Stimulation of *Drosophila* TrpL by capacitative Ca^{2+} entry

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Trp-like protein (TrpL, where Trp is transient receptor-potential protein) of *Drosophila*, a non-selective cation channel activated in photoreceptor cells by a phospholipase C-dependent mechanism, is thought to be a prototypical receptor-activated channel. Our previous studies showed that TrpL channels are not activated by depletion of internal Ca^{2+} stores when expressed in Sf9 cells. Using fura-2 to measure cation influx via TrpL, and cell-attached patch recordings to monitor TrpL single-channel activity directly, we have found a thapsigargin-induced increase in TrpL activity in the presence of extracellular bivalent cations, with $\text{Ca}^{2+} > \text{Sr}^{2+} \gg \text{Ba}^{2+}$. The increase in TrpL channel activity was blocked by concentrations of La^{3+} that completely inhibited endogenous capacitative Ca^{2+} entry (CCE), but have no effect on TrpL, suggesting that TrpL exhibits *trans*-stimulation by cation entry via CCE. TrpL has two putative calmodulin (CaM)-binding domains, designated CBS-1 and CBS-2. To determine which site

may be required for stimulation of TrpL by the cytosolic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$), a chimaeric construct was created in which the C-terminal domain of TrpL containing CBS-2 was attached to human TrpC1, a short homologue of Trp that is not activated by depletion of internal Ca^{2+} stores or by a rise in $[\text{Ca}^{2+}]_i$. This gain-of-function mutant, designated TrpC1-TrpL, exhibited *trans*-stimulation by Ca^{2+} entry via CCE. Examination of CaM binding in gel-overlay experiments showed that TrpL and the TrpC1-TrpL chimaera bound CaM, but TrpC1 or a truncated version of TrpL lacking CBS-2 did not. These results suggest that only CBS-2 binds CaM in native TrpL and that the C-terminal domain containing this site is important for *trans*-stimulation of TrpL by CCE.

Key words: Ba^{2+} , calmodulin, non-selective cation channel, patch clamp, Sr^{2+} .

INTRODUCTION

The transient receptor-potential protein (Trp) and the Trp-like protein (TrpL) form ion channels responsible for the light-induced conductance change in *Drosophila* photoreceptor cells [1]. We have functionally expressed both Trp and TrpL channels in Sf9 insect cells using the baculovirus expression system [2–5]. TrpL forms Ca^{2+} -permeable non-selective cation channels that exhibit spontaneous activity in both whole-cell and single-channel recordings and are relatively insensitive to lanthanides. TrpL channels are not activated by depletion of the internal Ca^{2+} stores by thapsigargin when expressed in Sf9 cells, but can be stimulated by a receptor-dependent mechanism involving inositol 1,4,5-trisphosphate [3,6,7] or by a direct $\text{G}\alpha_{11}$ -mediated event [8]. Single TrpL channels have been recorded in cell-attached, and excised inside-out and outside-out patches, from Sf9 cells expressing the TrpL protein [8,9]. Interestingly, TrpL-channel activity is dramatically increased by receptor stimulation when recorded in cell-attached mode, i.e. when the receptor agonist is applied outside the patch pipette [6], suggesting that activation occurs via a diffusible cytoplasmic messenger, possibly inositol 1,4,5-trisphosphate or some other downstream effector, such as Ca^{2+} .

A rise in the cytosolic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) of the photoreceptor cell is not sufficient to activate the light-induced conductance change. However, photolysis and release of ‘caged’ Ca^{2+} during the rising phase of the light response facilitates, whereas release of Ca^{2+} during the later phase suppresses, membrane current [10]. Thus it appears that a change in $[\text{Ca}^{2+}]_i$ of the photoreceptor cell can have both positive and negative effects on the light-induced channels. Since TrpL was originally cloned as a calmodulin (CaM)-binding protein [11], regulation

by Ca^{2+} may occur by either a direct effect of Ca^{2+} on the channels or indirectly via a Ca^{2+} –CaM-dependent mechanism. Warr and Kelly [12] measured CaM binding to fusion proteins or peptides containing the amino acid sequences thought to represent the CaM-binding domains in TrpL, designated CBS-1 and CBS-2. However, these investigators did not examine the functional consequences of CaM binding, nor did they define the binding domains in native TrpL. Furthermore, it is not clear which of the two putative CaM-binding domains is responsible for activation and/or inhibition of TrpL current. Barritt and colleagues [13] examined the effect of Ca^{2+} and CaM on TrpL using the *Xenopus* oocyte expression system. Although some evidence was obtained to suggest that Ca^{2+} –CaM plays a role in both activation and inhibition of Ca^{2+} fluxes in TrpL-expressing oocytes, ion currents via TrpL channels were not reported and the Ca^{2+} fluxes observed were completely inhibited by $5 \mu\text{M}$ Gd^{3+} , a concentration of lanthanide that fails to inhibit TrpL in either photoreceptor cells [14] or other heterologous expression systems [3,15,16]. In a subsequent study, Lan et al. [17] generated TrpL mutants with point mutations in the putative CaM-binding domains. Measurement of Ca^{2+} influx in oocytes expressing these mutant TrpL proteins led to the conclusion that CBS-1 is responsible for activation of TrpL by Ca^{2+} ; however, CaM binding to either wild-type or mutant TrpL proteins expressed in the oocytes was not reported. In contrast, Scott et al. [18] created mutants in which the putative CaM-binding domains of TrpL were deleted. Expression of these deletion mutants in a *trp/trpL* double-mutant fly produced light-induced currents with slow inactivation kinetics consistent with inhibition of TrpL by Ca^{2+} –CaM and the rapid turn-off of current seen in *trp* mutant flies. Again, however, CaM binding to either wild-type or mutant TrpL channel proteins expressed in the eye was not reported. In

Abbreviations used: $[\text{Ca}^{2+}]_i$, cytosolic free Ca^{2+} concentration; CaM, calmodulin; CCE, capacitative Ca^{2+} entry; MBS, Mes-buffered saline; BK cell, Sf9 cell expressing human B_2 bradykinin receptor; Trp, transient receptor-potential protein; TrpL, Trp-like protein; TrpL cell, TrpL-expressing Sf9 cell.

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Drosophila Sf9 cells expressing the TrpL protein, whole-cell TrpL currents were either increased, decreased or unaffected by release of caged Ca^{2+} , but the role of CaM in this response was unclear [19]. Lastly, Obukhov et al. [20] reported that single TrpL channels recorded in excised patches from TrpL-expressing Sf9 cells are inhibited by Ca^{2+} , independent of CaM, with an IC_{50} of 2.3 μM . These results suggest that feedback inhibition is unrelated to direct CaM binding to TrpL, but leave open the possibility that Ca^{2+} -CaM affects channel activation.

The purpose of the present study was to determine if TrpL expressed in Sf9 insect cells could be regulated by $[\text{Ca}^{2+}]_i$. Using fura-2 to measure $[\text{Ca}^{2+}]_i$ and cell-attached patch recordings to monitor single-channel activity directly, the results of the present study demonstrate that TrpL exhibits *trans*-stimulation by Ca^{2+} entry via the endogenous capacitative Ca^{2+} entry (CCE) pathway in the Sf9 cell. Comparing the responses using Ca^{2+} , Sr^{2+} and Ba^{2+} suggests modulation consistent with the properties of bivalent cation binding to CaM. To determine what part of the TrpL protein is needed for this regulation, a chimaeric channel was created in which the C-terminal domain of TrpL containing the putative CBS-2 was attached to TrpC1, a short human homologue of Trp that is not activated by depletion of the internal Ca^{2+} store or by a rise in $[\text{Ca}^{2+}]_i$ [21]. This chimaera was shown to be a 'gain-of-function' mutant exhibiting stimulation by an increase in $[\text{Ca}^{2+}]_i$. Furthermore, evaluation of CaM binding in gel-overlay experiments demonstrated that the CaM-binding site was transferred from TrpL to TrpC1 by addition of this C-terminal domain. These results provide evidence at the single-channel level that TrpL is stimulated by a rise in $[\text{Ca}^{2+}]_i$ and identify a region of the TrpL protein that may be involved in this effect.

EXPERIMENTAL PROCEDURES

Solutions and reagents

Mes-buffered saline (MBS) contained the following: 10 mM NaCl, 60 mM KCl, 17 mM MgCl_2 , 10 mM CaCl_2 , 4 mM D-glucose, 110 mM sucrose, 0.1% BSA and 10 mM Mes, pH-adjusted to 6.2 at room temperature with Trizma-base. The total osmolarity of MBS was ≈ 340 mosM. Nominally Ca^{2+} -free MBS was identical with MBS with the exception that CaCl_2 was iso-osmotically replaced by MgCl_2 . Thapsigargin was obtained from Calbiochem (San Diego, CA, U.S.A.). Fura-2 acetoxymethyl ester was obtained from Molecular Probes (Eugene, OR, U.S.A.).

Cell culture

Spodoptera frugiperda Sf9 cells were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). They were cultured as described previously [3,22,23] using Grace's insect medium supplemented with 2% lactalbumin hydrolysate/2% yeastolate solution/2 mM L-glutamine/10% heat-inactivated fetal bovine serum/1% penicillin/streptomycin solution (stock solutions of the supplements were from Gibco, Grand Island, NY, U.S.A.).

Creation of mutant channel constructs

The cDNA clone for TrpC1 was obtained from Dr. Craig Montell (Johns Hopkins University, Baltimore, MD, U.S.A.), and first 49 amino acids, which resulted from a cloning artifact, were removed as described previously [21]. A unique *Bgl*II restriction site at amino acid 734 of TrpC1 was chosen as the junction site for construction of the TrpC1-TrpL chimaera (see below, Figure 7). Since no corresponding site was present in

TrpL, PCR primers were used to amplify a region of TrpL corresponding to amino acids 759–878. The forward primer was prefixed with the sequence AGATCT which, following amplification, adds a *Bgl*II site to the 5' end of the TrpL fragment. The amplified fragment contained a *Sac*I site at amino acid 833. TrpC1 (in pVL1392) was digested with *Bgl*II and *Xba*I to remove amino acids 735–759. The PCR fragment was digested with *Bgl*II and *Sac*I to create a linker region, and native TrpL (in pBluescript) was digested with *Sac*I and *Xba*I to create a fragment containing amino acids 833–1124. The three DNA fragments were gel-purified and ligated, yielding a chimaeric construct in which the C-terminal 292 amino acids of TrpL were appended to TrpC1. The nucleotide sequence of TrpC1-TrpL was confirmed by the dideoxynucleotide method using Sequenase Version 2.0 (U. S. Biochemical Corp., Cleveland, OH, U.S.A.).

To create truncated TrpL (see below, Figure 7), PCR primers were used to amplify the region corresponding to amino acids 675–803. The reverse primer included a stop codon and the sequence GCGGCCGC to create a *Not*I site in the amplified fragment; the fragment contained a unique *Dra*III site at amino acid 682. Native TrpL (in pVL1393) was digested with *Dra*III and *Not*I to remove the C-terminal region; the latter was replaced with the *Dra*III/*Not*I-digested PCR fragment, resulting in a net deletion of 322 amino acids from the C-terminal end of TrpL.

Generation of recombinant baculovirus

Recombinant baculoviruses containing the cDNA encoding the human B_2 bradykinin receptor and the various channel constructs were produced using the BaculoGoldTM Transfection Kit (PharMingen, San Diego, CA, U.S.A.) and used for infection of Sf9 insect cells as described previously [3].

Isolation of membrane-associated FLAG proteins and immunoblotting

To monitor expression at the protein level, the FLAG epitope (DYKDDDDK) was attached to the N-terminus of each construct. Membrane preparations and epitope-FLAGged channel constructs were isolated and identified by Western-blot analysis following SDS/PAGE as described previously [5].

Determination of CaM binding

CaM binding to the various channel constructs was determined by gel-overlay technique [24]. Briefly, following SDS/PAGE and transfer of proteins to PVDF membranes on ice, the membranes were blocked for 1 h in overlay buffer [10 mM imidazole/150 mM KCl/1 mM CaCl_2 /0.1% Tween 20 (pH 7.4)] containing 5% non-fat dry milk. Blocked membranes were incubated for 4 h at room temperature in overlay buffer containing 5% BSA and 0.5 $\mu\text{g}/\text{ml}$ biotinylated CaM (Calbiochem). Following incubation, membranes were rinsed and incubated for 1 h in overlay buffer containing 0.5 $\mu\text{g}/\text{ml}$ avidin-conjugated horseradish peroxidase (Pierce, Rockford, IL, U.S.A.). CaM-bound proteins were revealed by addition of horseradish peroxidase substrates in peroxidase buffer (Pierce). Membranes were scanned digitally with a Hewlett-Packard ScanJet IIcx and the acquired image processed for presentation using Designer software.

Measurement of $[\text{Ca}^{2+}]_i$

$[\text{Ca}^{2+}]_i$ was measured at 22 °C in Sf9 cells using the fluorescent indicator fura-2, as described previously [3,23]. In some experiments, $[\text{Ba}^{2+}]_i$ and $[\text{Sr}^{2+}]_i$ were estimated using the fura-2 fluorescence ratio (excitation wavelengths of 350 and 390 nm) as described previously [3,25]. Unless otherwise indicated, the results

shown are representative of at least three independent infections. For comparison, the Figures show experiments that were performed sequentially on the same day. For statistical purposes, the means $\pm$ S.E.M. were determined at selected times and significant differences ($P < 0.05$) were determined by Student's t test or by analysis of variance followed by Tukey's multiple-comparison test.

Electrophysiological techniques

The patch-clamp technique for single-channel recording was utilized in cell-attached mode [2,26]. All experiments were performed on Sf9 cells attached to glass coverslip chambers at room temperature. Unless otherwise indicated, the bath (extracellular) and pipette solution contained 100 mM sodium gluconate and 10 mM Mes (pH 6.5). The stock Ca^{2+} solution contained 50 mM calcium gluconate and 10 mM Mes (pH 6.5). Extracellular solution with intermediate Ca^{2+} concentrations were obtained by mixing the calcium gluconate solution with the appropriate amount of sodium gluconate. Strontium gluconate and barium gluconate solutions were prepared in the same manner. The osmolarity of all solutions was adjusted to 340 mosM with mannitol. Data were obtained using an Axopatch 1D amplifier (Pacer Scientific, Los Angeles, CA, U.S.A.), sampled on line at 10 kHz using pClamp 5.5 software, and recorded on VCR tape via a VR-10B Digital Data Recorder interface (Instrutech Corp., Great Neck, NY, U.S.A.) for subsequent computer analysis. The cell-attached patches were recorded with a pipette potential of -50 mV. Single-channel records were filtered at 2 kHz, digitized and analysed using pClamp6 and EDA (Event Dynamic Analysis utility, [27]). Where indicated, n equals the number of cells examined under each condition. Statistical differences were determined by analysis of variance followed by Tukey's multiple-comparison test; $P < 0.05$ was considered significant.

RESULTS

Stimulation of TrpL channels following inhibition of the endoplasmic reticular Ca^{2+} -pumps with thapsigargin

Our previous fura-2 and whole-cell current measurements have shown that TrpL channels are not activated by depletion of the internal Ca^{2+} stores by thapsigargin [2,3]. However, during the course of these studies, we noticed that the thapsigargin-induced change in $[\text{Ca}^{2+}]_i$ in TrpL-expressing Sf9 cells (TrpL cells) always occurred faster and to a greater extent than in control Sf9 cells expressing the human B_2 bradykinin receptor (BK cells). Representative responses of TrpL and BK cells are shown in Figure 1. Addition of thapsigargin to BK cells produced a biphasic response; $[\text{Ca}^{2+}]_i$ increased ≈ 2 -fold within the first minute and subsequently increased more slowly with time, reaching maximum levels within 6–8 min of thapsigargin addition. As previously reported, basal $[\text{Ca}^{2+}]_i$ was significantly increased in TrpL cells (Figure 1A). Addition of thapsigargin to TrpL cells produced a rapid increase in $[\text{Ca}^{2+}]_i$ that reached a peak level that was 2–3-fold greater than that observed for the BK cells. To distinguish Ca^{2+} influx from release, the effect of thapsigargin was examined in the absence of extracellular Ca^{2+} (Figure 1B). Addition of thapsigargin to both BK and TrpL cells caused an increase in $[\text{Ca}^{2+}]_i$, indicative of Ca^{2+} mobilization from internal stores. Re-addition of Ca^{2+} to the extracellular buffer produced an increase in $[\text{Ca}^{2+}]_i$ that was significantly greater in the presence of thapsigargin (Figure 1B, traces a and b versus c and d, respectively) in both cell types. Furthermore, the thapsigargin-stimulated component was 2–3-fold greater in TrpL cells com-

Figure 1 Effect of thapsigargin on $[\text{Ca}^{2+}]_i$ in TrpL cells

(A) Fura-2-loaded Sf9 cells were suspended in MBS. At the indicated time, thapsigargin (TG, 200 nM; in this, and all subsequent Figures, final concentrations are indicated) was added to the cuvette. Two traces are superimposed; trace a was obtained from TrpL cells, and trace b was obtained from BK cells. The symbols shown represent means $\pm$ S.E.M., $n = 5$. (B) Four traces are shown superimposed. TrpL cells (traces a and c) or BK cells (traces b and d) were suspended in nominally Ca^{2+} -free MBS. At the times indicated, thapsigargin (200 nM) and Ca^{2+} (10 mM) were added to the cuvette and the fluorescence recorded (traces a and b); traces c and d received Ca^{2+} only (no thapsigargin). (C) Thapsigargin-stimulated Ca^{2+} influxes for TrpL cells (trace a minus c) and BK cells (trace b minus d) were calculated from the values shown in (B); $n = 3$.

pared with control BK cells (Figure 1C). These results might suggest that TrpL is activated by thapsigargin, i.e. by depletion of the internal Ca^{2+} stores. However, as seen in Figure 2, thapsigargin-stimulated Ca^{2+} influx in TrpL cells is completely blocked by addition of $10 \mu\text{M}$ La^{3+} during the sustained phase of the response. This concentration of La^{3+} is sufficient to completely block endogenous CCE in Sf9 cells, but is without effect on TrpL [2,3,5,9,23], suggesting that the observed influx does not arise from a thapsigargin-mediated increase in TrpL activity, i.e. TrpL is not activated by depletion of the internal Ca^{2+} store. Alternatively, TrpL may be stimulated by the rise in $[\text{Ca}^{2+}]_i$ that occurs via the endogenous CCE pathway, i.e. *trans*-stimulation by CCE.

To determine if Ba^{2+} entry via CCE also stimulates TrpL, the effect of thapsigargin on Ba^{2+} influx was determined in TrpL cells and control BK cells. Ba^{2+} will pass through most known Ca^{2+} channels (including the CCE pathway [3,25]), but is a poor

Figure 2 Blockade of thapsigargin-stimulated Ca^{2+} influx by La^{3+} in TrpL cells

Fura-2-loaded TrpL cells were suspended in nominally Ca^{2+} -free MBS. Two traces are shown superimposed. Thapsigargin (TG, 200 nM), Ca^{2+} (10 mM) and La^{3+} (10 μM) were added to trace a at the times indicated; trace b received Ca^{2+} and La^{3+} , but no thapsigargin.

Figure 3 Thapsigargin-stimulated Ba^{2+} influx

(A) Four traces are shown superimposed. TrpL cells (traces a and c) or BK cells (traces b and d) were suspended in nominally Ca^{2+} -free MBS. At the times indicated, thapsigargin (TG, 200 nM) and Ba^{2+} (10 mM) were added to the cuvette and the fluorescences recorded (traces a and b); traces c and d received Ba^{2+} only (no thapsigargin). (B) Thapsigargin-stimulated Ba^{2+} influxes for TrpL cells (trace a minus c) and BK cells (trace b minus d) were calculated from the values shown in (A); the symbols shown represent means $\pm$ S.E.M., $n = 5$; $P < 0.08$.

Figure 4 Thapsigargin-stimulated Sr^{2+} influx

(A) Fura-2-loaded BK cells were suspended in nominally Ca^{2+} -free MBS. Two traces are shown superimposed. Thapsigargin (TG, 200 nM) and Sr^{2+} (10 mM) were added to trace a at the times indicated; trace b received Sr^{2+} , but no thapsigargin. (B) Same as in (A) except with TrpL cells. The insets in (A) and (B) show the effect of adding La^{3+} (10 μM) after Sr^{2+} influx in each cell type. (C) The thapsigargin-stimulated Sr^{2+} influx was calculated from the values shown in (A) and (B); $n = 6$, $P < 0.015$. The symbols shown represent means $\pm$ S.E.M.

substrate for the pumps that normally remove Ca^{2+} from the cytosol. Ba^{2+} will, however, bind to fura-2 and produce the same spectral changes as seen with Ca^{2+} . As seen in Figure 3, thapsigargin stimulates Ba^{2+} influx in both cell types. However, unlike the response observed in the presence of Ca^{2+} , thapsigargin-stimulated Ba^{2+} influx was essentially the same for both BK and TrpL cells (Figure 3B). Thus a large increase in $[\text{Ba}^{2+}]_i$ does not stimulate TrpL to a significant degree. Experiments were also performed to examine the effect of Sr^{2+} on TrpL. Like Ca^{2+} and Ba^{2+} , Sr^{2+} permeates all known Ca^{2+} channels and will produce the same spectral changes in fura-2 as Ca^{2+} and Ba^{2+} [25]. Unlike Ba^{2+} , which is a poor surrogate for Ca^{2+} , Sr^{2+} is a good substrate for the Ca^{2+} pumps and will be sequestered into intracellular organelles [25,28,29]. Indeed, Sr^{2+} can substitute for Ca^{2+} in many cellular reactions, including activation of CaM [30]. However, the affinity of CaM for Sr^{2+} is 30-fold less than that for Ca^{2+} [31]. As seen in Figure 4(A), there is a significant thapsigargin-induced Sr^{2+} influx in control BK cells that is

Figure 5 Regulation of single TrpL channels by Ca^{2+} in the presence of thapsigargin

Left-hand panels: single channels were recorded in cell-attached patches from TrpL cells. The bath solution contained 100 mM sodium gluconate, 10 mM Mes (pH 6.5) and sufficient mannitol to bring the total osmolality to 340 mosM. The pipette contained the bath solution plus 1 mM EGTA. Single channels were recorded at a pipette potential of -50 mV. The average open probability (nP_o) binned at 1-s intervals as a function of time after seal formation is shown. Thapsigargin (TG, 200 nM) and Ca^{2+} (A), Sr^{2+} (B) or Ba^{2+} (C) were added to the bath at the times indicated by the bars. In these and all subsequent nP_o plots, gaps in the record reflect periods of time when the bath was exchanged or when the channel activity was monitored at different potentials. Insets: traces shown are recordings of channel activity at the times indicated during the experiment; horizontal and vertical scale bars are 200 ms and 4 pA, respectively. Right-hand panels: the average nP_o values for 30–60 s before (pre) and 90–120 s after (post) addition of the indicated bivalent cation in the presence of thapsigargin are shown for individual experiments performed as on the left. The bars show the means $\pm$ S.E., $n = 4, 6$ and 6 for Ca^{2+} , Sr^{2+} and Ba^{2+} , respectively. Patches exhibiting high initial activity of TrpL, or patches exhibiting transient stimulation followed by rapid inhibition by Ca^{2+} , were excluded from this analysis (see text and Figure 6 for details).

reversed by addition of low-micromolar La^{3+} (Figure 4A, inset). This result is consistent with those obtained in mammalian cells demonstrating that the CCE pathway is permeable to Sr^{2+} [25,32]. Sr^{2+} influx in TrpL cells is also stimulated by thapsigargin

(Figure 4B, trace c). Additionally, basal Sr^{2+} influx is increased in TrpL cells relative to that seen in BK cells (Figure 4B, trace d). These results suggest that TrpL channels are permeable to Sr^{2+} and that Sr^{2+} can substitute for Ca^{2+} in the stimulation of TrpL

Figure 6 Effect of high concentrations of Ca^{2+} on TrpL-channel activity

Single TrpL channels were recorded in cell-attached mode as described in the legend to Figure 5. Ca^{2+} was added to the bath solution at the indicated concentrations following addition of thapsigargin (TG, 200 nM), as indicated by the bars.

seen in thapsigargin-treated cells. Furthermore, the La^{3+} -sensitivity (Figure 4B, inset) suggests that Sr^{2+} influx occurs via the endogenous CCE pathway in the Sf9 cells. As seen in Figure 4(C), the thapsigargin-stimulated component of Sr^{2+} influx is, on average, 1.3-fold greater than that seen in the BK cells. Thus although the general profile for Sr^{2+} is similar to that seen for Ca^{2+} , the effect of Ca^{2+} appears to be greater in magnitude (compare Figures 1C with 4C). Overall, these experiments suggest that TrpL exhibits *trans*-stimulation by bivalent cation entry via CCE with $\text{Ca}^{2+} > \text{Sr}^{2+} \gg \text{Ba}^{2+}$.

Effect of cytoplasmic divalent cations on single TrpL channels

To test the *trans*-stimulation hypothesis directly, single TrpL channels were recorded in cell-attached patches with sodium gluconate in both the pipette and bath solutions. Under these conditions, inward and outward currents through TrpL were carried by univalent cations. Importantly, in cell-attached recording mode, the extracellular surface of the TrpL channel(s) is isolated from the bath solution. Thus changes in activity reflect changes in the cytoplasmic environment. Addition of thapsigargin to the bath had little or no effect on TrpL single-channel activity in the absence of bivalent cations (Figure 5). Addition of Ca^{2+} (10 mM) after thapsigargin produced an increase in TrpL single-channel activity. The channels had all the characteristics of TrpL channels reported previously, including a short mean

open time (1–2 ms), multiple conductance states and voltage-dependent changes in open probability, P_o [9]. The primary effect of Ca^{2+} was to increase the frequency of openings with little change in opening time or single-channel conductance. A smaller, but resolvable, increase in P_o was observed when Sr^{2+} (10 mM) was added after thapsigargin (Figure 5B), but not when Ba^{2+} was added to the bath solution (Figure 5C). A summary of experiments showing the average open probability, nP_o , before and after Ca^{2+} , Sr^{2+} or Ba^{2+} addition in the presence of thapsigargin is shown on the right in each panel of Figure 5. The activity after addition of Ca^{2+} or Sr^{2+} was significantly different when compared with the activity observed beforehand ($P < 0.05$), but no significant difference was observed for Ba^{2+} . The increase in channel activity with Sr^{2+} was significantly less ($P < 0.005$) than that observed with Ca^{2+} , consistent with the fura-2 results. The change in activity induced by Ca^{2+} or Sr^{2+} addition was dependent upon the presence of thapsigargin, since activity did not significantly increase upon addition of either ion alone ($n = 3$) and was blocked by prior addition of La^{3+} (10 μM) to the bath ($n = 3$). Overall, these results confirm that this increase in TrpL channel activity requires Ca^{2+} or Sr^{2+} entry via the endogenous CCE pathway in Sf9 cells, and that Ba^{2+} entry has little or no effect.

As seen in Figure 5(A), TrpL single-channel activity increased initially after Ca^{2+} addition to the bath after thapsigargin, but subsequently decreased with time back towards basal levels, suggesting that Ca^{2+} may have both positive and negative effects on TrpL activity. We also noted that, in some high-activity patches, addition of 10 mM Ca^{2+} after thapsigargin produced a pronounced inhibition of TrpL channel activity (Figure 6A). To examine this in greater detail, higher concentrations of Ca^{2+} were added to the bath solution during TrpL recording from low-activity patches (Figures 6B and 6C). Addition of Ca^{2+} at 20 mM ($n = 3$) caused a transient increase in TrpL channel activity followed by a time-dependent inhibition. Addition of 50 mM Ca^{2+} also produced an increase in TrpL activity ($n = 5$) followed by inhibition, but the time-dependent inactivation was often incomplete. These results suggest that although an increase in $[\text{Ca}^{2+}]_i$ activates TrpL, Ca^{2+} has multiple complex effects; the level of activity seen at any time in cell-attached patches or in the fura-2 experiments undoubtedly reflects a combination of these effects.

Effect of Ca^{2+} on the activity of channel chimaeras

Previous studies have shown that the human Trp homologue designated TrpC1 is insensitive either to thapsigargin or to elevated intracellular $[\text{Ca}^{2+}]_i$ when expressed in the Sf9 cell [21]. TrpC1 is shorter in length than TrpL (759 versus 1124 amino acids respectively). To determine the region of TrpL required for activation by $[\text{Ca}^{2+}]_i$, we created a chimaeric construct, designated TrpC1-TrpL, in which the C-terminal region of TrpL containing the second putative CaM-binding domain, CBS-2, was attached to the C-terminal domain of TrpC1 (Figure 7A). We also created a truncated version of TrpL, designated TrpL_{trunc}, in which the C-terminal region of TrpL was shortened to approximately the same length as TrpC1. This truncated version of TrpL lacked CBS-2, but had an intact CBS-1. Additionally, we created versions of each protein containing the FLAG epitope to monitor protein expression. As seen in Figure 7(B), all constructs were expressed in the Sf9 cells following infection with the individual recombinant baculoviruses. However, only TrpL and the TrpC1-TrpL chimaera bound CaM (Figure 7C); no binding of CaM to either TrpC1 or TrpL_{trunc} was observed in the gel-overlay experiments. CaM binding was Ca^{2+} -dependent; no CaM binding

Figure 7 Channel constructs, protein expression and CaM binding

(A) Schematic comparison of *Drosophila* TrpL, human TrpC1, the TrpC1-TrpL chimera and a truncated version of TrpL designated TrpL_{trunc}. Shown are the two putative CaM-binding domains on TrpL, designated CBS-1 and CBS-2, and the BglII restriction site used to create the chimaeric channel. Note that the chimera had an intact CBS-2 sequence, but lacked CBS-1. TrpL was truncated to exactly the same length as TrpC1, yielding TrpL_{trunc}. Note that TrpL_{trunc} has an intact CBS-1, but lacked CBS-2. (B) and (C) SDS/PAGE and immunoblotting were performed, as described in the Experimental procedures section, on membrane preparations isolated at 42 h post-infection from Sf9 insect cells infected with recombinant baculovirus containing the indicated construct; TrpL (10 µg of protein per gel, predicted molecular mass (*M*) = 127.6 kDa), TrpL_{trunc} (1 µg, *M* = 92.3 kDa), TrpC1 (1 µg, *M* = 87.6 kDa) and TrpC1-TrpL chimera (10 µg, *M* = 122.8 kDa). The first lane shows *M* standards. The upper gel was probed with anti-FLAG antibody and the lower gel with 50 µM biotinylated CaM in the presence of 1 mM Ca²⁺.

was seen if performed in the presence of EGTA (results not shown).

We next asked if the TrpC1-TrpL chimera could be activated by Ca²⁺ entry via CCE. Comparison of the changes in [Ca²⁺]_i induced by thapsigargin among TrpL-, TrpC1- and chimera-expressing cells is shown in Figure 8. In the presence of extracellular Ca²⁺, the chimera cells had a thapsigargin-stimulated change in [Ca²⁺]_i that is intermediate between TrpL- and TrpC1-expressing cells. Interestingly, the change in [Ca²⁺]_i in the chimera cells closely matched that for TrpC1 until ≈ 600 nM (Figure 8A), suggesting that this is the threshold concentration for activation by [Ca²⁺]_i. A similar profile was obtained in the Ca²⁺ add-back protocol (Figures 8B and 8C); thapsigargin-stimulated Ca²⁺ influx in the TrpC1-TrpL chimera-expressing cells was intermediate between TrpL and TrpC1 cells. Similar results were obtained when thapsigargin-stimulated Sr²⁺ influx was examined in these three cell types; however, Ba²⁺ influx in the TrpC1-TrpL chimera-expressing cells was not different from TrpC1 cells (results not shown). Thus, trans-stimulation of TrpL cells and the TrpC1-TrpL chimera cells exhibits the same bivalent cation selectivity. These results suggest that attachment of the C-terminal tail region of TrpL containing CBS-2 conveys trans-stimulation to TrpC1. If this is the case,

then deletion of this tail region from TrpL should remove trans-stimulation. Unfortunately, TrpL_{trunc} is poorly targeted to the plasmalemma and appears to accumulate in the endoplasmic reticulum. The presence of constitutively active TrpL_{trunc} in the endoplasmic reticulum causes the release of Ca²⁺ and spontaneous activation of CCE. For this reason, no further experiments were performed with this construct. Parenthetically, we also created a TrpC1-Trp chimera in which the C-terminal tail region of *Drosophila* Trp was attached to TrpC1. This construct did not express significant levels of protein in the Sf9 cells when examined up to 48 h post-infection.

DISCUSSION

We and others have previously provided both fura-2 and electrophysiological evidence that TrpL is not activated by depletion of internal Ca²⁺ stores by thapsigargin [2,3,7,17]. The present study confirms these results at the single-channel level. This is an important observation because, unlike whole-cell recording, cytoplasmic integrity and composition are essentially preserved when channels are recorded in cell-attached mode. Under these conditions, thapsigargin added to the bath solution had no effect on TrpL channels in the absence of Ca²⁺. Thus

Figure 8 Thapsigargin-induced Ca^{2+} influx in cells expressing the TrpC1-TrpL chimaera

(A) Three traces are superimposed. At the time indicated, thapsigargin (TG) was added to cells expressing the indicated channel construct, $n = 5$; $P < 0.05$ for cells expressing the chimaera versus TrpC1 cells. (B) Six traces are shown superimposed. TrpL cells (traces a and d), TrpC1-TrpL chimaera-expressing cells (traces b and e) and TrpC1 cells (traces c and f) were suspended in nominally Ca^{2+} -free MBS. At the times indicated, thapsigargin (200 nM) and Ca^{2+} (10 mM) were added to the cuvette and the fluorescence recorded (traces a–c); traces d–f received Ca^{2+} only (no thapsigargin). (C) Thapsigargin-stimulated Ca^{2+} influx for each cell type was calculated from the values shown in (B); $P < 0.05$ for chimaera-expressing cells versus TrpC1 cells. The symbols shown represent means $\pm$ S.E.M., $n = 3$ –4.

diffusible factors that might be generated during depletion of the Ca^{2+} stores and that may normally give rise to, or regulate, CCE [33], have no effect on TrpL activity. These same cell-attached patches, however, have TrpL channels, as shown by the activity seen before and after application of thapsigargin and by the dramatic increase in channel activity upon subsequent application of Ca^{2+} . Furthermore, it is clear that CCE is active during these recordings since the increase in channel activity produced by Ca^{2+} was dependent upon application of thapsigargin and was completely blocked by low-micromolar La^{3+} , a concentration sufficient to completely block CCE, but with no effect on cation flux via TrpL. Thus under a variety of conditions known to maximally activate CCE, and in cells where CCE is fully activated, TrpL channels are unaffected. This is in contrast with

the results obtained by Xu et al. [16]. These investigators reported that TrpL is constitutively active when heterologously expressed in 293T cells, and that outwardly rectifying whole-cell currents are increased by pretreatment for 7–10 min with thapsigargin in Ca^{2+} -free solutions. The reason for this difference is unknown, but it may be related to spontaneous activation of TrpL [6,9,34] during pretreatment with thapsigargin, or to activation by the rise in $[Ca^{2+}]_i$ induced by thapsigargin in the 293T cells. Alternatively, mammalian cells may donate subunits and/or accessory proteins to the TrpL channels and impart thapsigargin sensitivity. In this regard, TrpC1 appears to be a store-operated channel when expressed in mammalian cells [35], but not Sf9 cells [21]. In a recent study, Yagodin et al. [15] suggested that TrpL is activated by thapsigargin when expressed in *Drosophila* S2 cells. However, these investigators measured only the change in $[Ca^{2+}]_i$ with fura-2. One complication in determining sensitivity to depletion of the Ca^{2+} stores is the possibility that these channels may be directly regulated by a rise in $[Ca^{2+}]_i$. Regulation of whole-cell TrpL currents by $[Ca^{2+}]_i$ was reported recently in *Drosophila* S2 cells upon release of caged Ca^{2+} [19]. Whether this can explain the apparent thapsigargin-induced increase in TrpL activity reported in this cell line remains unknown.

The fura-2 and the electrophysiological results of the present study demonstrate clearly that Ca^{2+} entry via the endogenous CCE pathway in Sf9 cells causes *trans*-stimulation of TrpL. This regulation is also observed to a lesser extent when Sr^{2+} is used as a surrogate for Ca^{2+} , although Ba^{2+} has no significant effect. Thus the rank order of potency is $Ca^{2+} > Sr^{2+} \gg Ba^{2+}$, which is the order expected for regulation by a CaM- or troponin-like Ca^{2+} -binding site [30,36]. Ba^{2+} is often used to discriminate between CaM- and non-CaM-dependent regulation, since CaM has a low affinity for Ba^{2+} [30]. In this regard, TrpL was originally cloned as a CaM-binding protein [11], and two putative CaM-binding domains, designated CBS-1 and CBS-2, have been identified using fusion proteins [12]. However, the binding of CaM to native TrpL protein has not been described. Furthermore, Warr and Kelly [12] state that 'some difficulty was encountered' in CaM overlays for the fusion protein containing CBS-1. Thus the number and location of CaM-binding sites on TrpL remain unclear.

Likewise, the effect of CaM on TrpL functions is poorly defined. When expressed in *Xenopus* oocytes, basal Ca^{2+} influx via TrpL appears to be stimulated by low and inhibited by high concentrations of exogenous CaM [17]. Mutations of CBS-1 in this expression system led to the conclusion that CBS-1 is responsible for this stimulation of TrpL [13]. Interestingly, Lan et al. [17] report an IC_{50} of 5 μM for Gd^{3+} blockade of Ca^{2+} influx in TrpL-expressing oocytes. Thus it is possible that Ca^{2+} entry via CCE is responsible for sustaining TrpL activity in this expression system. In contrast, Zuker and colleagues [18] have shown that expression of mutant TrpL channels containing a deletion of either CBS-1 or CBS-2 gives rise to light-induced currents in *Drosophila* photoreceptor cells that have dramatically slowed inactivation, suggesting that CaM binding to either CBS-1 or CBS-2 is involved in termination of the light response. Unfortunately, CaM binding to these mutant channels was not reported and the deleted regions contain putative protein kinase C phosphorylation sites. Protein kinase C is known to be a component of the signal-transduction complex in photoreceptor cells [37] and to have a major impact on light-induced currents [38]. Thus the role of altered CaM binding in these mutant TrpL channels remains unclear. In the present study, both TrpL and the TrpC1-TrpL chimaera bound CaM in gel-overlay experiments. No CaM binding was detected for TrpC1 or a truncated version of TrpL lacking CBS-2, but retaining an intact CBS-1.

Although the lack of binding in a gel-overlay experiment should be viewed with caution, these results suggest that only CBS-2 binds CaM in the native TrpL protein and that this site may be important for *trans*-stimulation of TrpL by Ca^{2+} entry via CCE. Alternatively, there may be a bivalent cation binding site(s) associated with the cytoplasmic surface of the TrpL channels that is responsible for stimulation of TrpL. This binding site would have ionic selectivity similar to that of CaM, i.e. a site that binds Ca^{2+} and Sr^{2+} , but not Ba^{2+} . Irrespective of the exact mechanism, it is clear that a rise in $[\text{Ca}^{2+}]_i$ in intact cells stimulates TrpL and that this stimulation gives rise to 'apparent' thapsigargin sensitivity. It is important to note that other members of the Trp channel family, including Trp3 and Trp5, appear to be regulated by cytosolic Ca^{2+} [39,40]. Thus the recent report by Muallem and colleagues [41] of activation by thapsigargin and receptor stimulation of Trp3 recorded in cell-attached patches may not reflect depletion of the internal Ca^{2+} stores, but may simply reflect a rise in $[\text{Ca}^{2+}]_i$, i.e. *trans*-stimulation by Ca^{2+} entry via CCE.

At higher extracellular (and, presumably, intracellular) Ca^{2+} concentrations, stimulation of TrpL single channels was always followed by a time-dependent inhibition. The mechanisms of this inhibitory effect of Ca^{2+} are unknown. Recently, Obukhov et al. [20] reported that TrpL currents activated by receptor stimulation were inhibited by $[\text{Ca}^{2+}]_i$ with an IC_{50} of $\approx 2 \mu\text{M}$. Inhibition of TrpL by Ca^{2+} was also observed in inside-out patches, and was unaffected by CaM. Likewise, Hardie and Raghu [19] reported that TrpL currents in expressed in S2 cells are sometimes inhibited by a rise in $[\text{Ca}^{2+}]_i$. In preliminary studies, we have also seen inhibition of TrpL single channels by Ca^{2+} recorded in inside-out patches, but, in our hands, the inhibition observed at a $[\text{Ca}^{2+}]_i$ of $0.05\text{--}2 \mu\text{M}$ was enhanced by the presence of CaM (D. Salgado-Commissariat, A. Ramirez, D. L. Kunze, and W. P. Schilling, unpublished work). Although the molecular mechanisms responsible for the biphasic effect of Ca^{2+} on TrpL channel activity requires further investigation, it seems clear that the steady-state level of $[\text{Ca}^{2+}]_i$ observed in TrpL-expressing Sf9 cells reflects a balance between stimulatory and inhibitory influences. It will be important to determine the structural features of TrpL that give rise to these effects.

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