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

Christian Harteneck**, Alexander G. Obukhov**, Andrea Zobel, Frank Kalkbrenner, Günter Schultz*

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 spontanous 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 A_2, all known to cause phospholipase C-β activation in mammalian cells. Although the identity of the G-proteins 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 IP_3-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 IP_3-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 IP_3-induced calcium release and concomitant calcium influx from the extracellular space, connected to sustained depolarization and reponse 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 TNM-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 H_1, the rat histamine H_2, the human thrombin and the human thromboxane A_2 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

*Corresponding author. Fax: (49) (30) 831-5954.

**These two authors contributed equally to the work.

Abbreviations: $[Ca^{2+}]_i$, cytoplasmic Ca^{2+} concentration; EGTA, ethylene glycol-bis(β-aminoethyl ether)*N,N,N′,N′*-tetraacetic acid; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); IP_3, inositol-1,4,5-trisphosphate; NPPB, 5-nitro-2-(3-phenylpropylamino)benzoic acid; PCR, polymerase chain reaction; PIPES, piperazine-*N,N′*-bis(2-ethanesulfonic acid); U46619, 9,11-dideoxy-11α,9α-epoxymethano prostaglandin $F_{2\alpha}$.

0014-5793/95/$9.50 © 1995 Federation of European Biochemical Societies. All rights reserved.
SSDI 0014-5793(94)01455-8

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^{2+} concentration in infected Sf9 cells

Determination of $[Ca^{2+}]_i$ 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^{2+}. Thereafter, the cells were resuspended in buffer containing 0.5 mM Ca^{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). $[Ca^{2+}]_i$ was calculated according to the equation in [15], using a K_d value of 278 nM for Ca^{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-

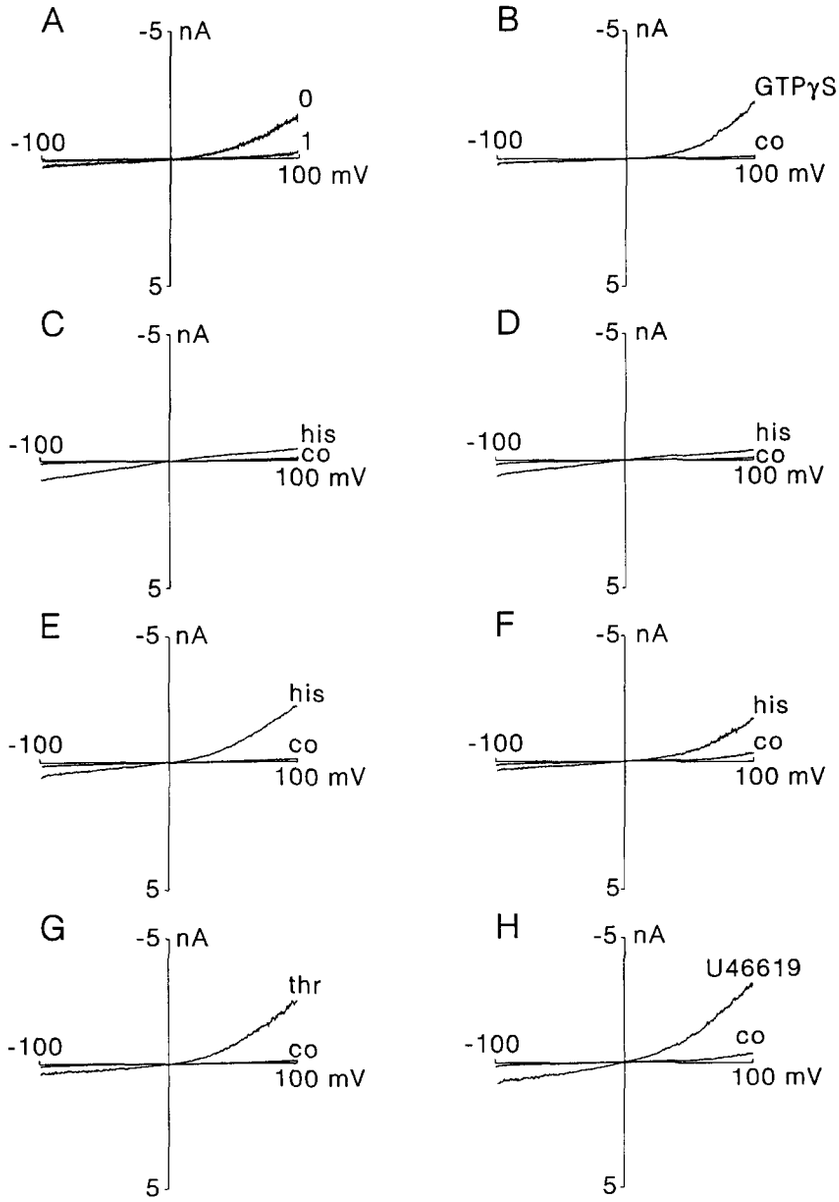

Fig. 1. Whole-cell membrane currents in baculovirus-infected *Sf9* cells. (A) An outwardly rectifying current was observed in cells infected with *trpl* virus, which was blocked by Gd^{3+}; numbers at the curves indicate mM Gd^{3+} concentrations. Cells were infected with *trpl* virus for 48 h. For experiments 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 GTPγS plus 5 mM Mg^{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_1 and H_2 receptors, respectively. (E) to (H) Cells were coinfected with viruses encoding *trpl* plus histamine (H_1, H_2), thrombin, and thromboxane A_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, $CaCl_2$ 1.8, $MgCl_2$ 1, sucrose 70, glucose 10, PIPES 10; *E2*: sodium isethionate (2-hydroxyethanesulfonate) 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 Ca^{2+} was present, infection of *Sf9* cells with recombinant viruses containing the *trpl* cDNA increased the cytoplasmic Ca^{2+} concentration from about 50 to 300 nM or more; the effect increased with time, starting around 24 h after infection. This elevation of $[Ca^{2+}]_i$ was largely reduced by adding 1 mM Gd^{3+} 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 Ca^{2+} influx recorded by the fura-2 technique, the current was blocked by 1 mM Gd^{3+} (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 Ca^{2+}, histamine, thrombin (or thrombin receptor peptide) and U46619 (a thromboxane A_2 receptor agonist) induced rapid and transient increases in $[Ca^{2+}]_i$ in *Sf9* cells infected with histamine H_1, thrombin and thromboxane A_2 receptor viruses, respectively (Fig. 2A,B). In contrast, the effect of histamine was delayed when the cells were infected with histamine H_2 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 $[Ca^{2+}]_i$ (see

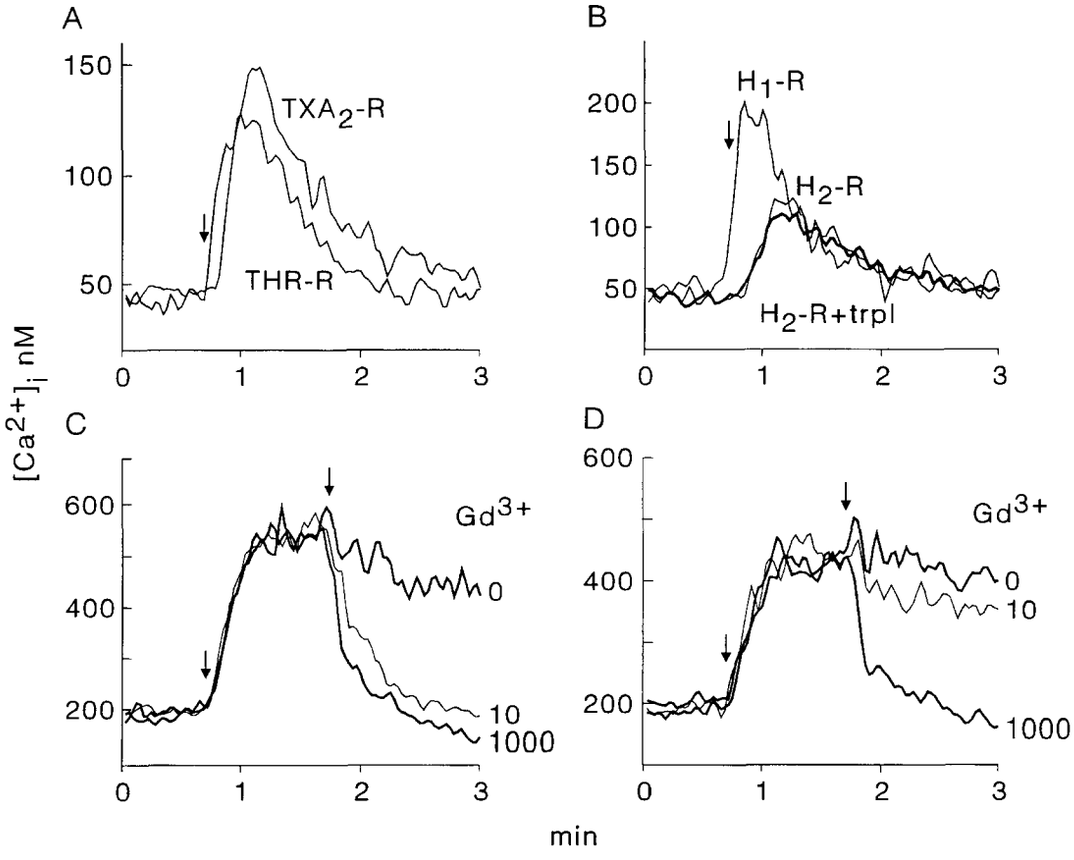

Fig. 2. Time courses of intracellular Ca^{2+} concentrations of baculovirus-infected *Sf9* cells by the fura-2 method. (A) *Sf9* cells were infected with viruses encoding the thrombin receptor (THR-R) or the thromboxane A_2 receptor (TXA_2-R). The arrow indicates the addition of 100 μM thrombin receptor peptide (SFLLRN-NH_2) and 5 μM U46619, respectively. Experiments were performed after 2 independent infections in 2 parallel $[Ca^{2+}]_i$ determinations. (B) *Sf9* cells were infected with viruses encoding the histamine H_1 receptor (H_1-R), the histamine H_2 receptor (H_2-R) or were coinfected with H_2-R and *trpl* viruses (H_2-R + *trpl*, bold line). The arrow indicates the addition of 100 μM histamine. In (A) and (B), measurements were performed by complexing free Ca^{2+} with 5 mM EGTA, added 20 s before start of the recording. (C) *Sf9* cells were infected with histamine H_2-R viruses or, in (D), were coinfected with H_2-R and *trpl*. The experiments were performed in the presence of extracellular Ca^{2+} adjusted to 10 mM one minute before agonist addition. The first arrow indicates addition of 100 μM histamine, the second arrow indicates the addition of $GdCl_3$ at the μM concentrations indicated in the figure. Time courses of $[Ca^{2+}]_i$ are given in bold lines for 0 and 1000 μM Gd^{3+}, in fine lines for 10 μM Gd^{3+}. Experiments shown in (B) to (D) were performed after 3 independent infections and 2 parallel recordings.

Fig. 2B for the histamine H_2 receptor; data for other receptors and agonists are not shown).

Since an unphysiological $[Ca^{2+}]_i$ elevation occurred due to the spontaneous activity of *trpl* in *Sf9* cells, we measured hormonal effects on $[Ca^{2+}]_i$ 22–26 h after infection when the *trpl* expression was presumably low. Application of Gd^{3+} at maximal $[Ca^{2+}]_i$ elevation allowed to discriminate contribution of *trpl* and endogenous currents. In cells infected only by receptor virus, 10 μM Gd^{3+} largely reduced the hormone-stimulated $[Ca^{2+}]_i$ (Fig. 2C); in contrast, 1 mM Gd^{3+} was needed for full suppression of $[Ca^{2+}]_i$ in receptor/*trpl*-coinfected cells (Fig. 2D); These results demonstrate that the endogenous Ca^{2+} influx pathway could be blocked by 10 μM Gd^{3+} whereas the *trpl* channel was sensitive to Gd^{3+} only at high (i.e. 1 mM) concentrations.

Whole-cell currents were studied in *Sf9* cells infected with histamine H_1, H_2, thrombin and thromboxane A_2 receptor viruses. Stimulation by agonists of all four receptors resulted in activation of an endogenous conductance (0.32 ± 0.05 nA, $n = 8$ for H_1, 0.27 ± 0.05 nA, $n = 8$ for H_2, 0.28 ± 0.05 nA, $n = 4$ for thrombin, 0.66 ± 0.1 nA, $n = 5$ for thromboxane A_2 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 H_1 and H_2 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 for 24 h with viruses containing *trpl* and histamine H_1 receptor cDNAs, most cells (25 of 35 cells studied) showed a large current with outward rectification (1.92 ± 0.24 nA at +90 mV) starting after 10–20 s and reaching a maximum 40–120 s after application of histamine (Fig. 1E). This currrent 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 H_2 (2.32 ± 0.66 nA, $n = 8$, Fig. 1F), thrombin (2.18 ± 0.33 nA, $n = 4$, Fig. 1G) or thromboxane A_2 (2.34 ± 0.48 nA, $n = 5$, Fig. 1H) receptor viruses.

The stimulatory effects of these hormones were mimicked by intracellular application of GTPγS (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-β stimulation in mammalian systems. G-proteins appear to be involved in the agonist- and GTPγS-induced stimulations of endogenous and *trpl* currents. Histamine H_1, thrombin and thromboxane A_2 receptors are known to interact in vertebrate systems with G-proteins of the G_q family, the thrombin receptor in addition interacts with G-proteins of the G_i family [18,19]. In contrast, agonists of histamine H_2 receptors are capable of stimulating G_s-dependent and G_s/cAMP-independent pathways [20,21]. Therefore, G_q- and G_i-like G-proteins may be involved in the stimulations of endogenous and *trpl* currents in *Sf9* cells. Differentiation of G_q- and G_i-mediated effects by treatment of *Sf9* cells with pertussis toxin is not possible since the toxin does not interfere with G_i-mediated reactions in intact cells [22] even at very high concentrations. Whether activated α-subunits or free $\beta\gamma$-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 infusions and isolated patches of *trpl*-expressing cells.

Acknowledgements: We thank Jürgen Malkewitz for *Sf9* culture. cDNAs of histamine (H_1, H_2), thromboxane A_2 and thrombin receptors were kindly provided by Drs. J.-C. Schwartz and E. Traiffort (Paris, France), P.V. Halushka and C.J. Allan (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

[1] Putney, J.W. and Bird, G.S. (1993) Endocr. Rev. 14, 610–631.
[2] Fasolato, C., Innocenti, B. and Pozzan, T. (1994) Trends Pharmacol. Sci. 15, 77–83.
[3] Gollasch, M., Kleuss, C., Hescheler, J., Wittig, B. and Schultz, G. (1993) Proc. Natl. Acad. Sci. USA 90, 6265–6269.
[4] Hoth, M., Fasolato, C. and Penner, R. (1993) Ann. NY Acad. Sci. 707, 198–209.
[5] Penner, R., Fasolato, C. and Hoth, M. (1993) Curr. Opin. Neurobiol. 3, 368–374.
[6] Hardie, R.C. and Minke, B. (1993) Trends Neurosci. 16, 371–376.
[7] Hardie, R.C. and Minke, B. (1992) Neuron 8, 643–651.
[8] Phillips, A.M., Bull., A. and Kelly, L.E. (1992) Neuron 8, 631–642.
[9] Jan, L.Y. and Jan, Y.N. (1992) Cell 69, 715–718.
[10] Vaca, L., Sinkins, W.G., Hu, Y., Kunze, D.L. and Schilling, W.P. (1994) Am. J. Physiol. 266, C1501–C1505.
[11] Hu, Y.L., Vaca, X., Zhu, L., Birnbaumer, L., Kunze, D.L. and Schilling, W.P. (1994) Biochem. Biophys. Res. Commun. 201, 1050–1556.
[12] Maniatis, T., Fritsch, E.F. and Sambrook, J. (1989) Molecular Cloning: a Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[13] Felgner, P.L., Gadek, T.R., Holm, M., Roman, R., Chan, H.W., Wenz, M., Northop, J.P., Ringold, G.M. and Danielsen, M. (1987) Proc. Natl. Acad. Sci. USA 84, 7413–7417.
[14] O'Reilly, D.R., Miller, L.K. and Luckow, V.A. (1992) Baculovirus Expression Vectors: a Laboratory Manual, Freeman, New York.
[15] Grynkiewicz, G., Poenie, M. and Tsien, R.Y. (1985) J. Biol. Chem. 260, 3440–3450.
[16] Shuttleworth, T.J. and Thompson, J.L. (1991) J. Biol. Chem. 266, 1410–1414.
[17] Hamill, O.P., Marty, A., Neher, E., Sakmann, B. and Sigworth, F.J. (1981) Pflügers Arch. 391, 85–100.
[18] Leurs, R., Traiffort, E., Arrang, J.M., Tardivel-Lacombe, J., Ruat, M. and Schwartz, J.C. (1994) J. Neurochem. 62, 519–527.
[19] Offermanns, S., Laugwitz, K.-L., Spicher, K. and Schultz, G. (1994) Proc. Natl. Acad. Sci. USA 91, 504–508.
[20] Traiffort, E., Ruat, M., Arrang, J.M., Leurs, R., Piomelli, D. and Schwartz, J.C. (1992) Proc. Natl. Acad. Sci. USA 89, 2649–2653.
[21] Seifert, R., Höer, A., Schwaner, I. and Buschauer, A. (1992) Mol. Pharmacol. 42, 235–241.
[22] Mulheron, J.G., Casanas, S.J., Arthur, J.M., Garnovskaya, M.N., Gettys, T.W. and Raymond, J.R. (1994) J. Biol. Chem. 269, 12954–12962.