Previous studies have shown that the Drosophila cation channels designated Trp and Trpl can be functionally expressed in Sf9 insect cells using baculovirus expression vectors. The trp gene encodes a Ca\(^{2+}\)-permeable channel that is activated by thapsigargin, blocked by low micromolar Gd\(^{3+}\), and is relatively selective for Ca\(^{2+}\) versus Na\(^{+}\) and Ba\(^{2+}\). In contrast, trpl encodes a Ca\(^{2+}\)-permeable cation channel that is constitutively active, not affected by thapsigargin, blocked by high micromolar Gd\(^{3+}\), and non-selective with respect to Ca\(^{2+}\), Na\(^{+}\), and Ba\(^{2+}\). The region of lowest sequence identity between Trp and Trpl occurs in the COOH-terminal domain. To test the hypothesis that this region is responsible for the differential sensitivity of these channels to thapsigargin, chimeric constructs of Trp and Trpl were created in which the COOH-terminal tail region of each protein was exchanged. The Trp construct with the Trpl COOH-tail was constitutively active, insensitive to thapsigargin, but retained selectivity for Ca\(^{2+}\) over Na\(^{+}\) and Ba\(^{2+}\). In contrast, the Trpl construct with the Trp COOH-tail was not constitutively active, could be activated by thapsigargin, but remained non-selective with respect to Ca\(^{2+}\), Ba\(^{2+}\), and Na\(^{+}\). These results suggest that the COOH-terminal domain of Trp plays an important role in determining constitutive activity, whereas the COOH-terminal region of Trp contains the structural features necessary for activation by thapsigargin.

The Drosophila proteins encoded by the trp and trpl genes (Trp and Trpl) are thought to form Ca\(^{2+}\)-permeable cation channels responsible for depolarization of photoreceptor cells following stimulation by light (1, 2). Since phototransduction in Drosophila requires phospholipase C activity (for review, see Refs. 2–4), Trp and Trpl may be insect homologues of the channel responsible for I\(_{\text{c resistant}}\), the elusive store-operated, capacitative Ca\(^{2+}\) entry channels that are activated by receptor-mediated phosphoinositide hydrolysis in mammalian non-excitatory cells. The first studies demonstrating that trp and trpl encode ion channels were performed in a heterologous expression system using baculovirus expression vectors (5–8). Evaluation of whole cell membrane currents in Sf9 insect cells following infection with recombinant baculovirus containing the cDNA for trp and trpl under control of the polyhedrin promoter demonstrated that trp encodes a cation channel that is selective for Ca\(^{2+}\) over Na\(^{+}\), can be activated by depletion of the internal Ca\(^{2+}\) store by thapsigargin, and is a poor conductor of Ba\(^{2+}\) (8). In contrast, trpl encodes a cation channel that is constitutively active, is relatively non-selective with respect to Ca\(^{2+}\), Ba\(^{2+}\), and Na\(^{+}\), is unaffected by thapsigargin (7, 8), but can be activated by a receptor-mediated increase in inositol-1,4,5-trisphosphate (6, 9).

The functional studies on Trp and Trpl allow for specific predictions concerning the structural features of Trp that may be responsible for the differential sensitivity of these two channel proteins to thapsigargin. Trp and Trpl exhibit substantial amino acid identity in their NH\(_{2}\)-terminal regions and in their proposed membrane spanning segments but differ in their COOH-terminal domains (1, 10, 11). We hypothesized that the COOH-terminal domain of Trp is necessary for thapsigargin sensitivity. To test this hypothesis, chimeric proteins were created in which the COOH-terminal domains of Trp and Trpl were exchanged, and the resulting channels were functionally expressed using the baculovirus-Sf9 insect cell expression system. The results demonstrate that the COOH domain of Trp confers thapsigargin sensitivity to Trpl and that the COOH domain of Trpl confers constitutive activity to Trp. Although the relative selectivity of the chimeric channels for Ca\(^{2+}\), Na\(^{+}\), and Ba\(^{2+}\) appears to be unchanged from the native channels, the sensitivity of the chimeric channels to Gd\(^{3+}\) blockade is intermediate between that seen for Trp and Trpl. Thus, the COOH-terminal domain may influence pore characteristics.

EXPERIMENTAL PROCEDURES

Solution and Reagents—MES\(^{1}\)-buffered saline (MBS) contained the following: 10 mM NaCl, 60 mM KCl, 17 mM MgCl\(_2\), 4 mM CaCl\(_2\), 110 mM sucrose, 0.1% bovine serum albumin, and 10 mM MES, pH adjusted to 6.2 at room temperature with Trizma-base. The total osmolarity of MBS was ~340 mosm. Taq DNA polymerase was purchased from Perkin-Elmer, and thapsigargin was obtained from Calbiochem. Fura-2-acetoxymethylester (fura-2/AM) was obtained from Molecular Probes (Eugene, OR).

Cell Culture—Sf9 cells were obtained from Invitrogen and were cultured as described previously (5, 6, 13) using Grace’s insect medium supplemented with lactalbumin hydrolysate, yeastolate, l-glutamine, 10% heat-inactivated bovine serum, and 1% penicillin-streptomycin solution (Life Technologies, Inc.). Cells were grown either in spinner flasks (Belco Glass, Vineland, N J) or in 35- or 100-mm plastic tissue culture dishes. The cell cultures were incubated at 27°C in a humidified air atmosphere.

Construction of Chimeric cDNAs—The cDNA for trp and trpl was

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The COOH-terminal Domain of Drosophila TRP Channels Confers Thapsigargin Sensitivity*

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1 The abbreviations used are: MES, 2-[N-morpholino]ethanesulfonic acid; MBS, MES-buffered saline.
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subcloned using standard techniques (14) into the baculovirus transfer vector pVL1393 (Invitrogen) yielding pVL-trp and pVL-trpl. The trp and trpl sequences have in common a unique cleavage site for the restriction endonuclease DraII, 14 amino acids downstream from the final (S6) putative transmembrane domain (Fig. 1), and the pVL vector contains a cleavage site for NotI in the downstream multiple cloning site. Both pVL-trp and pVL-trpl were digested with DraII and NotI, and the resulting fragments were purified by agarose gel electrophoresis. The COOH-terminal fragment of trpl was ligated with the pVL-trp fragment to produce the trp/trpl chimera; the trpl/trp chimera was produced in an analogous fashion. The nucleotide sequence for each construct was confirmed by the deoxyxynucleotide method using Sequenase version 2.0 (US Biochemical Corp.).

To monitor expression at the protein level, the nucleotide sequence encoding the FLAG epitope (DYKDDDDK) was attached to the NH2 terminus of each construct (pVL-trp, pVL-trpl, pVL-trp/trpl, and pVL-trp/trpl/ trp) using the following general procedure. Oligonucleotides (Ran- som Hill Bioscience, Ramona, CA) were synthesized consisting of a methionine start codon, the FLAG sequence, and several base pairs encoding the NH2 terminus of either trp or trpl. A second set of oligo- nucleotides was synthesized consisting of sequence within the coding region of trp and trpl that included a unique restriction site. These oligonucleotides were used as primer sets for amplification by polymerase chain reaction of the FLAG-attached NH2 terminus of trp and trpl. The polymerase chain reaction products were subcloned into plasmid (PHC) and subsequently transferred to the -trp and -trpl constructs using convenient restriction sites. All constructs were sequenced to confirm that the nucleotides encoding the FLAG epitope were attached and that the trp and trpl coding sequence remained in frame with the new start codon.

Generation of Recombinant Baculovirus—Recombinant baculovi- ruses were produced using the BaculoGold™ transfection kit (Phar- mingen, San Diego, CA) by cotransferring SF9 cells with pVL-trp, pVL-trpl, and -chimeric constructs and linearized BaculoGold viral DNA as de- scribed in the instructions provided with the kit. Single viral plaques were isolated and amplified two to four times to obtain a high titer viral stock solution, which was stored at 4°C until use.

In Vivo Isolation of Membrane-associated FLAG Proteins—Cells were infected with the BaculoGold™ transfection kit (Phar- mingen, San Diego, CA) by cotransferring SF9 cells with pVL-trp, pVL-trpl, and -trp constructs using convenient restriction sites. All constructs were sequenced to confirm that the nucleotides encoding the FLAG epitope were attached and that the trp and trpl coding sequence remained in frame with the new start codon.

Isolation of Membrane-associated FLAG Proteins—Cells were harvested at 48 h postinfection and subjected to centrifugation at 500 x g for 5 min, and resuspended at a density of 5 x 10^6 cells/ml in lysis buffer containing 20 mM Tris-Cl, 5 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 0.5 μM aprotinin, and 2 μM leupeptin. The cell suspension was sonicated on electrothermal sonicator for 15 min, and resuspended at a density of 10^6 cells/ml in MBS containing 2% SDS, 10% glycerol, 100 mM dithiothreitol, and 0.025% bromphenol blue. The resulting supernatants were centrifuged at 1000 x g for 10 min at 4°C. The resulting pellets were discarded, and the supernatants were centrifuged at 42,000 x g for 30 min. The microsomal pellets were resuspended in lysis buffer at a protein concentration of 5–10 mg/ml and stored at –80°C until use.

Proteins were transferred electrophoretically from the polyacrylamide gels to Millipore polyvinylidi- dene difluoride membranes (100–150 mA for 12 h on ice). The mem- branes were blocked with dry milk for 1 h and probed with mouse M2 antihuman M2 (Covance, Madison, WI), Bridgeport, CO, 1:500 dilution. After washing, the membranes were probed for 1 h with goat anti-mouse IgG conjugated with horseradish peroxidase and with avin- conjugated horseradish peroxidase for reaction with biotinylated molecular weight standards. Protein bands were visualized by addition of horseradish peroxidase substrates in peroxidase buffer (Pierce).

Immunoblotting of the FLAG Proteins—Proteins were transferred electrophoretically from the polyacrylamide gels to Millipore polyvinylidi- dene difluoride membranes (100–150 mA for 12 h on ice). The mem- branes were blocked with dry milk for 1 h and probed with mouse M2 antihuman M2 (Covance, Madison, WI), Bridgeport, CO, 1:500 dilution. After washing, the membranes were probed for 1 h with goat anti-mouse IgG conjugated with horseradish peroxidase and with avin- conjugated horseradish peroxidase for reaction with biotinylated molecular weight standards. Protein bands were visualized by addition of horseradish peroxidase substrates in peroxidase buffer (Pierce).

Measurement of Free Cytosolic Ca²⁺ Concentration—[Ca²⁺], was measured in Sf9 cells using the fluorescent indicator, fura-2, as de- scribed previously (5, 6). Briefly, cells were dispersed, washed, and resuspended at a concentration of 1.5–2 x 10⁶ cells/ml in MBS contain-
Effect of Protein Expression on Basal \([\text{Ca}^{2+}]_i\) — Previous studies have shown that Trp is constitutively active when expressed in Sf9 insect cells. This activity gives rise to an increase in basal \([\text{Ca}^{2+}]_i\) as a function of postinfection time (7). As seen in Fig. 1C, basal \([\text{Ca}^{2+}]_i\) was significantly increased 225% at 48 h postinfection in Trp/Trpl-expressing cells relative to that in cells expressing native Trp (\(p < 0.00001\)). Likewise, basal \([\text{Ca}^{2+}]_i\) was significantly decreased 54% in cells expressing Trpl/Trp chimeric protein relative to that observed in cells expressing native Trpl (\(p < 0.00001\)). These results suggest a correlation between the presence of the Trpl COOH-terminal domain and constitutive activity.

Expression of Channel Activity — Trp-expressing cells exhibit substantial membrane current under basal non-stimulated conditions (7), which is unaffected by thapsigargin (8). In contrast, membrane currents observed in Trp-expressing cells are small under basal conditions but increase dramatically following application of thapsigargin (8). Whole cell currents were recorded in symmetrical sodium gluconate solutions in cells expressing either Trp/Trpl or Trpl/Trp chimeric proteins (Fig. 2). Inward and outward membrane currents were observed in Trp/Trpl-expressing cells under basal, non-stimulated conditions (n = 16). The current-voltage relationship was linear with a reversal potential near 0 mV. In 13 out of 16 cells, addition of thapsigargin (200 nM) to Trp/Trpl expressing cells had no effect on inward or outward currents. In three cells, current increased ~5% after thapsigargin addition. In sharp contrast, currents recorded in Trpl/Trp cells were small under basal conditions but increased dramatically following the addition of thapsigargin to the bath (14 out of 17 cells). Thapsigargin-induced currents were linear and reversed close to 0 mV when recorded in symmetrical sodium gluconate solutions. In 3 of 17 cells expressing Trp/Trpl, current was seen within 1 min after establishment of the whole cell recording mode and did
FIG. 2. Effect of thapsigargin on whole cell membrane currents in cells expressing Trp/Trpl and Trpl/Trp chimeric proteins. Whole cell membrane currents were recorded in Sf9 cells infected with recombinant baculovirus containing the cDNA for Trp/Trpl (left) or Trp/Trpl (right) chimeric proteins. Current traces shown were recorded before (control) or after application of 200 nM thapsigargin. Voltage steps (400 ms) were applied at 2-s intervals from a holding potential of 0 mV to potentials ranging from −100 to +60 mV. Average current-voltage relationship in the absence (○) or presence (●) of thapsigargin is shown below the respective current traces. All values represent the mean ± S.E. current amplitudes obtained at time 200 ms during each voltage pulse (n = 16 and 17 for Trp/Trpl and Trpl/Trp, respectively). Dotted line in the current records indicates the zero current level.

not increase after thapsigargin.

Chimeric Channel Ion Selectivity—Trp channels are relatively selective for Ca\(^{2+}\) versus Na\(^+\) and Ba\(^{2+}\). In contrast, Trpl channels are non-selective with respect to Ca\(^{2+}\), Na\(^+\), and Ba\(^{2+}\) (8). To determine the ionic selectivity for the chimeric channels, currents were first recorded in symmetrical sodium gluconate solutions and the bath solution was subsequently changed to one containing either 50 mM calcium gluconate or barium gluconate (Fig. 3). The I-V relationships for basal currents in the Trp/Trpl chimera were linear in symmetrical Na\(^+\) solutions with reversal potentials near 0 mV. Changing the bath to Ca\(^{2+}\) decreased inward current and produced a positive shift in reversal potential to approximately 35 mV; the current was inwardly rectifying with sodium gluconate in the pipette and calcium gluconate in the bath. Changing the bath solution to one containing Ba\(^{2+}\) decreased both inward and outward current without affecting the reversal potential. The thapsigargin-induced membrane currents in the Trp/Trpl chimera were also linear over a wide range of membrane potentials when recorded in symmetrical Na\(^+\) solutions. Changing the bath to one containing Ca\(^{2+}\) had little effect on the currents in the Trp/Trpl chimera-expressing cells. Inward current was reduced with Ba\(^{2+}\) bath solution, but the reversal potential remained near 0 mV. These results suggest that the relative permeability of the chimeras for Na\(^+\), Ca\(^{2+}\), and Ba\(^{2+}\) is not affected by switching the COOH-terminal domains.

Effect of Gd\(^{3+}\) on Chimeric Channel Currents—The effect of Gd\(^{3+}\) was determined on basal, non-stimulated currents in Trp and Trp/Trpl-expressing cells and on thapsigargin-induced currents in Trp and Trp/Trpl-expressing cells (Fig. 4). Trp and Trpl inward currents exhibit profound differences in sensitivity to blockade by Gd\(^{3+}\); inward Na\(^+\) currents in Trp-expressing cells are almost completely blocked by 10 \(\mu\)M Gd\(^{3+}\) added to the extracellular bath, whereas currents in Trp cells required 1 mM Gd\(^{3+}\) to achieve the same level of inhibition. The sensitivity of the Trp/Trpl and Trp/Trpl chimera cells is intermediate, with 10 \(\mu\)M Gd\(^{3+}\) producing 50% inhibition of inward Na\(^+\) currents. Thus, exchanging the COOH-terminal domains of Trp and Trpl affects sensitivity of both channels to Gd\(^{3+}\) blockade.

DISCUSSION

Previous studies on the functional expression of Trp in Sf9 insect cells suggest that although Trp appears to be a Ca\(^{2+}\)-store-operated channel that can be activated by thapsigargin, the ionic selectivity is different from both the endogenous I\(_{\text{crac}}\) recorded in the Sf9 insect cells (8) and I\(_{\text{crac}}\) observed in several mammalian cell types (23–26); I\(_{\text{crac}}\) appears to be highly selective for Ca\(^{2+}\) over Na\(^+\) and to be inwardly rectifying even in the presence of symmetrical Na\(^+\) solutions (25). Thus, Trp may not be the insect homologue of the I\(_{\text{crac}}\) channel. Trp does, however, appear to be a member of a large protein family found in Drosophila, Calliphora, Xenopus, mouse (22), and human (27, 28), although there is no functional information on the Trp homologues from sources other than insect. Petersen et al. (22) recently reported that expression of Drosophila Trp in Xenopus oocytes following injection of cRNA gives rise to an enhanced thapsigargin-induced increase in Ca\(^{2+}\) influx estimated by the magnitude of the Ca\(^{2+}\)-activated Cl\(^{-}\) current, consistent with the activation of Trp by depletion of the internal Ca\(^{2+}\) stores. Thus, it seems likely that although Trp may not be identical to I\(_{\text{crac}}\) channels, it may be regulated in a fashion similar to I\(_{\text{crac}}\).

Understanding the mechanism by which Trp is regulated by thapsigargin and identification of the structural domain of Trp necessary for this regulation could provide important clues to structure and function of mammalian I\(_{\text{crac}}\) channels. Toward this end, the purpose of the present study was to determine the general region of Trp that is necessary or sufficient for regulation of channel activity by thapsigargin. To accomplish this goal, we exploited the differences in sensitivity of Trp and Trpl to thapsigargin. With exception of amino acid residues 330–500, Trp and Trpl are very similar over the first two-thirds of the predicted amino acid sequence. We therefore focused our attention on the COOH-terminal domain as the region that gives rise to the differential sensitivity of these two channel proteins to thapsigargin.

Exchanging the COOH-terminal domains of Trp and Trpl produced several important functional changes. First, and most importantly, thapsigargin sensitivity was conveyed to Trp by the COOH domain of Trp, and the presence of the Trp COOH-terminal domain on Trp eliminated thapsigargin sensi-
Fig. 3. Ionic selectivity of Trp/Trpl and Trpl/Trp chimeric channels. Whole-cell membrane currents were recorded in baculovirus-infected Sf9 cells as described in the legend to Fig. 2. Currents were recorded under basal non-stimulated conditions for Trp/Trpl, whereas currents were recorded in the presence of 200 nM thapsigargin for Trp/Trp. The average current-voltage relationship was first determined in each cell with 100 mM sodium gluconate in both the bath and pipette solution (○). The bath solution was then changed for one containing 50 mM calcium gluconate or barium gluconate (●) as indicated in each panel. All values represent the mean ± S.E. current amplitudes obtained at time 200 ms during each voltage pulse (n = 4).

Fig. 4. Blockade of inward current by Gd3+. Whole-cell membrane currents were recorded in baculovirus-infected Sf9 cells as described in the legend to Fig. 2. Currents were first determined in each cell with 100 mM sodium gluconate in both the bath and pipette solution. EGTA was omitted from the bath solution for this set of experiments. Currents were recorded under basal, non-stimulated conditions for Trp (○) and Trp/Trpl (●), and in the presence of 200 nM thapsigargin for Trp (●) and Trp/Trpl (●) expressing cells. The bath solution was then changed for one containing 100 mM sodium gluconate plus the indicated concentration of Gd3+. All values represent the mean ± S.E. (n = 4–5) current amplitudes taken at 200 ms after a step change in membrane potential from 0 to −100 mV. Currents in the presence of Gd3+ were normalized to the control current amplitudes obtained in each cell before application of Gd3+. In all cells reported, currents returned to control levels following washout of Gd3+.

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...and Trpl may have subtle influences on pore characteristics. One possibility is that the COOH-terminal domain of Trp forms an extension of the pore structure into the cytoplasm beyond the inner leaflet of the phospholipid membrane in a fashion analogous to the nicotinic acetylcholine receptor, where a large extracellular domain forms the vestibule of the channel, which extends out from the membrane structure. Presumably this region would contain the sequence responsible for binding Gd3+. Alternatively, the COOH-terminal domain may influence the conformation of the pore region producing subtle alterations in the Gd3+ binding site. A similar phenomenon has been observed in the inwardly rectifying K+ channel, where the COOH-terminal domain appears to have a major role in specifying pore properties (29).

The mechanism by which the COOH-terminal domain affects constitutive activity is unknown. This does not reflect variation in protein expression, since a difference in basal [Ca2+]i is seen between Trp and the Trp/Trpl chimera, yet both are expressed to approximately the same levels in the Sf9 cell. Furthermore, the constitutive activity (or lack thereof) of the chimeras is clear from the whole cell current recordings; the COOH-terminal domain of Trp maintains the channel in a non-conducting state. In a fashion analogous to other channel types, part of COOH-terminal domain of Trp may act as the “gate,” which is closed in the absence of thapsigargin but opens in response to depletion of the internal Ca2+ store.

The present structure-function study may also provide insight into the mechanism by which store depletion activates surface membrane channels like I_{ca}. In this regard, there are basically two hypotheses. The conformational coupling hypothesis suggests that close association between the endoplasmic reticulum and the plasmalemma allows for direct physical coupling between the inositol 1,4,5-trisphosphate receptor and Ca2+ entry channels and that information concerning the depletion status of the internal store is related to surface membrane channels via a conformational change (30). Alternatively, a soluble, diffusible messenger may be generated and/or released upon depletion of the Ca2+ store, which then either...
directly or indirectly activates the surface membrane channels (31, 32). The COOH-terminal domain of Trp contains a unique proline-rich region in which the dipeptide KP is repeated 27 times at relatively even intervals and includes a highly charged segment where the sequence DKDKKP(G/A)D is repeated 9 times. Interestingly, the bacterial protein TonB also has a proline-rich region in which a string of EP and KP repeats is thought to form a mechanical linkage between the inner and outer bacterial membranes (33, 34). By analogy, the highly charged proline-rich segment of Trp may perform the same function linking proteins of the endoplasmic reticulum to the Trp channel. Alternatively, another region of the COOH-terminal domain of Trp may be responsive to depletion of the Ca\(^{2+}\) store. In this regard, Trp has 5 tyrosine residues in the COOH-terminal domain at positions 665, 687, 745, 756, and 922. The first three tyrosine residues are conserved in Trpl. It is possible that phosphorylation of Tyr-756 or Tyr-922 may play a role in activation or regulation of Trp by thapsigargin. Additional chimeric constructs in which different regions of the COOH-terminal domains of Trp and Trpl are exchanged may help determine the specific regions necessary for thapsigargin sensitivity, for constitutive activity, and for determination of pore characteristics.

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