Drugs Affecting Phospholipase C-Mediated Signal Transduction Block the Olfactory Cyclic Nucleotide-Gated Current of Adult Zebrafish

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Ma, Li and William C. Michel. Drugs affecting phospholipase C-mediated signal transduction block the olfactory cyclic nucleotide-gated current of adult zebrafish. J. Neurophysiol. 79: 1183–1192, 1998. Amino acid and bile salt odorants are detected by zebrafish with relatively independent odorant receptors, but the transduction cascade(s) subsequently activated by these odorants remains unknown. Electro-olfactogram recording methods were used to determine the effects of two drugs, reported to affect phos-
pholipase C (PLC)/inositol trisphosphate (IP3)-mediated olfactory transduction in other vertebrate species, on amino acid and bile salt-evoked responses. At the appropriate concentrations, either an IP3-gated channel blocker, ruthenium red (0.01–0.1 μM), or a PLC inhibitor, neomycin (50 μM), reduced amino-acid–evoked responses to a significantly greater extent than bile salt-evoked responses. Excised patch recording techniques were used to measure the effects of these drugs on second-messenger–activated currents. Ruthenium red and neomycin are both effective blockers of the olfactory cyclic nucleotide-gated (CNG) current. Both drugs blocked the CNG channel in a voltage-dependent and reversible manner. No IP3-activated currents could be recorded. The differential effects of ruthenium red and neomycin on odor-evoked responses suggest the activation of multiple transduction cascades. The nonspecific actions of these drugs on odor-activated transduction pathways and our inability to record an IP3-activated current do not permit the conclusion that zebrafish, like other fish species, use a PLC/IP3-mediated transduction cascade in the detection of odorants.

INTRODUCTION

Olfactory transduction is initiated when membrane-associated odorant receptors, located on the cilia of olfactory receptor neurons (ORN), bind an appropriate odorant. Results obtained using a variety of experimental approaches suggest that one of several distinct transduction cascades may be activated upon ligand binding (Ache and Zainazarov 1995; Dionne and Dubin 1994). The most thoroughly studied transduction pathways are GTP dependent and use either cyclic nucleotides (Buck 1996; Zagotta and Siegelbaum 1996; Zufall et al. 1994) or inositol trisphosphate (IP3) (Bruch 1996; Honda et al. 1995; Restrepo et al. 1996) as second messengers. A general strategy often used to associate an odor-evoked response with a specific transduction cascade requires that one or more drugs specifically perturb both odor and second-messenger–activated responses. A problem with this approach is that the drugs affecting olfactory transduction are frequently not specific (Klee 1994). For example, the olfactory cyclic nucleotide-gated (CNG) channel can be blocked by divalent cations (Frings et al. 1992; Zufall and Firestein 1993), calcium/calmodulin antagonists (W-7, trifluoperazine) (Klee 1994), soluble guanylate cyclase inhibitors [6-anilino-5,8-quinolinedione] (Leinders-Zufall and Zufall 1995), and epithelial sodium (amiloride) and calcium channel blockers (diltiazem) (Frings et al. 1992). The olfactory IP3-activated current also is blocked by divalent cations (Restrepo et al. 1992) and by ruthenium red (Restrepo et al. 1990), a drug that interacts with many calcium-binding proteins (Amann and Maggi 1991) and blocks voltage-gated calcium channels (Hamilton and Lundy 1995). Because few studies have exhaustively screened drug effects on all components of odor-activated transduction pathways and because drug effects may vary across species, it is essential to experimentally confirm that drug effects described in one species account for perturbed odor-evoked responses in other species.

The zebrafish is emerging as an increasingly popular model for studies of the organization of the olfactory system (Baier and Korsching 1994; Baier et al. 1994; Byrd and Brunjes 1995; Friedrich and Korsching 1997; Weth et al. 1996) and its development (Barth et al. 1996; Byrd et al. 1996; Hansen and Zeiske 1993; Vogt et al. 1997). Functional properties of the zebrafish olfactory system have received less attention. Zebrafish respond behaviorally (Algranati and Perlmuter 1981; Bloom and Perlmuter 1977; Dill 1974; Steele et al. 1990, 1991; Van Den Hurk and Lambert 1983) and electrophysiologically (Michel and Lubomudrov 1995) to most of the common water-soluble odorants detected by other fish species (Hara 1992). Most of the amino acid and bile salt odorants tested appear to interact with at least partially independent odorant receptors (Michel and Derbridge 1997) but the transduction cascades subsequently activated remain unknown. Odorants stimulate phospholipase C (PLC) activity and inositol trisphosphate production in the channel catfish (Restrepo et al. 1990, 1993) and Atlantic salmon (Lo et al. 1994). Components of the CNG pathway are present in these fish species [and the zebrafish (Barth et al. 1996)], but at least in the catfish, odorants do not stimulate rapid cyclic nucleotide synthesis (Restrepo et al. 1993). Toward understanding the nature of transduction cascade(s) present in zebrafish ORNs, we conducted two experiments. In the first experiment, electro-olfactogram (EOG) methods were used to determine if drugs affecting olfactory transduction in other vertebrates affect the amino acid and bile salt-evoked responses of zebrafish. The two drugs tested, ruthenium red and neomycin, are reported to block IP3-gated channel (Honda et al. 1995; Restrepo et al. 1990) and PLC (Slivka and Insel 1988) activity, respectively. To explore the mechanistic basis of any drug effects, a second series of experiments examined the ac-
Solution contents are in millimolar. The osmolarity of all solutions was adjusted to 290–300 milliosmolar, the pH was adjusted to 7.4. Ca$^{2+}$ free solution (free Ca$^{2+}$ ~10 nM) was used in earlier studies (noted in text), HEPES, N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid; EGTA, ethylene glycol-bis(β-aminoethyl ether)-N,N,N’,N’-tetraacetic acid. * The calculated chloride reversal potential using the low Cl$^-$ solution and Ca$^{2+}$ free solution was −60 mV.

**Methods**

**Animal care and maintenance**

Zebrafish (*Danio rerio*) were purchased from a commercial supplier (Steve Lambourne), housed in recirculating 10–20 gal aquaria equipped with under-gravel and charcoal filtration, and fed a commercially available flake food diet (Tetramin).

**Electro-olfactogram methods**

EOG responses were obtained using methods as previously described (Michel and Lubomudrov 1995). Fish were immobilized with an intramuscular injection of gallamine triethiodide (Flaxedil; 60 μg/g body wt) and secured to a silicone elastomer (Sylgard) recording chamber. The olfactory organ immediately was provided with a continuous flow of artificial freshwater (AFW; see Solutions for composition), and the gills were irrigated with a flow of ~5 mL/min of AFW containing the general anesthetic 3-aminobenzoic acid ethyl ester (MS-222; 20 mg/l in AFW). The level of anesthesia was monitored by observation of reflexive movement of the gills and eyes and increased if required. Anesthesia was not provided before immobilization to minimize the loss of afferent sensory activity associated with topical application of anesthetic (Speth and Schweickert 1977). While under general anesthesia, the small flap of epithelium covering the olfactory organ was removed surgically to facilitate positioning of the recording electrode. The recording electrode (3 M KCl in 1% agar, 5–10 μM tip diam) was positioned between olfactory lamellae near the midline raphe in a location that maximized the response to 100 μM cysteine (Cys).

An identical electrode positioned on the head served as the differential electrode and a silver/silver chloride ground electrode positioned beneath the fish in the AFW bath completed the electrical circuit. Responses to olfactory stimuli were amplified (2,000 ± 250 times gain) and filtered (2 kHz) by a low-noise differential DC amplifier and displayed on an oscilloscope. The amplified signal also was digitized (100 Hz), stored, and displayed using Axotape software (Axon Instruments). Each digital record contained ~8 s prestimulation and 32 s of poststimulation time. Preparations were rejected if 100 μM Cys did not elicit a response ≥250 μV.

**In situ odorant delivery**

Methods for odorant delivery have been described previously (Michel and Lubomudrov 1995). Briefly, the olfactory epithelium was bathed by a carrier flow of AFW (or drugs prepared in AFW) that was supplied by gravity-flow from polyethylene bottles, selected by a six-way valve and regulated to 3 mL/min with a flow-meter. Olfactory stimuli (50 μl) were introduced into the carrier flow of AFW through a rotary loop injector (Rheodyne). The bolus of odor reached a peak concentration of ~84% of the loaded concentration by 8 s after injection and decayed to the baseline concentration after ca. 12 s (Michel and Lubomudrov 1995). The concentrations reported have not been corrected for dilution. To determine the effects of ruthenium red and neomycin on olfactory sensitivity, odor-evoked responses measured in the presence of these drugs were normalized to responses obtained in normal AFW.
frequently vesiculated, no attempt was made to determine the proportion of patches containing second-messenger–activated currents. The magnitude of the second-messenger–activated current was measured from the average of four replicate voltage ramps from \(-60\) to \(+60\) mV (duration \(= 30\) ms). The intracellular surface of the excised patch was exposed to cAMP for \(3\)–\(5\) s before initiating the ramp protocol. Leak currents, measured using an identical ramp protocol in the absence of second messenger, were subtracted from the second-messenger–activated current before calculating the slope conductance. The

before drug exposure. Odors tested in a drug background were diluted with the appropriate background solution.

**Dissociation of the olfactory epithelium**

Fish were killed by decapitation and the olfactory rosettes were dissected into ice-cold Ringer (see Table 1). After rinsing, the rosettes were held in fresh, oxygenated Ringer at 4°C until use. To dissociate ORNs, a rosette was transferred into divalent-free Ringer containing L-cysteine–activated (1.25 mg/ml) papain (0.25 mg/ml) for 15–30 min. The rosette was rinsed in divalent-free Ringer, placed onto a concanavalin A-coated glass coverslip containing a single drop of divalent-free Ringer, and dissociated by teasing with fine insect pins or by trituration through fire-polished glass pipettes. The cells were allowed to settle and attach to the coverslip for \(\approx 10\) min before use.

**Excised patch recording techniques**

Coverslips containing dissociated ORNs were placed in a recording chamber situated on the stage of a Nikon MM1 upright microscope and viewed through a \(\times 40\) water immersion lens with an Optizoom image inverter/variable magnifier (\(\times 0.8–2\)) and \(\times 10\) eye pieces. Recordings were obtained with borosilicate glass patch electrodes (\(>1\) \(\mu\)m tip diam) filled with the appropriate pipette solution and positioned on the soma or dendrite of an isolated ORN under visual control. Signals were amplified with a commercial patch clamp amplifier (Axopatch 200A), displayed on an analog oscilloscope, and stored digitally using pClamp software (Axon Instruments). Recordings were filtered at 1 kHz and digitized at 5 kHz. All reported voltages refer to the intracellular surface of the membrane relative to the extracellular surface.

Membrane patches were excised from the soma or dendrite of acutely dissociated ORNs in the inside-out configuration. The use of low [Ca\(^{2+}\)] solutions and a brief air exposure often resulted in successful inside-out patches. The presence of one or many second-messenger–activated channels was confirmed by exposing the intracellular face of the patch to either adenosine 3’5’-cyclic monophosphate (cAMP) or IP\(_3\), and noting either single- or multichannel activity. Only excised patches estimated to have five or more second-messenger–activated channels were used in this study. Since excised patches

**FIG. 2.** Phospholipase C inhibitor, neomycin (50 \(\mu\)M), reduces the responses to amino acid odorants to a significantly greater extent than the responses to the bile salt odorants (1-way ANOVA, Fisher’s LSD test \(P \approx 0.05\)). Amino acid odorants were tested at a concentration of 100 \(\mu\)M, and bile salt odorants were tested at a concentration of either 1 \(\mu\)M (TCA) or 10 \(\mu\)M (all others). Data from 5 preparations are plotted as means ± SE. Gln, L-glutamine; GCA, glycocholic acid; His, L-histidine; LCA, lithocholic acid; Lys, L-lysine.

**FIG. 3.** Application of adenosine 3’5’-cyclic monophosphate (cAMP) to the intracellular surface of membrane patches excised from the dendrite or soma of many zebrafish olfactory receptor neurons (ORNs) activates a dose-dependent cyclic nucleotide-gated (CNG) current. A: CNG current in 1 inside-out patch from the soma is activated by low micromolar concentrations of cAMP and saturates at cAMP concentrations \(\approx 10 \mu\)M. Patch was stimulated with 0.4, 0.8, 1.6, 2, 3, 5, 10, 20, 30, and 40 \(\mu\)M cAMP. In this and all subsequent patches, the CNG-specific current was calculated by subtracting the “leak” currents (measured in the absence of cAMP; inset shows an example from another patch) from the total currents measured in the presence of cAMP. B: plot of normalized slope conductances of the data shown in A were fit with the Hill equation to yield a \(K_{1/2}\) for cAMP activation and Hill coefficient for this patch of 2.4 \(\mu\)M and 2.1, respectively. Slope conductance for each trace was normalized to the highest slope conductance. Data was obtained from inside-out patches in symmetrical NaCl solutions.
slopes and conductances were calculated from a linear regression of the average of the leak-corrected current over the voltage range of −30 to +30 mV. Plots of the slope conductance versus second-messenger concentration were fit with the Hill equation to calculate the $K_{1/2}$ and Hill coefficient for second messenger binding. Bi-ionic methods were used to measure the potassium/sodium ($K^+/Na^+$) permeability ratio (Frings et al. 1992). Second messengers and drugs were applied to excised patches using a flow microswitching device (Warner Instruments, Hamden, CT). Unless otherwise indicated, all data are reported as means ± SE.

**Solutions**

The compositions of all solutions are listed in Table 1. Stock solutions of IP$_3$ and cAMP were prepared in distilled water and stored at −20°C. Stock solutions of ruthenium red, (+)-cis-diltia-
zem, and amiloride were prepared in the NaCl bath solution and stored at 4°C. Stock solutions of N-(6-aminohexyl)-5-chloro-1-naphthalenesulphonamide (W7) and trifluoperazine were prepared in ethanol and stored at 4°C. 6-anilino-5,8-quinolinedione (LY-83583) was obtained from Calbiochem (San Diego, CA), and a 10 mM stock solution, prepared in dimethyl sulfoxide, was stored at 4°C. All other chemicals were obtained from Sigma Chemical (St. Louis, MO).

RESULTS

Effects of ruthenium red and neomycin on odor-evoked responses

Odor-evoked responses were affected significantly by both of the drugs tested. Submicromolar ruthenium red significantly reduced the response to the amino acid L-cysteine without affecting the response to the bile salt taurocholic acid. Ten and 100 nM ruthenium red reduced the response to 100 μM Cys by ca. 50 and 80%, respectively (Fig. 1A). In contrast, responses elicited by 1 μM taurocholic acid (TCA) were unaffected by 10 mM ruthenium red and reduced by <15% by 100 nM ruthenium red. When the ruthenium red concentration was increased to either 1 or 10 μM, the Cys-evoked response was eliminated and TCA-evoked responses were attenuated significantly. Cys-evoked responses recovered slower than TCA-evoked responses regardless of the ruthenium red concentrations tested (data not shown). At 10 μM ruthenium red, the response to cysteine did not recover within 30 min. The responses to three other amino acids, thought to interact with odorant receptors independent of the receptor(s) mediating the cysteine response (Michel and Derbidge 1997), also were attenuated significantly by 100 nM ruthenium red (Fig. 1B).

Odorants stimulate the olfactory PLC activity of several fish species (Boyle et al. 1987; Har Lo et al. 1993; Huque and Bruch 1986; Restrepo et al. 1993). If PLC activation is essential to odor transduction, then drugs blocking PLC activity also should block odor-evoked responses. The PLC inhibitor, neomycin (50 μM) reduced amino acid-evoked responses to a significantly [2-way analysis of variance (ANOVA), Fisher’s least significant difference (LSD) test, P < 0.05] greater extent than bile salt-evoked responses (Fig. 2). The responses elicited by the eight amino acids tested were reduced by >80–90%, whereas the responses to the four bile salts tested were only reduced by <20% to ~50%.

The selective reduction of amino acid-evoked, but not of bile salt-evoked, responses by submicromolar ruthenium red and by neomycin is consistent with the presence of a PLC/IP₃ transduction cascade activated by amino acids but not by bile salts. However, the specific effects of ruthenium red and neomycin on any potential odorant-activated transduction cascades in zebrafish are unknown. Consequently, in the second phase of this investigation, we tested the effects of these drugs on second-messenger–activated currents in excised patch recordings.

Second-messenger–activated currents in excised patch recordings

Second-messenger–activated currents were identified by application of cAMP or IP₃ to the intracellular surface of membrane patches excised from the soma or dendrites of isolated ORNs and recording currents during voltage clamp protocols. A cAMP-activated current was recorded routinely, but we failed to observe an IP₃-activated current in excised patches from >40 zebrafish ORNs, including eight patches that were sensitive to cAMP (data not shown). Our failure to identify an IP₃-activated current might have been due to an inappropriate calcium concentration, the use of patches generally excised from somatic membrane, or the absence of these channels in zebrafish ORNs. The effects of ruthenium red and neomycin on the CNG current are considered in the following sections.

General properties of zebrafish olfactory cng channel

Low micromolar concentrations of cAMP activated the zebrafish olfactory CNG channel (Fig. 3). The average Kᵢ₀ for cAMP was 3.0 ± 0.6 μM (n = 10, range 0.94–6.6 μM). A Hill coefficient of 2.8 ± 0.5 (n = 10) indicates cooperativity of cAMP binding. At concentrations of cAMP sufficient to elicit a saturating response, the average slope conductance was 1.2 ± 1.2 nS, (n = 102, range, 0.09–6.3 nS). The calculated K⁺/Na⁺ permeability ratio of 0.9 ± 0.01 (n = 5) indicates that the zebrafish olfactory CNG channel is a relatively nonspecific cation channel. Shifting Eᵥ from 0 to −60 mV changed the reversal potential of the CNG current by only 1 mV (n = 3 patches; data not shown).

Before determining the effects of ruthenium red and neomycin on the zebrafish olfactory CNG current, the basic pharmacology of the zebrafish CNG current was compared with other vertebrate olfactory CNG currents. Four of five drugs previously reported to block other vertebrate olfactory CNG channels reversibly blocked the zebrafish olfactory CNG channel (Fig. 4, Table 2). At 1–10 μM, the calcium/calmodulin antagonists W-7 and trifluoperazine partially blocked the zebrafish olfactory CNG channel.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Concentrations Tested, μM</th>
<th>Percent Block (Most Effective Dose)</th>
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<tbody>
<tr>
<td></td>
<td>−60 mV</td>
<td>+60 mV</td>
</tr>
<tr>
<td>W7</td>
<td>1 (3), 10 (3), 100 (4)</td>
<td>73.5 ± 6.6</td>
</tr>
<tr>
<td>Trifluoperazine</td>
<td>1 (9), 10 (3), 100 (5)</td>
<td>83 ± 5.1</td>
</tr>
<tr>
<td>Diltiazem</td>
<td>20 (3), 40 (3), 1000 (5)</td>
<td>38.2 ± 16.3</td>
</tr>
<tr>
<td>Amiloride</td>
<td>50 (3), 100 (3), 1000 (5)</td>
<td>51.2 ± 7.1</td>
</tr>
<tr>
<td>LY-83583</td>
<td>10 (16), 80 (7)</td>
<td>0.0 ± 0.1</td>
</tr>
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All drugs were applied with adenosine 3',5'-cyclic monophosphate (cAMP) to the intracellular membrane surface. The cAMP concentration tested was always ≥10 μM. Percent Block is the average current measured in the presence of a blocker at Vᵥ = −60 and +60 mV expressed as a proportion of the current elicited under control conditions. The percent block reported is for the highest concentration tested. Extracellular (included in the recording pipette) diltiazem (40 μM), amiloride (20 μM) and 6-anilino-5,8-quinolinedione (LY-83583; 20 μM) failed to block the cyclic nucleotide-gated (CNG) current in 3 patches each. Parentheses enclose number of patches. W7, N-(6-aminohexyl)-5-chloro-1-naphthalenesulphonamide.
CNG channel. At 100 μM, either calcium/calmodulin antagonist effectively blocked the CNG current (Fig. 4, A and B). Amiloride and diltiazem partially blocked the zebrafish olfactory CNG channel at millimolar concentrations (Fig. 4, C and D) but were ineffective at concentrations <200 μM (not shown). LY-83583 (10 μM) blocked the tiger salamander olfactory CNG channel (Leinders-Zufall and Zufall 1995) but failed to block the zebrafish olfactory CNG channel when concentrations ranging from 10 to 80 μM were applied to either the intracellular or extracellular surface of the membrane (Table 2). The pharmacological data indicates that the zebrafish olfactory CNG channel has properties similar to other vertebrate CNG channels.

Effect of ruthenium red on the zebrafish olfactory CNG current

To determine if ruthenium red affected the CNG current, we coapplied 40 μM cAMP and one of several concentrations of ruthenium red to the intracellular surface of the excised membrane patch (Fig. 5, A, C, and D). The lowest concentration of ruthenium red tested (100 nM) blocked ~25% of the outward component of the CNG current. At an intermediate concentration of ruthenium red (1 μM), nearly 90% of the outward component was blocked. At the highest ruthenium red concentrations (10 and 100 μM), the block of the outward component of the CNG current was generally complete. In some patches, at the intermediate and higher ruthenium red concentrations, a negative slope conductance at positive membrane potentials was observed, indicating the ruthenium red block is voltage dependent. The inward component of the CNG current also was blocked but only ruthenium red concentrations ≥10–100 μM (Fig. 5D). The block of the inward component of the CNG current was also voltage dependent. To determine if extracellular ruthenium red blocked the CNG current, we included ruthenium red in the recording pipette solution. Although the block developed slowly, extracellular ruthenium red also affected the CNG current (Fig. 5B). Initially, only the outward component of the CNG current was partially blocked by ruthenium red (the block was most notable at positive transmembrane potentials). After 30 min, both inward and outward components of the CNG current were blocked by ruthenium red.
To investigate the recovery from ruthenium red block, ruthenium red was applied to the intracellular surface of an excised patch and 40 μM cAMP was applied in ruthenium red until a stable level of block was achieved. To start the recovery period, ruthenium red was removed and cAMP was tested alone. At each concentration of ruthenium red tested, the magnitude of the CNG current was measured before, in the presence of, and at several recovery intervals after the removal of ruthenium red (Fig. 6). The data plotted are representative of data obtained from at least three patches at each concentration. In general, recovery from ruthenium red block was relatively rapid and complete. Recoveries from 100 nM and 1 μM ruthenium red were complete in <5 min. Recoveries from 10 and 100 μM ruthenium red exposure were incomplete. Nearly 80% of the outward current recovered in <5 min, but no further recovery was measured even after 30 min.

**Effect of neomycin on the zebrafish olfactory CNG current**

Application of neomycin to the intracellular surface of the membrane partially blocked the olfactory CNG current. The block was dose dependent (Fig. 7A). Neomycin (50–200 μM) blocked ~50% of the outward current at +60 mV. The effect of neomycin concentration on the average CNG currents of three excised patches is illustrated in Fig. 7B. Extracellular neomycin (included in the pipette solution, n = 3 patches) blocked only a small portion (<10%) of the peak outward current (Fig. 7C). The most pronounced neomycin block was observed when it was applied to both sides of the membrane patch (Fig. 7D). In three patches with 50 μM neomycin bathing the intracellular surface and 50–100 μM neomycin bathing the extracellular surface, nearly all of the outward component and ~50% of the inward component of the CNG current was blocked.

**DISCUSSION**

Two significant findings are reported in this study. First, drugs, previously reported to block phospholipase C activity (neomycin) and IP₃-gated channel activity (ruthenium red), differentially affected odor-evoked responses of the zebrafish olfactory epithelium. Both drugs reduced the responses evoked by amino acids to a significantly greater extent than the responses evoked by bile salts. Submicromolar concentrations of ruthenium red (10–1,000 nM) selectively blocked amino acid-evoked responses, 10 μM ruthenium red blocked all odor-evoked responses. Fifty micromolar neomycin selectively blocked amino acid-evoked responses. This neomycin concentration is 20- to 200-fold lower than generally used to perturb PLC activity. Collectively, these results are consistent with the interpretation that a PLC/IP₃ transduction cascade mediates amino acid-evoked responses.

The second significant finding of this study was that ruthenium red and neomycin both blocked the zebrafish olfactory CNG current. The later finding renders any conclusion about the transduction pathway(s) used by amino acid and bile salt odorants premature. To our knowledge, we are the first to directly examine the actions of these drugs on the olfactory CNG channel.

Ruthenium red is an organometallic dye that reportedly affects the function of many calcium-binding proteins (Amann and Maggi 1991), blocks voltage-gated calcium channel activity (Hamilton and Lundy 1995) and nociceptor function (Dray et al. 1990), and associates with negatively charged phospholipids (Voekler and Smejtek 1996). In ol-
Phospholipase C (PLC) inhibitor neomycin blocked the CNG channel. A: intracellular neomycin blocked the CNG current in a dose-dependent manner. Membrane patches became very unstable when exposed to concentrations of neomycin >200 μM. B: average ± SE block of the outward component of the CNG current, measured in 3 excised patches, is plotted as a function of intracellular neomycin concentration. C: extracellular neomycin (100 μM) weakly blocks the outward component of the CNG current (neomycin included in the recording pipette). D: neomycin nearly completely blocked the outward component of the CNG current when present on both sides of the membrane. In all patches, the CNG current was activated with 40 μM cAMP. Data was obtained from inside-out patches in symmetrical NaCl solutions.

Ruthenium red originally was used to block norleucine-evoked responses of the channel catfish olfactory epithelium as well as IP$_3$-activated channel activity of enriched catfish olfactory cilia membranes incorporated into lipid bilayers (Restrepo et al. 1990). Ruthenium red has similar affects on the olfactory IP$_3$-activated currents/channels of other vertebrate (Honda et al. 1995; Okada et al. 1994; Restrepo et al. 1990, 1992) and invertebrate (Fadool and Ache 1992) species. The olfactory CNG channel must be added to the list of cellular substrates affected by ruthenium red.

The block of the zebrafish olfactory CNG current by ruthenium red was dose dependent and effective at submicromolar concentrations. One hundred-fold lower concentrations of ruthenium red were required block the outward component of the CNG current. Extracellular ruthenium red also blocked the outward component of the CNG current to a greater extent than the inward component. Collectively, these findings suggest that the primary site of ruthenium red action is on the intracellular surface. Ruthenium red association with negatively charged phospholipid residues (Voekler and catfish olfactory cilia membranes incorporated into lipid bilayers (Restrepo et al. 1990)) near the CNG channel presumably would reduce the local monovalent cation concentration, resulting in a block similar to that produced during intracellular acidification (Frings et al. 1992). If the adsorption of ruthenium red to these membrane phospholipids is strong, ruthenium red levels might be expected to persist even after ruthenium red application is stopped. The slow and incomplete recovery of both odor responsiveness and CNG currents after exposure to the higher ruthenium red concentrations might reflect its adsorption to phospholipids.

Neomycin is an aminoglycoside antibiotic that reportedly affects many cellular substrates. Neomycin blocks phospho-
Pharmacology of Zebrafish CNG Channel

Lipase C activity by binding to the enzyme’s substrate, phosphoinositide bisphosphate (PIP₂) (Slivka and Insel 1988). A variety of ion channel types, including skeletal muscle L-type Ca²⁺ channels, Ca²⁺-activated K⁺ channels, and mechanosensitive channels are blocked by neomycin and by other aminoglycoside antibiotics (Haws et al. 1996; Winegar et al. 1996). In isolated rat ORNs, 200 μM neomycin blocked the [Ca²⁺], increase elicited by an IP₃-activating odor mixture, presumably by inhibiting PLC activity (Tareilus et al. 1995), but also appears to have partially blocked the response to cAMP-activating odor mixture. Our finding that neomycin blocks olfactory CNG channel current suggests that the partial block of the response to the cAMP-activating odor mixture previously noted may have been a result of a direct action of neomycin upon the CNG channel. Although higher concentrations of neomycin frequently are used to perturb PLC function, we found that excised patches became the transduction of amino acid odorants by zebrafish is affected primarily by these drugs. For example, at low ruthenium red concentrations the block of amino acid-evoked responses might be due to drug interactions with an anionic binding site present on amino acid receptors but not on bile salt receptors. At higher concentrations, ruthenium red might eliminate all odor-evoked responses through its action on the CNG current. Alternately, the block of amino acid-evoked responses observed at lower drug concentrations might be due to an action of these drugs on an independent transduction cascade, perhaps mediated by PLC and an IP₃-gated current. Our failure to identify an IP₃-activated current may have been due to any number of factors, including recording from the soma rather than cilia or an inappropriate calcium concentration. Because bile salt-evoked EOG responses and CNG currents are both blocked 10 μM ruthenium red but only slightly affected by 50 μM neomycin, it is perhaps likely that bile salts activate a CNG transduction cascade.

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A variety of ion channel types, including skeletal muscle L-type Ca²⁺ channels, Ca²⁺-activated K⁺ channels, and mechanosensitive channels are blocked by neomycin and by other aminoglycoside antibiotics (Haws et al. 1996; Winegar et al. 1996). In isolated rat ORNs, 200 μM neomycin blocked the [Ca²⁺], increase elicited by an IP₃-activating odor mixture, presumably by inhibiting PLC activity (Tareilus et al. 1995), but also appears to have partially blocked the response to the cAMP-activating odor mixture. Our finding that neomycin blocks olfactory CNG channel current suggests that the partial block of the response to the cAMP-activating odor mixture previously noted may have been a result of a direct action of neomycin upon the CNG channel. Although higher concentrations of neomycin frequently are used to perturb PLC function, we found that excised patches became the transduction of amino acid odorants by zebrafish is affected primarily by these drugs. For example, at low ruthenium red concentrations the block of amino acid-evoked responses might be due to drug interactions with an anionic binding site present on amino acid receptors but not on bile salt receptors. At higher concentrations, ruthenium red might eliminate all odor-evoked responses through its action on the CNG current. Alternately, the block of amino acid-evoked responses observed at lower drug concentrations might be due to an action of these drugs on an independent transduction cascade, perhaps mediated by PLC and an IP₃-gated current. Our failure to identify an IP₃-activated current may have been due to any number of factors, including recording from the soma rather than cilia or an inappropriate calcium concentration. Because bile salt-evoked EOG responses and CNG currents are both blocked 10 μM ruthenium red but only slightly affected by 50 μM neomycin, it is perhaps likely that bile salts activate a CNG transduction cascade.

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