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ARTICLE

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Adenophostin A and imipramine are two activators of the olfactory inositol 1,4,5-trisphosphate-gated channel in fish olfatory cilia

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Abstract Binding of an odorant to its receptor activates the cAMP-dependent pathway, and also leads to inositol 1,4,5-trisphosphate ($InsP_3$) production. This induces opening of a plasma membrane channel in olfactory receptor cells (ORCs). We investigated single-channel properties of this channel in the presence of a phospholipase C (PLC) activator (imipramine) and of a potent activator of the $InsP_3/Ca^{2+}$ release channel (adenophostin A) by reconstituting carp olfactory cilia into planar lipid bilayers. In the presence of 53 mM barium as a charge carrier, the addition of 50 μM imipramine induced a current of 1.53 ± 0.05 pA at 0 mV. There were two different mean open times (6.0 ± 0.6 ms and 49.6 ± 6.4 ms). The I/V curve displayed a slope conductance of 50 ± 2 pS. Channel activity was transient and was blocked by neomycin (50 μM). These observations suggest that imipramine may activate the olfactory $InsP_3$-gated channel through PLC. Using the same ionic conditions, the application of 0.5 μM adenophostin A triggered a current of 1.47 ± 0.04 pA at 0 mV. The I/V curve displayed a slope conductance of 60 ± 2 pS. This channel showed only a single mean open time (15.0 ± 0.3 ms) and was strongly inhibited by ruthenium red (30 μM) and heparin (10 μg/mL). These results indicate that adenophostin A and imipramine may act on the ciliary $InsP_3$-gated channel and are potentially valuable pharmacological tools for studying olfactory transduction mechanisms.

Keywords Ciliary membrane · Adenophostin A · Imipramine · Lipid bilayers · Phospholipase C

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Abbreviations *CNGc* cyclic nucleotide-gated channel · *EDTA* ethylenediaminetetraacetic acid · *EGTA* ethyleneglycol-bis(β-aminoethyl)-*N*,*N*,*N*,*N*'-tetraacetic acid · *ER* endoplasmic reticulum · *HEEDTA* *N*-(2-hydroxyethyl)ethylenediaminetriacetic acid · *HEPES* *N*-2-(hydroxyethyl)piperazine-*N*'-(2-ethanesulfonic acid) · $InsP_3$ inositol 1,4,5-trisphosphate · *ORCs* olfactory receptor cells · $PdtIns(4,5)P_2$ phosphatidylinositol 4,5-bisphosphate

Introduction

In olfactory receptor cells (ORCs), the interaction between odorant molecules and G-coupled receptors in the ciliary membrane triggers a complex biochemical cascade. The key steps are the activation of specific enzymes, the formation of second messenger molecules and the opening of cation channels, causing the ORC to depolarize (reviewed in Schild and Restrepo 1998; Paysan and Breer 2001). The best-characterized pathway for olfactory signal transduction involves production of cAMP and opening of cyclic nucleotide-gated channels (CNGc) (Nakamura and Gold 1987). However, certain odorant molecules also stimulate phospholipase C (PLC), allowing hydrolysis of phosphatidylinositol 4,5-bisphosphate ($PdtIns(4,5)P_2$) and production of inositol 1,4,5-trisphosphate ($InsP_3$) (Boekhoff et al. 1990; Breer et al. 1990; Ronnett et al. 1993; Lo et al. 1994). Olfactory PLC has been characterized (Abogadie et al. 1995; Bruch et al. 1995; Noe and Breer 1998), but investigations into its nature have been limited. It was suggested that the $InsP_3$ produced binds to specific cation channels in the plasma membrane of ORCs of both vertebrates and invertebrates. Confirmation of this by biophysical and electrophysiological techniques has been reported (Restrepo et al. 1990; Lischka et al. 1999; Cadiou et al. 2000; Kaur et al. 2001). Immunohistochemical (Cunningham et al. 1993), biochemical (Kalinoski et al. 1992) and molecular

approaches (Munger et al. 2000) have suggested the presence of an $InsP_3$ receptor-like protein in the apical compartment of ORCs. This type of ion channel appears to be directly involved in odorant response mechanisms (Fadool and Ache 1992; Wekesa and Anholt 1997) and in olfactory adaptation (Deshpande et al. 2000). However, several groups failed to find evidence of this channel in the plasma membrane of ORCs and of its involvement in olfactory transduction mechanisms. Its contribution thus remains controversial (see Gold 1999 for review). We recently demonstrated that the ciliary olfactory $InsP_3$-gated channel from carp ORCs differs from the $InsP_3/Ca^{2+}$ release channel in the endoplasmic reticulum (ER) in terms of conductance, kinetics, calcium dependence and immunoreactivity (Cadiou et al. 2000). Most previous studies involving the $InsP_3$ receptor from the ER have used either $InsP_3$ or related inositol phosphates; however, the demonstration that adenophostins are the most potent agonists of the $InsP_3$ receptor known (Takahashi et al. 1994) has provided additional opportunities to study this receptor. Recently, adenophostin analogues were used on turtle ORCs to mimic $InsP_3$-induced inward currents in whole cell mode (Kashiwayanagi et al. 2000).

The aim of our study was to identify pharmacological agents that could activate directly or indirectly the olfactory $InsP_3$-gated channel. First, we wanted to use this channel to detect any $InsP_3$ production resulting from PLC activation. Functional analysis of the channel was performed after incorporation of carp olfactory cilia into planar lipid bilayers using the tip-dip method, as described previously (Cadiou et al. 2000). Secondly, using the same technique, we investigated the ability of adenophostin A to activate the olfactory $InsP_3$-gated channel. Our findings suggest that these compounds are powerful tools for investigating the inositides pathway in vertebrates ORCs.

Materials and methods

Purified olfactory cilia were isolated from the olfactory epithelium of the carp *Cyprinus carpio* by the calcium shock method, as previously described (Cadiou et al. 2000). Briefly, after removal, the olfactory rosettes were rinsed in Ringer solution [112 mM NaCl, 3.4 mM KCl, 2.4 mM $NaHCO_3$, 2 mM ethylenediaminetetraacetic acid (EDTA), 10 mM *N*-2-(hydroxyethyl)piperazine-*N*'-(2-ethanesulfonic acid) (HEPES), pH 7.4] to which the following protease inhibitors were added: 2 μg/mL leupeptin, 1 μg/mL pepstatin A, 2 μg/mL aprotinin and 100 μg/mL PMSF. A calcium shock was then given by applying Ringer solution without EDTA and containing 10 mM $CaCl_2$ during 20 min. After homogenization, the supernatant of two subsequent low-speed centrifugations (1500×*g*, 5 min) was layered on a sucrose cushion (45%, w/w) and ultracentrifuged at 141,000×*g* for 1 h. The band at the surface of the cushion was collected and centrifuged again at 141,000×*g* during 1 h. The resulting pellet was resuspended in Ringer solution containing 2 mM ethyleneglycol-bis(*β*-aminoethyl)-*N*,*N*,*N'*,*N'*-tetraacetic acid (EGTA) and adjusted to 2 mg of protein/mL. Samples were store at 80 °C in small aliquots.

Tip-dip methodology and single channel recording

Patch pipettes were made from borosilicate glass capillaries (Biologic Science Instruments, Claix, France) pulled in two steps using a Narishige pipette-puller (Tokyo, Japan). Planar lipid bilayers were formed at the tip of fire-polished patch pipettes with a mixture of 1-palmitoyl-2-oleoylglycerophosphatidylcholine and 1,2-dioleoylglycero-3-phosphatidylethanolamine (7:3, 0.1% w/v in hexane) from Avanti Polar Lipids (Birmingham, Ala., USA) according to the tip-dip technique (Hanke et al. 1984). Ionic conditions were as described by Thrower et al. (1996) and Cadiou et al. (2000). The interior of the patch pipette acts as the extracellular compartment (Restrepo et al. 1990). The pipette was filled with a solution containing 25 mM KCl and 53 mM $Ba(OH)_2$/HEPES, pH 7.35. In this case, barium acts as the main current carrier. The bath forms the cytoplasmic compartment and contained 25 mM KCl, 1 mM *N*-(2-hydroxyethyl)ethylenediaminetriacetic acid (HEDTA or HEEDTA), 100 nM free calcium and 250 mM HEPES/KOH, pH 7.35.

Only stable bilayers with high resistance (>10 GΩ) were used for recordings. Diluted cilia corresponding to 5–10 μg of protein were introduced to the bath under continuous stirring. After 10 min, the electric silence of the bilayer was tested and adenophostin A (Calbiochem, Darmstadt, Germany), imipramine, ruthenium red, heparin and neomycin (Sigma-Aldrich, St Louis, Mo., USA) were added to the bath, as indicated in the legends to the figures. First, the lack of effects of these compounds on lipid bilayers alone was established (data not shown).

Currents were amplified and potentials were applied simultaneously by a patch clamp amplifier (RK 300, Biologic Science Instruments). Current traces were stored on a DAT recorder (DTR 1202, Biologic Science Instruments) and analysed with Satori (v3.1, Intracel Software, Royston, UK) and Sigmaplot (SPSS, Chicago, Ill., USA) software. Opening events were detected from the filtered records using the half-amplitude threshold crossing technique (Colquhoun and Sigworth 1995). All experiments were performed at room temperature. Data are expressed as mean ± SEM with the number of observations, *n*, in parentheses. Traces were filtered at 1 kHz before digitizing at 4 kHz for analysis; additional filtering could be done for presentation (additional filtering values indicated in the legends to the figures). Openings are defined as upward deflections from the baseline, as indicated by the bar on the right of each trace.

Results

Imipramine

We have previously described the single-channel properties of $InsP_3$-gated channels from carp olfactory cilia (Cadiou et al. 2000). Here we investigate whether this preparation contains proteins, such as PLC, involved in inositide metabolism. Imipramine, a PLCβ1 activator in rat brain (Fukuda et al. 1994), was used to determine whether carp olfactory cilia contain a functional PLC. Its activity was detected indirectly by measuring the activity of the ciliary $InsP_3$-gated channel.

No channel activity was observed in the absence of imipramine using 53 mM barium as a current carrier. Addition of 50 μM imipramine to the bath induced single-channel activity in 7 of 46 (15%) of the lipid bilayers formed (Fig. 1Ab, B). This activity persisted for at least 2 min. Channel characteristics in three of these imipramine-responsive bilayers were further investigated by adding neomycin and then applying $InsP_3$ and ruthenium red (Fig. 1A). The single-channel events

possessed an amplitude of 1.53 ± 0.05 pA ($n = 7$) at 0 mV (Fig. 1C). Further analysis revealed two mean open times of 6.0 ± 0.6 ms and 49.6 ± 6.4 ms ($n = 7$) (Fig. 1D) and two mean closed times of 3.8 ± 0.6 ms and 42.9 ± 5.5 ms ($n = 4$). The open probability was 0.45 ± 0.04 ($n = 7$).

Neomycin, an aminoglycoside antibiotic, is a potent inhibitor of PLC that acts by binding to the enzyme's substrate, PdtIns(4,5)P_2 (Slivka and Insel 1988). Neomycin (50 μM) suppressed channel activity caused by 50 μM imipramine (inhibition 100%, $n = 3$, Fig. 1Ac). Neomycin interacts with several ion channels, including the L-type calcium channel (Haws et al. 1996) and the CNGc of zebrafish ORCs (Ma and Michel 1998). We tested whether the effects of neomycin result from a direct action on the olfactory $InsP_3$-gated channel. $InsP_3$ (5 μM) was added to the bath (Fig. 1Ad) and single-channel openings were then observed without interruption, despite the presence of neomycin; the single current level was 1.62 ± 0.14 pA ($n = 3$) and the open probability was 0.37 ± 0.02 ($n = 3$). Application of 30 μM ruthenium red, a potent inhibitor of the olfactory $InsP_3$-gated channel (Restrepo et al. 1990; Cadiou et al. 2000), resulted in cessation of channel activity (Fig. 1Ae). Note that the channel activity induced by imipramine was also blocked by application of 30 μM ruthenium red (inhibition 100%, $n = 3$), or by heparin (inhibition 98%, $n = 3$) applied separately (data not shown). Heparin is a potent inhibitor of the $InsP_3$-gated channel from the ER (Ehrlich et al. 1994) and of the olfactory $InsP_3$-gated channel from carp olfactory cilia (Cadiou et al. 2000).

The amplitudes of the unitary current induced by 50 μM imipramine were measured at several applied potentials (Fig. 2A) and the I/V curve was plotted (Fig. 2B). The I/V relationship is linear and the slope value gives a single-channel conductance of 50 ± 2 pS. The reverse potential (−23.1 mV) indicates that the channel was selective for barium.

Adenophostin A

Adenophostin A is a potent agonist of the $InsP_3/Ca^{2+}$ release channel from the ER. We investigated its ability to activate the olfactory $InsP_3$-gated channel using carp olfactory cilia reconstituted into planar lipid bilayers obtained by the tip-dip method.

In the absence of adenophostin A, no channel activity was observed (Fig. 3A). Addition of adenophostin A (0.4 μM) to the bath (considered as the cytoplasmic side) induced channel activity in 15% of bilayers formed (Fig. 3A), using barium as a current carrier. No more than one channel was observed in any experiment. At 0 mV, the channel amplitude was 1.64 ± 0.04 pA ($n = 3$) and the open probability (P_o) was 0.10 ± 0.03 ($n = 3$). Further analysis revealed a single mean open time of 13.0 ± 1.5 ms ($n = 3$) and a mean closed time of 40.0 ± 5.2 ms ($n = 3$).

In another set of experiments, the addition of 0.5 μM adenophostin A gave a higher frequency of single-channel events compared to the precedent experiment (Fig. 3A). The amplitude of the current at 0 mV was 1.47 ± 0.04 pA ($n = 4$) (Fig. 3B) and the open probability increased to 0.57 ± 0.02 ($n = 4$). The open dwell time was 15.0 ± 0.3 ms ($n = 4$) and two mean closed

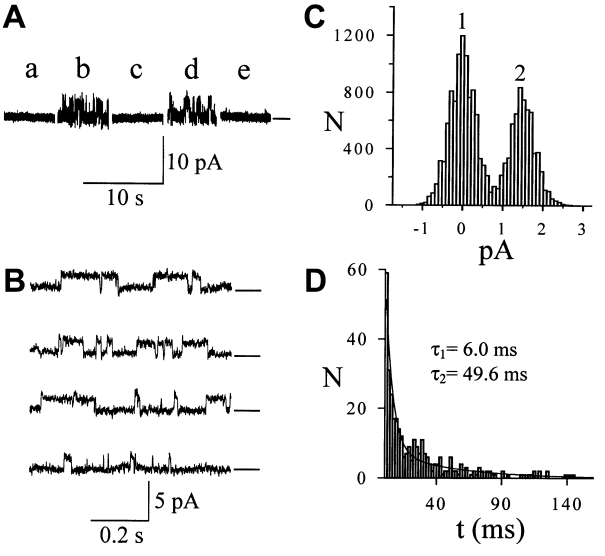

Fig. 1A–D Effect at the single-channel level of imipramine on carp olfactory cilia reconstituted into planar lipid bilayers. **A** Channel activity was recorded in the presence of 100 nM Ca^{2+} at 0 mV. (*a*) In the absence of imipramine, no channel activity was observed. (*b*) Addition of 50 μM imipramine activated one channel. The closed state is represented by the *bar* at the *right* of each trace (*c*) The application of 50 μM neomycin inhibited the channel. (*d*) Recovery of single channel activity with 5 μM $InsP_3$. (*e*) Inhibition of the $InsP_3$-elicited activity by the addition of 30 μM ruthenium red. *Gaps* between traces represent approximately 1 min. **B** Enlargements of the trace presented in **A**(*b*). **C** Amplitude histogram showing the closed (*1*) and the open state (*2*). Data were obtained from the same channel as shown in **A**(*b*) and **B**. **D** Lifetimes histogram for traces presented in **A**(*b*) and **B** showing two open dwell times. Traces were filtered at 1 kHz

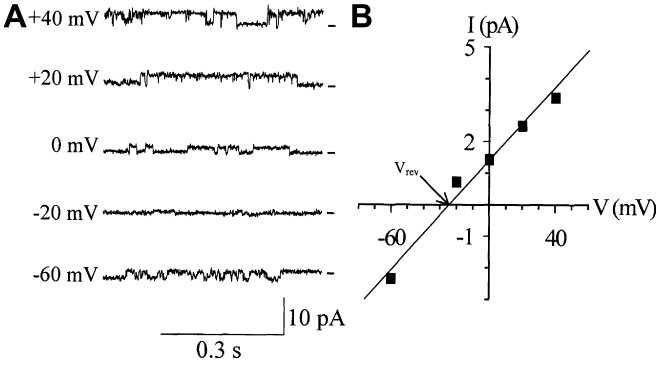

Fig. 2A, B Single-channel activity induced by imipramine at several applied potentials. **A** Traces were recorded under the conditions described in Fig. 1A and filtered at 700 Hz. **B** I/V plot (V_{rev} = reverse potential). The *line* is the sum of seven independent experiments

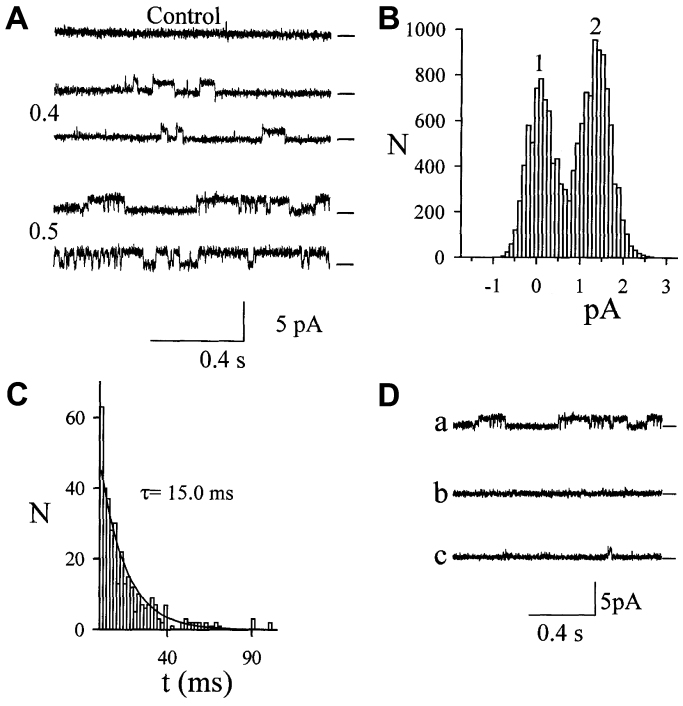

Fig. 3A–D Effect at the single-channel level of adenophostin A on carp olfactory cilia reconstituted into planar lipid bilayers. **A** Channel activity was recorded in the presence of 100 nM Ca^{2+} at 0 mV. In the absence of adenophostin A (*top*), no channel activity was observed. In two separated experiments, addition of 0.4 μM (*middle*) or 0.5 μM adenophostin A (*bottom*) activated one channel. The *bars* on the *right* represent the closed state. **B** All-points histogram showing the closed (*1*) and open state (*2*) of the channel for 0.5 μM adenophostin A. Data were taken from the same channel as shown in **A**. **C** Lifetimes histogram for traces presented in **A** (0.5 μM adenophostin A), showing two open dwell times obtained with 0.5 μM adenophostin A. **D** Effect of inhibitors against the single-channel activity induced by adenophostin A: (*a*) traces obtained in the presence of 0.5 μM adenophostin A; addition of 30 μM ruthenium (*b*) or 10 μg/mL heparin (*c*) inhibited the channel. Traces were filtered at 1 kHz

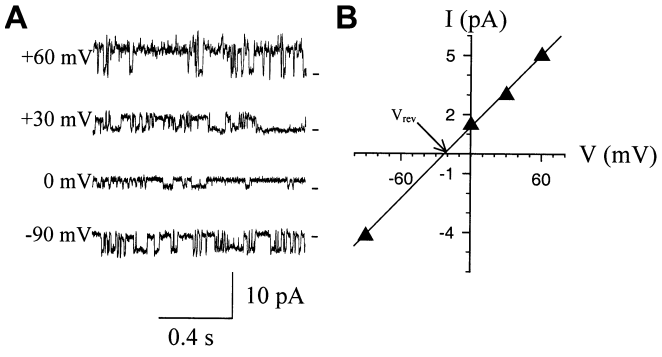

Fig. 4A, B Single-channel activity induced by adenophostin A at several applied potentials in carp olfactory cilia reconstituted into planar lipid bilayers. **A** Traces were recorded in the presence of 100 nM Ca^{2+} and 0.5 μM adenophostin A and filtered at 700 Hz. **B** I/V plot (V_{rev} = reverse potential). The *line* is the sum of six independent experiments

times were found (3.2 ± 0.3 ms and 42.7 ± 11.5 ms; $n = 4$) (Fig. 3C). The higher concentration of adenophostin A induced an increase in the open probability of the channel by the emergence of a short mean closed time.

Because adenophostin A is a by-product of nucleotides, this compound may interact with CNGc. However, high concentrations of barium blocked this channel in carp olfactory cilia reconstituted in lipid planar bilayers (data not shown). Inhibition studies were used to test whether the channel activity observed was due to the olfactory $InsP_3$ receptor (Fig. 3D). Addition of 30 μM ruthenium red in the presence of 0.5 μM adenophostin A totally inhibited the channel activity (100% inhibition, $n = 3$) (Fig. 3Da, b). Similarly, 10 μg/mL heparin also inhibited the channel activity (99% inhibition, $n = 3$) (Fig. 3Da–c).

The amplitudes of the unitary current at several applied potentials (Fig. 4A) were used to draw the I/V plot (Fig. 4B). The curve was linear and the slope corresponds to a single-channel conductance of 60 ± 2 pS. The reverse potential (−28.1 mV) indicated that the channel was highly selective for barium.

Discussion

In these experiments, imipramine triggered single-channel activity in carp olfactory cilia reconstituted in planar lipid bilayers obtained by the tip-dip method. The imipramine-activated channel possesses a similar current amplitude at 0 mV as the $InsP_3$-gated channel from carp olfactory cilia measured under the same conditions (Cadiou et al. 2000) (1.43 ± 0.05 pA and 1.60 ± 0.10 pA, respectively). Both display two mean open times and have a similar conductance (50 ± 2 pS and 45 ± 5 pS, respectively); also, both are inhibited by ruthenium red

and heparin. Thus, the $InsP_3$-activated channel and the imipramine-activated channel may be the same channel. However, it is likely that imipramine does not gate the channel directly but via PLC. First, neomycin, a PLC inhibitor, suppresses the imipramine-gated current without inhibiting the $InsP_3$-gated current. In addition, the imipramine-gated current is transient, indicating that its activation depends on a restricted amount of substrate [PdtIns(4,5)P_2]. The lipids used to form the bilayers were pure and did not contain PdtIns(4,5)P_2; therefore this lipid may be associated with PLC (Romoser et al. 1996). Also, it has been demonstrated that several ion channels could be associated with this lipid (Zeng et al. 2002). Our findings confirm that the olfactory $InsP_3$ receptor is localized in the plasma membrane. The percentages of bilayers which respond to $InsP_3$ (12%, Cadiou et al. 2000) and imipramine (data presented here) are close. If the olfactory $InsP_3$ receptor was a contamination from the ER, the ratios would be different. In addition, the size of the olfactory cilia (0.15 μm thick; 1–2 μm diameter) and the diameter of the tip-dip pipette (1–2 μm) are such that only a small number of cilia are present in any one bilayer. As a result, the $InsP_3$-gated channel and PLC may be co-localized in carp olfactory cilia.

In contrast to the results obtained by Gomez et al. (2000), imipramine alone was able to induce activity in carp olfactory membranes. This contrasts with whole rat and human ORCs, for which both imipramine and a PKC inhibitor are required for induction. In our planar lipid bilayer experiments, the cytoplasmic elements are dialysed and some features of the regulation in living cells may be lost. However, carp olfactory cilia may maintain their organization in planar lipid bilayers (Cadiou et al., unpublished data).

As imipramine activates PLCβ1 in rat brain, but not PLCγ1 or PLCδ1 (Fukuda et al. 1994), it is likely that carp olfactory cilia may contain PLCβ. In invertebrates, PLCβ was found in the olfactory organ of lobster (Xu and McClintock 1999) and in *Drosophila* a PLCβ-related protein, *norpA*, mediates odorant responses (Riesgo-Escovar et al. 1995). In vertebrates, Noe and Breer (1998) identified a PLCβ2-related subtype in rat ORCs. As PLCβ is mainly located in the plasma membrane and is Gq associated, we suggest that carp olfactory cilia PLC may mediate odorant responses, as already demonstrated for salmon and taurocholic acid (Lo et al. 1994). Despite this evidence, several authors report PLCδ1 in rat (Bruch et al. 1995) and in catfish olfactory epithelium (Abogadie et al. 1995), which is inconsistent with an involvement in olfactory transduction mechanisms.

To test whether adenophostin A would serve as a pharmacological agent able directly to open the olfactory $InsP_3$-gated channel, we applied it to carp olfactory cilia reconstituted into planar lipid bilayers. The carp $InsP_3$-gated channel from carp olfactory cilia measured under the same conditions requires 10 μM $InsP_3$ to induce a channel activity (Cadiou et al. 2000), whereas the adenophostin A-gated channel is activated in a 0.4–0.5 μM range. The 0.5 μM adenophostin A-gated channel and the $InsP_3$-sensitive channel display similar current amplitudes at 0 mV (1.47 ± 0.04 pA and 1.60 ± 0.10 pA, respectively) but different conductances (60 pS and 45 pS, respectively). At 0.5 μM, the adenophostin A-gated channel possesses a single mean open time (15.0 ± 0.3 ms), whereas the olfactory $InsP_3$-gated channel has two mean open times of 3.0 ± 0.4 ms and 42.0 ± 0.6 ms (Cadiou et al. 2000). However, both channels are inhibited by ruthenium red and heparin in the same ratio, suggesting that the carp olfactory $InsP_3$-gated channel and the adenophostin A-sensitive channel could be the same channel.

Adenophostin A is a potent agonist of the $InsP_3$ receptor from the ER. Experiments with platelets and isolated membranes from the cerebellum have shown that adenophostin A is 50-fold more potent than $InsP_3$ at inducing calcium release (Murphy et al. 1997). For carp ORCs, adenophostin A is 20 times more potent than $InsP_3$ to obtain a similar open probability (cf. Cadiou et al. 2000). Adenophostin A is also a potent activator of the $InsP_3$ type 3 expressed in 16HBE14o cells (Missiaen et al. 1998), of the $InsP_3$ receptor type 2 (Marchant et al. 1997) and of all the three subtypes expressed in insect Sf9 cells (Correa et al. 2001). Thus, the work presented here confirms that adenophostin A appears to be a non-selective $InsP_3$ receptor subtype agonist. Mak et al. (2001) recently investigated the action of adenophostin A on the $InsP_3$ receptor from the outer membrane of isolated *Xenopus laevis* oocyte nuclei at the single-channel level. Adenophostin A did not alter the conductance properties, whereas a slight increase of the conductance of the $InsP_3$-gated channel from carp olfactory cilia was observed. However, adenophostin modified the kinetics of both channels.

Kashiwayanagi and co-workers (2000) using patch-clamp whole-cell techniques showed that adenophostin analogues were able to induce inward currents in turtle ORCs. These currents had properties similar to those observed with $InsP_3$ and were calcium dependent. However, they used high concentrations of adenophostin analogues (1–100 μM). In addition, they demonstrated that adenophostin analogues with two phosphate groups are more potent activators than the ones with three phosphate groups. In our experiments, adenophostin A possessed a purine moiety and three phosphate groups. Thus, it is possible that the lower activating concentration thresholds found in carp ORCs are due to the presence of the purine group despite the presence of the three phosphate groups. In fact, the removal of the adenine moiety substantially decreases the efficiency of adenophostin analogues on Ca^{2+} release from the intracellular stores of permeabilized hepatocytes (Correa et al. 2001).

Although it has been suggested that adenophostin A could bind to ATP binding sites, it is now acknowledged that adenophostin A binds only to the $InsP_3$ binding site (Maes et al. 1999). Although antibodies do not recognize

the $InsP_3$ binding site of the olfactory $InsP_3$-sensitive channel (Cadiou et al. 2000), the results presented here suggest that the $InsP_3$ binding site of olfactory channels and of calcium channels from the ER could have the same conformation.

Our observations indicate that both adenophostin A and imipramine could be potent pharmacological tools for the study of the inositides pathway in ORCs from various species.

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