Receptor-mediated activation of recombinant Trpl expressed in Sf9 insect cells

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The *Drosophila* proteins, Trp and Trpl, are suggested to be cation channels responsible for depolarization of the receptor potential associated with stimulation of insect photoreceptor cells by light. Consistent with this hypothesis, we recently showed that recombinant Trpl forms Ca^{2+}- and Ba^{2+}-permeable non-selective cation channels when expressed in Sf9 cells using the baculovirus expression vector. As Trpl may be activated in the photoreceptor cell after stimulation of phospholipase C, we hypothesized that a similar regulation of recombinant Trpl may be observed in the Sf9 cell after activation of heterologous membrane receptors linked to Ca^{2+}-signal-transduction pathways. To test this hypothesis, Ca^{2+} signalling was examined in Fura-2-loaded Sf9 cells infected with baculovirus containing cDNA for the M5 muscarinic receptor alone (M5 cells) or in cells co-infected with both M5 and Trpl-containing baculoviruses (M5–Trpl cells). Addition of carbachol (100 μM) to M5 cells produced an increase in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) (mean $\pm$ S.D.; $n = 17$) from 101 ± 20 to 762 ± 178 nM which declined to a sustained elevated level of 384 ± 102 nM after 3 min. The sustained component was eliminated by removal of extracellular Ca^{2+} or by addition of La^{3+} or Gd^{3+} (10 μM). In M5–Trpl cells, basal $[Ca^{2+}]_i$ increased as a function of time after infection. To evaluate the contribution of Ca^{2+} influx to the overall profile observed, Ba^{2+}, a Ca^{2+} surrogate that is not a substrate for the Ca^{2+} pump, was used. The increase in basal $[Ca^{2+}]_i$ seen in M5–Trpl cells was associated with an increase in basal Ba^{2+} influx. Addition of carbachol to M5–Trpl cells at 30–36 h after infection produced a large increase in $[Ca^{2+}]_i$ to a sustained value of 677 ± 143 nM. This change in $[Ca^{2+}]_i$ was (1) blocked by atropine, (2) attenuated in the absence of extracellular Ca^{2+}, and (3) relatively insensitive to La^{3+}, but blocked by Gd^{3+} in the 0.1–1 mM range. In the presence of 10 μM Gd^{3+} to block the endogenous-receptor-mediated Ca^{2+}-influx pathway, carbachol produced a dramatic increase in Ba^{2+} influx in M5–Trpl cells. In sharp contrast, neither Ca^{2+} nor Ba^{2+} influx through Trpl was affected by thapsigargin, a selective inhibitor of the endoplasmic reticulum Ca^{2+}-ATPase pump. These results suggest that recombinant Trpl (1) is constitutively active under basal non-stimulated conditions, (2) can be further activated by a receptor-dependent mechanism and (3) is not affected by depletion of the internal Ca^{2+} stores by thapsigargin.

INTRODUCTION

In a variety of non-excitable cell types, specific receptor stimulation results in a biphasic increase in free Ca^{2+} concentration ($[Ca^{2+}]_i$); an initial transient component reflects the release of Ca^{2+} from internal stores, whereas a more long-lasting elevation in $[Ca^{2+}]_i$ reflects the influx of Ca^{2+} from the extracellular space. Although both phases normally require receptor-mediated production of Ins(1,4,5)P_3, selective inhibition of the endoplasmic-reticulum Ca^{2+}-ATPase pump by thapsigargin or 2,5-di-*t*-butylhydroquinone causes depletion of the Ins(1,4,5)P_3-sensitive internal Ca^{2+} store and stimulation of Ca^{2+} influx in the absence of phosphoinositide hydrolysis (Foder et al., 1989; Takemura et al., 1989; Kwan et al., 1990; Thastrup et al., 1990; Mason et al., 1991; Schilling et al., 1992). These results support the hypothesis that Ca^{2+} influx is related to the level of Ca^{2+} within the internal store, i.e. the so-called capacitative Ca^{2+}-entry model (Putney, 1990, 1992). Recent studies suggest that Ca^{2+} entry activated by depletion of the internal Ca^{2+} store occurs via a channel (Hoth and Penner, 1992, 1993; Vaca and Kunze, 1993; Zweifach and Lewis, 1993); however, the molecular mechanism(s) by which the repletion status of the internal store controls the activity of the surface membrane channel remains unknown.

A clue to the identity of the Ca^{2+}-influx channel comes from studies on *Drosophila* phototransduction. Light stimulation of the photoreceptor cells of *Drosophila* and other arthropods initiates a cascade of events that culminates in the opening of cation-selective channels in the plasmalemma and depolarization of the receptor potential (Hardie and Minke, 1993). As in vertebrate phototransduction, photons activate rhodopsin which in turn catalyses the dissociation of a heterotrimeric GTP-binding protein (G-protein) into α- and $\beta\gamma$-subunits. However, in contrast with vertebrate cells, G-protein activation in *Drosophila* is thought to stimulate phospholipase C. The rise in cytosolic Ins(1,4,5)P_3 causes the release of Ca^{2+} from internal storage sites and a concomitant activation of Ca^{2+} influx from the extracellular space. This gives rise to prolonged depolarization of the receptor potential as a result of activation of inward membrane current (Minke and Selinger, 1991). The biochemical similarity between insect phototransduction and receptor-mediated Ca^{2+} signalling in non-excitable cells of mammalian origin suggests that similar proteins may be involved in regulation of Ca^{2+} influx.

A substantial amount of information on *Drosophila* phototransduction has been deduced from studies of mutant flies. In the *transient receptor potential* mutant (*trp*), low-level light stimulation of the photoreceptor cell produces a near-normal response where prolonged stimulation with intense light causes only a transient change in receptor potential; the prolonged depolarization seen in the wild-type cell is eliminated as is the sustained inward current (Cosens and Manning, 1969; Minke and Selinger, 1991; Hardie and Minke, 1992). Although the actual function

Abbreviations used: $[Ca^{2+}]_i$, cytosolic free Ca^{2+} concentration; MBS, Mes-buffered saline; Fura-2/AM, Fura-2 acetoxymethyl ester.

of the protein responsible for the *trp* mutant has not been determined, it has recently been proposed that Trp is a light-activated Ca^{2+} channel (Hardie and Minke, 1992; Phillips et al., 1992). The *trp* gene has been cloned and the cDNA codes for a protein of 1275 amino acids with eight putative membrane-spanning regions (Montell and Rubin, 1989; Wong et al., 1989). Another protein initially identified as a calmodulin-binding protein has been cloned from *Drosophila* and designated as Trp-like or Trpl as it shares substantial sequence homology with Trp (Phillips et al., 1992). Trpl has six putative transmembrane regions. These segments of both Trp and Trpl show homologies to membrane-spanning regions of voltage-gated Ca^{2+} and Na^{+} channels although they lack the arginine residues of S4 which are thought to act as voltage sensors. Interestingly, some light-activated membrane current is observed in the *trp* mutant during intense light stimulation although it is only transiently activated (Hardie and Minke, 1992). This has led to speculation that *trp* encodes a Ca^{2+}-selective channel responsible for the sustained current component, whereas *trpl* encodes a Ca^{2+}-activated non-selective cation channel responsible for the transient change in membrane current (Phillips et al., 1992; Hardie and Minke, 1993).

Functional characterization of Trp and Trpl as cation channels and definition of their putative role in Ins(1,4,5)P_3-dependent Ca^{2+}-signalling mechanisms in both *Drosophila* and mammalian cells would be greatly facilitated by the availability of an expression system that could provide a consistent source of cells expressing functional protein. A variety of plasmalemmal proteins including ion channels and carriers have been functionally expressed in Sf9 insect cells after infection with the recombinant baculovirus, *Autographa californica* nuclear polyhedrosis virus (Luckow and Summers, 1988; Klaiber et al., 1990; Fafournoux et al., 1991; Li *et al.*, 1992). Studies have now shown that rat brain M5 muscarinic receptors (Hu et al., 1994a), human B2 bradykinin receptors (Schilling et al., 1994) and human thrombin receptors (Chen and Schilling, 1994) can be functionally expressed in Sf9 cells. Stimulation of these receptors by their respective agonist agents causes a change in Sf9-cell $[Ca^{2+}]_i$ that is essentially identical with that observed in mammalian non-excitable cells. Likewise, depletion of the internal Ca^{2+} stores by thapsigargin activates Ca^{2+} influx in the Sf9 cell (Hu et al., 1994a).

In a recent study, we measured $[Ca^{2+}]_i$ and whole-cell-membrane currents in Sf9 insect cells infected with recombinant baculovirus containing the cDNA for Trpl (Hu et al., 1994b). Basal $[Ca^{2+}]_i$ increased as a function of time after infection consistent with expression of a protein under the control of the polyhedrin promoter. The increased basal $[Ca^{2+}]_i$ was specific for expression of Trpl and was associated with an elevated plasmalemmal permeability to both Ca^{2+} and Ba^{2+} and the appearance of a Ca^{2+}-permeable non-selective cation channel. This spontaneous channel activity was observed under basal non-stimulated conditions, and was inhibited by the inorganic Ca^{2+}-channel blocker, Gd^{3+}, in the 0.1–1 mM range. This sensitivity to Gd^{3+} was 1000-fold lower than the endogenous-receptor-mediated Ca^{2+}-influx pathway observed in the Sf9 cell. Together these results suggested that Trpl forms a novel Ca^{2+}-permeable cation channel consistent with its proposed role in insect photoreception.

Although it is clear that membrane currents are activated after initiation of the phototransduction cascade in *Drosophila*, the downstream regulators of channel function remain unknown. A useful characteristic of the baculovirus–Sf9 cell expression system is that it can be used to express simultaneously multiple foreign proteins in the same cell. This can be accomplished by co-infection with two independent baculoviruses containing the cDNA for each protein. The purpose of the present study was to determine if recombinant Trpl could be activated by a receptor-dependent mechanism and/or by depletion of the internal Ca^{2+} store. To address this question, we examined the response to carbachol and thapsigargin of Fura-2-loaded Sf9 insect cells co-infected with baculoviruses containing the M5 muscarinic receptor and the Trpl cDNA. As Trpl may be activated in the photoreceptor cell after activation of phospholipase C, we hypothesized that a similar regulation of recombinant Trpl may be observed in the Sf9-cell-expression system after activation of heterologous receptors linked to Ca^{2+}-signal-transduction pathways. The results clearly demonstrate that, although Trpl is constitutively active in the Sf9 cell under basal non-stimulated conditions, it is further activated by receptor stimulation. In sharp contrast, Trpl is not affected by thapsigargin, suggesting that the activity of Trpl is unrelated to depletion of the internal Ca^{2+} store. Part of this work has been published in abstract form (Hu et al., 1994c).

MATERIALS AND METHODS

Solutions and reagents

Unless otherwise indicated, 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. Nominally Ca^{2+}-free MBS contained the same salts and sugars as MBS without $CaCl_2$ and with $MgCl_2$ increased to 25 mM. The total osmolarity of MBS was approx. 340 mosM. Fura-2 acetoxymethyl ester (Fura-2/AM) was obtained from Molecular Probes (Eugene, OR, U.S.A.). The full-length cDNA for Trpl (pAB3.14/Z9) (Phillips et al., 1992) was generously provided by Dr. Leonard E. Kelly (Department of Genetics, University of Melbourne, Parkville, Victoria, Australia). The cDNA for a rat brain M5 muscarinic acetylcholine receptor was provided by Dr. Lutz Birnbaumer (Department of Cell Biology, Baylor College of Medicine, Houston, TX, U.S.A.). The human B2 bradykinin receptor was cloned from a skin fibroblast cell line using reverse transcriptase PCR based on the published sequence (Hess et al., 1994). Details of the cloning and characterization of the recombinant B2-receptor in Sf9 cells will be reported elsewhere (X. Chen and W. P. Schilling, unpublished work, but see Schilling et al., 1994).

Culture of Sf9 cells

Sf9 cells were obtained from Invitrogen (San Diego, CA, U.S.A.) and cultured as previously described (O'Reilly et al., 1992) using Grace's insect medium supplemented with lactalbumin hydrolysate, yeastolate, L-glutamine, 10% heat-inactivated fetal bovine serum and 1 % penicillin/streptomycin solution (Gibco). Cell were grown in either spinner flasks (Bellco Glass, Vineland, NJ, U.S.A.) or 100 mm plastic tissue culture dishes (Falcon).

Production of recombinant baculoviruses and infection of Sf9 cells

The cDNAs encoding the rat brain M5 muscarinic receptor, the human fibroblast B2 bradykinin receptor or Trpl were subcloned into baculovirus transfer vectors, pVL1392 or pVL1393, using standard techniques (Sambrook et al., 1989). Recombinant viruses were produced using the BaculoGold Transfection Kit (PharMingen, San Diego, CA, U.S.A.). Single plaques were isolated and amplified two to four times to obtain a high-titre viral stock which was stored at 4 °C until use. For routine infection, Sf9 cells in Grace's medium were allowed to attach to the bottom of a 100 mm plastic culture dish (10^7 cells/dish).

After incubation for 15 min to 1 h, a portion of viral stock (multiplicity of infection was 10 for the M5 and B2 receptor and 3 for the TrpI-containing baculovirus) was added and the cultures were maintained at 27 °C in a humidified air atmosphere. Unless otherwise indicated, cells were used at 30–36 h after infection.

Measurement of free cytosolic Ca^{2+} concentration in dispersed Sf9 cells

$[Ca^{2+}]_i$ was measured using the fluorescent indicator, Fura-2, as previously described (Schilling et al., 1989; Tian et al., 1994; Hu et al., 1994a). Briefly, cells were dispersed, washed and re-suspended at a concentration of 1.5×10^6–2×10^6 cells/ml in MBS containing 2 μM Fura-2/AM. After 30 min incubation at room temperature (22 °C), the cell suspension was subjected to centrifugation, resuspended in an equal volume of MBS and incubated for an additional 30 min. The cell suspension was again subjected to centrifugation and the cells resuspended in fresh MBS. Portions of this final suspension were centrifuged and washed twice immediately before fluorescence measurement using an SLM 8000 spectrophotofluorimeter. Excitation wavelength alternated between 340 and 380 nm and fluorescence intensity was monitored at an emission wavelength of 510 nm. All measurements were performed at room temperature (22 °C) and were corrected for autofluorescence using unloaded cells. Calibration of the Fura-2 associated with the cells was accomplished using Triton lysis in the presence of saturating bivalent cation concentration followed by addition of EGTA (pH 8.5). $[Ca^{2+}]_i$ was calculated by the equation of Grynkiewicz et al. (1985) using the K_d value for Ca^{2+} binding to Fura-2 of 278 nM determined for 22 °C (Shuttleworth and Thompson, 1991). Cytosolic free Ba^{2+} concentration was estimated using Fura-2 as previously described (Schilling et al., 1989). The Figures show representative traces from experiments performed at least three times.

RESULTS

Receptor-mediated changes in $[Ca^{2+}]_i$ in Sf9 insect cells

Previous studies have shown that, except for octopamine, an endogenous insect neurotransmitter, Sf9 cells are unresponsive to a number of mammalian hormones and neurotransmitters typically linked to changes in $[Ca^{2+}]_i$ (Hu et al., 1994a). Sf9 cells become responsive to carbachol after infection with baculovirus containing cDNA for the M5-muscarinic receptor (M5 cells; Figure 1a). Addition of carbachol (100 μM) to M5 cells produced a 6–8-fold increase in $[Ca^{2+}]_i$ from a basal level of 101 ± 20 to 762 ± 178 nM (mean ± S.D.; $n = 17$). $[Ca^{2+}]_i$ subsequently decreased to a sustained level of 384 ± 102 nM after 3 min and remained elevated over the basal value for several minutes. Addition of La^{3+} (10 μM) before carbachol abolished the sustained component, but was without effect on the initial transient phase. A similar profile was obtained using Gd^{3+} (1 or 10 μM) as the blocking ion (results not shown). Both phases of the response were blocked by the muscarinic receptor antagonist, atropine.

To confirm that carbachol activates both the release of Ca^{2+} from internal stores and the influx of Ca^{2+} from the extracellular space, experiments were performed in the absence of extracellular Ca^{2+} (Figure 1b). Addition of carbachol under these conditions produced a transient 3–4-fold increase in $[Ca^{2+}]_i$ that was unaffected by extracellular La^{3+}. Readdition of Ca^{2+} to the extracellular buffer after carbachol produced an increase in $[Ca^{2+}]_i$ that peaked and slowly declined to a steady elevated phase. Addition of La^{3+} during this phase of the response rapidly returned $[Ca^{2+}]_i$ to the basal level. Addition of Ca^{2+} to the extracellular buffer after carbachol, but in the presence of La^{3+}, had no effect on $[Ca^{2+}]_i$. Similar results were obtained using Gd^{3+} (10 μM) as the blocking ion (results not shown). These results confirm that stimulation of recombinant M5 receptors expressed in Sf9 cells activates Ca^{2+} influx and that Ca^{2+} influx is blocked by low concentrations of La^{3+} and Gd^{3+}. Furthermore, the Sf9 cells clearly have Ca^{2+} pumps necessary for the maintenance of low cytosolic Ca^{2+} as $[Ca^{2+}]_i$ declines rapidly in the absence of extracellular Ca^{2+} after carbachol-induced release of Ca^{2+} from internal stores or after blockade of carbachol-induced Ca^{2+} influx by La^{3+}.

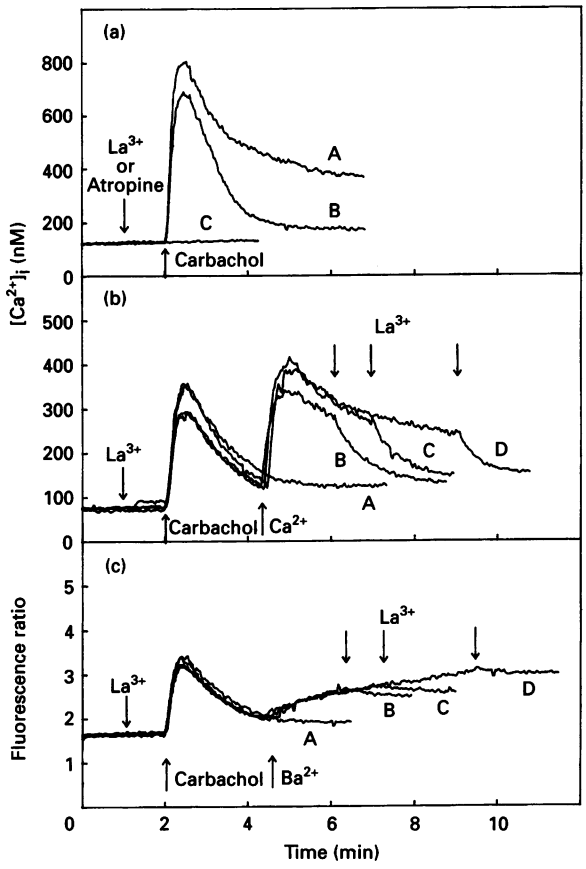

Figure 1 Effect of carbachol on $[Ca^{2+}]_i$ in Sf9 cells expressing the M5 muscarinic receptor

Sf9 insect cells were infected with recombinant baculovirus containing the cDNA for a rat brain M5 muscarinic acetylcholine receptor (M5 cells). At 30–36 h after infection, the cells were harvested and loaded with the intracellular fluorescent Ca^{2+} indicator, Fura-2, as described in the Materials and methods section. (**a**) Three traces are shown superimposed. Carbachol (100 μM) was added at the time indicated by the arrow, in the control (trace A) and in the presence of either $LaCl_3$ (10 μM; trace B) or atropine (10 μM; trace C) added at the time indicated. (**b**) Four traces are superimposed. M5 cells were incubated in Ca^{2+}-free MBS. Carbachol (100 μM) and $CaCl_2$ (10 mM) were added for each trace at the times indicated by the arrows. $LaCl_3$ (10 μM) was added before carbachol (trace A) or at various times after carbachol (traces B–D). (**c**) Same as in (**b**) with $BaCl_2$ (10 mM) added rather than $CaCl_2$.

The agonist-activated Ca^{2+}-influx pathway in mammalian non-excitable cells has been shown to permeate other bivalent cations, in particular, Ba^{2+} and Sr^{2+} (Schilling et al., 1989; Kwan and Putney, 1990). Ba^{2+} is useful for investigation of Ca^{2+} influx as it will pass through Ca^{2+} channels, bind to Fura-2 and produce similar fluorescence changes, but is not a substrate for the carriers or pumps normally responsible for removal of Ca^{2+} from the cytosol of mammalian cells. Ba^{2+} appears to behave in the

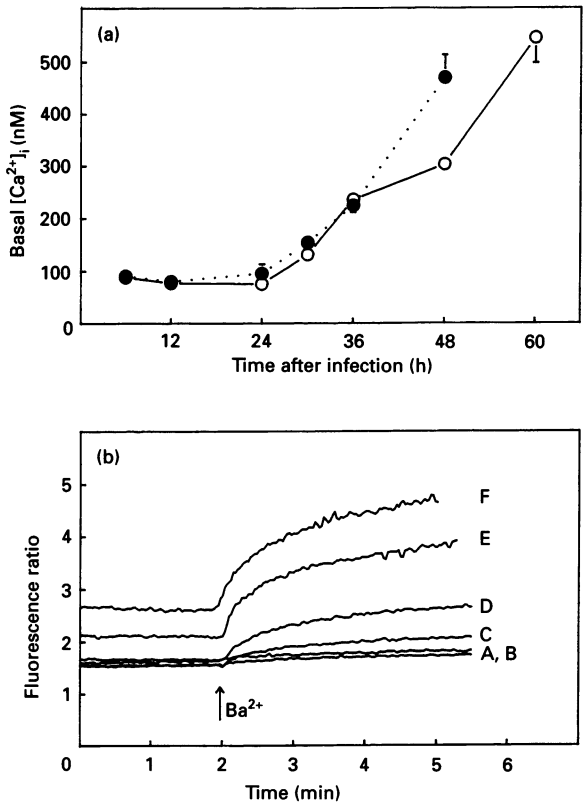

Figure 2 Effect of time after infection on TrpI-induced increase in basal $[Ca^{2+}]_i$ and Ba^{2+} influx

Sf9 insect cells were co-infected with recombinant baculoviruses containing the cDNA for a rat brain M5 muscarinic acetylcholine receptor and for the TrpI protein (M5–TrpI cells). At the indicated times after infection, the cells were harvested and loaded with the intracellular fluorescent Ca^{2+} indicator, Fura-2. (**a**) Basal $[Ca^{2+}]_i$ was measured in M5–TrpI cells at various times after infection. All values are means ± S.D. of three determinations at each time point from two independent infections (○ and ●). (**b**) Six traces are superimposed. At the time indicated by the arrow, $BaCl_2$ (10 mM) was added to Fura-2-loaded M5–TrpI cells incubated in Ca^{2+}-free MBS. Traces A–F show the response of the cells to added $BaCl_2$ at 6, 12, 24, 30, 36 and 48 h after infection respectively. Results are representative of two independent infections.

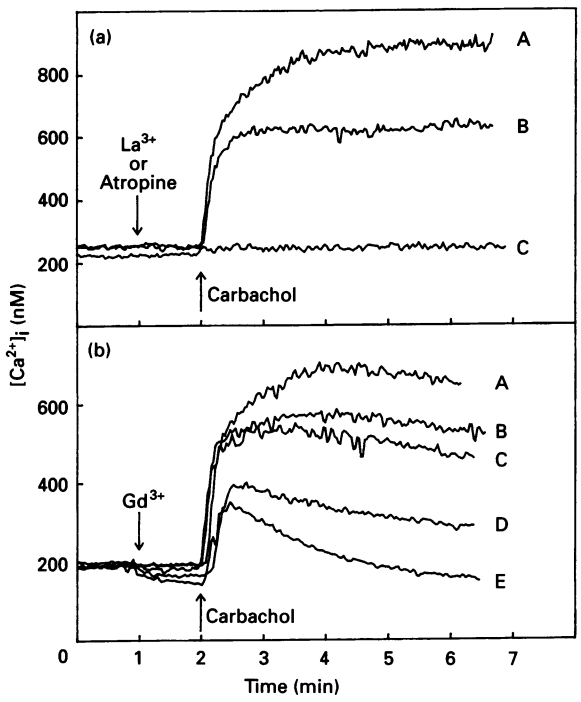

Figure 3 Effect of carbachol on $[Ca^{2+}]_i$ in Sf9 cells expressing both the M5 muscarinic receptor and TrpI

At 30–36 h after infection, M5–TrpI cells were harvested and loaded with Fura-2. (**a**) Three traces are shown superimposed. Carbachol (100 μM) was added at the time indicated by the arrow, in the control (trace A) and in the presence of either $LaCl_3$ (10 μM; trace B) or atropine (10 μM; trace C) added at the time indicated. (**b**) Five traces are superimposed. Carbachol was added at the time indicated in the absence (trace A) or in the presence of 1, 10, 100 or 1000 μM $GdCl_3$ (traces B–E respectively).

same fashion in M5-infected Sf9 cells (Figure 1c). Addition of Ba^{2+} to the extracellular bath after carbachol stimulation produced a change in fluorescence indicative of Ba^{2+} influx into the cell. Ba^{2+} influx was blocked by the addition of La^{3+} to the extracellular bath before carbachol stimulation. However, addition of La^{3+} during carbachol-induced Ba^{2+} influx did not return fluorescence to the basal level as was seen for Ca^{2+}; La^{3+} simply prevented further influx of Ba^{2+} into the cell. These results suggest that, like mammalian cells, the agonist-activated Ca^{2+}-influx pathway in Sf9 cells is permeable to Ba^{2+}, but Ba^{2+} cannot be removed from the cytosol by intracellular pumping mechanisms.

Effect of receptor stimulation on TrpI-induced Ca^{2+} influx

To test the hypothesis that TrpI can be activated and/or regulated by endogenous signal-transduction mechanisms, Sf9 cells were co-infected with baculoviruses containing the cDNA for the M5 receptor and the TrpI protein (M5–TrpI cells). Basal $[Ca^{2+}]_i$ in M5–TrpI cells increased as a function of time after infection (Figure 2a); it was unchanged from time zero to 24 h, but progressively increased thereafter. In contrast, basal $[Ca^{2+}]_i$ was unchanged in M5 cells up to 48 h after infection (Hu et al., 1994a). Previous studies have shown that TrpI is permeable to both Ca^{2+} and Ba^{2+} (Hu et al., 1994b). The increase in basal $[Ca^{2+}]_i$ in M5–TrpI cells was also associated with an increase in basal Ba^{2+} influx as judged by an increase in fluorescence ratio seen after addition of Ba^{2+} to cells incubated in Ca^{2+}-free buffer (Figure 2b). Basal Ba^{2+} influx was not detectable until 24 h after infection, but increased in a time-dependent fashion between 24 and 48 h after infection. Thus, as in Sf9 cells infected with TrpI alone (Hu et al., 1994b), TrpI channels in co-infected cells appear to be constitutively active giving rise to elevated basal $[Ca^{2+}]_i$.

Addition of carbachol to M5–TrpI cells produced a large increase in $[Ca^{2+}]_i$ to a sustained value of 677 ± 143 nM (Figure 3a; $n = 17$). This change in $[Ca^{2+}]_i$ was completely inhibited by prior addition of atropine, but was only partially blocked by extracellular La^{3+}. Thus it appears that a large component of the response of M5–TrpI cells was dependent on receptor stimulation, but insensitive to low concentrations of La^{3+}. The sustained component was, however, sensitive to Gd^{3+} (Figure 3b). Addition of 1–10 μM Gd^{3+} before carbachol had little effect on basal $[Ca^{2+}]_i$ and produced only a small inhibition of the sustained component of the $[Ca^{2+}]_i$ response. Increasing Gd^{3+} from 10 μM to 1 mM produced a graded decrease in the basal $[Ca^{2+}]_i$ in the M5–TrpI cells and inhibition of the sustained change in $[Ca^{2+}]_i$ observed after addition of carbachol. These results suggest that carbachol stimulates Ca^{2+} influx in M5–TrpI cells through two pathways: (1) the endogenous pathway that is blocked by low

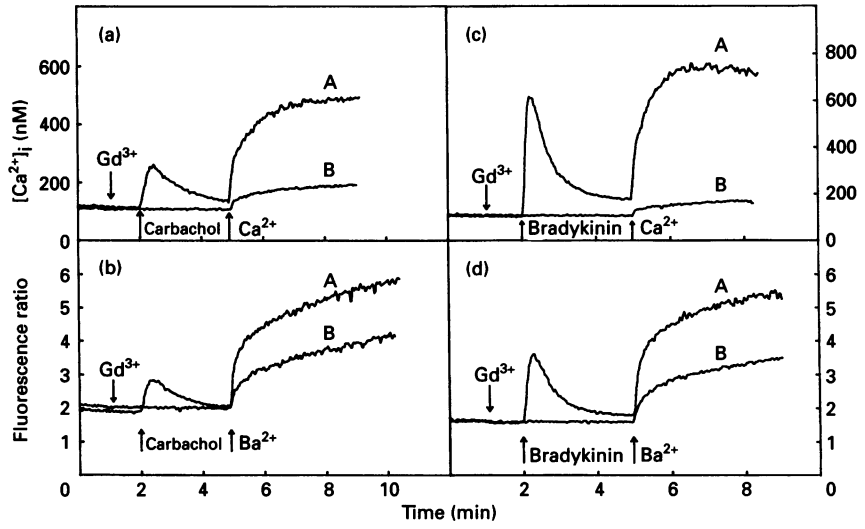

Figure 4 Receptor-stimulated influx of Ca^{2+} and Ba^{2+} through Trpl

(**a** and **b**) Fura-2-loaded M5–Trpl cells were incubated in Ca^{2+}-free buffer. Two traces are superimposed in each panel. At the times indicated, $GdCl_3$ (10 μM) and $CaCl_2$ (10 mM; **a**) or $BaCl_2$ (10 mM; **b**) were added for each trace. Carbachol (100 μM) was added only for trace A in each panel at the time indicated by the arrow. (**c** and **d**) Sf9 insect cells were co-infected with recombinant baculoviruses containing the cDNA for a human bradykinin receptor and for the Trpl protein (BK–Trpl cells). Fura-2-loaded BK–Trpl cells were incubated in Ca^{2+}-free MBS. At the times indicated, $GdCl_3$ (10 μM) and $CaCl_2$ (10 mM; **c**) or $BaCl_2$ (10 mM; **d**) were added for each trace. Bradykinin (50 nM) was added only for trace A in each panel at the time indicated by the arrow.

concentrations of Gd^{3+} and (2) through Trpl which is blocked by high concentrations of Gd^{3+}.

To confirm that receptor stimulation activates Trpl we examined Ba^{2+} influx in M5–Trpl cells incubated in Ca^{2+}-free buffer (Figure 4). These experiments were performed in the presence of 10 μM Gd^{3+} to eliminate any contribution from the endogenous-receptor-activated pathway. Addition of carbachol under these conditions produced an increase in $[Ca^{2+}]_i$ indicative of Ca^{2+} release from internal stores (Figure 4a). Subsequent readdition of Ca^{2+} to the extracellular buffer produced a large increase in Ca^{2+} indicative of carbachol-induced Ca^{2+} influx through Trpl. In the absence of carbachol, readdition of Ca^{2+} produced only a small increase in $[Ca^{2+}]_i$. The difference between traces A and B in Figure 4(a) therefore represents carbachol-induced Ca^{2+} influx through Trpl. In Figure 4(b), identical experiments were performed with Ba^{2+} added in the presence or absence of M5-receptor stimulation. As reported previously (Hu et al., 1994b), Ba^{2+} influx was elevated in Trpl-infected cells under basal non-stimulated conditions (Figure 4b, trace B). However, Ba^{2+} influx through Trpl was clearly stimulated by prior addition of carbachol (Figure 4b, trace A). A similar profile for Ca^{2+} influx and Ba^{2+} influx through Trpl is observed in Sf9 cells co-infected with baculoviruses containing the cDNA for the bradykinin receptor and the Trpl protein (BK–Trpl cells; Figures 4c and 4d), suggesting that receptor stimulation of Trpl is not dependent on the type of receptor expressed. It is important to emphasize that this stimulation of influx is unrelated to the endogenous-receptor-activated Ca^{2+}-influx pathway which is blocked in these experiments by the presence of 10 μM Gd^{3+}.

Effect of thapsigargin on Trpl-induced Ca^{2+} influx

As in mammalian non-excitable cells, inhibition of the endoplasmic-reticulum Ca^{2+}-ATPase pump by thapsigargin is associated with depletion of the internal Ca^{2+} store and concomitant activation of Ca^{2+} influx. To determine whether depletion of the store also activates Trpl, the effect of thapsigargin was examined

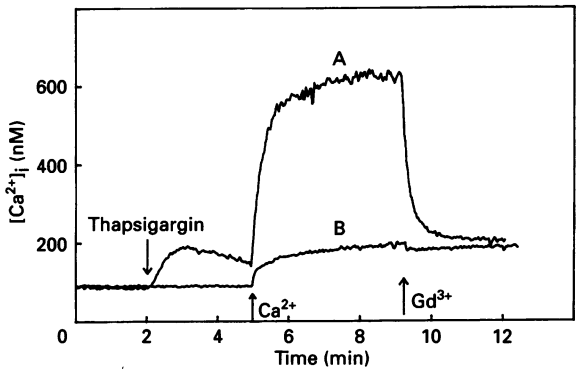

Figure 5 Effect of thapsigargin on $[Ca^{2+}]_i$ in M5–Trpl cells

Fura-2-loaded M5-Trpl cells were incubated in Ca^{2+}-free MBS. $CaCl_2$ (10 mM) and $GdCl_3$ (10 μM) were added at the times indicated in the presence (trace A) or absence (trace B) of thapsigargin (200 nM).

in M5–Trpl cells (Figure 5). Addition of thapsigargin to M5–Trpl cells incubated in Ca^{2+}-free buffer produced a 2-fold increase in $[Ca^{2+}]_i$. Subsequent readdition of Ca^{2+} to the extracellular buffer produced a dramatic increase in $[Ca^{2+}]_i$ that was rapidly reversed by addition of 10 μM Gd^{3+}. Addition of Ca^{2+} to M5–Trpl cells in the absence of thapsigargin (Figure 5, trace B) produced only a small increase in $[Ca^{2+}]_i$ that was insensitive to subsequent addition of 10 μM Gd^{3+}. These results suggest that, although thapsigargin stimulates Ca^{2+} influx through the endogenous Ca^{2+}-influx pathway, depletion of the internal Ca^{2+} stores has no effect on Ca^{2+} influx through Trpl. To confirm this result, the effect of thapsigargin on Ba^{2+} influx was examined in M5–Trpl cells and in BK–Trpl cells (Figure 6). Again, these experiments were performed in the presence of low Gd^{3+} to eliminate the endogenous influx component. In both cell types, thapsigargin was without effect on Ba^{2+} influx through Trpl.

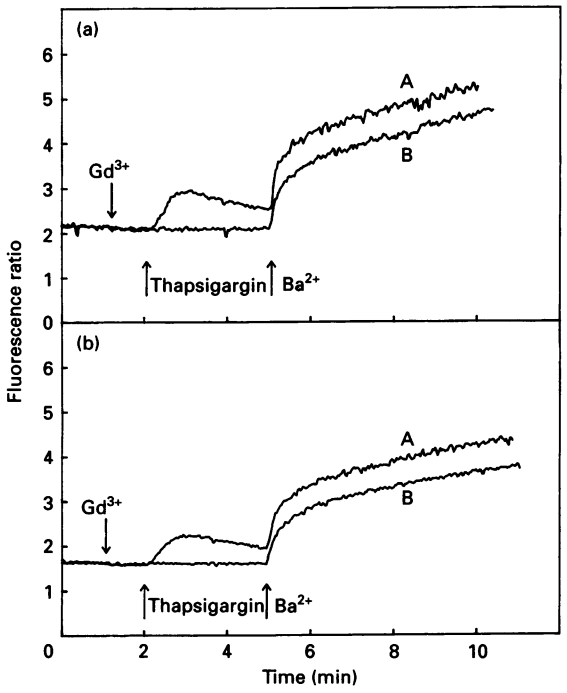

Figure 6 Effect of thapsigargin on Ba^{2+} influx in M5–Trpl cells and BK–Trpl cells

(**a**) Fura-2-loaded M5–Trpl cells were incubated in Ca^{2+}-free MBS. $GdCl_3$ (10 μM) and $BaCl_2$ (10 mM) were added at the times indicated in the absence (trace B) or presence (trace A) of thapsigargin (200 nM). (**b**) Fura-2-loaded BK–Trpl cells were incubated in Ca^{2+}-free MBS. $GdCl_3$ (10 μM) and $BaCl_2$ (10 mM) were added at the times indicated in the absence (trace B) or presence (trace A) of thapsigargin (200 nM).

DISCUSSION

The results of the present study extend our observations on the functional expression of the *Drosophila* protein, Trpl, using the baculovirus–Sf9 insect cell expression system. We have previously shown that Fura-2 is useful as an intracellular indicator of $[Ca^{2+}]_i$ in Sf9 cells and that mammalian receptors linked to changes in $[Ca^{2+}]_i$ are functional after infection with recombinant baculoviruses containing the cDNA for these receptors (Chen and Schilling, 1994; Hu et al., 1994a; Schilling et al., 1994). Furthermore, we recently demonstrated that expression of Trpl is associated with an increase in basal $[Ca^{2+}]_i$ indicative of increased plasmalemmal permeability to Ca^{2+} (Hu et al., 1994b). Trpl was found to be permeable to Ba^{2+} and was also shown to have a low sensitivity to the inorganic Ca^{2+}-channel blockers, La^{3+} and Gd^{3+} (Hu et al., 1994a,b). In contrast, the endogenous receptor-mediated Ca^{2+}-influx pathway in Sf9 cells is completely blocked by low concentrations of La^{3+} and Gd^{3+}. This difference in sensitivity to Gd^{3+} was used in the present study to evaluate selectively the effect of receptor stimulation of Trpl activity. The results clearly show that Trpl is activated by a receptor-dependent mechanism, but is unaffected by thapsigargin suggesting that depletion of the internal Ca^{2+} store is not sufficient to activate Trpl.

To date, there is no information in the literature about the function or regulation of Trpl in *Drosophila* photoreceptor cells. Thus the identity between Trpl and the non-selective cation channels responsible for the transient light-activated currents found in the *trp* mutant remains highly speculative. This proposal is supported, however, by the similarity between Trpl and voltage-gated Ca^{2+} and Na^+ channels (Phillips et al., 1992; Hardie and Minke, 1993). Furthermore, it is clear that the response of the photoreceptor cell to light consists of two distinct current components, one of which is selective for Ca^{2+}, is blocked by low concentrations of La^{3+} and is absent from the *trp* mutant (Hardie and Minke, 1992). The current observed after light stimulation in the *trp* mutant is, however, similar to the La^{3+}-insensitive current seen in wild-type cells with respect to ion selectivity and activation time course (Hardie and Minke, 1992). Thus the apparent low sensitivity of recombinant Trpl to La^{3+} and Gd^{3+} is consistent with the proposal that Trpl is responsible for the residual current seen in the *trp* mutant. One note of caution, this interpretation is further complicated by the fact that the actual subunit structure of the light-sensitive channels of *Drosophila* is unknown. Voltage-gated Na^+ and Ca^{2+} channels are large monomeric structures composed of four homologous motifs, each containing six putative transmembrane regions [for a review see Catterall (1993)]. However, in a fashion analogous to K^+ channels, the light-sensitive channels may be heterotetrameric structures composed of both Trp and Trpl monomers. In the Sf9 cells, expression of Trpl alone presumably results in channels composed of only *trpl* gene product, but this remains to be confirmed.

Although the actual subunit structure of the light-activated channels in the photoreceptor cell remains unknown, these channels are normally closed in the absence of light. Thus it seems unusual that recombinant Trpl is constitutively active in the Sf9 cell under basal non-stimulated conditions. The activity of Trpl increased in a time-dependent fashion after infection with recombinant baculovirus as determined by both an increase in basal $[Ca^{2+}]_i$ and a progressive increase in plasmalemmal permeability to Ba^{2+}. There are a number of possible explanations for this constitutive activity. First, some protein or factor not present in the Sf9 cell may be necessary to maintain low channel activity in *Drosophila*. Alternatively, some factor in the Sf9 cell that is normally low or absent in *Drosophila* may activate Trpl. Trpl was originally cloned as a calmodulin-binding protein, and two putative calmodulin-binding domains have been identified from sequence analysis (Phillips et al., 1992). It is possible that Trpl may be a Ca^{2+}/calmodulin-regulated protein and that Ca^{2+} activates in a positive feedback mechanism. Early after infection, Trpl may be only partially active and produce only a small change in $[Ca^{2+}]_i$, but as more and more of the channels are inserted into the membrane, basal $[Ca^{2+}]_i$ may increase, further activating Trpl channels. It is clear, however, that a rise in $[Ca^{2+}]_i$ *per se* is not sufficient for activation of Trpl as thapsigargin produced a large sustained increase in $[Ca^{2+}]_i$, but did not activate Trpl (see below). Another possibility is that the apparent constitutive activity reflects the 'overexpression' of Trpl in the Sf9 cell. There may be two pools of expressed Trpl channels, those that are closed until activated by receptor-dependent mechanisms and those that are constitutively active. The proportion of channels in the two pools may change as a function of time after infection. Early after infection, Trpl channels may be regulated (i.e. inactivated or closed) by endogenous mechanisms within the Sf9 cell. However, at later times, an 'excess' channel density may exceed the regulatory capacity of the Sf9 cell leading to the constitutively active Trpl channels. Electrophysiological studies in *Drosophila* photoreceptor cells support the hypothesis that the light-activated channels are normally inactive, but become spontaneously active during the first 7 min after establishment of the whole-cell recording mode (Hardie and Minke, 1994). This 'rundown current' has a reversal potential and sensitivity to La^{3+} similar to the light-activated current and is absent from or reduced and altered in the *trp* mutant. These results suggest that the rundown current is mediated by Trp-

dependent channels. The mechanisms responsible for spontaneous activation of Trp in *Drosophila* photoreceptor cells and the possible role for Trpl channels in this response remains unknown.

Irrespective of the mechanism responsible for constitutive Trpl activity, the results of the present study clearly demonstrate that recombinant Trpl can be activated by a receptor-dependent mechanism. Addition of carbachol to M5–Trpl cells produced a large sustained increase in $[Ca^{2+}]_i$ that was blocked by prior addition of atropine. The large sustained increase in $[Ca^{2+}]_i$ was little affected by a low concentration of lanthanides, but could be inhibited by Gd^{3+} in the 0.1–1 mM range. Furthermore, stimulation by carbachol in M5–Trpl cells or by bradykinin in BK–Trpl cells increased Ba^{2+} influx. These results strongly suggest that Trpl is regulated by receptor stimulation. There are four possible mechanisms by which this can occur. The Trpl channels may be regulated by (1) the change in $[Ca^{2+}]_i$, (2) the rise in Ins(1,4,5)P_3 or some other cytosolic messenger, (3) depletion of the internal Ca^{2+} store or (4) a membrane-delimited mechanism perhaps involving G-protein-mediated activation. It seems unlikely that depletion of the store *per se* activates Trpl as neither Ca^{2+} nor Ba^{2+} influx were affected by thapsigargin. Likewise, it seems unlikely that a rise in $[Ca^{2+}]_i$ *per se* is responsible for the activation of Trpl as thapsigargin did produce a large increase in $[Ca^{2+}]_i$ (Figure 5), but did not activate Trpl. In preliminary studies, we have found that the phospholipase C inhibitor, U73122 (Smith et al., 1990), blocks the response of M5–Trpl cells to carbachol (Y. Hu and W. P. Schilling, unpublished work) suggesting that activation of Trpl is distal to the production of either Ins(1,4,5)P_3 or diacylglycerol.

The effect of thapsigargin on membrane currents in insect photoreceptor cells has not been reported; however, the finding that Trpl is not activated by thapsigargin suggests that it is not the insect counterpart of the mammalian channel responsible for the Ca^{2+}-release-activated current (Hoth and Penner, 1992, 1993; Vaca and Kunze, 1993; Zweifach and Lewis, 1993). In a recent study, however, we found that expression of Trp in the Sf9 cell was associated with the appearance of a Ca^{2+}-permeable cation channel (Vaca et al., 1994). The Trp channels were not constitutively open, but could be activated by depletion of the internal Ca^{2+} store by thapsigargin. These results suggest that the proposed C-terminal cytoplasmic tail of Trp, which is absent from Trpl, may be important for capacitative Ca^{2+} entry. The baculovirus–Sf9 insect cell expression system may prove useful for the functional expression of Trp/Trpl chimaeric channel proteins and evaluation of those structural features necessary for regulated Ca^{2+} influx.

We thank Dr. Leonard E. Kelly for providing the *trpl* clone. This study was supported in part by grants HL44119 and HL47876 from the National Institutes of Health, Bethesda, MD, U.S.A. and performed during the tenure of an Established Investigatorship awarded to W.P.S. by the American Heart Association.

Received 20 June 1994/17 August 1994; accepted 26 August 1994

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