TRPγ Channels Are Inhibited by cAMP and Contribute to Pacemaking in Neurosecretory Insect Neurons*

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From a neuronal cDNA library of the cockroach Periplaneta americana we isolated a 3585-bp cDNA sequence encoding Periplaneta transient receptor potential γ (pTRPγ), a protein of 1194 amino acids showing 65% identity to the orthologous Drosophila channel protein dTRPγ. Heterologous expression of pTRPγ in HEK293 cells produced a constitutively active, non-selective cation channel with a Ca2⁺:Na⁺ permeability ratio of 2. In contrast to dTRPγ-mediated currents, pTRPγ currents were partially inhibited by 8-bromo-cAMP, and this effect was not mediated by protein kinase A (PKA) activation. pTRPγb, a truncated pTRPγ splice variant missing most of the C terminus, was insensitive to 8-bromo-cAMP. Thus, the critical CAMP-binding site seems to be located in the C-terminal part of pTRPγ, although there is no common CAMP-binding consensus sequence. While dTRPγ is only expressed in the photoreceptors, pTRPγ is expressed throughout the nervous system. In particular it is expressed in dorsal unpaired median (DUM) neurons. In these octopamine-releasing, neurosecretory cells a Ca2⁺ background current contributing to pacemaker activity was found to be up-regulated by the reduction of cAMP level. In contrast, the Ca2⁺ background current was inhibited by LOE-908, 2-APB, and La3⁺, which similarly affected the pTRPγ current. We thus propose that the pTRPγ protein is involved in forming the channel passing the Ca2⁺ pacemaking background current in DUM neurons.

Proteins of the TRP family (transient receptor potential) form cation-selective and Ca2⁺-permeable ion channels with multiple functions (1). The first TRP channel was described in Drosophila photoreceptors where mutants lacking this channel showed a decreased receptor potential upon continuous light stimulation (2). In the fly photoreceptors two further TRP proteins, TRPL and TRPβ (3), contribute to visual transduction (4). TRP proteins have six transmembrane segments, and most of them probably assemble to homo- or heterotetramers to form ion channels.

The superfAMILY of TRP channels has been grouped into several subfamilies (1, 5). Members of the mammalian TRPC (canonical TRP) family show the highest homology with the Drosophila TRP/TRPL/TRPγ variants. These channels appear to be receptor-operated in that they are activated by a variety of stimuli that lead to activation of phospholipase C (PLC).

The modulation of pacemaker conductances is a basic principle to adjust the neuronal activity to the physiological requirements. Certain peptide hormones affect ion channels involved in the regulation of pacemaking. Previous investigations on identified insect neurosecretory neurons, namely efferent dorsal unpaired median (DUM) neurons in the cockroach Periplaneta americana, have shown that a voltage-independent Ca2⁺ background current providing for non-capacitative Ca2⁺ entry (NCCE) changes the spike frequency of these cells. For example, up-regulation of this current by the Periplaneta adipokinetic hormone AKH I (pAKH I: pQ-V-N-F-S-P-N-W) variants. These channels appear to be receptor-operated in that they are activated by a variety of stimuli that lead to activation of phospholipase C (PLC).

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We were interested in identifying the molecular basis for the Ca2⁺ background current in DUM neurons and thus looked for a channel that (i) may conduct a voltage-independent Ca2⁺ current and (ii) that is sensitive to cAMP. Since TRP channels are known to fulfill the first criterion, we screened a Periplaneta cDNA library for putative members of the TRP family. We succeeded in finding a Periplaneta homolog to the Drosophila TRPγ channel. In the present study, we report an unusual regulation of this TRP channel. We demonstrate that the Periplaneta but not the Drosophila TRPγ channel is down-regulated by cAMP. Based on an analysis of the pharmacological profile of the Periplaneta TRPγ channel we propose that it is involved in forming the channel that conducts the Ca2⁺ background current in DUM neurons.

MATERIALS AND METHODS
Degenerated RT-PCR and RACE Protocols—RNA from isolated Periplaneta thoracic and abdominal ganglia were isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and reverse-transcribed using Superscript II reverse transcriptase (Invitrogen, Karlsruhe, Germany). According to sequence homologies between the three Drosophila melanogaster TRP channels dTRP (GenBank™ accession number M34394), dTRPL (GenBank™ accession number M88185), and dTRPγ (GenBank™ accession number AJ277968)

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2 The abbreviations used are: TRP, transient receptor potential; AKH I, adipokinetic hormone I; 8-Br-cAMP, 8-bromo-cAMP; 8-Br-cGMP, 8-bromo-cGMP; dTRPγ, Drosophila transient receptor potential; DUM, dorsal unpaired median; ETYA, 5,8,11,14-eicosatetraynoic acid; KLH, keyhole limpet hemocyanin; LOE-908, (R,S)-2-(3,4-dihydroxy-6,7-dimethoxyisochinolin-1-yl)-2-phenyl-N,N-dic(2,3,4-trimethoxyphenyl)ethylacetamide; NCCE, non-capacitative Ca2⁺ entry; PLC, phospholipase C; PKI, myristoylated PKA-inhibiting peptide 14–22 amide; pTRPγ, Periplaneta transient receptor potential; Rp-cAMPS, adenosine 3’,5’-cyclic phosphorothioate-Rp; RT, reverse transcription; RACE, rapid amplification of cDNA ends.
several degenerated primers were designed. Among these primers one pair (5′-tgtatgtggattgcc-3′ coding for WYEGLP; 5′-ggctccccagggc-3′ coding for NPHLG) amplified a ~650-bp fragment from the ganglial cDNA with strong homology to dTRP using AmpliTaq Gold polymerase (Applied Biosystems, Darmstadt, Germany). To obtain the full-length pTRPγ sequence we performed nested touch-down 5′- and 3′-RACE experiments as described previously (10) using pTRPγ-specific primers and a Periplaneta americana mixed tissue Marathon RACE library (RACE-F1, 5′-tggctcggggactcctgactcag-3′; RACE-F2, 5′-gccgcagggacagccattc-3′; RACE-B1, 5′-cgacacactcccttgctcagca-3′; RACE-B2, 5′-tgcagccagctgctcagagc-3′). PCR and RACE products were either sequenced directly or cloned into pGEM-T (Promega, Mannheim, Germany) for sequencing.

**RT-PCR Analysis of Periplaneta Tissues**—A nested RT-PCR approach was developed for testing ganglion-specific expression and single-cell analysis of isolated DUM neurons. The following intron-spanning PCR primers were designed: Sc-F1, 5′-cagaagaaactggtggtcctc-3′; Sc-F2, 5′-caactgccctcagcagc-3′; Sc-B1, 5′-gagctggtggtagttgct-3′; Sc-B2, 5′-cagacctctgctgctcagagc-3′. For single-cell RT-PCR analysis of pTRP expression the cytosol of single DUM neurons was harvested with patch pipettes and directly transferred to the RT reaction mixture. RT reaction was performed with either Superscript II reverse transcriptase (Invitrogen) and ganglion RNA or with Sensiscript reverse transcriptase (Qiagen) and isolated cytosol of single DUM neurons as described by the manufacturer, respectively. The two nested PCR reactions were performed with AmpliTaq Gold polymerase (Applied Biosystems) with initial 6 min at 94 °C enzyme activation followed by 40 cycles 30 s at 94 °C; 30 s at 55 °C; 1 min at 72 °C. PCR products of the second PCR reaction were visualized on a 2% agarose gel. As a positive control we amplified part of a ubiquitously expressed actin gene from P. americana.

**Construction of a pTRPγ pIRE2-EGFP Expression Vector (HEK293 Expression)**—Full-length pTRPγ was amplified using Advantage TaqII DNA polymerase mixture (Clontech, Palo Alto, CA) and ganglion cDNA using the following three primers: FL-for1, 5′-aaggctcgcagtagtgagactcg-3′; FL-for2, 5′-agacccctctgagcttgaggaagagaaacactg-3′; and FL-rev, 5′-ctgcctgctgctgctcagagc-3′. PCR was performed for 35 cycles with 30 s at 94 °C, 1 min at 64 °C (or 58 °C during initial two cycles), and 12 min at 68 °C. Finally, PCR products were cloned into the pIRE2-EGFP vector using Xhol sites incorporated into primer sequences (shown in italics). Among these clones a novel splice variant pTRPγyb was found, harboring a novel exon and introducing a stop codon resulting in a shorter C-terminally truncated splice variant.

**Production and Specificity of pTRPγ Antibodies**—Two pTRPγ antisera were raised in rabbits against the synthetic peptides (1) QPVSGH-NMSAGW and (2) QNKSRNG representing two different C-terminal motifs. The peptides were cross-linked to keyhole limpet hemocyanin (KLH) by means of glutaraldehyde. The peptides, the peptide-KLH conjugates, and the antisera (1) were produced by Sigma Genosys (Cambridge, UK). The specificity of antisera (1) was tested by Western blot (Fig. 2B). For immunocytochemical control of antisera (2) we used the preimmune serum (Fig. 3, B and E), and we performed experiments without the primary antibody (data not shown). In both control experiments there was no staining.

**Western Blot**—Freshly isolated P. americana thoracic and abdominal ganglia from five animals were homogenized in 100 μl of lysis buffer (50 mM Tris, 150 mM NaCl, 5 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 tablet Complete protease inhibitor mixture (Roche Diagnostics) per 50 ml). After electrophoretical separation of 20 μg protein per lane (SDS-PAGE) and blotting on polyvinylidene difluoride transfer membranes (Millipore), standard wash and detection procedures were used. Anti-pTRPγ antibody (1) was 1:2000 diluted, and peroxidase-coupled secondary antibodies (anti-rabbit IgG or anti-mouse IgG) were applied in 1:1000 dilution, followed by chemoluminescence detection with the ECL Plus kit (Amersham Biosciences). For control we used pTRPγ-transfected and mock-transfected HEK293 cells. 2 days after transfection about 1 × 10⁵ cells were harvested and immediately lysed in 200 ml of lysis buffer, and 10 μl were used for SDS-PAGE. To exclude unspecific bands due to antibodies directed against KLH, control blots were performed with antisera (5 μl) that was preincubated with 100 μg of KLH protein in 100 μl of phosphate-buffered saline, pH 7.4. This procedure prevented the Western blot detection of KLH protein by the serum but did not affect the 129 kDa signal in Periplaneta tissues or in transfected HEK293 cells.

**Immunocytochemistry**—Adult P. americana L. were taken from laboratory colonies at the University of Jena (maintained at 27 °C under a 12 h light/12 h dark regime). Animals were anesthetized by cooling under crushed ice and decapitated. Brains and the sixth abdominal ganglia were dissected from animals of both sexes under ice-chilled Tris-HCl saline and transferrerd into freshly prepared fixative: 4% formaldehyde in 0.1 M Millionig buffer (pH = 7.3–7.4) overnight at room temperature. The preparations were washed in Tris-HCl buffer (146 mM NaCl, 50 mM Tris-OH, pH = 7.4, for 12 h at 4 °C on a shaker. 40-μm-thick sections of agar-embedded tissues were cut on a vibratome (Technical Products, St. Louis, MO). The washed free-floating vibratome sections were then incubated in rabbit anti-TRP polyclonal antibody (cf. above) diluted 1:1000 in a solution of Tris-HCl buffer containing 2% normal goat serum, 0.25% Triton-X, 3% skim milk powder, and 0.25% bovine serum albumin (MPB). Subsequently, the tissue was washed in MPB overnight and incubated in the Cy3-tagged secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) for 3 h at a dilution of 1:800 in MPB. The vibratome sections were mounted and embedded in Mowiol. For the photographic documentation we used the bright-field optics on a Zeiss Axioshot microscope, a Hamamatsu digital camera C4742-95, and the software OpenLab (Improvision Ltd., Coventry, England). HEK293 cells growing on coverslips were treated as the vibratome sections.

**Electrophysiology**—HEK293 cells were cultured at a density of ~2 × 10⁴ per 35-mm dish and transfected with 1 μg of pTRPγ/pIRE2-EGFP using SuperFect (Qiagen). For some experiments we transfected cells with pTRPγ/pIRE2-EGFP or with dTRPγ/pCDNA3 (a generous gift of Drs. C. Montell and L. Salkoff) and 0.8 μg pEGFP-C1 (Clontech). Ion currents in HEK293 cells and in DUM neurons (isolated according to Ref. 7) were measured at room temperature using whole-cell patch clamp with appropriate compensation of series resistance and of capacitative and leakage currents. Some experiments were performed in the inside-out configuration. Pipettes having resistances of 2–4 MΩ (HEK293 cells) or 0.5–0.8 MΩ (DUM neurons) were pulled from borosilicate capillaries. Current measurements and data acquisition were performed using an EPC9 patch clamp amplifier controlled by PULSE software (HEKA Elektronik, Lambrecht, Germany).

**HEK293 Cells**—The pipette solution contained (in mM) 140 CsCl, 1 mM Mg-ATP, 10 EGTA, 10 HEPES (pH = 7.3), and the bath solution contained 140 CsCl, 15 glucose, 15 HEPES (pH = 7.4). For inside-out measurements the pipette contained the bath solution and the bath solution was exchanged by the pipette solution after establishing the seal. To
evaluate the permeability of pTRPγ channel to Na⁺ and Ca²⁺ the bath solution contained 140 NaCl, 15 glucose, 15 HEPES, and 140 N-methyl-d-glucamine-Cl, 5 CaCl₂, 10 glucose, 15 HEPES, respectively. The permeability ratios were calculated according to Equations 1 and 2.

\[
P_{Na^+}P_{Cs} = \frac{[Cs^+]_o}{[Na^+]_o} \times \exp(V_{rev}/F/RT) \quad \text{Eq. 1}
\]

\[
P_{Ca^2+}P_{Cs} = \frac{[Cs^+]_o}{4[Ca^{2+}]_o} \times \exp(V_{rev}/2F/RT) \quad \text{Eq. 2}
\]

where \(P_{ion}\) are the permeabilities, \([ion]_o\) and \([ion]_i\) are the internal and external concentration of the respective ion, and \(V_{rev}\) is the reversal potential (11).

To rule out an effect on endogenous HEK293 channels (12), 8-Br-cAMP and all other substances used below were first tested in mock-transfected cells (\(n = 5-8\)). In no case we found any significant effect indicating that all reported effects of the agents relied on the expressed pTRPγ channels. On the other hand, it cannot be excluded that the heterologous expression of TRP proteins in HEK293 cells may lead to the formation of new TRP heteromultimers including endogenous TRPCs (13) or to up-regulation of TRPC expression. As outlined under “Discussion,” this is rather unlikely in the case of the TRPγ proteins used in this study.

**Results**

**Sequence Analysis of D. americana TRPγ**—Using a combined degenerated PCR and RACE protocol, we were able to isolate the full-length sequence of the *D. americana* cDNA. The 3585-bp cDNA sequence (GenBank™ accession number AY387857) is coding for a protein of 1154 amino acids showing all typical features of a TRP-like channel: four ankyrin-like repeats at the N terminus, a coiled-coil domain, six transmembrane regions, a TRP-pore sequence typical for a nonspecific cation channel, and a long C terminus harboring several conserved TRPC-specific sequences. The pTRPγ sequence is 66% identical and 75% homologous to the orthologous *D. melanogaster* sequence (dTTPγ), whereas sequence similarity to paralogous *Drosophila* sequences (dTTP and dTRP) is significantly lower and spans only the very conserved core region of ~900 amino acids of the protein, with ~50% identity and 65% similarity. An alignment of pTRPγ and dTRPγ indicating several conserved protein features is shown in Fig. 1. The C terminus is less conserved between *Periplaneta* and *Drosophila* TRP γ as seen in various gaps in the alignment and in a C-terminal extension of pTRPγ. A comparison with the *Drosophila* genomic sequence (3) indicated that alternative splicing only in one case might be responsible for these variations, i.e. the pTRPγ sequence lacked one dTRPγ exon (indicated in Fig. 1). In addition, when amplifying the full-length pTRPγ sequence, a short alternatively spliced variant, pTRPγb, was discovered. This splice variant harbored the homologous dTRPγ exon missing in the full-length form, but it introduced a stop codon thereafter, resulting in a truncated pTRPγ version with only 791 amino acids, i.e. lacking most of the C-terminal sequence. In addition, a short variable region was identified in the C terminus, resulting in either deletion of a single amino acid (alanine 827) or in an expansion by four residues (GTNTS preceding alanine 827). The mechanism of this sequence variation is unclear but may represent alternative usage of exon-intron splice sites or polymorphisms.

**Expression Analysis of pTRPγ**—The expression of pTRPγ channels in neuronal tissue was tested by a nested RT-PCR approach. An intron-spanning 355-bp PCR fragment was amplified from *Periplaneta* brain as well as from all thoracic and abdominal ganglia indicating a widespread expression in cockroach neuronal tissue. In addition, in four out of ten investigated DUM neurons a pTRPγ signal was found on single-cell level as well (Fig. 2A). A Western blot analysis of neuronal tissues showed a distinct band with the expected molecular mass of 129 kDa in abdominal and thoracic ganglia with very low background staining (Fig. 2B). A protein of the same size was detected by the same antiserum (1) in transiently transfected HEK293 cells expressing pTRPγ but not in mock-transfected cells.

**Distribution of pTRPγ in the Nervous System**—Control immunostainings were performed with both pTRPγ antibodies in HEK293 cells transfected with pTRPγ DNA. 24 h after transfection, HEK293 cells showed significant immunofluorescence (Fig. 2, C and E), which was missing in non-transfected cells (Fig. 2, D and F). Immunostainings within the *Periplaneta* nervous system revealed a frequent but distinct expression of pTRPγ both in brain (Fig. 3, A–H) and abdominal ganglia (Fig. 3, J–L). Both antibodies, which were designed to recognize different C-terminal pTRPγ motifs, were seen to stain the same structures (Fig. 3, A, C, D, and F). Generally less intense immunofluorescence was seen in neuronal somata ranging from virtually absent to clearly pronounced staining as shown in Fig. 3, J and K, for the DUM neurons. Within the brain, immunostaining was found in the protocerebrum, in the deutocerebrum, and in the optic lobes. In the protocerebrum longitudinal and commissural fibers are stained. Within the central complex there is strong immunoreactivity in fibers around the upper (Fig. 3, D and F) and the lower (Fig. 3D) part of central body. Interestingly, the arborization areas within the central body do not show any immunoreactivity. This might indicate that pTRPγ is expressed in fibers except for their terminal branches including synaptic areas (Fig. 3, D and F). Similarly, there was clear staining around the olfactory glomeruli in the antennal lobe but not at all within the glomeruli where olfactory signals are processed via manifold synaptic contacts (Fig. 3G). The antennocerebral tract containing the projection neuron fibers was extensively stained (Fig. 3H). Besides the regions of olfactory information processing there is prominent staining in the optic lobes, particularly within the medulla (Fig. 3D). In the abdominal ganglia the somata of DUM neurons (Fig. 3, J and K) and those of a few smaller, not identified neurons (data not shown) appear stained. As in the brain, some fiber tracts are prominently stained while others such as the giant axons lack any staining (Fig. 3, J and L).

**cAMP Down-regulates pTRPγ but Not dTRPγ Currents**—Expression of pTRPγ in HEK293 cells produced a constitutively active non-selective cation conductance, which was not found in mock-transfected cells. The pTRPγ current showed outward rectification, even with symmetrical ionic concentrations (Fig. 4, A and B). The ratio of ion permeabilities was estimated from the dependence of the reversal potential on the composition of bath versus pipette solutions. With 5 mM Ca²⁺ and 140 mM Na⁺ in the bath versus 140 mM Cs⁺ in the pipette the reversal potential was \(-16.0 \pm 0.9\) mV (\(n = 5\)) and \(-0.4 \pm 1.1\) mV (\(n = 6\)), respectively. This yields, according to Equations 1 and 2, a permeability...
ratio of $P_{\text{Na}}:P_{\text{Ca}}:P_{\text{Na}} = 1:1.01:2.05$, which is very similar to the corresponding ratio reported for dTRPγ (3). HEK293 cells expressing pTRPγ were thus exposed to sustained influx of ions including Ca$^{2+}$ and showed considerably shortened survival time in culture compared with mock-transfected cells. 24 h after transfection the ratio of green fluorescing cells to non-fluorescing cells was $0.19 \pm 0.08$ ($n = 200$). Among

FIGURE 1. Sequence alignment of *Periplaneta* and *Drosophila* TRPγ. Ankyrin-like repeats are underlined and in bold; the coiled-coil domain is shown in italics; transmembrane regions are shown in bold and are labeled with a +. The dTRPγ exon missing in pTRPγ is indicated by *; the C-terminal sequence of the truncated pTRPγ splice variant is shown in bold italics. A second variable sequence stretch is found near alanine 787 (PGT-A787-GAG, shown in bold italics and underlined), where sequences can vary to PG-T-GAG (deletion) or PG-T-GNTSA-GAG (insertion).
The cells expressing pTRPγ the morphology of 26 ± 7 (n = 38) cells was typical for HEK293 cells; the remaining cells appeared spherical. 48 h after transfection only 9 ± 3% of cells (n = 200) showed green fluorescence, and all these cells were of spherical shape.

With the given sustained Ca²⁺ permeability of pTRPγ the heterogeneous expression system this channel meets one of the criteria for a channel involved in NCCE in DUM neurons. The most intriguing question was whether pTRPγ would be inhibited by cAMP. To test this we used symmetric Cs⁺ solution to avoid Ca²⁺ overload of cells. Application of the membrane-permeant cAMP analog 8-Br-cAMP (2 μM) caused a significant reduction of the pTRPγ current but no total block, since application of the TRP channel blocker 2-APB (100 μM) in the presence of 8-Br-cAMP blocked the remaining current nearly completely (Fig. 4, A and C). The fraction of current resistant to 8-Br-cAMP was 0.63 ± 0.06 at -100 mV and 0.67 ± 0.04 at +100 mV (n = 16), i.e. the 8-Br-cAMP effect was not apparently voltage-dependent. The 8-Br-cAMP-induced inhibition of the pTRPγ current started to develop within the first minute after application and saturated within a couple of minutes (Fig. 4B). After removal of 8-Br-cAMP the current recovered within 3–5 min (Fig. 6A). Taken together, 8-Br-cAMP seemed to act like a channel blocker. To support this more directly we performed experiments in the inside-out configuration. Bath application of cAMP again reduced the current, but even at a concentration of 1 mM more than 50% of current was resistant to cAMP (Fig. 4D). The dose-response curve of the cAMP effect shown in Fig. 4E is characterized by an IC₅₀ of 74 nM and a Hill coefficient of 0.83. At [cAMP] = 10 μM the effect saturates. Thus, the channel appears to be regulated in a physiological cAMP concentration range.

To our knowledge, no TRP channel has been found to be down-regulated by cAMP. Therefore, we were interested to see whether Drosophila TRPγ (dTRPγ), the only other presently known TRPγ channel, was also sensitive to cAMP. When expressing dTRPγ in HEK293 cells we obtained, similarly to pTRPγ, an outwardly rectifying current that was constitutively active. But this current was resistant to cAMP (Fig. 5, A and B). Application of 8-Br-cAMP (2 μM) only yielded a current reduction by 4% (n = 8). For comparison, 2-APB (100 μM) strongly reduced the dTRPγ current (Fig. 5, A and B).

To characterize the selectivity of the putative cyclic nucleotide-binding site in the pTRPγ channel, we applied the membrane-permeant cGMP analog 8-Br-cGMP (2 μM). However, cGMP failed to affect the pTRPγ and dTRPγ currents (Fig. 5C). Thus, the down-regulation of TRPγ channels is specific for cAMP.

The depressing action of cAMP on the NCCE channel in DUM neurons was a direct cAMP effect and not mediated by channel phosphorylation via PKA (8). To confirm that the down-regulation of pTRPγ current directly relies on cAMP, we had to rule out a possible contribution of pTRPγ channel phosphorylation by endogenous PKA. We thus tested the effect of 8-Br-cAMP in the presence of the PKA inhibitors KT5720, Rp-cAMPS, and myristoylated PKA-inhibiting peptide 14–22 amide (PKI). If the regulation of pTRPγ was PKA-dependent, the presence of an inhibitor should abolish the effect of cAMP. There was, however, neither a direct effect of PKA inhibitors on pTRPγ current (Fig. 6A) nor a significant change in the reducing effect of 8-Br-cAMP (Fig. 6B).

Searching for a putative constituent of a neuronal NCCE channel we thus found the pTRPγ channel, which displayed some of the expected properties including the unusual sensitivity to cAMP. For comparing the pharmacological profiles of TRPγ channels from Periplaneta and Drosophila we tested, besides typical TRP channel blockers such as 2-APB and La³⁺ (14) and SKF96365, which inhibits a variety of channels providing Ca²⁺ influx (15, 16), and LOE-908, which blocks NCCE in various preparations including DUM neurons (17, 2). The pTRPγ current was reduced by LOE-908 (10 μM) by 26 ± 5% (n = 5), while dTRPγ was insensitive (Fig. 7). 2-APB (100 μM) reduced the pTRPγ current by 71 ± 6% (n = 9) and the dTRPγ current by 49 ± 6% (n = 7). SKF96365 (10 μM) did not affect either of the currents, and La³⁺ (1 mM) reduced the pTRPγ current by 50 ± 9% (n = 5) and the dTRPγ current by 23 ± 9% (n = 8).
FIGURE 4. pTRP\textsubscript{γ} current is attenuated by cAMP. pTRP\textsubscript{γ} was expressed in HEK293 cells, and currents were measured in the whole-cell mode using symmetric CsCl solutions. A, families of currents obtained by 100-ms lasting voltage jumps from -80 to +100 mV from a holding potential of -50 mV. The currents measured before (Control) and 5 min after application of 2 mM 8-Br-cAMP are shown. Further application of 100 \mu M 2-APB caused nearly completely current block after 5 min (2-APB). B, time course of the current reduction by 8-Br-cAMP. Currents were measured at +100 mV and normalized to the current I\textsubscript{Control} that was obtained before application of 8-Br-cAMP (arrow). Data represent means ± S.E. of n = 5 cells. C, pTRP\textsubscript{γ} currents obtained by voltage ramps from -100 to +100 mV (in 400 ms). The pTRP\textsubscript{γ} current shows outward rectification, the reversal potential is near 0 mV. 8-Br-cAMP (2 \mu M, 5 min) caused a reduction of both inward and outward current. In the presence of 2-APB (5 min) only a residual current remained. D, pTRP\textsubscript{γ} currents obtained as described for C in the inside-out configuration. cAMP (1 mM, 5 min) reduced the inward and outward current. E, concentration dependence of the cAMP-effect at +100 mV. Filled squares represent means of 8–10 experiments. For comparison, the open square represents data obtained with 8-Br-cAMP (2 \mu M) in the whole-cell configuration (n = 9). The dose-response curve is described by an IC\textsubscript{50} of 74 nM and a Hill coefficient of 0.83. The saturating current reduction amounts to 48%.
The most marked difference of pTRPγ and dTRPγ is the cAMP-mediated current inhibition in the Periplaneta variant suggesting that one may find a cAMP-binding site by comparing both channel sequences. Since there is no typical consensus cAMP-binding motif in the channel protein, we tested a pTRPγ splice variant (pTRPγb) lacking most of the C terminus (see Fig. 1). Expression of pTRPγb yielded an outwardly rectifying current that was, however, insensitive to cAMP (Fig. 8, A and B). Therefore, the C terminus seems to play a critical role for cAMP binding and channel modulation. Interestingly, LOE-908 also failed to affect the pTRPγb current, and 2-APB was less effective than in the case of pTRPγ (Fig. 8C).

Does pTRPγ Contribute to the NCCE Conductance in DUM Neurons? —Our immunocytochemical investigations have shown that DUM neurons express pTRPγ channels, and the properties of the pTRPγ current in the heterologous expression system are compatible with the suggestion that this channel may be involved in forming the NCCE channel in DUM neurons. To obtain further support for this hypothesis, we tested whether LOE-908, 2-APB, and La³⁺, which attenuated the pTRPγ current in HEK293 cells (Fig. 7B), would also affect NCCE in DUM neurons. Using a bath solution containing only 2 mM Ca²⁺ as charge carrier, LOE-908 (10 μM) and 2-APB (100 μM) reduced the resting current density at −90 mV by 0.43 ± 0.15 pA/pF (n = 5) and 0.54 ± 0.09 pA/pF (n = 5), respectively (Fig. 9A). This reduction is similar to the effect of 8-Br-cAMP (2 μM) that caused a reduction by 0.42 ± 0.06 pA/pF (n = 7). This shows that the NCCE channel in DUM neurons, in addition to cAMP and LOE-908 (8), is sensitive to 2-APB. However, the relative effect of 2-APB appears to be weaker than on pTRPγ channels expressed in HEK293 cells (Fig. 7A). While 2-APB produced a stronger reduction of the TRP current than cAMP and LOE-908, there was no significant
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FIGURE 8. The truncated splice form pTRPγb is insensitive to cAMP. A, pTRPγb was expressed in HEK293 cells, and current families were measured as described in the legend to Fig. 4A. Currents measured 5 min after application of 2 μM 8-Br-cAMP are not reduced with respect to the control records. B, pTRPγb currents obtained by voltage ramps as described in Fig. 4B. The pTRPγb current shows outward rectification, and the reversal potential is near 0 mV. Currents registered before (Control) and 5 min after 8-Br-cAMP application are identical. C, effect of 8-Br-cAMP (2 μM), LOE-908 (10 μM), and 2-APB (100 μM) on pTRPγb currents measured at +100 mV. Data represent means ± S.E. of n = 5–9 cells.

FIGURE 9. The inhibitors of pTRPγ affect the non-capacitative Ca2+ entry current in Periplaneta DUM neurons. A, reduction of current density at −90 mV with 2 mM Ca2+ (white) or 3 mM Sr2+ (black) as charge carrier by 8-Br-cAMP (cAMP, 2 μM), LOE-908 (10 μM), and 2-APB (100 μM). Data represent means ± S.E. of n = 5–9 cells. B, current density at −90 mV with 2 mM Ca2+ before (white bars) and upon application of 10 μM ETYA (black bars). The effect of ETYA alone (Control) is reduced after preincubation of cells with 8-Br-cAMP (cAMP, 2 μM), LOE-908 (10 μM), and 2-APB (100 μM). Data represent means ± S.E. of n = 5–7 cells.

DISCUSSION

TRP proteins form a large and diverse family of ion channels with multiple functions (3). The first TRP channel was found in Drosophila photoreceptors where two further members of the family, TRPL and TRPγ, contribute to visual transduction. In adult Drosophila, the TRPγ protein was identified only in the eyes, although the mRNA message was also found in the body (3). dTRPγ appears highly enriched in photoreceptors where it preferentially forms heteromultimeric channels with TRPL. The eye-specific expression of dTRPγ indicates that this channel protein in Drosophila is solely involved in phototransduction. By contrast, in adult Periplaneta we found the mRNA message of pTRPγ throughout the nervous system, but we also detected the protein in neuronal cell bodies (Fig. 3). This indicates that the physiological role of TRPγ in Periplaneta considerably extends that in Drosophila. Several attempts to amplify the two other TRPC homologs of Drosophila (TRP and TRPL) by a degenerated PCR strategy were unsuccessful, indicating that TRPγ is probably the only TRPC-like channel with significant expression in the nervous system.

The immunocytochemical findings have indicated that the Periplaneta TRPγ protein is frequently expressed within the nervous system. Most prominently, it occurs in nerve fibers involved in processing/conducting sensory, particularly olfactory and optic, information. The role
of pTRPy in these fibers remains elusive. In hippocampal neurons the insertion of TRPC5 channels in neurites terminates their outgrowth (18). An intriguing finding in the Periplaneta brain was the fact that branching regions of fibers do not express pTRPy (e.g. in the central body, Fig. 3D). Furthermore, we cannot exclude the possibility that pTRPy is also expressed in glial cells surrounding the neurites. This point needs to be analyzed in more detail, e.g. by using glial cell markers and by performing electron microscopy. The most important result of the immunocytochemical approach for the present study was to see that the pTRPy protein is located in the somata of DUM neurons.

Drosophila TRP, but also the mammalian counterparts that form the subfamily of canonical TRPs (TRPC), function as receptor-operated channels (3). Activation of PLC was found to be a key element in the signal transduction process leading to dTRP activation (19). On the other hand, heterologously expressed dTRP and dTRPy proteins form constitutively active channels. However, coexpression of dTRP with dTRPy produces regulated channels that can be activated by agonists for receptors that stimulate PLC (3).

In Periplaneta DUM neurons we previously found a Ca\(^{2+}\)-permeable conductance that was voltage-independent and constitutively active (8, 20). This Ca\(^{2+}\)-background conductance was up-regulated by the peptide hormone AKH I via PLC activation. It was further established that the regulation of this conductance was independent of intracellular stores, i.e. that it contributes to NCCE (8). One might speculate whether the channel responsible for the Ca\(^{2+}\)-background conductance might belong to the TRP family. A detailed analysis of the AKH I-signal transduction process yielded a complex picture. The diacylglycerol produced by PLC activation is metabolized by diacylglycerol lipase to AA. This activates, by an unknown mechanism, NO-sensitive guanylyl cyclase. The resulting increase in cGMP level activates the phosphodiesterase 2, which in turn lowers the cAMP level (8). The final step in the up-regulation of Ca\(^{2+}\)-background conductance was thus the reduction of an inhibition. Such kind of regulation is akin to that of ATP-sensitive K\(^+\) channels, which open upon a drop in ATP concentration (21).

Searching for the molecular substrate of the Ca\(^{2+}\)-background conductance we found pTRPy, a homolog of the Drosophila TRPy. This protein is expressed in DUM cells, and we have seen that channels formed by pTRPy in HEK293 cells show some properties expected for a channel responsible for the Ca\(^{2+}\)-background conductance in DUM neurons.

It has to be kept in mind that overexpression of TRPy proteins in HEK293 cells may lead to the formation of new TRP heteromultimers including endogenous TRPCs (13) or to up-regulation of TRPC expression. However, the difference in 8-Br-cAMP sensitivity of currents produced by expression of pTRPy and its C-terminally truncated version pTRPyb (compare Fig. 4, C and D; with Fig. 8) clearly demonstrates that the overexpressed and not an endogenous channel protein determines the channel properties. Moreover, expression of dTRPy, the Drosophila homolog to pTRPy, results in the generation of currents similar to those produced by pTRPy but with partially different pharmacological properties (cf. Fig. 7).

With respect to a putative role of pTRPy in forming Ca\(^{2+}\)-background channels, the most important feature, besides its permeability to Ca\(^{2+}\), is the sensitivity to cAMP. Similarly important is the insensitivity of pTRPy to cGMP, since in DUM neurons the phosphodiesterase 2-mediated decrease in cAMP concentration requires an increase in cGMP concentration (8). Sensitivity of pTRPy to both CAMP and cGMP would thus not be compatible with the regulation of the NCCE channel in DUM neurons. Furthermore, both pTRPy and the background conductance are inhibited by LOE-908, 2-APB, and by La\(^{3+}\). Taken together, our results are compatible with the hypothesis that pTRPy is involved in forming channels that conduct the Ca\(^{2+}\)-permeable background conductance conferring NCCE in DUM neurons.

If our assumption was correct we had to propose a new functional role for a TRP channel, namely to act as a pacemaker channel. In DUM neurons the firing frequency is regulated by a variety of ion currents including the Ca\(^{2+}\)-background current (22). Depression of this current, e.g. by FMRF-related peptides, decreases the firing rate (23), while potentiation, e.g. by AKH I, enhances the firing rate (8). Although AKH I modulates a set of DUM cell currents, i.e. Na\(^{+}\) and P/Q-type Ca\(^{2+}\) current as well as Na\(^{+}\)- and Ca\(^{2+}\)-dependent K\(^+\) currents, solely the up-regulation of the Ca\(^{2+}\)-background current produces faster spiking (24). Furthermore, the Ca\(^{2+}\)-background current in DUM neurons is involved in controlling the intracellular Ca\(^{2+}\) concentration (20) and in filling of intracellular Ca\(^{2+}\) stores (25). Up-regulation of this current can induce local as well as global Ca\(^{2+}\) signals (8).

From the viewpoint of ion channel regulation the most intriguing result of the present study is the down-regulation of pTRPy current by cAMP. Although the binding site of cAMP on pTRPy is currently unknown, two conclusions can be drawn from sequence comparisons, pattern searches, and electrophysiological measurements of the short pTRPyb splice variant. (i) The distal C-terminal 400 amino acids of pTRPy seem to contain the cAMP-binding site as the truncated pTRPyb splice variant does not show any cAMP effect, and (ii) no consensus cAMP-binding sites were found in pTRPy indicating an unusual cAMP binding. Further work will be dedicated to analyze the cAMP effect on the level of single channels and to identify putative cAMP-binding sites in the channel protein. The latter will not exclude a search for N-terminal cAMP-binding sites, since it might also be possible that cAMP binds at the N terminus, while the distal C terminus is necessary to transduce the conformational change to channel closure.

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