB code 1FIT) as a template [35], this template structure has been used for modeling of the C-terminal region in previous works. Sequence similarity between the target (TRPV1-CT) and template (FHIT) is 44%, here we use the truncated tail of the template (FHIT) as a template [35], this template structure has been used for modeling of the C-terminal region in previous works. Sequence similarity between the target (TRPV1-CT) and template (FHIT) is 44%, here we use the truncated tail of the target (TRPV1-CT) and template (FHIT) which possesses a 44% sequence similarity. The sequences were aligned with CLUSTALX 2.0.10 [36] (Fig. 8). The energy minimization of all models was performed using a Swiss-PdbViewer with the GROMOS96 force field [37] and checked with ProSA-web [38] for recognizing errors in the 3D protein structure. The docking of this ligand (PDB code 3SPI) [39] to the C-terminus of TRPV1 was performed to obtain a population of possible conformations and orientations for the ligand at the binding site using Autodock 4. The Lamarckian genetic algorithm method was employed for docking a flexible ligand and rigid protein. In order to obtain receptor–ligand complexes, a two step strategy was used. In the starting point for ligand docking less restrictive conditions were set up, the grid box was centered to the macromolecule allowing ligand to explore the whole macromolecule. The cluster with the highest population of suggested ligand-receptor conformation was then selected. This region served as a starting point for the second step of the docking procedure. To show the interactions more precisely a grid box with 40, 40 and 40 points in the x, y, and z directions was built to cover the entire suggested binding site and accommodate ligands to move freely with a grid spacing of 0.375 Å. The default settings were used for all other parameters. The best conformation with the lowest docked energy was chosen.

Supporting Information

Figure S1 A. Schema of Elisa. TRPV1 fusion protein was non-specifically immobilized via adsorption to the surface of a microtiter plate. After the immobilization, the liposomes were added, forming a complex with the fusion protein. Each liposome had incorporated the cholesterol/oligonucleotide conjugate, which is complementary to the biotinylated oligonucleotide. The plate was developed by adding an enzymatic substrate (streptavidin/horse radish peroxidase) to produce a visible signal. B. The graph compares liposomes of different composition (PC80%PPIP20% - triangles, PC97%PIP3% - white circles, PC – black circles) and the corresponding binding of the TRPV1-CT fusion protein. (DOCX)

Figure S2 TRPV1-CT/CaM complex formation. Chromatogram from size exclusion chromatography including Coomassie-stained 15% SDS-PAGE of fractions 7–9. (DOCX)

Figure S3 A. Molecular structure of PIP2 Bodipy® FL C5, C6-PtdIns(4,5)P2 molecular probe (Invitrogen, cat. n. B22627) B. Molecular structure of 16:0-06:0 NBD PC 1-palmitoyl-2-{6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)arnino]hexanoyl}-sn-glycero-3-phosphocholine in chloroform (NBD-PC), (Avanti Polar Lipids, Inc. Cat. n. 810130C). (DOCX)

Author Contributions

Conceived and designed the experiments: LG ZL JT. Performed the experiments: LG BH LB JB MJ. Analyzed the data: LG LB. Contributed reagents/materials/analysis tools: LG JT. Wrote the paper: LG ZL JT.
11. Rosenbaum T, Gordon-Shaag A, Munari M, Gordon SE (2004) Ca2+/calmodulin modulates TRPV1 activation by capsaicin. J Gen Physiol 123: 53–62.
12. Kwon Y, Hofmann T, Montell C (2007) Integration of phosphoinositide- and calmodulin-mediated regulation of TRPC6. Mol Cell 25: 491–503.
13. Zhu MX (2005) Multiple roles of calmodulin and other Ca(2+)/binding proteins in the functional regulation of TRP channels. Pflugers Arch 451: 105–115.
14. Grycova L, Langy Z, Friedlova E, Vlachova V, Kubala M, et al. (2007) ATP binding site on the C-terminus of the vanilloid receptor. Arch Biochem Biophys 465: 389–398.
15. Lukho PV, Procko E, Jin X, Phelps CB, Gaudet R (2007) The ankyrin repeats of TRPV1 bind multiple ligands and modulate channel sensitivity. Neuron 54: 905–918.
16. Hurley JH, Meyer T (2001) Subcellular targeting by membrane lipids. Curr Opin Cell Biol 13: 146–152.
17. DiNitto JP, Cronin TG, Lambright DG (2003) Membrane recognition and targeting by lipid-binding domains. Sci STKE 2003: re16.
18. Cho W, Stahelin RV (2005) Membrane-protein interactions in cell signaling and membrane trafficking. Annu Rev Biophys Biomol Struct 34: 119–151.
19. Lemmon MA (2003) Phosphoinositide recognition domains. Traffic 4: 201–213.
20. Dietrich A, Kalwa H, Rost BR, Gudermann T (2005) The diacylglycerol-sensitive TRPC3/6/7 subfamily of cation channels: functional characterization and physiological relevance. Pflugers Arch 451: 72–80.
21. Hilgemann DW, Feng S, Nasuhoglu C (2001) The complex and intriguing lives of PIP2 with ion channels and transporters. Sci STKE 2001: re19.
22. Ufret-Vincenty CA, Klein RM, Hua L, Anguerya J, Gordon SE (2011) Localization of the PIP2 sensor of TRPV1 ion channels. J Biol Chem 286: 9688–9698.
23. Kim AY, Tang Z, Liu Q, Patel KN, Maag D, et al. (2008) Pirt, a phosphoinositide-binding protein, functions as a regulatory subunit of TRPV1. Cell 133: 475–485.
24. Klein RM, Ufret-Vincenty CA, Hua L, Gordon SE (2008) Determinants of molecular specificity in phosphoinositide regulation. Phosphatidylinositol(4,5)-bisphosphate (PIP2) is the endogenous lipid regulating TRPV1. J Biol Chem 283: 26268–26276.
26. Lukacs V, Thagagarajan B, Varnai P, Balla A, Balla T, et al. (2007) Dual regulation of TRPV1 by phosphoinositides. J Neurosci 27: 7070–7080.
27. Stein AT, Ufret-Vincenty CA, Hua L, Santana LF, Gordon SE (2006) Phosphoinositide 3-kinase binds to TRPV1 and mediates NGF-stimulated TRPV1 trafficking to the plasma membrane. J Gen Physiol 128: 509–522.
28. Rhoads AR, Friedberg F (1997) Sequence motifs for calmodulin recognition. FEBS J 11: 331–340.
29. Suh BC, Hille B (2005) Regulation of ion channels by phosphatidylinositol 4,5-bisphosphate. Curr Opin Neurobiol 15: 370–378.
30. Friedlova E, Grycova L, Holakova B, Sihlan J, Janoušková H, et al. (2010) The interactions of the C-terminal region of the TRPC6 channel with calmodulin. Neurochem Int 56: 363–366.
31. McLaughlin S, Murray D (2005) Plasma membrane phosphoinositide organization by protein electrostatics. Nature 430: 605–611.
32. Holakova B, Grycova L, Bily J, Trisinger J (2011) Characterization of calmodulin binding domains in TRPV2 and TRPV3 C-tails. Amino Acids 40: 741–748.
33. Lakowicz J, (2006) Principles of fluorescence spectroscopy; Springer, editor. New York.
34. Sali A, Blundell TL (1995) Comparative protein modelling by satisfaction of spatial restraints. J Mol Biol 234: 779–813.
35. Luna CD, D’Amico KL, Naday I, Rosenbaum G, Westbrook EM, et al. (1997) MAD analysis of FHIT, a putative human tumor suppressor from the HIT protein family. Structure 5: 763–774.
36. Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22: 4673–4680.
37. Guex N, Peisch MC (1997) SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modeling: Electrophoresis 18: 2714–2723.
38. Wiederstein M, Sippl MJ (2007) ProSA-web: interactive web service for the recognition of errors in three-dimensional structures of proteins. Nucleic Acids Res 35: W407–410.
39. Hansen SB, Tao X, Mackinnon R (2011) Structural basis of PIP2 activation of the classical inward rectifier K(+) channel Kir2.2. Nature.