The *Drosophila* cation channel *trpl* expressed in insect Sf9 cells is stimulated by agonists of G-protein-coupled receptors


Institut für Pharmakologie, Freie Universität Berlin, Thielallee 69–73, D-14195 Berlin, Germany

Received 9 October 1994; revised version received 27 December 1994

Abstract Structures and regulations of vertebrate channels responsible for sustained calcium elevations after hormone stimulation are largely unknown. Therefore, the *Drosophila* photoreceptor channels, *trp* and *trpl*, which are assumed to be involved in calcium influx, serve as model system. *trpl* expressed in Sf9 cells showed spontaneous activity. Hormonal stimulations of calcium influx (detected by fura-2) and of an outwardly rectifying current were observed in Sf9 cells coinfected with baculoviruses encoding *trpl* and various heptahelical receptors for histamine, thrombin, and thromboxane A2, all known to cause phospholipase C-β activation in mammalian cells. Although the identity of the G-protein and of possible second messengers involved need to be clarified, it is clear that *trpl* represents a receptor/G-protein regulated cation channel.

Key words: Non-selective cation channel; G-protein; Hormonal effects; *trpl*; *Drosophila* ion channel; Baculovirus/Sf9 system

1. Introduction

Numerous hormones and neurotransmitters interacting with heptahelical, G-protein-coupled receptors increase the cytoplasmic calcium concentration in a biphasic manner with a rapid IP3-mediated release of calcium from intracellular storage sites and subsequent sustained influx of calcium from the extracellular space [1,2]. Stimulation of voltage-dependent calcium channels is involved in the calcium influx in endocrine [3] and possibly in vascular smooth muscle cells. In most other cellular systems, non-selective cation channels and/or calcium release-activated calcium (CRAC) currents mediate calcium influx and serve prolonged calcium elevation and refill IP3-sensitive calcium storage sites [4,5]. Neither one of these two channels has been isolated nor the cDNA been cloned from vertebrate systems.

In *Drosophila* photoreceptor cells, light induces phospholipase C stimulation with IP3-induced calcium release and concomitant calcium influx from the extracellular space, connected to sustained depolarization and response to light [6]. At least two channels are assumed to be involved in this calcium influx. One is selective for calcium and is absent in the transient receptor potential *Drosophila melanogaster* mutant *trp* [7]. The cloned *trp* gene shows some homology to voltage-dependent ion channels [8] and structural similarity to second messenger-gated ion channels found in mammalian systems [9], and it is assumed that the *trp* gene product is related to mammalian channels whose opening is underlying the capacitative calcium entry, i.e. the CRAC current [6]. Based on its primary structure, *trpl*, another *Drosophila* gene, is assumed to code for a calcium/calmodulin-stimulated non-selective cation channel [8], contributing with *trp* to the sustained response to light.

The *trp* channel was electrophysiologically characterized in *Drosophila* photoreceptor cells [7]. It was stimulated after baculovirus infection of insect Sf9 cells by extracellular application of thapsigargin, an inhibitor of the calcium ATPase in the endoplasmic reticulum [10]. In contrast, expression of *trpl* in Sf9 cells induced a non-selective cation channel with constitutive activity which was not stimulated by thapsigargin [10,11]. Here we provide evidence that *trpl* expressed in Sf9 cells can be activated by hormonal factors after coinfection with recombinant viruses coding for various heptahelical, G-protein-coupled receptors.

2. Materials and methods

2.1. Cloning of the *trpl* cDNA

A 1166 bp fragment corresponding to nucleotides 1170–2334 of the *trpl* cDNA was amplified by PCR from a *Drosophila* head cDNA library (from E. Buchner, Universität Würzburg) and was used to screen the same library by a plaque hybridization technique according to standard methods [12]. After screening, one lambda phage containing the complete *trpl* cDNA was isolated and confirmed by DNA sequencing.

2.2. Sf9 cell culture, cloning and expression of recombinant baculoviruses

Monolayer fall armyworm ovary cells (Sf9 from ATCC, Rockville, MD) were propagated in TMN-FH medium (Sigma, Deisenhofen) supplemented with 10% (v/v) fetal calf serum. When cells were grown in suspension, Lipid Concentrate (1:200; Life Technologies, Karlsruhe) was added. cDNAs encoding the guinea pig histamine H1, the rat histamine H2, the human thrombin and the human thromboxane A2 receptors and *trpl* were subcloned to baculovirus transfer vectors (pVL1392, pVL1393 from Invitrogen, Leek, Netherlands, and pAcMP2, pAcMP3 from Dianova, Hamburg). Recombinant viruses were generated by cotransfection of Sf9 cells with the cDNA constructs and BaculoGOLD Baculovirus DNA (Dianova, Hamburg) by the lipofection method [13]. The subsequent plaque assay and amplification procedure were performed according to standard techniques [14]. Cells were infected at a multiplicity of infection of about 3 viruses per cell.
in the case of receptor-coding viruses and 3 to 5 in the case of trpl-coding virus.

2.3. Determination of the cytoplasmic Ca\textsuperscript{2+} concentration in infected Sf9 cells

Determination of [Ca\textsuperscript{2+}] in a Perkin-Elmer Luminescence spectrometer LS 50B by the fura-2 method was performed as in [11] except for growing Sf9 cells in spinner cultures and loading the cells with fura-2 acetoxymethyl (Molecular Probes, Eugene, OR) after 22–28 h of infection in the presence of 10 mM Ca\textsuperscript{2+}. Thereafter, the cells were resuspended in buffer containing 0.5 mM Ca\textsuperscript{2+}. Fluorescence was recorded at room temperature. Fluorescence signals were calibrated after lysis of cells with 0.4% (v/v) Triton X-100 (maximal fluorescence) and subsequent addition of 50 mM EGTA (minimal fluorescence). [Ca\textsuperscript{2+}] was calculated according to the equation in [15], using a $K_d$ value of 278 nM for Ca\textsuperscript{2+} binding to fura-2 at 22°C [16].

2.4. Electrophysiological studies

Electrophysiological experiments were performed using the whole-cell patch-clamp technique [17]. To improve the adherence of the infected Sf9 cells to the glass surface, glass was coated with poly-L-lysine (Biochrom, Berlin). Sf9 cells were tested at time intervals of 22–48 h after infection. Data were analyzed using the pCLAMP software (version 6, Axon Instruments, Foster City, CA). The cells tested were held at 0 mV. The current–voltage relations were recorded using linear ramp pulses from −100 to +100 mV with a slope of 0.4 mV/ms. The composition of the pipette solution was (mM): CsCl 120, glucose 70, PIPES 10. The extracellular solutions contained (mM): E1 (standard extracel-

Fig. 1. Whole-cell membrane currents in baculovirus-infected Sf9 cells. (A) An outwardly rectifying current was observed in cells infected with trpl virus, which was blocked by Gd\textsuperscript{3+}; numbers at the curves indicate mM Gd\textsuperscript{3+} concentrations. Cells were infected with trpl virus for 48 h. For experiments shown in all other panels, cells were infected for 22–28 h. (B) Currents in trpl-infected cell were recorded before (co) and 5 min after infusion of 200 μM GTPyS plus 5 mM Mg\textsuperscript{2+}. (C) and (D) Endogenous currents were shown without (co) and 1 min after addition of 20 μM histamine (his) in cells infected with viruses encoding histamine H\textsubscript{1} and H\textsubscript{2} receptors, respectively. (E) to (H) Cells were coinfected with viruses encoding trpl plus histamine (H\textsubscript{1}, H\textsubscript{2}), thrombin, and thromboxane A\textsubscript{2} receptors, respectively. Currents before (co) and after stimulation (1 min) by 20 μM histamine (his; (E) and (F)), 10 U/ml human thrombin (thr; (G)), and 5 μM U46619 (U46619; (H)) are shown.
lular solution): NaCl 110, KCl 10, CaCl2 1.8, MgCl2 1, sucrose 70, glucose 10, PIPES 10; E2: sodium isethionate (2-hydroxyethanesulfon-ate) 110, KOH 10, calcium gluconate 1.8, magnesium gluconate 1, sucrose 70, glucose 10, PIPES 10. The solutions were adjusted to pH 6.5 with HCl (the pH of E2 was adjusted with methanesulfonic acid). In experiments in which Cl− was substituted with isethionate anion in the extracellular solution the ground electrode was connected to the bath through an agar bridge. Borosilicate glass pipettes had a resistance of 3–5 MΩ. All experiments were performed at room temperature.

3. Results and discussion

When extracellular Ca2+ was present, infection of Sf9 cells with recombinant viruses containing the trpl cDNA increased the cytoplasmic Ca2+ concentration from about 50 to 300 nM or more; the effect increased with time, starting around 24 h after infection. This elevation of [Ca2+]i was largely reduced by adding 1 mM Gd3+ to the extracellular solution (data not shown). Likewise, we observed a spontaneous cation permeability by recording whole-cell currents in Sf9 cells which had been infected with the trpl virus for 48 h (Fig. 1A). The amplitude of the current amounted to 1.13 ± 0.25 nA (mean ± S.E.M., n = 15) at +90 mV. The current showed outward rectification and reversed at about 0 mV. Similar to the Ca2+ influx recorded by the fura-2 technique, the current was blocked by 1 mM Gd3+ (n = 5, see Fig. 1A). The current had similar amplitude and reversal potential (about 0 mV) when Cl− was omitted from the extracellular solution, indicating that the current was carried by cations (n = 3). Intracellular CsCl (120 mM in all experiments) did not affect the current, making the involvement of K+ channels unlikely. These data indicate that trpl is functionally expressed in Sf9 cells and allows cation entry, similar to recently reported observations [11].

To study the possible regulation by hormones acting via heptahelical receptors and G-proteins, we infected Sf9 cells with different viruses containing receptor cDNAs. In the absence of extracellular Ca2+, histamine, thrombin (or thrombin receptor peptide) and U46619 (a thromboxane A2 receptor agonist) induced rapid and transient increases in [Ca2+]i in Sf9 cells infected with histamine H1, thrombin and thromboxane A2 receptor viruses, respectively (Fig. 2A,B). In contrast, the effect of histamine was delayed when the cells were infected with histamine H2 receptor virus (see Fig. 2B). Coinfection of the receptor viruses with the trpl virus did not change the shape or magnitude of the hormone-induced increases in [Ca2+]i (see
tors are capable of stimulating Gs-dependent and GfAMP-regulated endogenous and intracellular application of GTP-7S (0.76 ± 0.29 nA, n = 4) to H2 (2.32 ± 0.66 nA, n = 8, Fig. 1F), thrombin (2.18 ± 0.33 nA, n = 4, Fig. 1G) or thromboxane A2 (2.34 ± 0.48 nA, n = 5, Fig. 1H) receptor viruses. Stimulation by agonists of all four receptors resulted in activation of an endogenous conductance (0.32 ± 0.05 nA, n = 8 for H1, 0.27 ± 0.05 nA, n = 8 for H2, 0.28 ± 0.05 nA, n = 4 for thrombin, 0.66 ± 0.1 nA, n = 5 for thromboxane A2 receptors at +90 mV), starting after 20–40 s and reaching a maximum after 120–240 s. Activation of this current by histamine in cells infected with H1 and H2 receptor viruses is shown in Fig. 1C and D, respectively (data not shown for the other agonists). The current showed a slight inward rectification with a reversal potential of about 0 mV. Under Cl-‐free extracellular conditions (E2), the reversal potential shifted from about 0 mV by about +50 to +100 mV. NPPB (10 μM), a blocker of Cl- channels, inhibited this current, suggesting that the current mostly represents a Cl- conductance (data not shown).

When Sf9 cells were coinfected with 24 h with viruses containing trpl and histamine H1 receptor cDNAs, most cells (25 of 35 cells studied) showed a large current with outward rectification (1.92 ± 0.34 nA at +90 mV) starting after 10–20 s and reaching a maximum 40–120 s after application of histamine (Fig. 1E). This current was clearly different from the endogenous current activated by the agonist (observed in 6 of 35 cells, which were possibly not infected by the trpl virus). Some cells (10 of 35) showed a mixture of the two conductivities. We obtained similar results, i.e. fast hormonal stimulation of an outwardly rectifying current with or without apparent stimulation of the endogenous conductivity, in cells coinfected with trpl and histamine H2 (2.32 ± 0.66 nA, n = 8, Fig. 1F), thrombin (2.18 ± 0.33 nA, n = 4, Fig. 1G) or thromboxane A2 (2.34 ± 0.48 nA, n = 5, Fig. 1H) receptor viruses.

The stimulatory effects of these hormones were mimicked by intracellular application of GTPYs (0.76 ± 0.29 nA, n = 4) to cells infected for 24 h with the trpl virus. The effect developed at about 2 min after membrane rupture and reached its maximum at about 5 min (Fig. 1B).

These data show that trpl expressed in Sf9 cells can be stimulated by agonists interacting with receptors inducing phospholipase C-gamma stimulation in mammalian systems. G-proteins appear to be involved in the agonist- and GTPYs-induced stimulations of endogenous and trpl currents. Histamine H1, thrombin and thromboxane A2 receptors are known to interact in vertebrate systems with G-proteins of the Gq family, the thrombin receptor in addition interacts with G-proteins of the Gi family [18,19]. In contrast, agonists of histamine H2 receptors are capable of stimulating G2-dependent and G/CAMP-independent pathways [20,21]. Therefore, G2- and G1-like G-proteins may be involved in the stimulations of endogenous and trpl currents in Sf9 cells. Differentiation of G2- and G1-mediated effects by treatment of Sf9 cells with pertussis toxin is not possible since the toxin does not interfere with G-mediated reactions in intact cells [22] even at very high concentrations. Whether activated α-subunits or free βγ-complexes activate trpl channels directly or induce the formation of a cytosolic second messenger stimulating the channel's activity needs to be determined in future studies using insertions and isolated patches of trpl-expressing cells.

Acknowledgements: We thank Jürgen Mallikewitz for Sf9 culture. cDNAs of histamine (H1, H2), thromboxane A2, and thrombin receptors were kindly provided by Drs. J.-C. Schwartz and E. Traiffort (Paris, France), P.V. Halushka and C.J. Allain (Charleston, SC), and S.R. Coughlin (San Francisco, CA), respectively. NPPB was a gift from Dr. R. Greger (Freiburg). We thank Drs. T. Gudermann and A. Lückhoff for critical reading of the manuscript and valuable suggestions. The studies were supported by Bundesministerium für Forschung und Technologie, Deutsche Forschungsgemeinschaft and Fonds der Chemischen Industrie. A.G.O. was supported by the Alexander von Humboldt Stiftung.

References