Functional expression of TrpC1: a human homologue of the *Drosophila* Trp channel

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TrpC1 appears to be a store-operated channel (SOC) when expressed in mammalian cells. In the present study, TrpC1 was expressed in Sf9 insect cells using the baculovirus expression system. Expression of TrpC1 caused an increase in basal cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) as a function of post-infection time. Basal Ba^{2+} influx, an index of plasmalemmal Ca^{2+} permeability, was also increased and was blocked by La^{3+}. Although the thapsigargin-induced change in $[Ca^{2+}]_i$ was greater in TrpC1-expressing cells than controls, Ba^{2+} influx was unaffected by thapsigargin. Whole-cell membrane currents recorded in TrpC1-expressing cells increased as a function of post-infection time and were (1) inwardly rectifying in symmetrical sodium gluconate solutions, (2) non-selective with respect to Na^+, Ca^{2+} and Ba^{2+}, and (3) blocked by La^{3+}. Furthermore TrpC1 currents were unaffected by (1) thapsigargin, (2) dialysis of the cell with Ins(1,4,5)P_3 or (3) dialysis of the cell with solutions containing high concentrations of the Ca^{2+} chelator, EGTA. These results suggest that TrpC1 forms non-selective cation channels that are constitutively active when expressed in Sf9 cells, but insensitive to depletion of the internal Ca^{2+} stores. Thus TrpC1 may be a subunit of a SOC which alone can form functional channels in Sf9 cells, but which requires additional subunits or cytoplasmic factors present in mammalian cells for expression of SOC activity.

INTRODUCTION

In mammalian non-excitable cells, stimulation of hormone receptors causes an increase in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) through an Ins(1,4,5)P_3-dependent mechanism. An important component of this response is the activation of Ca^{2+}-permeable cation channels in the plasmalemma. Activation of these channels is thought to occur as a direct consequence of depletion of the internal Ca^{2+} store. Indeed, passive depletion of the Ca^{2+} stores, which occurs, for example, after selective inhibition of the sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase pumps by thapsigargin, activates Ca^{2+} influx in the absence of measurable phosphoinositide hydrolysis. This mechanism of Ca^{2+} influx has been referred to as capacitative Ca^{2+} entry (CCE; for review see [1,2]). The membrane current generated by depletion of the stores has been recorded and termed Ca^{2+}-release-activated current (I_{CRAC}) [3], and the channels responsible for this current have been designated more generically as store-operated channels (SOCs).

In *Drosophila* photoreceptor cells, light stimulates a change in membrane conductance that is dependent on the activity of phospholipase C (for a review see [4]). The *Drosophila* protein responsible for the *transient receptor potential* mutant (Trp) and another protein homologous to Trp called Trp-like, or Trpl, either form the ion channels or are subunits of the channels responsible for the light-induced conductance. Consistent with this hypothesis, heterologous expression of Trp and Trpl in Sf9 cells after infection with recombinant baculovirus causes the appearance of Ca^{2+}-permeable cation channels with many of the characteristics expected for channels activated by a receptor-dependent mechanism. Trp channels are activated by thapsigargin and are relatively selective for Ca^{2+} over Na^+ and Ba^{2+} [5,6]. In contrast, Trpl channels are constitutively active, non-selective with respect to Ca^{2+}, Na^+ and Ba^{2+}, and are not activated by depletion of the Ca^{2+} stores by thapsigargin [6–8]. Furthermore, evaluation of channel chimaeras in which the C-terminal domains of Trp and Trpl were exchanged led to the conclusion that the C-terminus of Trp is important for sensitivity to thapsigargin [5].

Full-length human clones with homology to Trp were first independently identified by Montell and Birnbaumer and their respective colleagues [9,10]. These clones exhibit 38 % overall identity with Trp or Trpl, but are substantially shorter in length. Functional expression of the human clones designated TrpC1 and TrpC3 in COS cells gives rise to a modest, but significant, enhancement of thapsigargin- and/or receptor-induced Ca^{2+} influx as measured by fura 2 [11]. Transfection of mouse L-cells with a mixture of plasmids containing antisense sequences for six different mouse Trp clones essentially eliminated thapsigargin-induced Ca^{2+} response, supporting the hypothesis that one or more of these clones is essential for capacitative Ca^{2+} entry. Furthermore Zitt *et al.* [12] measured whole-cell currents after expression of TrpC1 in CHO cells and found an increase in Ins(1,4,5)P_3- and thapsigargin-induced cation currents with similar permeability for Ca^{2+}, Na^+ and Cs^+. Likewise, expression of a bovine Trp homologue, designated bCCE (which appears to be the same as Trp4), in HEK cells gives rise to an inward current that is activated by thapsigargin and Ins(1,4,5)P_3 and relatively selective for Ca^{2+} and Ba^{2+} over Na^+ [13]. Together, these results suggest that the mammalian homologues (1) are themselves SOCs, (2) are subunits of SOCs, or (3) can regulate endogenous SOC activity. However, Zitt *et al.* [14] were unable to activate TrpC3 heterologously expressed in CHO cells with thapsigargin or Ins(1,4,5)P_3. Likewise, Boulay *et al.* [15] showed that, whereas Trp6 could be activated by a receptor-dependent mechanism when expressed in COS or HEK cells, it was not activated by thapsigargin. Thus designation of the Trp family of ion channels as SOCs remains controversial.

Since the actual subunit composition of Trp, Trpl, or the mammalian homologues is unknown, it is possible that en-

Abbreviations used: SOC, store-operated channel; $[Ca^{2+}]_i$, cytosolic calcium concentration; CCE, capacitative Ca^{2+} entry; I_{CRAC}, Ca^{2+}-release-activated current; Trp, transient receptor potential; MBS, Mes-buffered saline.

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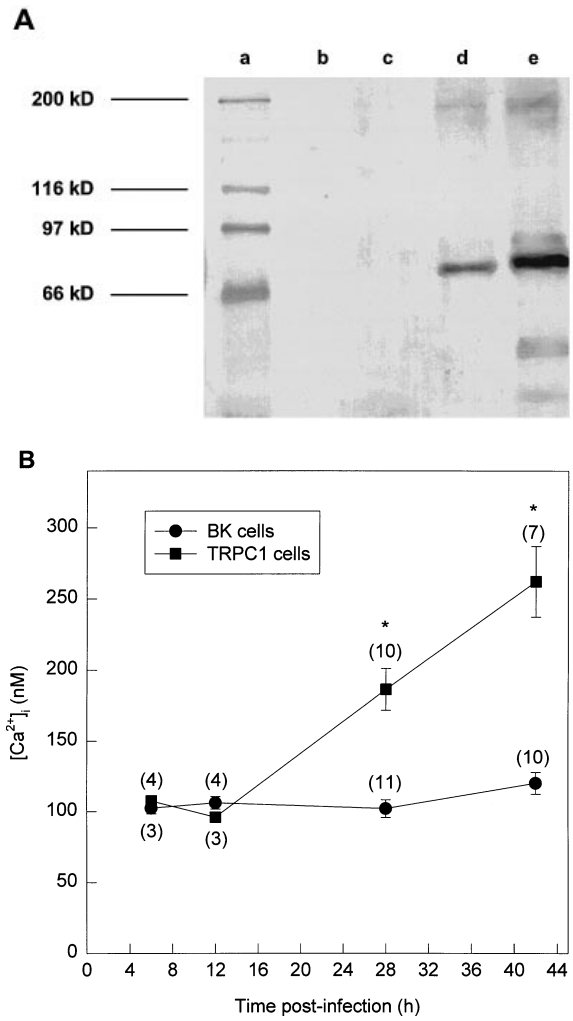

Figure 1 TrpC1 protein expression (A) and its effect on basal non-stimulated $[Ca^{2+}]_i$

(**A**) SDS/PAGE and immunoblotting were performed on membrane preparations isolated from non-infected Sf9 insect cells (lane *b*, 3 μg of protein) or from cells infected with recombinant baculovirus containing the cDNA for FLAG–TrpC1 at 12, 28 or 42 h after infection (lanes *c*–*e* respectively; 3 μg of protein/lane). Molecular-mass standards are also shown in lane *a* (kD, kDa). The Figure shows a digitally acquired picture of the immunoblot processed using Designer software, and printed on an HP LaserJet 4. (**B**) Sf9 insect cells were harvested and loaded with fura 2 at the indicated times after infection as described in the Materials and methods section. Basal $[Ca^{2+}]_i$ in cells expressing either the human bradykinin receptor (●) or TrpC1 (■) was determined in normal MBS. The values represent means ± S.E.M. $^*P < 0.001$.

dogenous proteins in mammalian cells heterologously expressing the various Trps may contribute to channel structure and impart thapsigargin sensitivity. In the present study, TrpC1 was expressed in Sf9 insect cells using recombinant baculovirus. A critical distinction between baculovirus-based expression in Sf9 cells and expression of foreign proteins in mammalian cells by transfection is that the machinery responsible for host cell protein synthesis in the Sf9 cell is commandeered by the baculovirus; host-cell protein synthesis decreases within 6 h of infection and is essentially zero after 24 h [16]. Thus problems associated with co-synthesis or up-regulation of host-cell proteins are minimized and the likelihood of recording homomeric channel activity is increased. Using fura 2 to monitor Ca^{2+} and Ba^{2+} influx and electrophysiological techniques to measure whole-cell membrane currents, the results suggest that TrpC1 forms ion channels that are constitutively active when expressed in the Sf9 cell, non-selective with respect to Ca^{2+}, Ba^{2+} and Na^+, and, contrary to results obtained in mammalian cells, not sensitive to depletion of the internal Ca^{2+} store.

MATERIALS AND METHODS

Solution 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 except that $CaCl_2$ was iso-osmotically replaced by $MgCl_2$.

Cell culture

Sf9 cells were cultured as previously described [7,16,17] using Grace's Insect Medium supplemented with lactalbumin hydrolysate, yeastolate, L-glutamine, 10 % heat-inactivated fetal bovine serum and 1 % penicillin/streptomycin solution.

Generation of recombinant baculovirus and measurement of protein expression

The human clone designated TrpC1 was a gift from Dr. Craig Montell (Johns Hopkins University, Baltimore, MD, U.S.A.). The first 49 amino acids encoded by this clone resulted from a cloning artifact [12]. For the present study, the bases encoding these amino acids were removed; the resulting construct encodes a protein identical with that previously described [12]. The human B_2 bradykinin receptor, TrpC1, and FLAG–TrpC1 were expressed in Sf9 cells using recombinant baculoviruses as previously described [5,7]. In the present study, Sf9 cells expressing the B_2 bradykinin receptor (BK cells) were used as infection controls for comparison with TrpC1-expressing cells. To monitor expression at the protein level, the nucleotide sequence encoding the FLAG epitope (DYDDDDDK) was attached to the N-terminus and protein was measured in isolated membrane preparations using mouse M2 anti-FLAG antibody as previously described [5].

Measurement of free $[Ca^{2+}]$

$[Ca^{2+}]_i$ was measured in Sf9 cells using fura 2 as previously described [7,17]. $[Ca^{2+}]_i$ was calculated by the equation of Grynkiewicz et al. [18] using a K_d value for Ca^{2+} binding to fura 2 of 278 nM for 22 °C [19]. Statistical differences were determined by Student's *t* test using Bonferroni's correction for multiple comparisons [20]. $[Ba^{2+}]_i$ was estimated using fura 2 fluorescence ratio (excitation wavelengths of 350 and 390 nm) as previously described [7,21]. Unless otherwise indicated, the results shown are representative of at least three independent infections.

Electrophysiological techniques

The patch-clamp technique for whole-cell recording in Sf9 cells was utilized as previously described [6,22]. The bath (extracellular) solution contained 100 mM sodium gluconate and 10 mM Mes, pH 6.5, and the pipette contained 100 mM sodium gluconate, 1 mM EGTA and 10 mM Mes, pH 6.5. In some experiments the sodium gluconate in the bath solution was iso-osmotically replaced with either calcium or barium gluconate. All recordings were made at room temperature (22 °C). Figures show representative traces uncorrected for leak currents; where

indicated n equals the number of cells examined under each condition.

RESULTS

Expression of TrpC1 protein in Sf9 insect cells

The FLAG epitope attached to the N-terminus of TrpC1 (FLAG–TrpC1) was used to monitor protein expression. Infection of Sf9 cells with recombinant baculovirus containing the cDNA encoding FLAG–TrpC1 under control of the polyhedrin promoter led to an increase in protein expression as a function of post-infection time. FLAG–TrpC1 was not observed at 12 h, but protein was clearly detectable at 28 and 42 h after infection (Figure 1A). This time course is appropriate for expression of a protein under control of the polyhedrin promoter, a late turn-on promoter in the baculovirus life cycle. FLAG–TrpC1 runs as a 79 kDa protein on SDS/PAGE, or about 10 % smaller than the predicted molecular mass of 87 kDa. Similar bands were not observed in membrane preparations obtained from non-infected Sf9 cells or from cells expressing the human B_2 bradykinin receptor (BK cells).

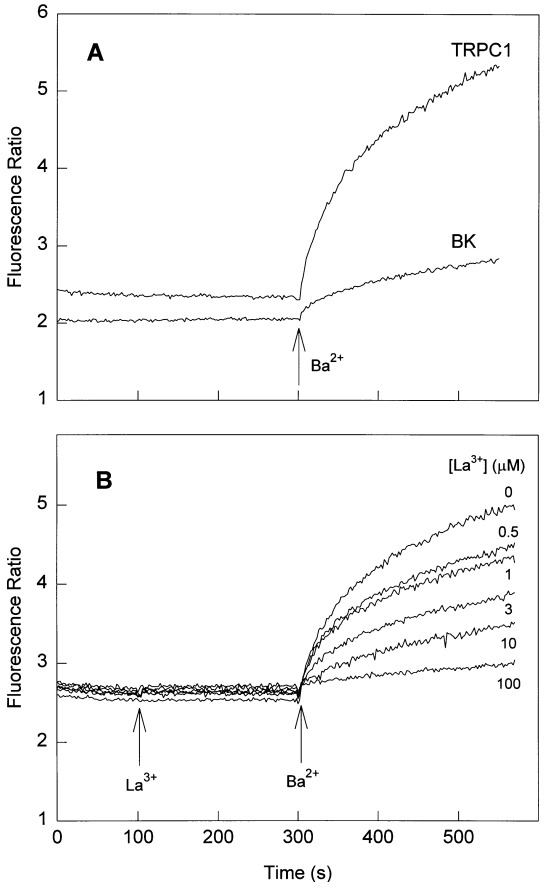

Figure 2 Effect of TrpC1 expression on basal Ba^{2+} influx

(**A**) Fura 2-loaded Sf9 cells (28 h after infection) were suspended in nominally Ca^{2+}-free MBS. At the indicated time, $BaCl_2$ (final concentration 10 mM) was added to the cuvette. Two traces are superimposed; the upper trace was obtained from TrpC1-expressing cells and the lower one from BK cells. (**B**) Six traces are superimposed. $BaCl_2$ was added at the time indicated by the second arrow to TrpC1-expressing cells suspended in nominally Ca^{2+}-free MBS in the absence or presence of 0.5, 1, 3, 5, 10 or 100 μM $LaCl_3$, added at the first arrow.

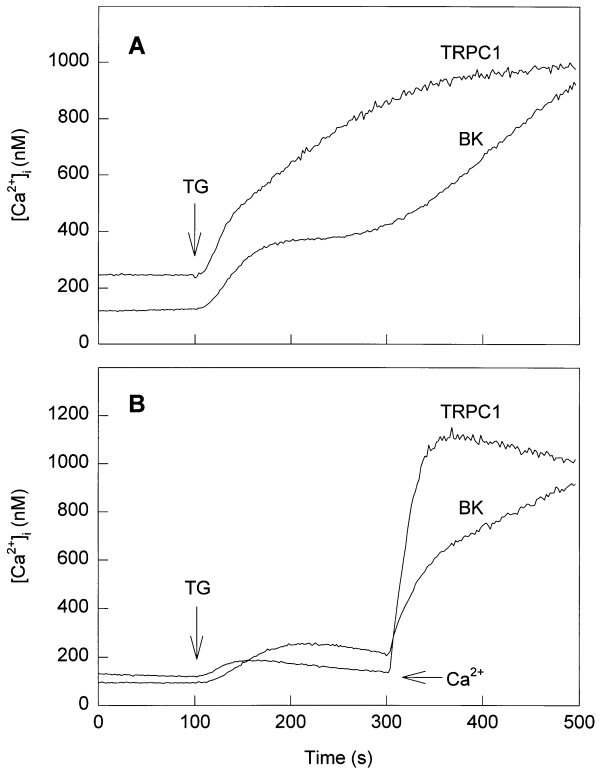

Figure 3 Effect of thapsigargin on TrpC1-expressing Sf9 cells

Two traces are superimposed in each panel. (**A**) Fura 2-loaded BK cells or TrpC1-expressing Sf9 cells (28 h after infection) were suspended in normal MBS. Thapsigargin (TG) was added to the cuvette at the time indicated (final concentration 200 nM) and the fluorescence recorded. (**B**) $CaCl_2$ (final concentration 10 mM) was added to cells suspended in nominally Ca^{2+}-free MBS after stimulation with thapsigargin.

Functional expression of TrpC1

Fura 2 fluorescence was used to monitor $[Ca^{2+}]_i$ in Sf9 cells. Expression of TrpC1 in Sf9 cells caused a time-dependent increase in basal non-stimulated $[Ca^{2+}]_i$ compared with control BK cells (Figure 1B). The elevated basal $[Ca^{2+}]_i$ in TrpC1-expressing cells may be related to (1) an increase in basal Ca^{2+} influx or (2) inhibition of Ca^{2+}-efflux mechanisms. To distinguish between these mechanisms, Ba^{2+} influx was examined in TrpC1-expressing cells. Ba^{2+} will pass through most known Ca^{2+} channels and produce a change in fura 2 fluorescence essentially identical with that produced by Ca^{2+}. However, Ba^{2+} is a poor substrate for the carriers and pumps normally responsible for extrusion of Ca^{2+} from the cytoplasm. Hence, Ba^{2+} influx can be used as an indicator of plasmalemmal Ca^{2+} permeability [7,21]. Expression of TrpC1 was associated with a large increase in basal Ba^{2+} influx relative to control BK cells (Figure 2A). Basal Ba^{2+} influx was blocked by La^{3+} with an IC_{50} of approx. 3 μM (Figure 2B). These results suggest that elevated basal $[Ca^{2+}]_i$ in TrpC1-expressing cells reflects an increase in the permeability of the surface membrane to Ca^{2+} and that this pathway for Ca^{2+} entry has a relatively low sensitivity to blockade by lanthanides (see below).

Stimulation of TrpC1 by thapsigargin

Application of thapsigargin to control BK cells produced a time-dependent change in $[Ca^{2+}]_i$ (Figure 3A). Initially $[Ca^{2+}]_i$ increased within 1 min from a basal resting level of 102 ± 6 nM to

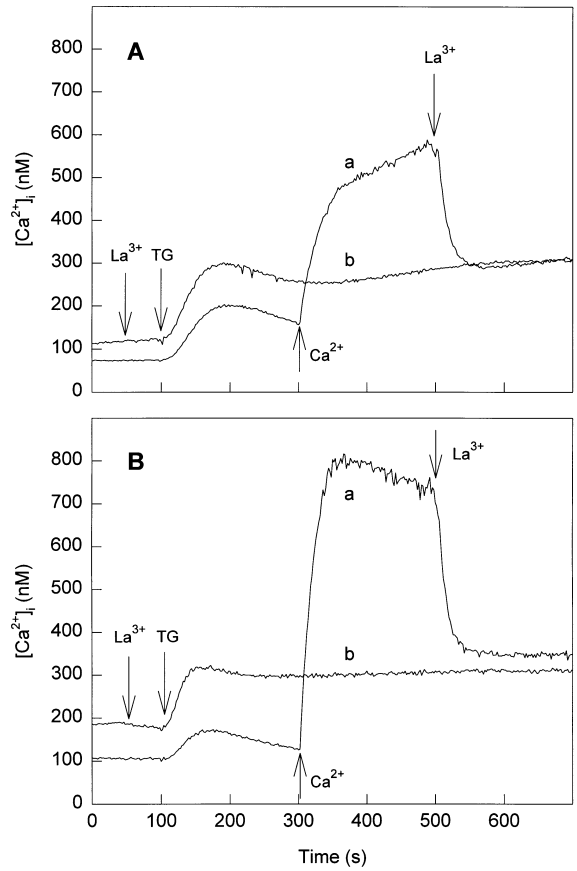

Figure 4 Effect of thapsigargin and La^{3+} on Ca^{2+} influx in TrpC1-expressing Sf9 cells

Two traces are superimposed in each panel. (**A**) In trace *a*, BK cells (42 h after infection) were suspended in nominally Ca^{2+}-free MBS. Thapsigargin (TG) was added to the cuvette at the time indicated (200 nM), followed by $CaCl_2$ (10 mM) and $LaCl_3$ (0.5 μM) at the indicated times. In trace *b*, the BK cells were suspended in normal MBS; $LaCl_3$ and thapsigargin were added at the indicated times. (**B**) As in (**A**) except in TrpC1-expressing cells.

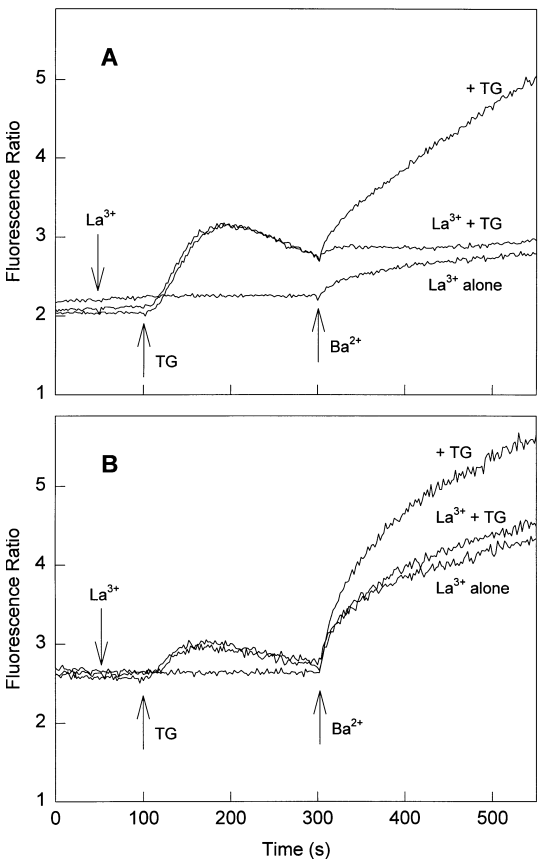

Figure 5 Effect of thapsigargin on Ba^{2+} influx in TrpC1-expressing Sf9 cells

Three traces are superimposed in each panel. (**A**) BK cells (42 h after infection) were suspended in nominally Ca^{2+}-free MBS. $BaCl_2$ (10 mM) was added in the absence and presence of thapsigargin (200 nM) and/or $LaCl_3$ (0.5 μM) as indicated in the Figure. (**B**) As in (**A**) except in TrpC1-expressing cells.

310 ± 14 nM (mean $\pm$ S.E.M., $n = 11$). This increase in $[Ca^{2+}]_i$ presumably reflects blockade of the Ca^{2+} pumps of the endoplasmic reticulum by thapsigargin, leakage of Ca^{2+} from the endoplasmic reticulum, and establishment of a new steady-state $[Ca^{2+}]_i$ as predicted from a pump-leak model. At 2.5 min after thapsigargin addition, $[Ca^{2+}]_i$ began to increase slowly with time, and peaked after 8 min. This increase corresponds to activation of the endogenous CCE pathway in the Sf9 cells. The thapsigargin-induced change in $[Ca^{2+}]_i$ in non-infected Sf9 cells was essentially identical with that observed in BK cells (results not shown). To clearly distinguish the Ca^{2+} influx component of the response to thapsigargin, these experiments were performed in Ca^{2+}-free buffer (Figure 3B). In the absence of extracellular Ca^{2+}, thapsigargin produced a small increase in $[Ca^{2+}]_i$ in both BK and TrpC1-expressing cells. Re-addition of Ca^{2+} caused a dramatic increase in $[Ca^{2+}]_i$ that was significantly faster in TrpC1 cells than in control BK cells. Thus it would appear that TrpC1 is stimulated by thapsigargin. However, there are two possible explanations for these results. First, in the presence of constitutive Ca^{2+} influx via TrpC1, a pump-leak model predicts that the rise in $[Ca^{2+}]_i$ produced by thapsigargin will be greater when compared with a cell without TrpC1 influx (BK cells). Secondly, TrpC1 may be activated by thapsigargin. To distinguish between these possibilities, we compared the effect of La^{3+} on thapsigargin-stimulated Ca^{2+} influx in BK and TrpC1-expressing cells. Figure 4(A) shows the effect of 0.5 μM La^{3+} on thapsigargin-stimulated change in $[Ca^{2+}]_i$ in BK cells; in trace *a*, La^{3+} was added after thapsigargin whereas in trace *b* La^{3+} was added before thapsigargin. Both experimental protocols result in the establishment of the same final $[Ca^{2+}]_i$. These results demonstrate that endogenous CCE is substantially blocked by 0.5 μM La^{3+}, a concentration that has little effect on TrpC1 (Figure 2B). The results for TrpC1-expressing cells are shown in Figure 4(B). Although reapplication of Ca^{2+} to stimulated cells results in significantly greater influx compared with that observed in BK cells, influx is almost completely blocked by 0.5 μM La^{3+}, suggesting that the majority of thapsigargin-stimulated Ca^{2+} influx occurs through the endogenous CCE pathway and not through TrpC1. To test this hypothesis further, we examined thapsigargin-stimulated Ba^{2+} influx. As seen in Figure 5, addition of Ba^{2+} after thapsigargin produced a dramatic increase in fluorescence ratio in BK cells that was completely blocked by the prior addition of 0.5 μM La^{3+} to the extracellular buffer. Thus Ba^{2+} influx via the endogenous CCE pathway in Sf9 cells is almost completely blocked by 0.5 μM La^{3+}. A large increase in Ba^{2+} influx is also observed in the TrpC1-expressing cells after thapsigargin. However, in the presence of 0.5 μM La^{3+}, Ba^{2+} influx after thapsigargin addition

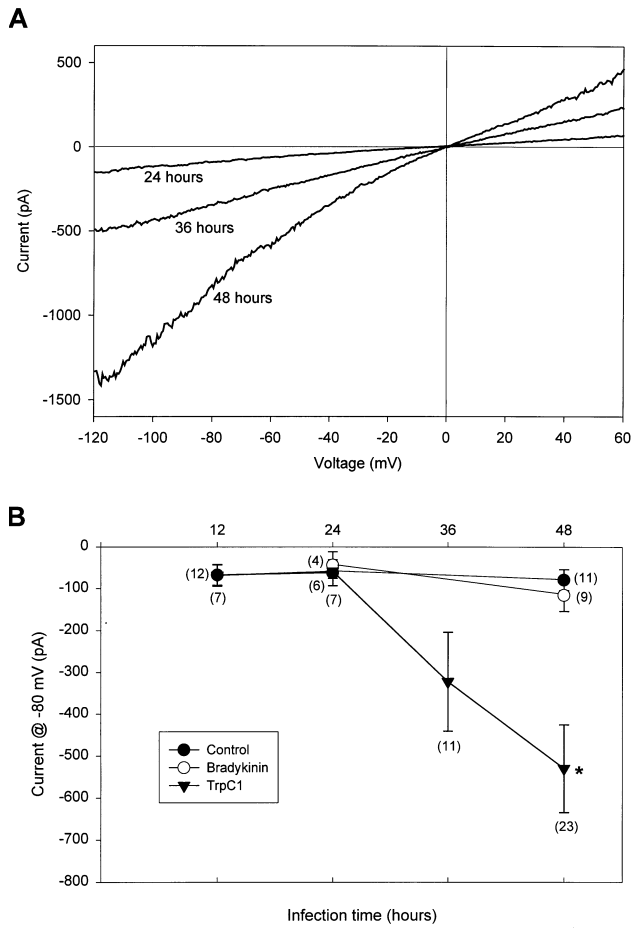

Figure 6 Membrane currents in TrpC1-expressing Sf9 cells

(**A**) Membrane currents were recorded from TrpC1-expressing Sf9 cells in symmetrical sodium gluconate solutions. The holding potential was 0 mV. The current–voltage relationship was generated within 2 min of establishment of the whole-cell recording mode by ramping the voltage over 200 ms from −120 to +60 mV. (**B**) Currents recorded at −80 mV from multiple cells were averaged and are shown as a function of post-infection time in non-infected Sf9 cells (●), or in cells infected with recombinant baculovirus containing the human B_2 bradykinin receptor (○) or TrpC1 (▼). Values represent means ± S.E.M. (*n*).

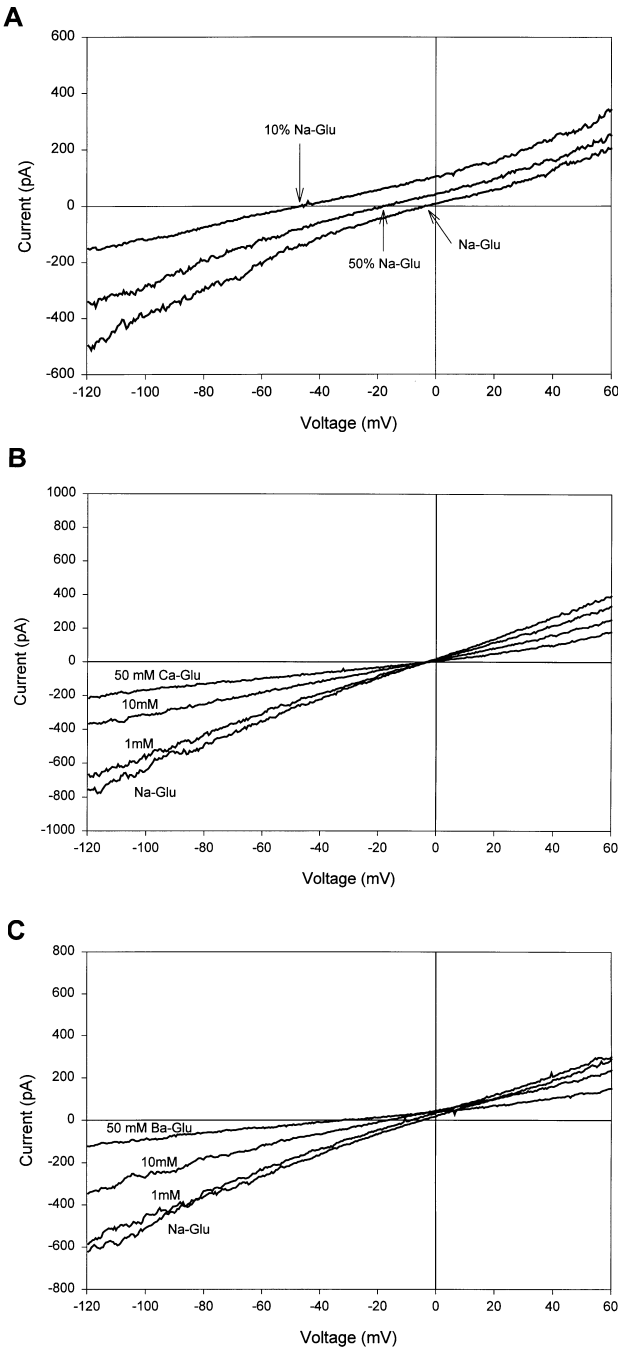

Figure 7 Ion selectivity of membrane current in TrpC1-expressing Sf9 cells

Membrane currents were recorded from TrpC1-expressing Sf9 cells 48 h after infection initially in sodium gluconate bath solution. The pipette contained sodium gluconate with 1 mM EGTA. Voltage ramps were applied from a holding potential of 0 mV every 20 s. (**A**) Sodium gluconate in the bath solution (initially 100 mM) was iso-osmotically reduced to 50% (50 mM) and 10% (10 mM) using mannitol; the apparent reversal potential shifted from −3 mV to −17 and −45 mV respectively. (**B**) and (**C**) Sodium gluconate in the bath solution was iso-osmotically replaced with the indicated concentrations of calcium and barium gluconate (respectively). Note that 50 mM calcium or barium gluconate reflects complete replacement of extracellular Na^+.

was essentially superimposable with basal Ba^{2+} influx observed in the absence of thapsigargin (Figure 5B). Thus thapsigargin-stimulated Ba^{2+} influx in TrpC1-expressing cells consists of two components: (1) endogenous CCE which is blocked by low concentrations of La^{3+}; (2) basal Ba^{2+} influx through TrpC1. Clearly, Ba^{2+} influx via TrpC1 is not stimulated by thapsigargin, and the apparent stimulation of 'Ca^{2+}' influx by thapsigargin seen in Figure 3 simply reflects constitutive basal activity of TrpC1 superimposed on pump inhibition.

Whole-cell membrane currents in TrpC1-expressing Sf9 cells

Whole-cell membrane currents were recorded in TrpC1-expressing Sf9 cells using the giga-seal technique (Figure 6A). Currents, measured immediately after establishment of the whole-cell recording mode, were inwardly rectifying in symmetrical sodium gluconate solutions; currents reversed at about 0 mV under these ionic conditions. Currents in TrpC1-expressing cells increased as a function of post-infection time and were significantly greater than those recorded in either non-infected Sf9 cells or control BK cells at 48 h (Figure 6B). The appearance of this current followed a time course that paralleled both protein expression and the increase in basal $[Ca^{2+}]_i$ shown in Figure 1. Even at 48 h,

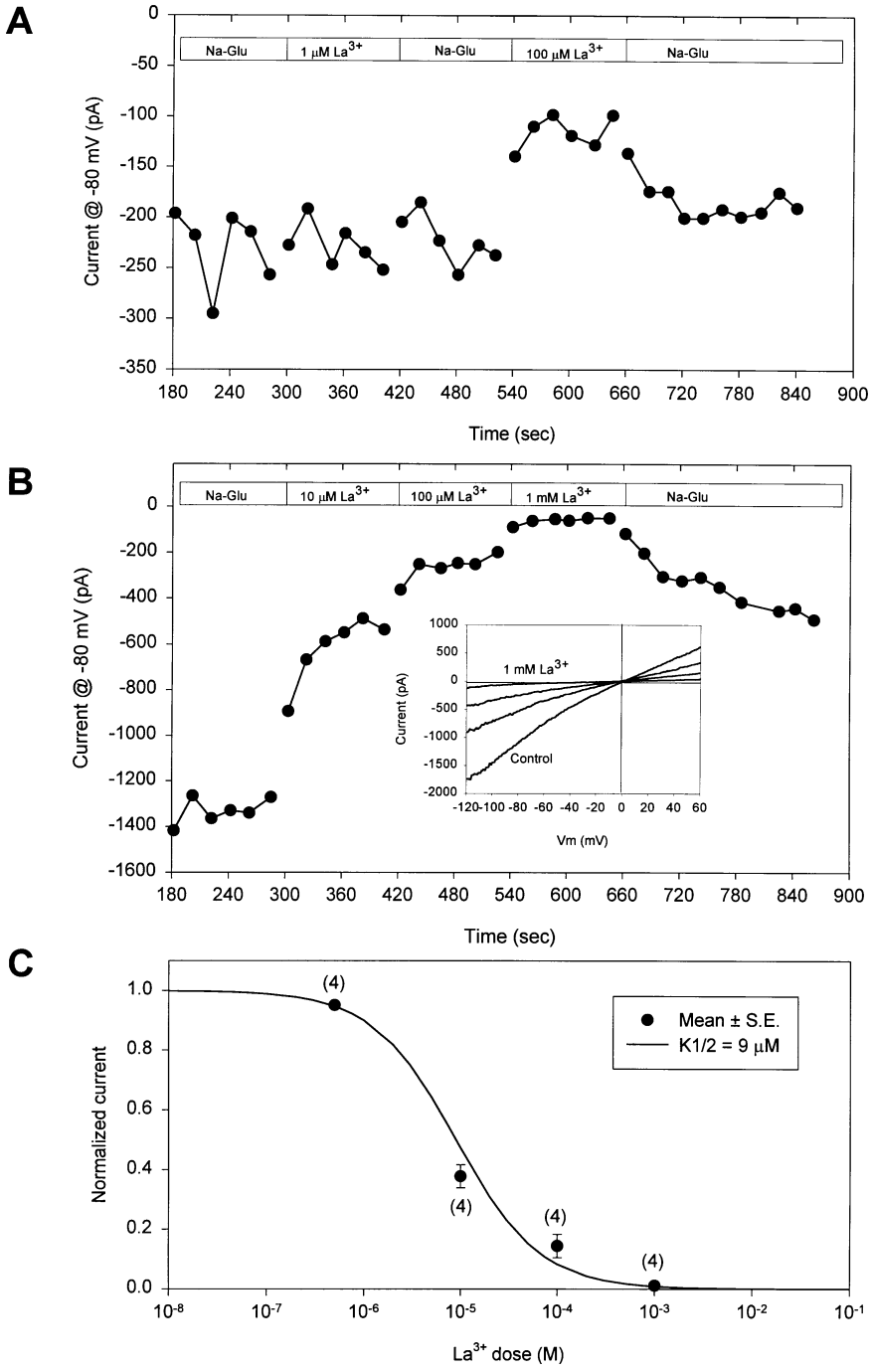

Figure 8 Blockade of TrpC1 currents by La^{3+}

(**A**) and (**B**) At the times indicated in the boxed area, the cell was superfused with sodium gluconate solution containing 1, 10, 100 or 1000 μM La^{3+}. (**C**) Steady-state membrane currents at -80 mV in the presence of the indicated La^{3+} concentrations were normalized to the current amplitude immediately before superfusion with the blocking ion. Values represent means $\pm$ S.E.M. (*n*). The line represents least-squares regression analysis to a single-site binding model; $IC_{50} = 9\ \mu$M.

however, a small proportion of cells ($\sim 10\%$) did not express currents above control, which could reflect a failure of infection. In the standard pipette solution, the expressed inwardly rectifying current is stable during whole-cell recording for at least 20 min.

To examine ionic selectivity, sodium gluconate in the bath was iso-osmotically replaced with increasing concentrations of mannitol. Under these conditions, the Na^+ equilibrium potential shifts to the left, whereas the gluconate equilibrium potential shifts to the right. Thus, if the TrpC1 channels are cation-selective, a shift to the left should be observed; if TrpC1 is anion-selective, a shift to the right is expected; and if TrpC1 has equal permeability to cations and anions, no shift should be seen on reducing the sodium gluconate in the bath solution. As seen in Figure 7(A), reducing sodium gluconate by 50 or 90% produced

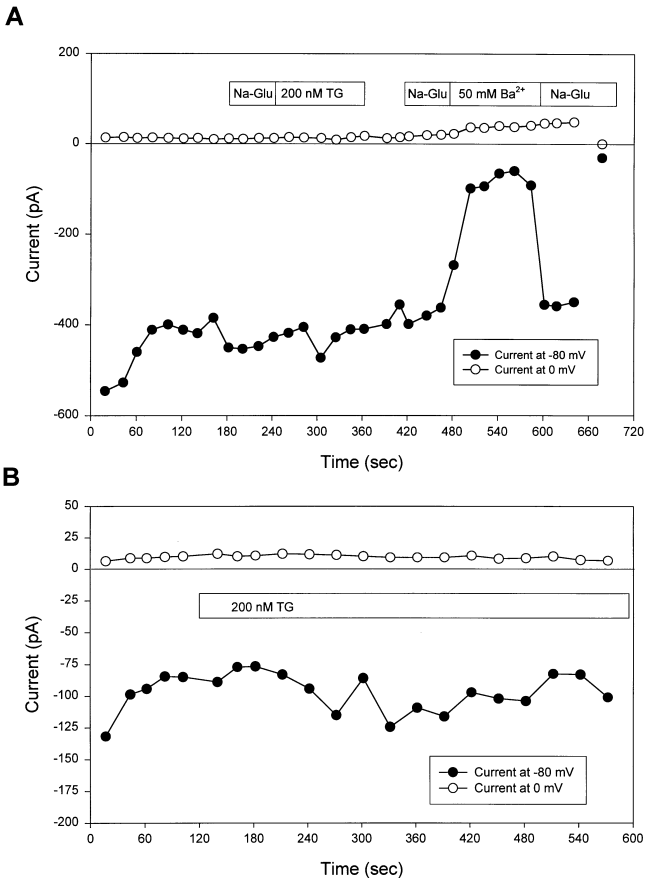

Figure 9 Effect of thapsigargin on TrpC1 currents

(**A**) At the time indicated in the boxed area the cell was superfused with standard sodium gluconate solution containing thapsigargin (final concentration 200 nM). Perfusion of iso-osmotic barium gluconate followed by a return to sodium gluconate solution is indicated. (**B**) As in (**A**) with TrpC1 current recorded in the continuous presence of thapsigargin for 8 min.

a leftward shift in the reversal potential, confirming that TrpC1 is a cation channel. To examine cation selectivity, sodium gluconate in the bath was iso-osmotically replaced with increasing concentrations of either calcium or barium gluconate (Figures 7B and 7C). Both bivalent cations support inward currents, but there is a concentration-dependent reduction in conductance in both the inward and outward direction. Similar results have been reported for Trp currents expressed in Sf9 cells [6]. Conductance returned to higher levels on reintroduction of sodium gluconate to the bath (see Figure 9). Very little change in the reversal potential was observed on changing to Ca^{2+} or Ba^{2+} bath solution (Figures 7B and 7C) consistent with the hypothesis that TrpC1 forms non-selective cation channels. The effect of La^{3+} on TrpC1 currents is shown in Figure 8. Currents were unaffected by 1 μM La^{3+} added to the bath solution, but were clearly reduced at higher concentrations. The effect of lower concentrations of La^{3+} on TrpC1 currents was reversible (Figure 8A), but currents blocked by 1 mM La^{3+} reversed slowly and never fully recovered to preblock levels during the time examined (Figure 8B; $n = 4$). The blocking effect of La^{3+} on TrpC1 currents was concentration-dependent with an IC_{50} of about 9 μM (Figure 8C; $n = 4$), confirming the fura 2 results and suggesting that TrpC1 has a relatively low sensitivity to La^{3+}.

To determine if TrpC1 currents could be regulated by depletion of the internal Ca^{2+} stores, cells were stimulated with thapsigargin in symmetrical sodium gluconate solutions. We previously reported that endogenous thapsigargin-induced currents in Sf9 cells are not recorded under these ionic conditions [6]. Thus, any currents seen will reflect the activity of TrpC1. Application of thapsigargin for either 2 min (Figure 9A) or 8 min (Figure 9B) had no effect on membrane currents in TrpC1-expressing cells ($n = 14$). Furthermore dialysis of the cytoplasm with solutions containing high concentrations of EGTA (10 mM) to buffer Ca^{2+} to very low levels (~ 10 nM), causing further depletion of the intracellular stores, did not itself activate membrane currents ($n = 12$), and the subsequent application of thapsigargin had no effect even in the presence of high EGTA in the pipette solution ($n = 10$). Finally, the dual manipulation of 10 mM EGTA and 5 μM Ins(1,4,5)P_3 in the pipette, which should completely empty the intracellular Ca^{2+} stores, failed to alter the TrpC1 currents ($n = 8$).

DISCUSSION

Time course of expression and activity

We have expressed TrpC1, a human homologue of *Drosophila* Trp, in Sf9 cells. Protein expression was not detected 12 h after infection, but was easily detectable at 28 h, and had increased further by 42 h. Functional data are in agreement with the biochemical observations; TrpC1 forms constitutively active cation channels, and the time course of activity parallels that of protein expression. Fura 2 measurements show that $[Ca^{2+}]_i$ is 1.8-fold higher than in controls 28 h after infection and 2.2-fold higher at 42 h. Similarly, whole-cell membrane currents in TrpC1 cells are comparable with those in control cells up to 24 h after infection, but thereafter are significantly greater. Taken together, these observations are consistent with heterologous expression of a gene under control of the polyhedrin promoter and provide strong evidence that the membrane currents observed are related to TrpC1 expression.

Ionic permeability and selectivity

Fura 2 data indicate that the channel is permeable to Ba^{2+} as well as Ca^{2+}; Ba^{2+} influx under basal unstimulated conditions is significantly greater in TrpC1 cells than in controls. Current measurements show that, in symmetrical Na^+ solutions, current is inwardly rectifying over the range -120 to $+60$ mV, reversing at 0 mV. Ca^{2+} at 50 mM in the bath reduces conductance, but there is little shift in reversal potential as would be expected for a Ca^{2+}-selective channel. Similarly, 50 mM Ba^{2+} reduces conductance with little shift in reversal potential. TrpC1 thus appears to form non-selective cation channels when expressed in Sf9 cells. TrpC1 activity is sensitive to blockade by La^{3+}, but less so than the endogenous CCE pathway. As measured by fura 2, La^{3+} antagonizes Ba^{2+} influx in TrpC1 cells with an IC_{50} of about 3 μM, whereas Ba^{2+} influx in control cells is completely blocked by 0.5 μM La^{3+}. Likewise, TrpC1 Na^+ current is relatively insensitive to La^{3+}, with an IC_{50} of about 9 μM. TrpC1 is therefore significantly less sensitive to La^{3+} blockade than endogenous CCE. Trp and Trpl expressed in Sf9 cells are also differentially sensitive to blockade by lanthanides with the IC_{50} for inhibition of Na^+ current by Gd^{3+} of 1 and 100 μM respectively [5]. Thus the sensitivity of TrpC1 to La^{3+} is intermediate between Trp and Trpl. The structural basis for this difference is unknown, but presumably reflects differences in the pore region of these channel proteins.

Regulation by store depletion

The data presented in Figure 3 suggests that TrpC1 channels can be stimulated by passive depletion of the intracellular Ca^{2+} store, i.e. Ca^{2+} influx after thapsigargin treatment is significantly greater in TrpC1 cells than in control cells. However, three lines of evidence argue that, under the conditions of the present investigation, TrpC1 is not activated by store depletion. First, the enhanced influx of Ca^{2+} in response to thapsigargin can be reversed by 0.5 μM La^{3+}, a concentration sufficient to block endogenous influx channels completely, but without effect on TrpC1 channels. Secondly, Ba^{2+} entry is stimulated by thapsigargin treatment in TrpC1-expressing cells, but all of the stimulated component is blocked by 0.5 μM La^{3+}, i.e. there is little or no thapsigargin-stimulated La^{3+}-insensitive Ba^{2+} influx, as would be expected if the activity of TrpC1 were enhanced by depleting the stores. Thirdly, there was no increase in whole-cell membrane current after application of thapsigargin, or during intracellular dialysis with buffers containing high concentrations of EGTA, or both. Furthermore inclusion of Ins(1,4,5)P_3 in the pipette solution (along with high EGTA) also failed to activate TrpC1 currents. Thus the apparent enhancement of Ca^{2+} permeability seen in Figure 3 does not represent store-dependent activation of TrpC1, but is a consequence of the pump-leak model of Ca^{2+} homoeostasis. The latter holds that $[Ca^{2+}]_i$ is maintained at a steady-state level that reflects a balance between the rate of leakage of Ca^{2+} into the cytoplasm versus the rate of extrusion by pump mechanisms. Blockade of the sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase pumps by thapsigargin therefore will produce a rise in $[Ca^{2+}]_i$, and the rise will be more pronounced if the rate of influx into the cytoplasm is enhanced by a constitutively active Ca^{2+} influx pathway, such as TrpC1. This underscores the importance of using another measure of plasmalemmal Ca^{2+} permeability such as Ba^{2+} or Mn^{2+} influx, or direct measurement of membrane currents when evaluating the effect of thapsigargin.

Comparison with other members of the Trp family

The peptide sequence of TrpC1 is 38 % identical with that of *Drosophila* Trp and Trpl; functionally, TrpC1 has properties common to each of the insect proteins. We have previously reported that, when expressed in Sf9 cells, Trpl is constitutively active under basal conditions, is non-selective with respect to Na^+, Ca^{2+} and Ba^{2+}, is not activated by store depletion, and gives rise to membrane currents that are outwardly rectifying. With the exception of outward rectification, TrpC1 shares all the characteristics of Trpl. Interestingly, the mammalian protein produces inwardly rectifying currents, which are a characteristic of Trp. Trp, however, is activated by thapsigargin, is highly selective for Ca^{2+}, and shows very low activity under basal conditions. TrpC1 then appears more like Trpl than Trp, but comparison of the primary sequences of the three proteins reveals no obvious reason for this distinction, that is, there are no regions of TrpC1 that are manifestly more like Trpl than Trp.

The initial reports that the mammalian homologues of Trp could be activated by depletion of the Ca^{2+} stores by thapsigargin (see the Introduction) were surprising, given that the C-terminal domain of *Drosophila* Trp, which is essentially absent from mammalian forms, appears to contain structural elements important for thapsigargin sensitivity [5]. On the basis of these findings we hypothesized that TrpC1 would not be sensitive to thapsigargin and, as discussed above, the results of the present study clearly support this conclusion. What then is the explanation for the apparently conflicting results? Since we have previously reported that *Drosophila* Trp is activated by thapsigargin, the discrepancy cannot be ascribed to an inherent inability of the Sf9 cell to support CCE. Furthermore CCE and the associated I_{CRAC} in Sf9 insect cells are essentially identical with that observed in mammalian cells. Thus it also seems unlikely that the signal generated on depletion of the Ca^{2+} stores in Sf9 differs from that in mammalian cells. We cannot eliminate the possibility that TrpC1 is misfolded when expressed in Sf9 cells, but clearly TrpC1 is expressed and forms functional channels that are targeted to the plasma membrane. Furthermore other channels have been successfully expressed in Sf9 cells including the plant K^+ channel, KAT1 [23], *Shaker* K^+ channels [24–26], ryanodine-sensitive Ca^{2+}-release channels [27], cystic fibrosis transmembrane conductance regulator Cl^- channels [28], L-type voltage-gated Ca^{2+} channels [29], delayed rectifier K^+ channels [30], inwardly rectifying ROMK1 channels [31,32], and P_{2x} purinergic receptor channels [33]. These studies suggest that synthesis, post-translational modification, targeting and regulation of channel proteins are normal in the Sf9 cell. However, there is one difference between the insect and mammalian expression systems. The baculovirus/Sf9 cell system allows expression of foreign protein under the control of a late promoter that is normally responsible for expression of a non-structural non-essential viral protein called the polyhedrin protein. Thus the foreign protein of interest is expressed as a normal viral protein and is expressed late ($>$ 12–24 h after infection) in the baculovirus life cycle. Importantly, host cell protein synthesis is dedicated at later times exclusively to the production of viral proteins, minimizing the possible co-expression of TrpC1 with endogenous channel subunits. Thus thapsigargin sensitivity may depend on an endogenous protein that is absent from baculovirus-infected Sf9 cells, but is supplied by COS or CHO cells. This is consistent with the hypothesis of Zhu et al. [11], that the five identified partial mouse Trp clones are subunits of the CCE channel. One or more of these partial clones may have an extended C-terminal region similar to *Drosophila* Trp which may impart thapsigargin sensitivity. Consistent with this hypothesis, Garcia and Schilling [34] have recently shown that all five mammalian Trp transcripts are ubiquitously, but differentially, expressed across tissues and cell lines. Furthermore Xu et al. [35] showed that TrpC1 and TrpC3 co-immunoprecipitate from homogenates of 293T cells heterologously expressing these proteins, suggesting that mammalian Trps form hetero-oligomeric structures. Thus membrane currents observed after expression of a single Trp homologue (e.g. TrpC1) in mammalian cells may not reflect the activity of homomeric channels.

Alternatively, an accessory protein, perhaps with structure similar to the C-terminal domain of *Drosophila* Trp, may be required for thapsigargin sensitivity. It is recognized that most ion channels are composed of multiple pore-forming subunits as well as one or more accessory proteins. Trp has been found to associate *in vivo* with the proteins NorpA, InaC and InaD. NorpA and InaC are the *Drosophila* forms of phospholipase C and protein kinase C respectively, and InaD is a putative 'scaffolding' protein containing two to five PDZ domains; it has been proposed that these proteins, together with Trp, form a functional signalling complex [36–39]. The foregoing suggests that the mammalian Trps may also require additional proteins to function correctly. It is possible that the Sf9 cell does not produce the correct accessory proteins, or that the Sf9 equivalents are sufficiently different that they do not form the required functional association with TrpC1. If accessory subunits are identified in mammalian cells, this hypothesis can be tested by co-expressing them with TrpC1 in the Sf9 cell, with the expectation that store-dependent activation can be reconstituted. The Sf9 system

therefore could be a valuable tool for elucidating the oligomeric nature of mammalian SOCs.

Lastly, the possibility that mammalian Trp homologues are not SOCs should be considered. In a recent study, Garcia and Schilling [34] found that mammalian Trps were differentially expressed across tissues and cell lines. Interestingly, all known Trp transcripts were barely detectable in liver and highly expressed in brain and nodose ganglia, a pattern of distribution unexpected for a channel involved in CCE. Furthermore Jurkat cells, which predominantly express Trp3 and Trp6 and lack Trp4, and RBL cells, which predominantly express Trp1 and Trp5, but lack Trp3, have remarkably similar I_{CRAC} currents [2]. Mammalian Trps also appear to be differentially regulated by hormone treatment in vascular endothelial cells [40]. Although *trans*-retinoic acid caused up-regulation of Trp5 and β-oestradiol caused down-regulation of Trp4, CCE was unaffected by treatment with these hormones. Recent heterologous expression studies have shown that Trp3 appears to be activated by Ca^{2+} and Trp6 appears to be activated by receptor, but neither are affected by depletion of the internal Ca^{2+} stores [14,15]. Although there are alternative hypotheses for these results, perhaps the simplest explanation is that the mammalian Trp homologues are not SOCs.

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REFERENCES

1 Putney, Jr., J. W. and Bird, G. S. J. (1993) Endocrine Rev. **14**, 610–631
2 Parekh, A. B. and Penner, R. (1997) Physiol. Rev. **77**, 901–930
3 Hoth, M. and Penner, R. (1992) Nature (London) **355**, 353–356
4 Hardie, R. C. and Minke, B. (1995) Cell Calcium **18**, 256–274
5 Sinkins, W. G., Vaca, L., Hu, Y., Kunze, D. L. and Schilling, W. P. (1996) J. Biol. Chem. **271**, 2955–2960
6 Vaca, L., Sinkins, W. G., Hu, Y., Kunze, D. L. and Schilling, W. P. (1994) Am. J. Physiol. **267**, C1501–C1505
7 Hu, Y. and Schilling, W. P. (1995) Biochem. J. **305**, 605–611
8 Obukhov, A. G., Harteneck, C., Zobel, A., Harhammer, R., Kalkbrenner, F., Leopoldt, D., Lückhoff, A., Nürnberg, B. and Schultz, G. (1996) EMBO J. **15**, 5833–5838
9 Wes, P. D., Chevesich, J., Jeromin, A., Rosenberg, C., Stetten, G. and Montell, C. (1995) Proc. Natl. Acad. Sci. U.S.A. **92**, 9652–9656
10 Zhu, X., Chu, P. B., Peyton, M. and Birnbaumer, L. (1995) FEBS Lett. **373**, 193–198
11 Zhu, X., Jiang, M. S., Peyton, M., Boulay, G., Hurst, R., Stefani, E. and Birnbaumer, L. (1996) Cell **85**, 661–671
12 Zitt, C., Zobel, A., Obukhov, A. G., Harteneck, C., Kalkbrenner, F., Lückhoff, A. and Schultz, G. (1996) Neuron **16**, 1189–1196
13 Philipp, S., Cavalié, A., Freichel, M., Wissenbach, U., Zimmer, S., Trost, C., Marquart, A., Murakami, M. and Flockerzi, V. (1996) EMBO J. **15**, 6166–6171
14 Zitt, C., Obukhov, A. G., Strubing, C., Zobel, A., Kalkbrenner, F., Lückhoff, A. and Schultz, G. (1997) J. Cell Biol. **138**, 1333–1341
15 Boulay, G., Zhu, X., Peyton, M., Hurst, R., Stefani, E. and Birnbaumer, L. (1997) J. Biol. Chem. **272**, 29672–29680
16 O'Reilly, D. R., Miller, L. K. and Luckow, V. A. (1992) Baculovirus Expression Vectors: A Laboratory Manual, W. H. Freeman and Co., New York
17 Hu, Y., Rajan, L. and Schilling, W. P. (1994) Am. J. Physiol. **266**, 1736–1743
18 Grynkiewicz, G., Poenie, M. and Tsien, R. Y. (1985) J. Biol. Chem. **260**, 3440–3450
19 Shuttleworth, T. J. and Thompson, J. L. (1991) J. Biol. Chem. **266**, 1410–1414
20 Winer, B. J. (1962) Statistical Principles in Experimental Design, McGraw-Hill Book Co., New York
21 Schilling, W. P., Rajan, L. and Strobl-Jager, E. (1989) J. Biol. Chem. **264**, 12838–12848
22 Hamill, P. P., Marty, A., Neher, E., Sakmann, B. and Sigworth, F. J. (1981) Pflügers Arch. **391**, 85–100
23 Marten, I., Gaymard, F., Lemaillet, G., Thibaud, J. B., Sentenac, H. and Hedrich, R. (1996) FEBS Lett. **380**, 229–232
24 Klaiber, K., Williams, N., Roberts, T. M., Papazian, D. M., Jan, L. Y. and Miller, C. (1990) Neuron **5**, 221–226
25 Gonez-Lagunas, F. and Armstrong, C. M. (1995) Biophys. J. **68**, 89–95
26 Perez-Cornejo, P. and Begenisich, T. (1994) Biophys. J. **66**, 1929–1938
27 Marks, A. R. (1996) Methods **9**, 177–187
28 Larsen, E. H., Price, E. M., Gabriel, S. E., Stutts, M. J. and Boucher, R. C. (1996) Pflügers Arch. **432**, 528–537
29 Puri, T. S., Gerhardstein, B. L., Zhao, X. L., Ladner, M. B. and Hosey, M. M. (1997) Biochemistry **36**, 9605–9615
30 Gubitos-Klug, R. A. and Gross, R. W. (1996) J. Biol. Chem. **271**, 32519–32522
31 Schwalbe, R. A., Wang, Z., Wible, B. A. and Brown, A. M. (1995) J. Biol. Chem. **270**, 15336–15340
32 Schwalbe, R. A., Wang, Z., Bianchi, L. and Brown, A. M. (1996) J. Biol. Chem. **271**, 24201–24206
33 Radford, K. M., Virginio, C., Surprenant, A., North, R. A. and Kawashima, E. (1997) J. Neurosci. **17**, 6529–6533
34 Garcia, R. L. and Schilling, W. P. (1997) Biochem. Biophys. Res. Commun. **239**, 279–283
35 Xu, X. Z. S., Li, H. S., Guggino, W. B. and Montell, C. (1997) Cell **89**, 1155–1164
36 Shieh, B. H. and Zhu, M. Y. (1996) Neuron **16**, 991–998
37 Huber, A., Sander, P., Gobert, A., Bähner, M., Hermann, R. and Paulsen, R. (1996) EMBO J. **15**, 7036–7045
38 Chevesich, J., Kreuz, A. J. and Montell, C. (1997) Neuron **18**, 95–105
39 Tsunoda, S., Sierralta, J., Sun, Y., Bodner, R., Suzuki, E., Becker, A., Socolich, M. and Zuker, C. S. (1997) Nature (London) **388**, 243–249
40 Chang, A. S., Chang, S. M., Garcia, R. L. and Schilling, W. P. (1997) FEBS Lett. **415**, 335–340

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