Receptor-induced Activation of *Drosophila* TRPγ by Polyunsaturated Fatty Acids*

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Cellular calcium homeostasis is regulated by hormones and neurotransmitters, resulting in the activation of a variety of proteins, in particular, channel proteins of the plasma membrane and of intracellular compartments. Such channels are, for example, TRP channels of the TRPC protein family that are activated by various mediators from receptor-stimulated signaling cascades. In *Drosophila*, two TRPC channels, TRP and TRPL, are involved in phototransduction. In addition, a third *Drosophila* TRPC channel, TRPγ, has been identified and described as an auxiliary subunit of TRPL. Beyond it, our data show that heterologously expressed TRPγ formed a receptor-activated, outwardly rectifying cation channel independent from TRPL co-expression. Analysis of the activation mechanism revealed that TRPγ is activated by various polyunsaturated fatty acids generated in a phospholipase C- and phospholipase A₂-dependent manner. The most potent activator of TRPγ, the stable analogue of arachidonic acid, 5,8,11,14-eicosatetraynoic acid, induced currents in single channel recordings. Here we show that upon heterologous expression TRPγ forms a homomeric channel complex that is activated by polyunsaturated fatty acids as mediators of receptor-dependent signaling pathways. Reverse transcription PCR analysis showed that TRPγ is expressed in *Drosophila* heads and bodies. Its body-wide expression pattern and its activation mechanism suggest that TRPγ forms a fly cation channel responsible for the regulation of intracellular calcium in a variety of hormonal signaling cascades.

The number of G-protein-coupled receptors functionally expressed in *Drosophila* is still elusive. However, the access to the sequence of the entire genome for *Drosophila melanogaster* allowed the categorization of *Drosophila* G-protein-coupled receptors (GPCR)² (1). More than 100 genes coding for putative GPCRs were identified, including 22 genes encoding receptors for biogenic amines and 32 genes encoding receptors for peptides (2, 3). Many of the *Drosophila* receptors have been characterized in human embryonic kidney (HEK) 293 cells showing that signaling cascades found in mammalian cells generating second messengers like cAMP or increases in intracellular Ca²⁺ concentrations are also induced by *Drosophila* GPCRs. The application of ligands like octopamine or leucokinin to HEK293 cells expressing the Dmoa1 or CG10626 receptors ubiquitously expressed in *Drosophila* resulted in increased intracellular Ca²⁺ (4, 5). Therefore, it is likely that not only sensory processes but also many other physiological functions in *Drosophila*, e.g. development (4) and hindgut motility and renal fluid secretion (5), depend on changes in intracellular Ca²⁺ concentration mediated by release from intracellular calcium stores or by influx mechanism.

*Drosophila* TRP was the first protein described mediating Ca²⁺ influx in *Drosophila* photoreceptor cells in response to activation of a GPCR (6). Since the identification of *Drosophila* TRP a large number of homologous proteins have been cloned. Today, TRP proteins form a superfamily of nonselective cation channels containing six putative transmembrane domains, a pore region between the fifth and sixth segment, and cytosolic C and N termini (7). Based on sequence analysis of genomic and expressed sequence tag data, three different groups of TRP channels, TRPC (C for “classic” or “canonical”), TRPV (V for “vanilloid receptor-like”), and TRPM for (M for “melastatin-like”), have been identified (8, 9). In addition, other related channel protein families have been classified as TRP channels by phylogenetic analysis (TRPN, TRPA, TRPML, TRPP) (10, 11).

Thirteen TRP channels, including the TRPC members TRP and TRPL, have been identified in the *Drosophila* genome so far with their biological functions mainly related to sensory systems (12). Mammalian and *Drosophila* TRPC channels are activated by mediators created by the GPCR-dependent stimulation of phospholipase C isoforms. Whereas the mammalian TRPC channels TRPC3, TRPC6, and TRPC7 (13, 14) and TRPC2 (15) are activated by diacylglycerols, *Drosophila* TRPL, and possibly TRP, are activated by the polyunsaturated fatty acids (PUFA), arachidonic acid (AA) and linoleic acid (16). A third *Drosophila* TRPC channel, TRPγ, was identified and characterized as an auxiliary subunit of TRPL that, when coexpressed with TRPL in HEK293 cells, forms a receptor-activated cation channel (17). However, the activation mechanism of TRPγ remained obscure. TRP and TRPL and possibly TRPγ...
participate in phototransduction. The distribution of TRPγ expression in *Drosophila*, however, is controversial. Xu et al. showed expression of TRPγ predominantly in *Drosophila* head (17). In contrast, two recent publications showed a much broader distribution of TRPγ (18, 19). A broad expression pattern of TRPγ and the fact that there are only three TRPC members (TRP, TRPL, TRPγ) in the genome of *Drosophila* make it likely that a TRPγ-mediated Ca^{2+} influx is integrated in many receptor-mediated signaling pathways outside the visual system.

In our study, we show that TRPγ is expressed in heads and in the bodies of fruitflies. When heterologously expressed in HEK293 cells, TRPγ forms a channel that is regulated via a hormone-induced, GPCR-activated, intracellular signaling pathway. Analysis of this signaling pathway revealed that TRPγ is activated by polyunsaturated fatty acids in a phospholipase C- and phospholipase A_2-dependent manner. The activation of TRPγ by 5,8,11,14-eicosatetraynoic acid (ETYA) excludes the participation of metabolites of arachidonic acid. In summary, we show, for the first time, the functional and biophysical characterization of TRPγ as a homomeric, non-selective cation channel that is activated by polyunsaturated fatty acids.

**EXPERIMENTAL PROCEDURES**

*Chemicals—*AA, linoleic acid (Sigma), and palmitoleic acid (MP Biochemical, Heidelberg, Germany) were diluted from 100-mM stock solutions in ethanol. 1-decanoyl-rac-glycerol (MDG), 1-oleoyl-rac-glycerol, 1,2-dioctanoyl-sn-glycerol (DOG), 1-oleoyl-2-acetyl-sn-glycerol (OAG) (Sigma) were used from 100-mM stock solution in dimethyl sulfoxide (Me2SO). The phospholipase A_2 inhibitors N-(p-amylcinnamoyl) arachidonic acid, arachidonylethanolamethoxyl ketone (Calbiochem), bromoenol lactone (Sigma) were diluted from 50-mM stock solutions in Me2SO. p-bromophenacyl bromide (pBPB) (Sigma), ETYA (Calbiochem) were used from 50-mM stock solution in ethanol.

*Extraction of mRNA and RT-PCR—*Total RNA was isolated from wild-type *D. melanogaster* using TriReagent (Ambion, Austin, TX) according to the standard protocol and subsequent incubation with Turbo RNase-free DNase I (Ambion). The heads were separated from the body on dry ice before RNA preparation. cDNAs were generated using M-MLV reverse transcriptase Rnase H Minus (Promega) and served as templates in a subsequent PCR, using TaqPCR Master Mix (Qiagen, Hilden, Germany) and specific oligonucleotides (TRPγ sense, 5’-AGTCGAAACGTGAGCAAATGTG-3’, and antisense, 5’-TGGAGTTCACTGACGTATTG-3’; TRPγ sense, 5’-CACCATGGGCCAGCAGC-3’, and antisense, 5’-GGGCCGGGTGTTGTACGATAGTTT-3’). The fragments were subcloned in the expression vector pcDNA3.1Directional/V5-His-TOPO (Invitrogen), resulting in constructs of fusion proteins with C-terminal V5 and His tags. For expression as yellow fluorescence protein fusion protein, the coding sequence of YFP was subcloned in-frame C-terminal of the TRPC channel proteins. Both strands of all cDNA fragments were sequenced using ABI Prism BigDye terminator cycle sequencing kits and an ABI Prism 377 DNA sequencer (Applied Biosystems, Weiterstadt, Germany). DNA for transient transfection was prepared using anion exchange columns (Qiagen).

*Cell Culture and Transfection of HEK293 Cells—*HEK293 cells were cultured in Earle’s minimal essential medium (Biochrom, Berlin, Germany), supplemented with 10% fetal calf serum (Biochrom), 100 μg/ml penicillin, and 100 μg/ml streptomycin under a 5% CO_2 humidified atmosphere at 37 °C. Cells were plated in 85-mm dishes onto glass coverslips and transiently transfected 2 days later by addition of a transfection mixture containing 2.5–3 μg of DNA and 7 μl of FuGENE 6 transfection reagent (Roche Diagnostics) in 93 μl of OptiMEM medium (Invitrogen). Fluorescence measurements and electrophysiological studies were carried out 1–2 days after transfection.

*Western Blot Analysis—*Transfected HEK293 cells were harvested by centrifugation (800 × g, 5 min, room temperature). Cells were resuspended in lysis buffer (50 mM Tris/HCl, 2 mM dithiothreitol, 0.2 μM benzamidine, 1 mM EDTA, pH 8.0) and homogenized by shearing through 26-gauge needles. After removal of nuclei (800 × g, 2 min, 4 °C), supernatants were mixed with gel loading buffer (62.5 mM Tris/HCl, 10% glycerol, 5% mercaptoethanol, 2% SDS, 0.02% bromphenol blue, pH 6.8). To detect TRPγ, TRP, and TRPL expressed in HEK293 cells, the membrane extracts were separated on an 8% SDS-PAGE (22). After electrophoresis the proteins were transferred on nitrocellulose membrane, and the fusion proteins were detected by incubating the membrane with an anti-tetra His monoclonal antibody 1:10000 at 4 °C overnight. The bound antibody was detected using an ECL Advance Western blotting detection kit (Amersham Biosciences).

*Fluorescence Measurements—*[Ca^{2+}], measurements in single cells were carried out using the fluorescence indicator Fura-2/AM in combination with a monochromator-based imaging system (T.I.L.L. Photonics, Martinsried, Germany) attached to an inverted microscope (Axiovert 100; Carl Zeiss, Oberkochen, Germany). HEK293 cells were loaded with 4 μM Fura-2/AM (Molecular Probes) and 0.01% Pluronic F-127 (Molecular Probes) for 60 min at room temperature in a standard solution composed of 138 mM NaCl, 6 mM KCl, 1 mM MgCl_2, 2 mM CaCl_2, 5.5 mM glucose, and 10 mM HEPES (adjusted to pH 7.4 with NaOH). The osmolality of the solution amounted to 300 mosmol−1 and was measured using a freezing point depression osmometer (Roebling, Berlin, Germany). Coverslips were then washed in this buffer for 20 min and mounted in a perfusion chamber on the microscope stage. For [Ca^{2+}], measurements, fluorescence was
excited at 340 and 380 nm. After correction for background fluorescence, the fluorescence ratio \( F_{340}/F_{380} \) was calculated. Fluorescence quenching by Mn\(^{2+}\) entry was studied using the Fura-2 isosbestic excitation wavelength at 360 nm, and the emitted light was monitored using the same filter system as for \([Ca^{2+}]_i\) measurements. In all experiments, transfected cells of the whole field of vision were identified by their YFP fluorescence at an excitation wavelength of 480 nm. Experiments were performed on at least 20 cells and were presented as the number of experiments for each experimental condition.

**Patch Clamp Measurements**—Membrane currents were recorded using the whole-cell, cell-attached or inside-out configurations of the patch clamp technique at room temperature. Pipettes were made from borosilicate glass capillary tubes. The resistance of the pipettes varied between 2 and 5 MΩ in whole-cell recordings and between 7 and 9 MΩ in channel recordings. Whole-cell currents were elicited by voltage ramps from -100 to +100 mV (400-ms duration) applied every 10 s from a holding potential of 0 mV. Currents through the pipette were recorded by an Axopatch 200B amplifier (Axon Instruments), filtered at 5 or 10 kHz (Bessel filter), and analyzed using pCLAMP software (version 9.2; Axon Instruments).

**RESULTS**

**Detection of TRPγ mRNA in Drosophila Heads and Residual Bodies**—To clarify whether or not TRPγ expression is restricted to the head of *Drosophila*, we performed RT-PCR analysis from RNA isolated from *Drosophila* heads and adult residual bodies (Fig. 1A). The synthetic oligonucleotides for PCR were designed to distinguish fragments of different lengths depending on the template being amplified. A 656-bp fragment indicated an amplification starting from genomic DNA, whereas the 545-bp fragment corresponded to the TRPγ cDNA as template. The RT-PCR repeatedly resulted in the amplification of the 545-bp fragment from head and body cDNA (see Fig. 1A). Parallel RT-PCR reactions amplifying a fragment of glyceraldehyde-3-phosphate dehydrogenase were performed as control to ensure that comparable RNA amounts were used for the reactions (see Fig. 1A).

**Heterologous Expression of TRP, TRPL, and TRPγ in HEK293 Cells**—To study the function of the *Drosophila* TRPC channels, we subcloned the cDNA fragments coding for TRP, TRPL, and TRPγ in a vector directing the expression of the channel proteins as C-terminal fusion proteins with either V5 tag and His tag, or YFP. We verified the capability of the constructs to direct expression of the TRPC proteins in HEK293 cells by Western blot analyses using an antibody directed against the C-terminal His tag (Fig. 1B). The apparent molecular masses of the expressed proteins were comparable with published...
During application of CCh, 2 mM Ca\(^{2+}\) in the presence of 2 mM EGTA and after addition of 2 mM extracellular Ca\(^{2+}\) with at least 20 cells each. Panels A–C represent the averaged traces of one independent experiment (out of three to eight, numbers in parentheses) with at least 20 cells each. D, CCh (100 μM)-induced and TRP\(^{\gamma}\)-mediated Mn\(^{2+}\) quench of Fura-2 fluorescence was inhibited by application of Gd\(^{3+}\) (n = 3), La\(^{3+}\) (n = 3), or SKF-96365 (n = 4) (100 μM each) at the start of the experiments. Black and gray columns represent the effect of Cch on TRP\(^{\gamma}\)-YFP-expressing cells; the open column represents the effect of Cch on YFP-expressing cells. Bars represent the TRP\(^{\gamma}\)-mediated Mn\(^{2+}\) quench as mean values ΔF\(_{360}^\text{rel}\) (%) 200 s after application of the blocker ± S.E. of at least three independent experiments with at least 20 cells each.

**FIGURE 2. Activation of receptor signaling-induced Ca\(^{2+}\) influx in TRP\(^{\gamma}\)-HEK293 cells.** A, shown are effects of 100 μM carbachol (CCh) on [Ca\(^{2+}\)]i in TRP\(^{\gamma}\)-YFP-expressing cells (n = 4) and YFP-expressing control cells (n = 3). During application of CCh, 2 mM Ca\(^{2+}\) was exchanged for 2 mM EGTA. B, carbachol (100 μM) was applied in the presence of 2 mM EGTA and after addition of 2 mM extracellular Ca\(^{2+}\). Shown are effects in TRP\(^{\gamma}\)-YFP-expressing cells (n = 4) and YFP-expressing cells (n = 3). C, influx of Mn\(^{2+}\) (1 mM extracellular) into TRP\(^{\gamma}\)-YFP-expressing cells (n = 8) was enhanced by addition of 100 μM CCh. YFP-expressing cells (n = 3) served as control. Panels A–C represent the averaged traces of one independent experiment (out of three to eight, numbers in parentheses) with at least 20 cells each. D, CCh (100 μM)-induced and TRP\(^{\gamma}\)-mediated Mn\(^{2+}\) quench of Fura-2 fluorescence was inhibited by application of Gd\(^{3+}\) (n = 3), La\(^{3+}\) (n = 3), or SKF-96365 (n = 4) (100 μM each) at the start of the experiments. Black and gray columns represent the effect of Cch on TRP\(^{\gamma}\)-YFP-expressing cells; the open column represents the effect of Cch on YFP-expressing cells. Bars represent the TRP\(^{\gamma}\)-mediated Mn\(^{2+}\) quench as mean values ΔF\(_{360}^\text{rel}\) (%) 200 s after application of the blocker ± S.E. of at least three independent experiments with at least 20 cells each.

In membrane extracts of TRP-expressing HEK293 cells, the antibody detected two proteins with differences in data (17). In membrane extracts of TRP-expressing HEK293 cells, the antibody detected two proteins with differences in apparent molecular mass. To test whether these differences resulted from posttranslational protein modifications, we incubated aliquots of the extracted membrane protein with Endo H glycosidase, PNGase F glycosidase, and λ protein phosphatase. Subsequent Western blot analysis demonstrated that the appearance of two TRP protein bands did not result from differences in glycosylation (supplemental Fig. S1) or from differences in protein phosphorylation.

**TRP\(^{\gamma}\) Forms a Receptor-regulated Cation Channel**—Next, we studied TRP\(^{\gamma}\) in transiently transfected HEK293 cells. Fluorescence energy transfer (FRET) experiments using TRP\(^{\gamma}\)-CFP and -YFP fusion proteins expressed in TRP\(^{\gamma}\)-HEK293 cells resulted in FRET signals of ~10% (supplemental Fig. S2), arguing for the formation of a homomeric channel complex. For functional characterization of this homomeric TRP\(^{\gamma}\) channel complex, we initially applied Ca\(^{2+}\)-imaging methods. In Fura-2-loaded cells, the expression of TRP\(^{\gamma}\) resulted in an increase in the basal Ca\(^{2+}\) signal (Fig. 2A), due to its spontaneous activity (17). To test whether TRP\(^{\gamma}\) is regulated by receptor activation, we applied carbachol to stimulate endogenous muscarinic receptors in HEK293 cells. To our surprise the application of carbachol increased the intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]i) in TRP\(^{\gamma}\)-expressing cells ~10-fold as compared with control cells (see Fig. 2A). The increase in [Ca\(^{2+}\)]i, resulted from TRP\(^{\gamma}\)-mediated Ca\(^{2+}\) entry, because it was suppressed by extracellular EGTA. The origin of the increased [Ca\(^{2+}\)]i, was further analyzed by modified protocols (Fig. 2B). In the absence of extracellular Ca\(^{2+}\) and in the presence of extracellular EGTA, carbachol induced only a small increase in [Ca\(^{2+}\)]i. The addition of extracellular Ca\(^{2+}\) induced a pronounced Ca\(^{2+}\) influx into TRP\(^{\gamma}\)-expressing cells.

Manganese quenching of intracellular Fura-2 allows a direct correlation of a fluorescence signal and the activity of a plasma membrane channel protein. Application of manganese to TRP\(^{\gamma}\)-expressing cells resulted in an instantaneous slow and progressive reduction of Fura-2 fluorescence due to the spontaneous activity of TRP\(^{\gamma}\) (17) (Fig. 2C). Subsequent activation of muscarinic receptors after application of carbachol further enhanced the manganese quenching of Fura-2 fluorescence (see Fig. 2C).

Based on the result that TRP\(^{\gamma}\) is receptor regulated, we tested common blockers of TRP channels for further functional characterization. The application of Gd\(^{3+}\) (100 μM), La\(^{3+}\) (100 μM), or SKF-96365 (100 μM) completely blocked TRP\(^{\gamma}\)-mediated manganese quench in TRP\(^{\gamma}\)-expressing HEK293 cells (Fig. 2D).

In good agreement with the increased basal intracellular Ca\(^{2+}\) concentration, we observed spontaneous currents in whole-cell recordings from TRP\(^{\gamma}\)-expressing cells. Application of carbachol (100 μM) increased current responses in TRP\(^{\gamma}\)-expressing cells. Currents returned to a level similar to that before the application of carbachol during wash-out phase and removal of the agonist (data not shown). The current-voltage relationship obtained from whole-cell currents before and after the application of carbachol displayed outward rectification (Fig. 3A) and a reversal potential of −4 ± 4 mV (n = 11). The
PUFA-dependent Activation of Drosophila TRPγ

mean current densities under carbachol were $-26 \pm 16$ pA/pF and $+47 \pm 7$ pA/pF ($n = 11$) at $-90$ and $+90$ mV, respectively (Fig. 3B). Identical current-voltage relationships were measured under a voltage step protocol (10 mV/step from $-70$ to $70$ mV) before (Fig. 3C) and after (Fig. 3D) application of carbachol. The plateau of maximal current was reached after carbachol application (Fig. 3E). The exchange of Na$^+$ for the large cation NMDG$^+$ in the bath solution resulted in a clear decrease in inward currents in TRPγ-transfected HEK293 cells (Fig. 3F). The NMDG$^+$-sensitive inward current was $-6 \pm 7$ pA pF$^{-1}$ at $-90$ mV ($n = 5$) using a Ca$^{2+}$-free, Mg$^{2+}$-containing (2 or 5 mM) pipette solution. The current-voltage relationship of the current of spontaneously active TRPγ showed no clear rectification and a reversal potential ($E_{\text{rev}}$) of $-60 \pm 20$ mV ($n = 10$). To study the selectivity of the current of TRPγ in transfected cells, we replaced the standard extracellular solution containing Na$^+$, Ca$^{2+}$, and Mg$^{2+}$ by solutions containing only one of the cations, NMDG$^+$, Na$^+$, Ca$^{2+}$, or Mg$^{2+}$. In Na$^+$-containing bath solution the removal of divalent cations induced a slight increase in inward and outward currents (data not shown). Permeability ratios of TRPγ were $P_{\text{Na}}/P_{\text{NMDG}} = 1.015$, and $P_{\text{Ca}}/P_{\text{Na}} = 1:1$ ($n = 6$).

Analysis of the Signaling Pathway Leading to TRPγ Activation —To clarify whether TRPγ is activated by phospholipase C-generated mediators, we used the phospholipase C inhibitor U73122. In the presence of U73122, the application of carbachol to TRPγ-transfected HEK293 cells did not result in an increase in [Ca$^{2+}$]$_i$, suggesting a phospholipase C-dependent activation of TRPγ (data not shown). As some members of mammalian TRPC channels are activated by diacylglycerol, we used cell-permeable diacylglycerol analogues in our Ca$^{2+}$-imaging experiments. We applied 1,2-dioctanoyl-sn-glycerol, OAG, as well as monoacylglycerols such as MDG, and 1-octoyl-rac-glycerol to test their ability to induce a TRPγ-mediated Ca$^{2+}$ entry in HEK293 cells transfected with TRPγ DNA (Fig. 4). Carbachol reproducibly induced an increase in intracellular Ca$^{2+}$ in TRPγ-transfected cells, whereas OAG did not result in increases in [Ca$^{2+}$]$_i$, either in TRPγ-transfected cells or in control cells (Fig. 4A). Due to the instability of OAG, we performed side-by-side experiments using TRPC6-expressing HEK293 cells as control to verify the integrity of OAG (Fig. 4B). Whereas the diacylglycerols selectively activated TRPC6, the monoacylglycerol analogues failed to stimulate TRPC6 as well as TRPγ (Fig. 4C).

To determine the putative phospholipase C-dependent and diacylglycerol-independent activation of TRPγ, we looked for additional inhibitors interfering with phospholipase C-triggered pathways. In several reports, receptor-induced increases in arachidonic acid have been described and linked to the receptor-mediated activation of phospholipase A$_2$ isoenzymes (23–25). Therefore, we tested four PLA$_2$ inhibitors (N[2-amlylcinnamoyl]antranilic acid, p-bromophenacyl bromide bromoenol lactone, and arachidonyltrimfluoromethyl ketone) (Fig. 5). In manganese-quench experiments, the four PLA$_2$ inhibitors blocked carbachol-induced activation of TRPγ with different efficacies (see Fig. 5). In summary, these data argue for a phospholipase C- and phospholipase A$_2$-dependent activation of TRPγ.

TRPγ Forms a Cation Channel Regulated by Polyunsaturated Fatty Acids—Because an activation of PLA$_2$ enzymes results in an increase in AA, we tested whether TRPγ-transfected HEK293 cells can be activated by AA. The application of AA increased [Ca$^{2+}$]$_i$, in Ca$^{2+}$-imaging experiments and attenuated the Fura-2 fluorescence in manganese quench experiments (Fig. 6, A and B). AA is intensively metabolized by lipooxygenases, cyclooxygenases, and cytochrome P$_{450}$ isoenzymes, resulting in a great variety of compounds with diverse function. To test whether the effect of AA is mediated directly or by a metabolite, we studied the effects of ETYA as a common inhibitor of AA-metabolizing enzymes (26). However, upon application of ETYA in the absence of AA, there were still responses measured (Fig. 6, C and D). This stimulating effect of ETYA was reproducibly detected in manganese quench and Ca$^{2+}$-imaging experiments.

To test whether or not TRPL and TRPγ are activated by different unsaturated fatty acids, we measured fluorescence changes in Fluo-4-loaded cells in a FLIPRTetra. The application of different fatty acids resulted in comparable increases in intracellular Ca$^{2+}$.
PUFA-dependent Activation of Drosophila TRPγ

FIGURE 4. TRPγ is insensitive to mono- and diacylglycerols. A, effects of either 100 μM OAG or 100 μM CCh on HEK293 cells expressing TRPγ-YFP. The lines represent mean values from six independent Fura-2 experiments with at least 20 cells each. Application of substances is indicated by an arrow. B, effect of either 100 μM OAG or 100 μM CCh on HEK293 cells expressing TRPC6-YFP. Shown are mean values from four independent experiments with at least 20 cells each. Application of substances is indicated by an arrow. C, effects of decanoyl-rac-glycerol (MDG, 100 μM), 1-octanoyl-rac-glycerol (MOG, 100 μM), 1,2-di-octanoyl-sn-glycerol (DOG, 100 μM), and 1-octanoyl-2-acetyl-sn-glycerol (OAG, 100 μM) on TRPγ-YFP-transfected cells (open columns), TRPC6-YFP-expressing cells (bright gray filled columns), and YFP-expressing cells (dark gray filled columns). Columns represent the increases in [Ca2+]i 200 s after application of lipids as means ± S.E. of independent Fura-2 experiments (numbers given in parentheses) with at least 20 cells each.

FIGURE 5. Effects of the PLA2 inhibitors on CCh-induced TRPγ stimulation. Carbachol-induced activation of TRPγ-YFP-expressing HEK293 cells was studied by Mn2+ influx experiments. After a preincubation period of 5 min in the presence of the PLA2 inhibitors N-(p-amylcinnamoyl)anthranilic acid (ACA, 10 μM; n = 6) (A), bromoepol lactone (BEL, 20 μM; n = 4) (B), p-bromphenacyl bromide (pBPB, 50 μM; n = 5) (C), or 10 min in the presence of arachidonyltrifluoromethyl ketone (AACOCF3, 20 μM; n = 5) (D), carbachol (100 μM) was applied. All traces represent mean values from n independent experiments with at least 20 cells each.

Electrophysiological Characterization of ETYA-induced TRPγ Currents—For electrophysiological characterization of the polyunsaturated fatty acid-dependent activation of TRPγ, we used ETYA, the most potent activator, being an enzymatically and chemically stable AA derivative. In standard extracellular solutions, the current-voltage relationship of the ETYA-induced current, measured from voltage ramps, had an outwardly rectifying form comparable with the curves resulting from carbachol application (see Figs. 3A and 7A). Maximal currents were transiently recorded 40 s after the application of ETYA (Fig. 7B). Stable steady states were obtained 60 s after application with mean current densities of ~42 ± 29 pA/pF and +114 ± 31 pA/pF (n = 12) at −90 and +90 mV, respectively (Fig. 7C). In contrast, ETYA had no effect on untransfected HEK293 cells (Fig. 7D). Enhanced inward currents and subsequent rapid desensitization were only found after application of ETYA. Note: The different current densities in carbachol- and ETYA-stimulated cells may result from different stimulus intensities.

For characterization of the single channel conductance, we performed inside-out recordings from TRPγ-expressing cells (n = 6) (Fig. 8). Spontaneous activity of TRPγ was also recorded in the inside-out configuration showing a relatively high open probability from the start of the recordings (Fig. 8A). The application of ETYA induced an increase in channel activity within a time scale of 10 s (see Fig. 8A). Exemplary traces in the absence and presence of ETYA are shown as insets. For further quantification of our single channel data before (Fig. 8B) and after (Fig. 8C) application of ETYA, we calculated amplitude histograms of single channel amplitudes from current traces of 4-s length. The calculation revealed that ETYA increased the open prob-
ability of TRP\(\gamma\) from 0.15 (see Fig. 8B) to 0.85 (see Fig. 8C). At the holding potential \(-50\) mV the amplitude of the current was 8.9 pA. The chord conductance calculated from the data of our experiments is 148 pS.

DISCUSSION

TRP\(\gamma\), as a member of the TRPC channel family, has been discussed as participating in Drosophila phototransduction (12, 17). In the first report describing TRP\(\gamma\), its expression was shown to specifically occur in Drosophila eyes and the head, but not in the body (17), suggesting a possible sensory function of TRP\(\gamma\). In contrast, a second report showed that the transcription of TRP\(\gamma\) mRNA not only occurs in the fruit fly’s head but also in the cells of the Malpighian tubules (18). Similarly, our present RT-PCR data support the notion that TRP\(\gamma\) expression is not restricted to Drosophila head. The fact that there are only three TRPC members (TRP, TRPL, TRP\(\gamma\)) in the genome of Drosophila and the expression of TRP\(\gamma\) in Drosophila heads and bodies make it likely that TRP\(\gamma\)-mediated \(\text{Ca}^{2+}\) influx is integrated in many receptor-mediated signaling pathways as shown for the mammalian TRPC channel proteins. Indeed, TRP\(\gamma\), when heterologously expressed in HEK293 cells, can be regulated by hormonal activation of the cells via endogenous muscarinic GPCR, because both \(\text{Ca}^{2+}\)-imaging and whole-cell patch clamp experiments showed an increased influx into TRP\(\gamma\)-expressing cells.

The signaling pathway leading to TRP\(\gamma\) activation was clarified with the help of pharmacological tools. The data in this study revealed that TRP\(\gamma\), like TRPL, is activated by various polyunsaturated fatty acids generated in a phospholipase C- and phospholipase A\(2\)-dependent manner. Whereas the expression of a phospholipase C isoenzyme in Drosophila has been known since 1988 (27, 28), a gene coding for a phospholipase A\(2\) isoenzyme has been found only recently (29). Its gene product,
PUFA-dependent Activation of Drosophila TRPγ

A

ETYA

NP

0.8

0.6

0.4

0.2

0.0

0 s

10 s

100 s

FIGURE 8. Fatty acid regulation of TRPγ channel activity in inside-out patches. ETYA stimulated TRPγ channels in inside-out patches. A, plot of NP, against time during a representative experiment shows the increase in channel activity after the application of the ETYA. Insets, currents recorded at the times indicated by (1) and (2). Channel activity was recorded in inside-out patches from HEK293 cells expressing TRPγ-YFP at the holding potential of −50 mV. Statistical analysis of single channel amplitudes were calculated from current traces of 4-s length using amplitude histograms fitted to Gaussian functions before (B) and after (C) application of ETYA. Note the change in probability of channel opening from 0.15 (B) to 0.85 (C).

called Radish, has been shown to be expressed in a specific subset of neurons involved in synaptic transmission necessary for olfactory memory. However, due to the lack of histological localization of TRPγ, it remains unclear whether TRPγ and Radish are involved in olfactory memory.

The PLA2 inhibitors used in this study have been shown to be specific for different PLA2 isoenzymes. N-(p-Amylcinnamoyl)lanthanilic acid has been used as a common inhibitor, whereas p-bromphenacetyl bromide, bromoeno lactone, and arachidonitrifluoromethyl ketone have been described to be specific for the secretory, inducible, and cytosolic PLA2, respectively (30). Differences in inhibiting the carbachol-induced and TRPγ- or TRPL-mediated Ca2+ influx by these PLA2 inhibitors are, therefore, likely due to their different efficacies in inhibiting the endogenously expressed PLA2 in HEK293 cells.

The concentration-response relations of PUFA channels show that TRPγ and TRPL are activated by polyunsaturated fatty acids with comparable efficacies, whereas palmitoleic acid, a monounsaturated fatty acid, is ineffective. Both channel proteins are stimulated not only by naturally occurring polyunsaturated fatty acids, ETYA, a synthetic PUFA analogue, also induced TRPγ currents. After the application of ETYA, we obtained current-voltage relationships similar to those measured under carbachol.

TRPγ in HEK293 cells was constitutively active, with a current-voltage relationship reversing around 0 mV and a permeability for cations \( P_{Na} : P_{Ca} : P_{NDG} = 1:1:0.15 \), characterizing TRPγ as a non-selective cation channel. This is in good agreement with earlier data of Xu et al. (17). The amplitudes of inward and outward currents increased within 30 s after the application of carbachol, indicating that TRPγ is activated by mediators generated by intracellular signaling cascades. The ETYA-induced currents showed a current-voltage relationship and single channel conductance that is well comparable with that of the spontaneous currents.

The data on TRPγ obtained in this study, together with those for TRPII and TRP obtained by Chyb et al. (16), demonstrate that all members of the Drosophila TRPC family are regulated by fatty acids. In terms of phylogeny of signaling cascades, the questions arise whether regulation by fatty acids is a conserved feature and can also be found in other species or whether this mechanism is specific for flies. At present, it appears that most of the mammalian TRPC channels are activated by diacylglycerols (TRPC2, TRPC3, TRPC6, and TRPC7), whereas activation by fatty acids is a still unknown principle for mammalian TRPC channels (13–15). This is in line with our study in which PUFA did not induce current signals in mock-transfected HEK293 cells, which endogenously express TRP channels. It therefore appears unlikely that endogenous TRP channels in HEK293 cells interfere with the activation of TRPγ by PUFA.

The presence of endogenously expressed TRP channels in HEK293 cells is long known. For example, the first cloned TRP-homologous channel protein, TRPC1, has been cloned from HEK293 cells (31) as well as TRPC3 (32). In the meantime, RT-PCR experiments confirmed the expression of nearly all mammalian TRPC channels in HEK293 cells (33–35). The presence of natively expressed TRPC channels in HEK293 accounts for the receptor-induced responses recorded in mock-transfected cells and is discussed as contributing to the channel complexes formed in HEK293 cells heterologously expressing TRPC channels (36).

The work by Xu et al. (17) showed that heterologously expressed TRPγ forms a functional heteroerotic channel complex together with TRPL. This ability would allow TRPγ and other TRP channel subunits to potentially generate a diversity of heteromeric channels, each with properties specifically tailored to a particular cellular function. This feature is a well-known principle in other non-selective cation channel families, e.g. the cyclic nucleotide-gated channel subunits (37), the P2X receptor channels (38), or the nicotinic acetylcholine receptor channels (39). Results of fluorescence energy transfer assays and co-localization and co-immunoprecipitation studies have shown that co-expression of closely related proteins of the mammalian TRPC, TRPV, or TRPM families resulted in the formation of heteromeric channel complexes in HEK293 cells (40–42). Despite these results, co-expression of TRP channels in heterologous systems, such as HEK293 cells, had little impact on the functional properties of the channels studied. So far, only
one study described that the co-expression of TRPC1 and TRPC5 resulted in a current-voltage relationship that differed from that of the homomeric channels (43). However, Nilius et al. (44) showed that the heterologous expression of TRPV4 in HEK293 resulted in the down-regulation of a TRP-unrelated channel, the natively expressed volume-regulated anion channel. Any contribution of endogenous TRP channels of HEK293 cells to the regulation of the heterologously expressed TRPγ channels remains to be proven.

Whereas biochemical data argue in favor of a promiscuous interaction among TRP, TRPL, and TRPγ channels (17), the analysis of fly phototransduction demonstrated that TRP channels alone translocated from the membrane of the rhabdomere to intracellular membrane compartments in response to light (45). The fact that TRPL and TRPγ in our hands formed unitary homomeric channels activated by fatty acids may support the notion that these channels are involved in selective cellular signaling cascades, beyond their heteromeric function in phototransduction.

In summary, our data show for the first time that TRPγ forms a Ca2+-permeable non-selective cation channel directly activated by polyunsaturated fatty acids generated from GPCR-dependent signaling pathways. Whereas a specific functional role of TRPγ in Drosophila phototransduction appears most attractive, its body-wide expression led us to anticipate, however, a more general role of TRPγ in the entire organism of the fruit fly.

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