Many olfactory receptor neurons use a cAMP-dependent transduction mechanism to transduce odorants into depolarizations. This signalling cascade is characterized by a sequence of two currents: a cation current through cyclic nucleotide-gated channels followed by a chloride current through calcium-activated chloride channels. To date, it is not possible to interfere with these generator channels under physiological conditions with potent and specific blockers. In this study we identified the styryl dye FM1-43 as a potent blocker of native olfactory cyclic nucleotide-gated channels. Furthermore we characterized this substance to stain olfactory receptor neurons that are endowed with cAMP-dependent transduction. This allows optical differentiation and pharmacological interference with olfactory receptor neurons at the level of the signal transduction.

The first step of odorant recognition in vertebrates begins at the level of the olfactory epithelium (OE). This consists of three principal cell types: olfactory receptor neurons (ORNs), glia-like sustentacular cells and basal cells. ORNs are primary sensory cells which transduce the binding of ligands to olfactory receptors through a second messenger pathway into sequences of action potentials. Although biophysically not entirely understood, a well known feature of olfactory transduction is the cascade of two generator channels, i.e., a \( \text{Ca}^{2+} \)-permeable cyclic nucleotide-gated (cng) channel driving a \( \text{Ca}^{2+} \)-dependent chloride channel (1). The transduction of odorants can be interfered with (i) at the level of olfactory receptors (2, 3, 4), (ii) at the level of receptor potential modulation or transformation (e.g. cannabinoids: (5); acetyl-choline: (6); carbachol: (7); adrenaline: (8)) or (iii) at the level of spike generation. Blocking olfactory transduction at the level of one or the other generator channel has proven difficult so far, due to the lack of specific chloride channel blockers and the lack of cng channel blockers that act at physiological membrane potentials. Pseudochetoxin, the only specific blocker of the cng channel so far reported (9), is presently not commercially available. Obviously, such blockers would be extremely useful to experimentally dissect the transduction cascade.

Here we set out to find a possibility to specifically block cng channels in ORNs. For the following reasons we speculated that FM1-43 might be a promising candidate:

While FM1-43 is presently better known as a means to monitor membrane trafficking (10, 11, 12), and vesicle endocytosis in cochlear hair cells (13, 14), FM1-43 has also been reported to stain several sensory and neuronal cells in an endocytosis-independent way, e.g., sensory hair cells in the lateral line organ, cochlea hair cells of various vertebrate species (15, 16, 17, 18), Merkel cells, taste buds, nociceptive fibers as well as primary sensory neurons in the trigeminal (V), geniculate (VII), petrosal (IX), nodose (X) and dorsal root ganglia (18, 19, 20). In addition, FM1-43 has been reported to label the lateral line organ and epidermal cells at the nasal pits in \textit{Xenopus laevis} tadpoles (15). Three years later (21) FM1-43 was shown to label dissociated ORNs. However, the question whether labelling with FM1-43 had any physiological effects in ORNs remained unanswered.

Apart from staining cells, FM1-43 has also been described as a blocker of cation currents. Gale and coworkers (17) observed that FM1-43 reversibly blocked mechanotransduction of cochlear hair cells, and Drew and Wood (19) reported that the dye blocked rapidly- and slowly-adapting mechanically activated cation currents in cultured dorsal root ganglion neurons. Additionally, FM1-43 has been known to permeate through mechanoelectric
transduction channels of hair cells and of dorsal root ganglion cells (18, 19) as well as through TRPV1 vanilloid receptors and purinergic P2X$_2$ receptors (18).

We therefore investigated the action of FM1-43 in the OE and characterized the mechanisms by which it acts therein. We found that FM1-43 stains the subset of ORNs that is endowed with the cAMP-dependent transduction cascade. Furthermore, extracellular FM1-43 turned out to inhibit cng currents in the physiological range of membrane potentials.

EXPERIMENTAL PROCEDURES

Ethical approval- This study was performed on tadpoles of *Xenopus laevis* (stage 51-54; (22)). For tissue slice preparations the animals were anaesthetized by chilling them in a mixture of ice and water and then sacrificed by decapitation. For electroporation experiments tadpoles were anaesthetized in 0.02 % MS-222 (Sigma). Both procedures were performed as approved by the University of Göttingen Committee for Ethics in Animal Experimentation. The number of tadpoles used for each experimental series is indicated in the corresponding results section.

In vivo labelling of ORNs with FM1-43- To stain ORNs with FM1-43, living tadpoles were transferred into distilled water for 5 min. Then they were put either for 7 min (standard staining) or for 1 min and 15 s (light staining) into 10 ml of distilled water with 2 µM FM1-43 (stock solution: 2 mM in methanol; Molecular Probes, Leiden, the Netherlands). In some experiments, where we were interested in the impact of certain substances on the staining efficiency, we added 2 mM CaCl$_2$, 1 mM MgCl$_2$, 200 µM LY-83583 or 1 mM amiloride to the solution that contained FM1-43. In these cases the exposure time in the respective incubation solution was 7 min.

OE slice preparation- OE tissue slices were made either from animals that had undergone an *in vivo* staining or from control animals, which were equally treated with the exception of FM1-43 being left out from the exposure solution. The tadpoles were chilled in a mixture of ice and water and were decapitated. A block of tissue containing the OE, the olfactory nerves, and the brain was cut out and kept in bath solution. The tissue was then glued onto the stage of a vibrotome (VT 1200S; Leica, Bensheim, Germany) and cut horizontally into 130 to 150 µm thick slices.

*Explant of a nose-brain preparation*- For imaging [Ca$^{2+}$], of glomeruli, ORNs were traced using electroporation of the OE with fluo-4 dextran. To this end larval *Xenopus laevis* were anesthetized in 0.02 % MS-222 (Sigma). Crystals of fluo-4 dextran potassium salt (10 kDa, Invitrogen) were inserted into the nasal cavities, where it dissolved in the residual water. Subsequently, two platinum electrodes (diameter: 250 µm) were placed 3 mm apart from each other into the nasal cavities, and the dye was electroporated by applying twelve 30 V pulses (20 ms) of alternating polarity.

After being kept under standard conditions for one to three days in a water tank the tadpoles were chilled in a mixture of ice and water and then decapitated. A block of tissue containing the OE, the olfactory nerves and the brain was cut out and kept in bath solution. The tissue surrounding the ventral part of the olfactory bulb was removed and the explant preparation was put under a grid in a recording chamber and viewed with a laser-scanning confocal microscope.

Imaging- The efficiency of staining with FM1-43 was assessed using a laser-scanning confocal microscope attached to an inverted microscope (LSM 510, Zeiss) with 10x/0.45 or 40x/1.3 objectives. The confocal pinhole was set to 120-150 µm to exclude fluorescence detection from more than one cell layer. Fluorescence images of FM1-43 (excitation at 488 nm; emission > 505 nm) in the OE were acquired together with a pseudo bright-field, scanned transmission image for better orientation in the tissue.

For imaging [Ca$^{2+}$], in ORN somata, tissue slices were incubated in 200 µl of a bath solution that contained 50 µM Ca$^{2+}$ indicator dye fluo-4 AM (Molecular Probes, Leiden, The Netherlands) and 50 µM MK571 (Alexis Biochemicals, Grünberg, Germany). Fluo-4 AM was dissolved in DMSO (Sigma, Deisenhofen, Germany) and Pluronic F-127 (Molecular Probes). The final concentrations of DMSO and Pluronic F-127 did not exceed 0.5 % and 0.1 %, respectively. To avoid multidrug resistance transporter-mediated destaining of the slices, MK571, a specific inhibitor of the multidrug resistance-associated proteins, was added to the incubation solution (23). After incubation at room temperature for 30 min, the tissue slices were put under a grid...
in a recording chamber, which was placed on the stage of the LSM 510 or a custom-built two photon excitation microscope (TPM). Before starting the calcium imaging experiments, the slices were rinsed with bath solution for at least 5 min.

Fluorescence images at the LSM 510 (excitation at 488 nm; emission > 505 nm for fluo-4 imaging, and emission from 505 to 530 nm and > 560 nm for fluo-4 and FM1-43 imaging, respectively) and at the TPM (excitation at 800 nm; emission from 470 nm to 550 nm for fluo-4) were acquired at 1 to 2 frames/s, with three to twenty images taken as control images before the onset of odor delivery. The fluorescence changes \( \Delta F / F \) of fluo-4 were calculated for individual ORNs (or glomeruli) as \( \Delta F / F = (F_1 - F_2) / F_2 \), where \( F_1 \) was the fluorescence averaged over the pixels of an ORN soma (or glomerulus), while \( F_2 \) was the average fluorescence of the same pixels prior to stimulus application, averaged over five images. A response was assumed if the following two criteria were met: (i) the first two intensity values after stimulus arrival at the mucosa, \( \Delta F / F(t_1) \) and \( \Delta F / F(t_2) \), had to be larger than the maximum of the prestimulus intensities; (ii) \( \Delta F / F(t_2) > \Delta F / F(t_1) \) with \( t_2 > t_1 \). Data analysis was performed with Matlab (Mathworks, USA). Paired t-tests were used to assess statistical significance.

**Uncaging of cAMP in ORNs viewed with confocal microscopy**- FM1-43-stained slices were incubated in 200 µl of Ca\(^{2+}\) indicator rhod-2 AM solution (50 µM rhod-2 AM (Molecular Probes, Leiden, The Netherlands) dissolved in DMSO (0.5 %) and Pluronic F-127 (0.1 %) and 50 µM MK571) at room temperature for 30 min. A glass fiber (HCG-M0200T 200 µm, Laser Components) coupled to a 378 nm diode laser (iPulse, 378 nm, 16 mW, Toptica Photonics) was positioned close to a 378 nm diode laser (iPulse, 378 nm, 16 mW, Toptica Photonics) was positioned close to a 378 nm diode laser. Pulse protocol and data acquisition programmes were written in Matlab (Mathworks, USA). Paired t-tests were used to assess statistical significance.

**Patch-clamp recordings of the cng current**- Patch clamp recordings (25) from ORNs were done in OE slices using an EPC7 patch-clamp amplifier (List, Darmstadt, Germany). The slices were viewed under Nomarski optics (Axioskop 2; Zeiss, Göttingen, Germany). Pipettes with a tip resistance of 6-10 MΩ were pulled from borosilicate glass (diam. 1.8 mm, Hilgenberg, Malsfeld, Germany) using a two-stage pipette puller (PC-10, Narishige) and filled with 4 µl of a cAMP- and cGMP-containing pipette solution. Pulse protocol and data acquisition programmes were written in C.

The responsiveness of a patch-clamped cell was assessed in the on-cell configuration \( (u_{hold} = 0 \text{ V}) \) by stimulating it with forskolin (50 µM; Sigma) dissolved in bath solution. Then the whole-cell configuration was established after setting the holding potential to -70 mV and replacing the external solution by Ca\(^{2+}\)- and Mg\(^{2+}\)-free bath solution with or without 10 µM FM1-43. The recorded currents were plotted using Matlab (Mathworks, USA). Variance analysis (ANOVA) was used to assess the statistical significance of the current response amplitude upon forskolin application.

**Solutions and stimulus application**- The compositions of the bath and pipette solutions (in mM) were as follows. Bath solution: NaCl, 98; KCl, 2; CaCl\(_2\), 1; MgCl\(_2\), 2; glucose, 5; sodium pyruvate, 5; HEPES, 10. Ca\(^{2+}\) - and Mg\(^{2+}\)-free bath solution: NaCl, 98; KCl, 2; glucose, 5; sodium pyruvate, 5; HEPES, 10; EGTA, 2. Pipette solution: NaCl, 2; KCl, 11; MgSO\(_4\), 2; K-Gluconat, 80; HEPES, 10; EGTA, 0.2; Na2ATP, 1; Na2GTP, 0.1; cAMP, 1; cGMP, 0.1. The pH was adjusted to 7.8,
osmolarities were 230 mOsmol/l for bath solutions and 190 mOsmol/l for the pipette solution.

The recording chamber was perfused with bath solution by gravity feed through a funnel applicator. The funnel’s outflow was through a syringe needle the outlet of which was placed in front of the OE. Changes of the external solution were done by starting the influx of a bath solution into the funnel applicator and simultaneously stopping the influx of another one.

Amino acids (26, 27, 28), amines (29, 30, 31, 32, 33), bile acids (34, 35) and alcohols (36) are known to be odorants for aquatic species. The odorants were dissolved in bath solution (stocks of 10 mM or 25 mM) and used at a final concentration of 100 µM in all of the experiments. Stimulus solutions were prepared immediately before use and were pipetted directly into the funnel for bath perfusion without stopping the flow. The time course of stimulus arrival at the OE was simulated by applying the fluorescent dye avidin Alexa Fluor 488 application to the funnel. The delay of stimulus arrival caused by the syringe, i.e., the time from pipetting the dye into the funnel to the resulting fluorescence increase in the OE was approximately 2 s. The minimum interstimulus interval between odorant applications was 2 min.

Fluorispectrometry- The fluorescence of FM1-43 mixed with amiloride or LY-83583 dissolved in pipette solution was assessed with a fluorescence spectrophotometer (F-2700, Hitachi). Excitation was at 488 nm, emission was observed from 505-700 nm with a slit width of 5 nm and a scan speed of 300 nm/min.

RESULTS

FM1-43 stains a subset of ORNs- In a first set of experiments, living Xenopus laevis tadpoles were put into water containing the styryl dye FM1-43 (2 µM). Thereafter the animals were sacrificed and tissue slices were prepared from the OE. When the slices were viewed with a confocal laser scanning microscope, a large number of cells were stained in the entirety of their cytosol (Fig. 1A, N = 20 slices), whereas control slices showed no fluorescence (Fig. 1C, N = 15). For a better orientation we overlayed the fluorescence images with the corresponding transmission images scanned through wide-field optics. Fig. 1B shows the magnified rectangular area of A as a z-projection to illustrate the fine structure of the cells stained. Dendrites running to the surface of the OE, where cilia or microvilli issued from dendritic knobs, and axons running into the opposite direction to join the olfactory nerve defined these cells as ORNs. No staining at all was found in the vomeronasal organ (not shown).

FM1-43 never stained the entire OE. It rather appeared to stain a certain subset of ORNs. To visualize this subset, tadpoles were bathed in FM1-43 for 7 minutes and ORNs were then backfilled with Alexa Fluor 488. The fluorescence intensities of the two dyes were spectrally unmixed (24), which allowed illustrating either dye individually (Fig. 1D). Only a fraction of the backtraced ORNs (green) were doublelabelled with FM1-43 (red).

As the staining protocol did not allow FM1-43 loading of slice preparations and FM1-43 severely interfered with [Ca²⁺]-imaging, we first labelled ORNs of living tissue and afterwards tried to characterize the ORNs of this subset by testing their sensitivity to amino acids, bile acids, amines, alcohols, and a mixture of all (100 µM for each substance). 156 out of 165 stained ORNs did not respond to any of the stimuli, which is in stark contrast to the high responsiveness of Xenopus tadpole ORNs as seen in previous studies (37, 38). Only nine ORNs were responsive to the mixture, one of them to alcohols and four to amines. Fig. 1E through 1G gives a typical example showing primarily two things. First, this ORN was sensitive to alcohols (E, G, H) but not to amino acids (F), and second, the response amplitudes to both the stimulus mixture (E, I) and to alcohols (G, H) rapidly declined over time and then vanished. The facts that FM1-43 stained only a subset of ORNs and that most of the stained ORNs did not respond at all, while those few which initially did lost their responsiveness rapidly, suggested that the responsiveness of the stained ORNs was severely compromised by FM1-43.

FM1-43 is selectively taken up by ORNs endowed with the cAMP cascade- As FM1-43 was taken up in the OE in-vivo, it certainly passed through the plasma membrane of the compartments exposed to the principal cavity,
i.e., through cilia, microvilli, and/or dendritic knobs. The interstitial space was never stained so that the possibility for dye molecules crossing the tight junction barrier could be excluded. Further, as the FM1-43 fluorescence was cytosolic and as it built up rapidly in the cytosol, FM1-43 permeated presumably via ion channels rather than via transport proteins. We therefore checked whether cng channels were permeable for FM1-43 whereby we took advantage of the well-known permeability properties of divalents in cng channels as well as of the effect of two non-specific blockers of cng channels.

When CaCl$_2$ (2 mM; N = 5) or MgCl$_2$ (1 mM; N = 5) was added to the water during the in-vivo incubation with FM1-43, the fluorescence intensity of ORNs was reduced to almost zero (Fig. 2, A (CaCl$_2$) and B (MgCl$_2$), control: Fig. 2C). This would be consistent with an uptake of FM1-43 through cng channels as Mg$^2+$ and Ca$^2+$ has been reported to exert a permeation block in these channels (39).

If FM1-43 permeates through cng channels its permeation should be affected by LY-83583 or amiloride. When LY-83583 (200 µM), which blocks cng channels and the soluble guanylyl cyclase (40), was added during dye incubation, the uptake of FM1-43 was completely blocked (Fig. 2D, N = 10, control, Fig. 2F). The presence of amiloride (1 mM), which blocks cng channels, Na$^+$ channels, T-type Ca$^{2+}$ channels and several transporters (41, 42, 43, 44), during incubation also reduced the FM1-43 uptake dramatically (Fig. 2E, N = 8). It can be excluded that the reduction of FM1-43 fluorescence is primarily due to quenching, since LY-83583 quenches the emission maximum of FM1-43 to about 60%, and amiloride even increases the emission maximum (Fig. 2G). These results suggest that cng channels have a sizable permeability for FM1-43. The ORNs stained by FM1-43 may thus correspond to the subset of ORNs endowed with the canonical cAMP-transduction cascade.

The direct test of this hypothesis would be to evoke responses to cAMP in FM1-43-stained cells. Of course, this is conflicting with the hypothesis itself as FM1-43 would suppress the responses. We tried to circumvent this problem by exposing the animals to FM1-43 for a relatively short time in order to have a correspondingly weak staining and at least some cng channels left functional. In fact, under these conditions, the ORN staining with FM1-43 was rather faint but forskolin, which is reported to activate the cAMP cascade (45) clearly induced reproducible responses (Fig. 2H, here: $\Delta F/F = 10 \%$). Similar results were obtained in ten out of 13 cells (five slices). The three non-responding cells came all from the same slice. Uncaging of caged cAMP in FM1-43-loaded ORNs also resulted in a small, transient fluorescence increase of the calcium indicator dye rhod-2 (Fig. 2I, here: $\Delta F/F = 5 \%$, five out of five cells; three slices).

Taken together, the blockage of FM1-43 uptake by divalents and by cng channel blockers as well as the responses of faintly stained ORNs to forskolin and cAMP is consistent with the hypothesis that FM1-43 enters ORNs through cng channels.

Cng-generator currents are inhibited by extracellular FM1-43. Patch-clamped ORNs in untreated OE tissue slices were first classified as cAMP-dependent or cAMP-independent by stimulation with forskolin in the on-cell mode of the patch clamp technique. Some ORNs responded to forskolin with a transient firing rate increase (Fig. 3, A and B, upper traces), while others, presumably due to the lack of cng channels, showed no response to forskolin (Fig. 3C, upper trace). Though the parameters (latency, frequency, duration) of the responses to forskolin commonly vary from cell to cell (46) responding and non-responding cells could always be clearly distinguished. In a second step of the experiment the same cells were recorded in the whole-cell mode, with cAMP and cGMP added to the pipette solution. The effect of the second messengers diffusing from the pipette into the cell was observed either with (Fig. 3B, lower trace) or without FM1-43 (Fig. 3A, lower trace) added to the bath solution. Without any FM1-43 in the bath an inward current set in immediately after breakthrough. To avoid, as much as possible, the activation of Ca$^{2+}$-activated Cl$^-$ channels downstream the cng channels, Ca$^{2+}$ was omitted from the bath in these experiments, so that the recorded current was a current through cng channels carried by Na$^+$ ions. Its average amplitude was 213.8 +/− 21.2 pA (SEM, N = 5). FM1-43 in the bath solution (10 µM) significantly (ANOVA, p < 0.001) reduced the inward current in cAMP-dependent cells upon breakthrough to 54.5 +/− 31.6 pA (Fig. 3B, lower trace; N = 6). In non-cAMP-dependent ORNs, cAMP and cGMP never had any effect on the current (Fig. 3C,
lower little Ca. Slice preparation and in that there was no or in that the recordings were made in a tissue slice preparation and in that there was no or very little Ca$^{2+}$ flux through the cng channels. On the one hand the generator current blockage shown in Fig. 3B does thus not necessarily mean that FM1-43 would block odor responses under physiological conditions, i.e. with Ca$^{2+}$ permeating cng channels. On the other hand, FM1-43 once having entered the cell in divalent-free medium could have blocked odor responses in many ways intracellularly. To exclude such possibility we carried out two further experiments regarding the effect of extracellularly applied FM1-43. Specifically, we imaged either ORN somata deep in the OE of an acute slice preparation using two photon excitation microscopy or axons of ORNs in the olfactory bulb of a whole-mount preparation with intact OEs under physiological saline including divalent ions. Washout of FM1-43 led to an increased [Ca$^{2+}$], transient amplitude for both conditions (0.67 ± 0.22, p<0.1 for washout of 10 µM FM1-43; 0.39 ± 0.20, p<0.1 for washout of 20 µM FM1-43).

**DISCUSSION**

The starting point of this work were the following observations. First, the styryl dye FM1-43 stained ORNs in the OE only when living tadpoles were exposed to the dye in distilled water. Second, only a subset of ORNs in the OE were stained and, third, ORNs that were stained, mostly failed to respond to odorants. We then carried out a number of experiments that demonstrated that FM1-43 entered and permeated through cng channels under divalent-free conditions. This result explains all of the observations made.

First, FM1-43 had not been observed before because most experiments with ORNs had been done on isolated ORNs or on ORNs in tissue slices, where physiological saline including divalent ions was used. In the numerous cases where odor stimuli were applied to aquatic mucosae, FM1-43 was usually not added to the stimulus. However, Nishikawa and Sasaki (15) reported that FM1-43 labelled epidermal cells at nasal pits, and three years later Rankin and coworkers (21) showed that FM1-43 labelled dissociated ORNs. In these experiments FM1-43 was internalized and appeared in cell body, dendrite and knob after stimulation with L-glutamate. Rankin et al. (21) postulated a novel endocytosis-like mechanism for dye uptake, which we have not found any evidence for in our experiments.

FM1-43 may have entered the OE through its tight junctions, though it had been shown that the tight junctions of the OE prevent most molecular species from crossing them (47, 48). However, in our experiments the FM1-43 staining was never extracellular. Instead it was consistently confined to the cytosol of ORNs, so that the OE tight junctions must be assumed to be impermeable for FM1-43. Therefore, dye
uptake had to occur at the level of the cilia which were exposed to the principal cavity. In agreement with this finding, FM1-43 uptake in hair cells also occurred at the stereocilia, and removal of the cilia prevented dye uptake (17, 18).

Second, the finding that only a subset of ORNs in the OE was stained can also be explained by FM1-43 permeating cng channels. It has been reported in a number of publications (26, 45, 49) that only a fraction of *Xenopus laevis* ORNs possess the canonical, cAMP-dependent olfactory transduction cascade. FM1-43 when permeating cng channels must thus be supposed to stain these ORNs. Other ORNs, in particular those responsive to amino acid, cannot be stimulated this way and are therefore believed to express a different kind of generator channel. If FM1-43 would permeate those channels, too, one would expect the vast majority of ORNs in the OE to be stained. As this was not the case, we conclude that the ORN generator channels involved in the detection of amino acids are not permeable for FM1-43.

The third of our initial observations was that ORNs that were stained by FM1-43 mostly failed to respond to odorants. This observation is also well explained by FM1-43 entering cng channels. Either an odorant acts on ORNs that don't possess the cAMP-dependent transduction cascade, then it does, per definition, not act on FM1-43-stained ORNs, or an odorant acts on ORNs that do possess the cAMP-dependent transduction cascade, then the cng channels are likely to be blocked by FM1-43 and no odor response would be detectable.

FM1-43 entered ORNs in the absence of a stimulus. Generally, it is hard if not impossible to perfectly exclude the presence of any olfactory stimul.. Apart from this caveat cng channels in ORNs are reported to gate spontaneously and ligand-independent, thereby producing a detectable macroscopic conductance (50). While Tibbs and coworkers (51) calculated an open probability of heterologously expressed cng channels with the α subunit to 0.002, Kleene (52) estimated the open probability of spontaneously gating cng channels in dissociated grass frog ORNs to be 0.03, which would be sufficient for a spontaneous dye uptake.

The uptake of dyes through plasma membrane channels seems to be a more general process than previously assumed. For example, YO-PRO permeates purinergic receptors (53). Besides cng and hair cell mechanotransducer channels, other sensory channels like the vanilloid receptor TRPV1, the purinergic receptor P2X3, and mechanoelectric transduction channel of dorsal root ganglion cells (18, 19) were shown to be permeable for FM1-43.

While FM1-43 permeates cng channels, it blocks the ionic current through these channels and thereby odorant responses. These properties are characteristic for permeation blockers (54). This is rather useful as there are virtually no specific blockers for cng channels. L-cis-diltiazem, amiloride and its derivates, dichloro-benzamil, LY-83583 or tetracain analogues are either unspecific or block at positive membrane potentials or both (55, 56, 57, 44, 58, 40, 59, 60). Because of these unfavorable properties the required concentrations of the unspecific cng channel blockers are usually rather high. In our study, e.g., LY-83583 and amiloride were used at 200 µM and 1 mM, respectively. In contrast to the mentioned inhibitors, FM1-43 blocks cng channels under physiological conditions. Cells can thus be stained in-vivo at resting membrane potential and without stimulation. 10 µM FM1-43 reduced the cng current to ~25 % at resting membrane potential. The cng current was measured in the absence of Ca²⁺ and Mg²⁺ and was therefore carried by monovalent ions only (39). FM1-43 has also been found to be a blocker of cation currents in two other studies: Gale et al. (17) observed that extracellular FM1-43 reversibly blocked mechanotransduction of cochlear hair cells in culture. FM1-43 reduced the currents in a voltage-dependent way, such that the block was most effective at ~4 mV (Kd = 1.2 µM) and less effective at large positive and negative potentials. Further, the block was strongly dependent on extracellular Ca²⁺, most effective at low Ca²⁺ concentrations. In a study by Drew and Wood (19) extracellular FM1-43 blocked both rapidly- and slowly-adapting mechanically activated cation currents in cultured dorsal root ganglion neurons. The Kd was reported to be 5 µM and 3 µM, respectively. The block was equally efficient at -70 and -35 mV. It was however significantly reduced at positive holding potentials. At low extracellular Ca²⁺ concentrations the FM1-43 block of the currents was more effective that at higher concentrations.
Taken together, FM1-43 appears to exert a permeation block of cng channels. It is a novel mechanism to label a distinct subset of ORNs, and conversely, to identify non-labelled cells such as sustentacular cells or ORNs that don’t use cAMP in their transduction cascade. Further it allows staining and blocking in-vivo and under physiological conditions. It seems therefore particularly useful for studies of olfactory transduction cascades. Finally the fluorescence of FM1-43 may turn out to be well-suited for studying ciliary processes and channel densities.

REFERENCES

1. Stephan, A. B., Shum, E. Y., Hirsh, S., Cygnar, K. D., Reisert, J., and Zhao, H. (2009) Proc Natl Acad Sci U S A 106, 11776–11781
2. Oka, Y., Nakamura, A., Watanabe, H., and Touhara, K. (2004) Chem Senses 29, 815–822
3. Oka, Y., Omura, M., Kataoka, H., and Touhara, K. (2004) EMBO J 23, 120–126
4. Sanz, G., Schlegel, C., Pernollet, J.-C., and Briand, L. (2005) Chem Senses 30, 69–80
5. Czesnik, D., Schild, D., Kuduz, J., and Manzini, I. (2007) Proc Natl Acad Sci U S A 104, 2967–2972
6. Bouvet, J.-F., Delaleu, J.-C., and Holley, A. (1988) Neurosci Res 5, 214–223
7. Frings, S. (1993) J Gen Physiol 101, 183–205
8. Kawai, F., Kurahashi, T., and Kaneko, A. (1999) Proc Nat Acad Sci U S A 96, 754–759
9. Oka, Y., Omura, M., Kataoka, H., and Touhara, K. (2004) EMBO J 23, 120–126
10. Sanz, G., Schlegel, C., Pernollet, J.-C., and Briand, L. (2005) Chem Senses 30, 69–80
11. Czesnik, D., Schild, D., Kuduz, J., and Manzini, I. (2007) Proc Natl Acad Sci U S A 104, 2967–2972
12. Bouvet, J.-F., Delaleu, J.-C., and Holley, A. (1988) Neurosci Res 5, 214–223
13. Frings, S. (1993) J Gen Physiol 101, 183–205
14. Kawai, F., Kurahashi, T., and Kaneko, A. (1999) Proc Nat Acad Sci U S A 96, 754–759
15. Oka, Y., Nakamura, A., Watanabe, H., and Touhara, K. (2004) Chem Senses 29, 815–822
16. Bouvet, J.-F., Delaleu, J.-C., and Holley, A. (1988) Neurosci Res 5, 214–223
17. Frings, S. (1993) J Gen Physiol 101, 183–205
18. Kawai, F., Kurahashi, T., and Kaneko, A. (1999) Proc Nat Acad Sci U S A 96, 754–759
19. Oka, Y., Omura, M., Kataoka, H., and Touhara, K. (2004) EMBO J 23, 120–126
20. Sanz, G., Schlegel, C., Pernollet, J.-C., and Briand, L. (2005) Chem Senses 30, 69–80
21. Czesnik, D., Schild, D., Kuduz, J., and Manzini, I. (2007) Proc Natl Acad Sci U S A 104, 2967–2972
22. Bouvet, J.-F., Delaleu, J.-C., and Holley, A. (1988) Neurosci Res 5, 214–223
23. Frings, S. (1993) J Gen Physiol 101, 183–205
24. Kawai, F., Kurahashi, T., and Kaneko, A. (1999) Proc Nat Acad Sci U S A 96, 754–759
25. Oka, Y., Nakamura, A., Watanabe, H., and Touhara, K. (2004) Chem Senses 29, 815–822
26. Bouvet, J.-F., Delaleu, J.-C., and Holley, A. (1988) Neurosci Res 5, 214–223
27. Frings, S. (1993) J Gen Physiol 101, 183–205
28. Kawai, F., Kurahashi, T., and Kaneko, A. (1999) Proc Nat Acad Sci U S A 96, 754–759
29. Oka, Y., Omura, M., Kataoka, H., and Touhara, K. (2004) EMBO J 23, 120–126
30. Sanz, G., Schlegel, C., Pernollet, J.-C., and Briand, L. (2005) Chem Senses 30, 69–80
31. Czesnik, D., Schild, D., Kuduz, J., and Manzini, I. (2007) Proc Natl Acad Sci U S A 104, 2967–2972
32. Bouvet, J.-F., Delaleu, J.-C., and Holley, A. (1988) Neurosci Res 5, 214–223
33. Frings, S. (1993) J Gen Physiol 101, 183–205
34. Kawai, F., Kurahashi, T., and Kaneko, A. (1999) Proc Nat Acad Sci U S A 96, 754–759
35. Oka, Y., Nakamura, A., Watanabe, H., and Touhara, K. (2004) Chem Senses 29, 815–822
36. Bouvet, J.-F., Delaleu, J.-C., and Holley, A. (1988) Neurosci Res 5, 214–223
37. Frings, S. (1993) J Gen Physiol 101, 183–205
38. Kawai, F., Kurahashi, T., and Kaneko, A. (1999) Proc Nat Acad Sci U S A 96, 754–759
39. Oka, Y., Omura, M., Kataoka, H., and Touhara, K. (2004) EMBO J 23, 120–126
40. Sanz, G., Schlegel, C., Pernollet, J.-C., and Briand, L. (2005) Chem Senses 30, 69–80
41. Czesnik, D., Schild, D., Kuduz, J., and Manzini, I. (2007) Proc Natl Acad Sci U S A 104, 2967–2972
42. Bouvet, J.-F., Delaleu, J.-C., and Holley, A. (1988) Neurosci Res 5, 214–223
43. Frings, S. (1993) J Gen Physiol 101, 183–205
44. Kawai, F., Kurahashi, T., and Kaneko, A. (1999) Proc Nat Acad Sci U S A 96, 754–759
45. Oka, Y., Nakamura, A., Watanabe, H., and Touhara, K. (2004) Chem Senses 29, 815–822
46. Bouvet, J.-F., Delaleu, J.-C., and Holley, A. (1988) Neurosci Res 5, 214–223
47. Frings, S. (1993) J Gen Physiol 101, 183–205
48. Kawai, F., Kurahashi, T., and Kaneko, A. (1999) Proc Nat Acad Sci U S A 96, 754–759
49. Oka, Y., Omura, M., Kataoka, H., and Touhara, K. (2004) EMBO J 23, 120–126
50. Sanz, G., Schlegel, C., Pernollet, J.-C., and Briand, L. (2005) Chem Senses 30, 69–80
51. Czesnik, D., Schild, D., Kuduz, J., and Manzini, I. (2007) Proc Natl Acad Sci U S A 104, 2967–2972
52. Bouvet, J.-F., Delaleu, J.-C., and Holley, A. (1988) Neurosci Res 5, 214–223
53. Frings, S. (1993) J Gen Physiol 101, 183–205
54. Kawai, F., Kurahashi, T., and Kaneko, A. (1999) Proc Nat Acad Sci U S A 96, 754–759
55. Oka, Y., Nakamura, A., Watanabe, H., and Touhara, K. (2004) Chem Senses 29, 815–822
FOOTNOTES

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The abbreviations used are: cng channel, cyclic nucleotide-gated channels; FM1-43, N-(3-triethylammoniumpropyl)-4-(4-(dibutylamino)styryl)pyridinium dibromide; OE, olfactory epithelium; ORN, olfactory receptor neuron.

FIGURE LEGENDS

FIGURE 1. FM1-43-stained ORNs in the OE and their responses to odorants. A, OE of a tadpole after a 7 min exposure to FM1-43. B, Z-projection of a number of zoomed cells shown in A illustrates the morphology of FM1-43-labelled ORNs. C, Control OE. D, The spectrally different fluorescence intensities of ORNs labelled with FM1-43 (red) and backfilled with Alexa Fluor 488 (green) were unmixed (upper, combined fluorescence; middle, backtraced ORNs in green; lower, FM1-43-stained ORNs in red). E through I, [Ca\(^{2+}\)], transients of an FM1-43-labelled ORN evoked by the odorant mixture but recorded at different times (E, I), amino acids (F) and alcohols (G, H), shown in
chronological order. Scale bars: A and C = 50 µm; B = 10 µm; D = 150 µm; E through I, black lines under the traces indicate odorant applications. Time scale [s] and change of fluorescence intensity ∆F/F [%] are indicated by the bars in the lower right corner. The boundaries of the OEs are shown by white dotted lines.

FIGURE 2. Evidence for FM1-43 passing cng channels. Incubation of the tadpoles in FM1-43 solution with 2 mM CaCl\textsubscript{2} (A) or 1 mM MgCl\textsubscript{2} (B) almost completely blocked FM1-43 uptake. The unspecific channel blockers (D) LY-83583 and (E) amiloride also blocked FM1-43 uptake. C and D, show two control OEs each with a large number of ORNs labelled by FM1-43 (2 µM). G, Fluorescence emission of FM1-43 alone (red traces) and mixed with 200 µM LY-83583 or 1 mM amiloride. The emission maximum of FM1-43 decreases to 60 % in the presence of LY-83583 and slightly increases with amiloride. H, Forskolin-evoked [Ca\textsuperscript{2+}] transients and I, [Ca\textsuperscript{2+}], transients induced by uncaging of cAMP in individual FM1-43-stained ORNs. Similar results were obtained in ten out of 13 cells (five slices; the non-responding cells came all from the same slice) for stimulation with forskolin and five out of five cells (three slices) for stimulation with uncaging of caged cAMP. Scale bars: A-F = 200 µm. OE boundaries are illustrated by white dotted lines. H and I, The black line under the trace indicates the application of forskolin and the black dot the time point of uncaging. Time scale [s] and change of fluorescence intensity ∆F/F [%] are indicated by the bars in the upper right corners.

FIGURE 3. Cng currents are inhibited by FM1-43. A-C, Current traces of patch-clamped cells in bath solution. The upper traces show action potential-associated capacitive currents in the voltage clamp, each current pulse indicating one action potential. Forskolin increased the spiking rate in cAMP-dependent (A and B, upper black traces), but not in cAMP-independent ORNs (C, upper black trace). (A, lower trace) Inward current after breakthrough into the whole-cell mode and after changing to the Ca\textsuperscript{2+}-and Mg\textsuperscript{2+}-free bath solution (0 Ca / 0 Mg). (B, lower trace) Markedly reduced inward current with FM1-43 being present in the 0 Ca / 0 Mg bath solution. (C, lower trace) No inward current was observed in ORNs that were not responsive to forskolin. Same current and time scale bars for A, B and C. D, Bar graph of inward current amplitudes with (left) and without (right) FM1-43 in the bath solution. ***: p < 0.001.

FIGURE 4. Extracellular FM1-43 blocks odorant transduction. A, Overview over the OE and the principal cavity (PC), the black rectangle indicates the position of the area of the OE measured in B using two photon excitation microscopy. Forskolin-induced fluorescence changes of fluo-4 are illustrated at the response peak. C, Forskolin-evoked [Ca\textsuperscript{2+}] transients of the ORN encircled ORN in B (black trace) were reduced upon FM1-43 in the bath (10 µM; red trace). Recovery after wash-out of the dye (grey). D, Average amplitudes and their S.E.M. for 10 µM FM1-43 and the wash-out (N = 61, 4 animals) are quantified in the bar graph. E, Overview over the olfactory bulb (OB), the black rectangle indicating the position of the area of the olfactory bulb shown in F, i.e., the medial cluster of the olfactory bulb explant preparation shown. Forskolin-induced fluorescence changes of fluo-4 are illustrated at the response peak. G, Forskolin-evoked [Ca\textsuperscript{2+}] transients of glomeruli in this cluster (black trace, corresponding to the glomerulus marked in F) were reduced upon FM1-43 in the bath (10 µM; red trace). Recovery of the response amplitude after wash-out of the dye (grey trace). The black line indicates the application of forskolin. H, Reduction of response amplitudes (and S.E.M.) for FM1-43 bath concentrations of either 10 µM (N = 11, 3 animals) or 20 µM (N = 17, 3 animals). **: p < 0.01; ***: p < 0.001. Scale bars: A, 250 µm, B, 20 µm , E, 250 µm, F, 30 µm.
Figure 3

A

B

C

D

forskolin

bath solution

forskolin

bath solution

0 Ca 0 Mg

0 Ca 0 Mg + 10 µM FM1-43

0 Ca 0 Mg

0 Ca 0 Mg + 10 µM FM1-43

I (µA)

0 50 100 150 200 250

0 Ca 0 Mg 0 Ca 0 Mg + 10 µM FM1-43

***

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Figure 4

A

B

C

D

E

F

G

H
The styryl dye FM1-43 suppresses odorant responses in a subset of olfactory neurons by blocking cyclic nucleotide-gated (cng) channels
Esther Breunig, Eugen Kludt, Dirk Czesnik and Detlev Schild

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