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Calcium Mediates the NO-induced Potassium Current in Toad and Rat Olfactory Receptor Neurons

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Abstract. Nitric oxide (NO) activates a K^+ current in dissociated amphibian olfactory receptor neurons. Using the patch-clamp technique in its whole-cell mode and stimulation with puffs of the NO-donor sodium nitroprusside, we further studied this effect and show that it was sensitive to the K^+-channel blockers tetraethylammonium and iberiotoxin, indicating the activation of a Ca^{2+}-dependent K^+ conductance. The Ca^{2+}-channel blockers nifedipine and cadmium abolished the NO-induced current, and lowering external Ca^{2+} reduced it significantly. Ca^{2+} imaging showed a transient fluorescence increase upon stimulation with NO, and after blockade of K^+ currents, an NO-induced inward current could be measured, suggesting that the activation of the Ca^{2+}-dependent K^+ conductance is mediated by Ca^{2+} influx. LY83583, a blocker of the ciliary cAMP-gated channels, did not affect the current, and experiments with focal stimulation indicated that the effect is present in the soma, therefore Ca^{2+} is unlikely to enter via the transduction channels. Finally, we show that NO exerts an effect with similar characteristics on olfactory receptor neurons from the rat. These data represent the first evidence that NO activates a Ca^{2+}-dependent K^+ conductance by causing a Ca^{2+} influx in a sensory system, and suggest that NO signaling plays a role in the physiology of vertebrate olfactory receptor neurons.

Key words: Nitric oxide — Olfactory receptor neuron — Potassium channel — Calcium — Toad — Rat

Introduction

Nitric oxide (NO) is a small and labile, membrane-permeant free radical that is produced by the enzyme NO-synthase (NOS). Of the three identified and cloned NOS-isoforms, the one abundant in neurons has been termed *neuronal*, or nNOS, but there is evidence for the expression of the other NOS-isoforms in the nervous system as well. Whereas nNOS has been detected in the olfactory epithelium of embryonal rats (Bredt & Snyder, 1994; Roskams et al., 1994), the presence of NOS in the adult olfactory epithelium is still a matter of controversy (*see* Schild & Restrepo, 1998).

From its source, NO may reach nearby inter- or intracellular targets by free diffusion or protein-guided transport (reviewed by Stamler et al., 1997), but its half-life, high reactivity and spatial dilution limit its field of action (Garthwaite & Boulton, 1995). One function of NO is the activation of soluble guanylyl cyclase, which leads to an elevation of cGMP-levels (Bredt & Snyder, 1989). A stimulation of soluble guanylyl cyclase by NO has been found in ciliary preparations of rat olfactory receptor neurons (ORNs), where odor-induced rises of cGMP levels could be abolished by the NOS-inhibitor l-nitroarginine and the NO-scavenger hemoglobin (Breer, Klemm & Boekhoff, 1992), suggesting the odor-induced synthesis of NO in these cells.

Lischka & Schild (1993) reported an induction of inward currents by the NO-donor sodium nitroprusside (SNP) in voltage-clamped isolated *Xenopus* ORNs. As these currents were similar to those elicited by cGMP, the authors proposed a NO/cGMP-system in ORNs. More recently, comparable results have been obtained in the turtle (Inamura, Kashiwayanagi & Kurihara, 1998).

In contrast, using isolated ORNs from the two amphibian species *C. caudiverbera* and *X. laevis*, we recently reported that pulses of the NO-donors SNP and NOC-12 cause transient outward currents which hyperpolarize the cell under current-clamp (Schmachtenberg & Bacigalupo, 1999). This current was characterized by its sensitivity to the K^+ channel blocker charybdotoxin and dependence on external K^+, indicating the activation

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of a K^+ conductance. The current-voltage relationship is reminiscent of a Ca^{2+}-activated K^+ (K_{Ca}) conductance. The effect is rapid (latencies as short as ~30 msec), independent from the presence of cilia and insensitive to the soluble guanylyl cyclase-inhibitors ODQ and LY83583, suggesting a mechanism independent from cGMP.

Here we extend our previous study on the NO-induced K^+ current in the toad, show that it depends on the influx of external Ca^{2+} and document that a similar effect is present in the rat.

Materials and Methods

Cell Preparation

ORNs were isolated from *C. caudiverbera* and the rat. Toads were cooled down to 0°C, sacrificed and pithed before opening the nose chamber. The olfactory epithelium was removed with fine scissors, cut into pieces of ~1 mm^2 and stored in Ringer supplemented with 1% bovine albumin at 4°C for up to 48 hr. Adult Whistar rats, bred in the laboratory, were anesthetized by CO_2-inhalation and decapitated. The head capsule was opened by a sagittal section and the olfactory epithelium was removed from the dorsal posterior part of the nasal septum and from the turbinates. The epithelium was cut into small pieces, stored in Leibovitz L-15 medium at 4°C and used only on the day of the preparation. Dissociation was achieved by trituration through a fire-polished Pasteur pipette, without the use of enzymes. Cells were let settle for 20 min on coverslips coated with Pegotin (BiosChile) and washed with mammalian saline. In a few experiments, Pegotin was omitted, with identical results.

Patch-Clamp Recordings

Electrical recordings were obtained using a PC-501A amplifier (Warner Inst.) and pClamp 6.0 software (Axon Instruments). Recording pipettes were drawn from Blu Tip capillary tubes (Oxford Labware) in a horizontal Puller (Sutter P 80/PC) to a tip resistance of 3–6 MΩ. Whole-cell mode was established by suction and capacitance was compensated. Only experiments with seal resistances >1 GΩ (typical ⩾4 GΩ) were considered. *Caudiverbera* ORNs were held at −70 mV; rat ORNs at −80 mV.

Solutions

Amphibian Ringer (mM): 110 NaCl, 1 $CaCl_2$, 1.5 $MgCl_2$, 2.5 KCl, 10 HEPES, 3 glucose, pH 7.6. Low Ca^{2+}-Ringer contained 0.1 mM $CaCl_2$. Mammalian saline: 137 NaCl, 1.26 $CaCl_2$, 1 $MgCl_2$, 5.36 KCl, 0.73 $MgSO_4$, 0.3 KH_2PO_4, 1.2 Na_2HPO_4, 32 Sucrose, pH 7.6. Internal solution (mM): 120 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 2 EGTA, 4 HEPES, 0.1 GTP, 1 ATP, 13 sucrose, pCa 8.0, pH 7.6. To measure Ca^{2+} currents, the internal solution contained: 110 Cs methanesulfonate, 10 CsFl, 15 CsCl, 5 Cs-Hepes, 4 Mg-ATP, 10 phosphocreatin, pH 7.5, and the bath solution was supplemented with 10 mM TEA. In some experiments, external sodium was replaced by N-methyl-d-glucamine, but this diminished the viability of the cells. The NO-donor sodium nitroprusside (SNP; 10 mM) was prepared freshly and protected from light. To find out if the applied concentrations of NO were in a physiological range, we measured NO liberation by 10 mM SNP in Ringer with a Sievers NOA 280 nitric oxide analyzer (courtesy of M. Bóric and X. Figueroa) under conditions similar to those of the stimulus pipette during experiments (22–24°C, indirect dim neon light). After dissolving SNP, [NO] quickly rose to ~1 μM and remained relatively constant for at least 45 min (*see* Fig. 1*A*), which corresponds to our typical usage time of a stimulus pipette. 1 μM is considered a peak value at an endogenous NO source and is 1,000 times higher than an estimated biologically relevant threshold value (*see* Garthwaite & Boulton, 1995).

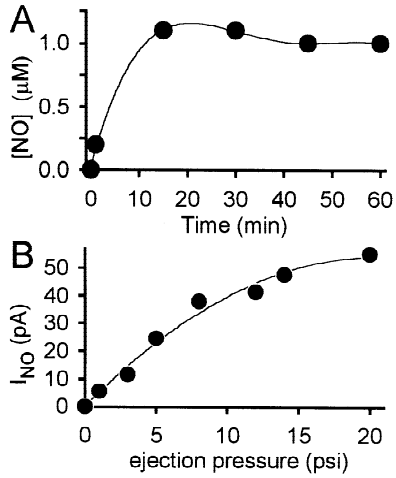

Fig. 1. (*A*) Time course of NO-liberation by 10 mM SNP. SNP was dissolved in Ringer at $t = 0$ min. The curve represents a 4th-order regression. (*B*) Dose-dependence of the NO-effect. Net NO-induced currents of subsequent trials (intervals: 30 sec) in a *Caudiverbera* ORN are plotted against the ejection pressure of the picospritzer. ORNs were stimulated by 1.5 sec SNP-pulses during 3 sec voltage steps to −30 mV. The solid line represents a second-order regression.

Stimulation

Stimuli were applied to the entire cell in pulses of varying duration using a picospritzer and triple-barreled glass pipettes (Sutter Instruments, tip diameter ca. 1.5 μM), positioned at 30–40 μM from the cell. Pressure ejection (5–15 psi) was monitored visually. The NO concentration at the cell is difficult to calculate with the use of stimulus puffs; therefore we display the relation between the NO-induced current and the ejection pressure in Fig. 1*B*. The current-pressure dependence is almost linear up to 10 psi, approaching a maximum at higher pressures, presumably because the NO concentration at the cell reaches the value in the stimulus pipette, estimated to be ~1 μM. The curve shows the concentration dependence of the NO effect and suggests approximate dilution factors between 3 and 1 for the pressures used in this work.

Iberiotoxin (IbTX; a gift from R. Latorre) was used at 100 nM, apamin (from RBI) at 2 μM and tetraethylammonium (TEA) at 2 mM. To block calcium channels, $CdCl_2$ was applied at 100 μM and nifedipine at 50 μM. LY83583 (RBI) was used at 20 and 10 μM. All values correspond to the solutions in the stimulus pipette. Concentrations reaching the cell were lower because of diffusion in the bath and due to the unstirred layer around the cell membrane. In the experiments with TEA, $CdCl_2$, nifedipine and low Ca^{2+}, the cells were microsuperfused using one of the stimulus pipette barrels. The second barrel contained the respective solution with 10 mM SNP and the third barrel SNP in normal Ringer. In all other experiments, drugs were coejected with SNP and compared with SNP- and drug-effects alone.

Ca^{2+} Imaging

Olfactory epithelium from *Caudiverbera* was loaded with 15 μM Fluo-3,AM (Molecular Probes) in 0.1% pluronic acid at 4°C for 30 min. After dissociation, cells were transferred to a Pegotin-coated coverslip. Images were obtained with a Zeiss Axiovert 135M confocal laser scanning microscope implemented with a 40× oil-immersion objective. SNP was applied with a picospritzer as in the electrophysiological experiments.

All chemicals were purchased from Sigma, unless otherwise indicated.

Results

This study is principally based on results from the Chilean toad *C. caudiverbera*. Complementary data from the rat are summarized in the last Results section.

The NO Effect is Sensitive to TEA and IbTX, But Not to Apamin

To characterize the conductances underlying the NO effect, we applied several channel blockers together with the NO-donor SNP. 2 mM tetraethylammonium (TEA) abolished the NO-induced current (Figs. 2*A* and 4). TEA blocks large-conductance K_{Ca}-channels in submillimolar concentrations ($K_d \approx 0.14$–0.29; *see* Latorre, 1994), but not small-conductance K_{Ca}-channels, which are insensitive to TEA. Iberiotoxin (IbTX), a specific blocker of large-conductance Ca^{2+}-dependent K^+-channels (K_{Ca}-channels; Candia, Garcia & Latorre, 1992) significantly reduced the NO effect at 100 nM (Figs. 2*B* and 4). The fact that IbTX did not cause a total block at this concentration in spite of a reported K_d of ~1 nM in muscle cells (Candia et al., 1992) might be indicative of a lower IbTX sensitivity of the K_{Ca}-channels in this tissue, or of the participation of other K_{Ca}-channels, possibly of intermediate conductance. 2 μM apamin, a potent specific blocker of small-conductance K_{Ca}-channels (Hille, 1992), did not reduce the NO-induced current significantly (Figs. 2*C* and 4), arguing against a contribution of these channels to the observed effect.

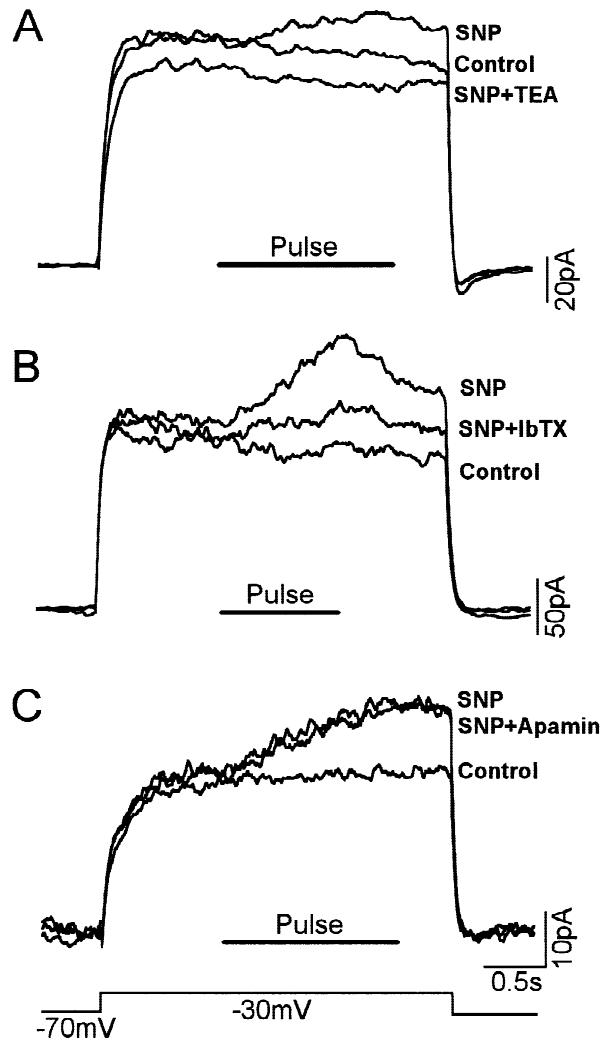

Fig. 2. The NO-effect is blocked by TEA and IbTX, but not by apamin. (*A*) Perfusion with 2 mM TEA. TEA also reduced the voltage-activated currents, as shown by the downward displacement of the lower trace. (*B*) Co-ejection of SNP with 100 nM IbTX. (*C*) Co-ejection of 2 μM apamin. Recordings were obtained from voltage-clamped *Caudiverbera* ORNs during 3 sec voltage-steps to −30 mV. Control traces were obtained by depolarizing steps in the absence of a puff.

The NO-induced K^+ Current is Dependent on Ca^{2+} Influx

Possible mechanisms by which NO might open K_{Ca}-channels include a direct, covalent activation of these channels and an increase of intracellular Ca^{2+}. To test the latter possibility, we stimulated the neurons during perfusion with the Ca^{2+} channel blockers cadmium and nifedipine. As demonstrated by representative traces in Fig. 3*A* and *B* and summarized in Fig. 4, both cadmium and nifedipine largely eliminated the NO effect, which nonetheless recovered completely after the end of perfusion. Experiments with low-Ca^{2+} perfusion provided further evidence for an influx of that ion, as a tenfold reduction of external Ca^{2+} significantly reduced the NO-induced current (Figs. 3*C* and 4).

The previous results predict that an inward current should be associated with the activation of the NO-induced K^+ current, but in our experiments, stimulation with SNP under normal ionic conditions never caused inward currents, irrespective of the holding potential (Fig. 5*A*). Yet, NO has been reported to induce inward currents in *Xenopus* (Lischka & Schild, 1993) and the turtle (Inamura et al., 1998). In those experiments, ORNs were subjected to prolonged bath perfusion with

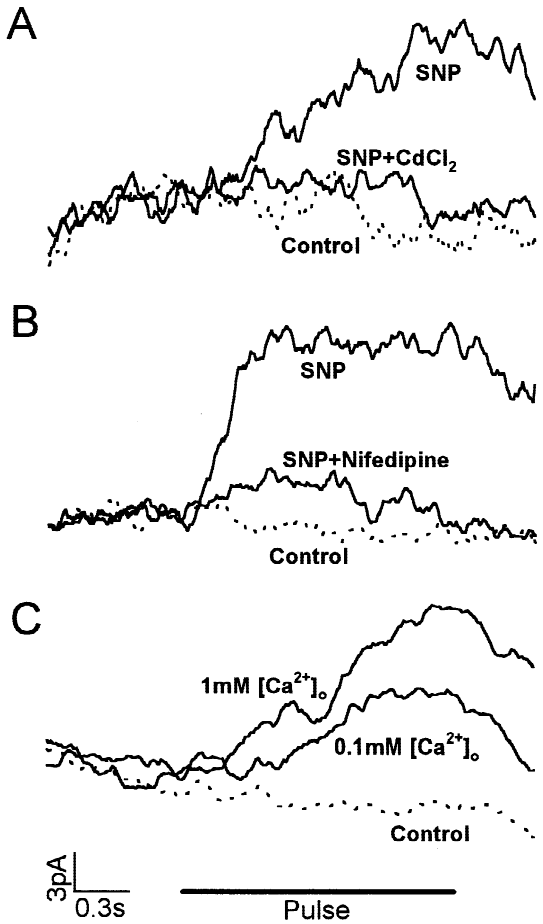

Fig. 3. The NO-effect is dependent on Ca^{2+} influx. (*A*) Perfusion with 100 μM $CdCl_2$. (*B*) Perfusion with 50 μM nifedipine, a blocker of L-type Ca^{2+} channels. (*C*) Exchange of 1 mM external Ca^{2+} by 0.1 mM reduced the effect of a SNP-pulse applied in the same respective Ringer. In all cases, the NO-effect recovered. Controls (dotted lines) are in normal Ringer, without SNP.

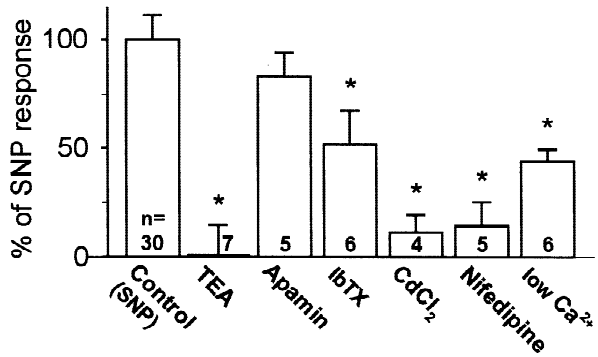

Fig. 4. Relative SNP-responses of *Caudiverbera* ORNs in the presence of ion channel blockers and reduced external Ca^{2+}. Bars (+SEM) display the relative net current amplitude induced by SNP at −30 mV under each tested condition, compared to SNP alone (control). Only experiments with at least partial recovery were considered, with numbers representing the total of cells analyzed. Asterisks indicate significant differences to the control according to the unpaired Student's *t*-test ($P < 0.05$).

10 mM SNP. To examine if continuous application of NO modified the cellular responses, we perfused *Caudiverbera* ORNs with SNP during one minute (Fig. 5*B*). No inward currents could be discerned at −70 mV, a potential at which an activation of the CNG channels is expected to produce a large current (equilibrium potential ~0 mV), but where K^+ currents are very small due to their voltage-dependence and their equilibrium potential of ~−100 mV. At −40 mV, the NO-induced outward current is clearly visible.

Since the putative Ca^{2+} current was not observable under normal conditions, we replaced K^+ with Cs^+ in the internal solution and added 10 mM TEA to the bath. Under these conditions, NO caused a small inward current at −30 mV in 9 out of 11 cells (Fig. 6*A*). This inward current could also be seen after replacement of external sodium by N-methyl-*d*-glucamine ($n = 2$, *not shown*), indicating that Ca^{2+} was indeed the entering ion.

To visualize a rise in intracellular Ca^{2+}, we used Ca^{2+}-imaging of *Caudiverbera* ORNs loaded with Fluo-3,AM during stimulation with SNP (Fig. 6*B*). In 6 out of 24 neurons, NO caused a transient fluorescence increase, indicative of an increment in intracellular Ca^{2+}. The fact that a rise in intracellular Ca^{2+} could only be observed in 25% of the cells, as opposed to 78% displaying the NO-induced K^+ current (Schmachtenberg & Bacigalupo, 1999), might be explained by the small magnitude of the Ca^{2+} current.

Altogether, these results support the interpretation that NO activates K_{Ca}-channels by triggering a Ca^{2+} influx.

Localization of the NO Effect

The fluorescence increase caused by NO seemed evenly distributed throughout the cell body and was also present in cells without cilia (fluorescence in the cilia was not resolved), in agreement with our previous electrophysiological observation of the NO effect in deciliated cells (Schmachtenberg & Bacigalupo, 1999). We further investigated the localization of the activated conductance by means of focal stimulation of intact isolated neurons (Fig. 7*A*). Puffs of SNP directed to the soma generally yielded slightly larger currents than stimulation of the dendritic knob and the cilia, but the NO effect was present in all cases. ORNs have been shown to express a ciliary K_{Ca}-conductance which participates in inhibitory odor responses (Morales, Labarca & Bacigalupo, 1995), thus we cannot exclude the possibility that this conductance is activated by NO as well and contributes to the overall NO-induced current. Alternatively, puffs

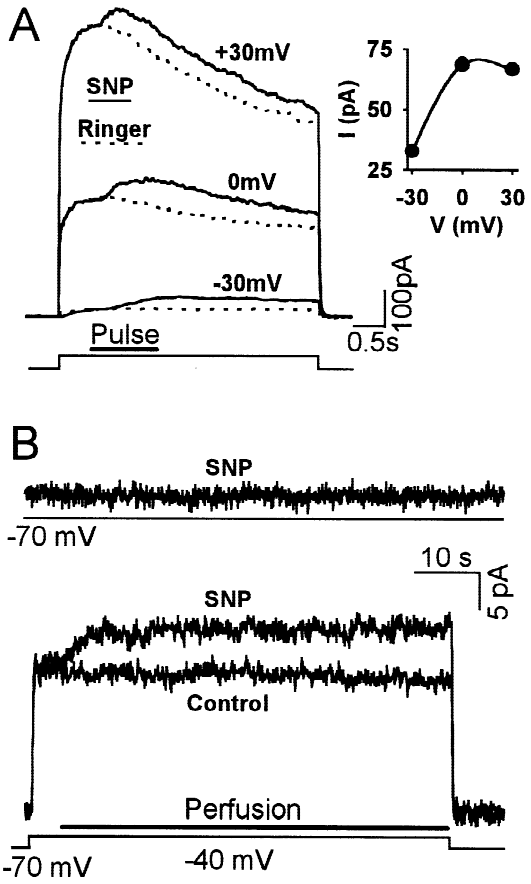

Fig. 5. NO-induced inward currents cannot be detected under normal ionic conditions. (*A*) At −30, 0 and + 30 mV, SNP induced outward currents. The absence of a tail current upon returning to the holding potential of −70 mV indicates that the CNG conductance was not activated (reversal potential ≈ 0 mV). (*B*) An ORN was held at −70 mV and microperfused with SNP during 1 min. No current was induced (n = 8). (*B*) The same ORN was stimulated at −40 mV, resulting in the activation of a sustained outward current. Experiments with ORNs from the rat yielded the same result (n = 4; *not shown*).

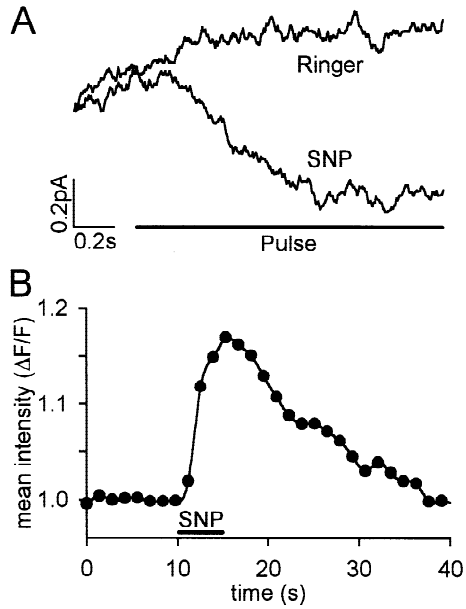

Fig. 6. NO causes Ca^{2+} influx. (*A*) Puffs of 10 mM SNP induced inward currents in *Caudiverbera* ORNs voltage-clamped at −30 mV after replacement of internal K^+ by Cs^+ and addition of 10 mM TEA to the external solution. Traces represent the average of eight experiments in one cell. Control puffs with Ringer had no effect. (*B*) NO causes a rise in intracellular Ca^{2+}. A 5 sec pulse of SNP induced a fluorescence increase in a Fluo-3,AM-loaded ORN under confocal Ca^{2+} imaging (n = 6 out of 24). Mean pixel intensities integrated from the entire cell were recorded in 1 sec intervals and normalized with a bleaching curve constructed from two nonresponsive cells.

directed at the cilia might reach the rest of the cell by diffusion, opening potassium channels in the dendrite and soma.

The CNG transduction channels are principally localized to the cilia, but can be found in the soma at much lower densities (Kurahashi & Kaneko, 1991). These channels are permeable to Ca^{2+} and have been reported to be directly activated by NO (Broillet & Firestein, 1996*a*, 1997). Although the NO effect does not predominate in the cilia, it cannot be ruled out that the NO-induced Ca^{2+} influx involves the CNG conductance. To test this hypothesis, we added 20 μM LY83583, a blocker of CNG channels ($K_d \approx 1.4$ μM; Leinders-Zufall & Zufall, 1995), to the bath. Yet, the blocker did not impair prominent NO effects (Fig. 7*B*), arguing against a significant involvement of the CNG conductance in the NO-induced response.

The NO Effect is also Present in Rat ORNs

Pulses of the NO-donor SNP induced outward currents in 42% of the analyzed ORNs from the rat (30 out of 72 cells from 20 animals; Fig. 8). Although less frequent, the effect seemed similar to the one described for *Caudiverbera.* A tail current upon returning to the holding potential (−80 mV) was generally not seen, indicating that no large inward current was activated. Currents elicited by NO were positive over the whole voltage range tested and their averaged current-voltage relationship displays the N-shape typical of K_{Ca}-conductances (Hille, 1992; Fig. 8*A* inset). The effect could be blocked by IbTX (Fig. 8*B*), but not by LY83583 (Fig. 8*C*). The presence of 2 μM ODQ, a potent inhibitor of soluble guanylyl cyclase, to the internal solution had no effect upon the NO-induced current (*not shown*), indicating that cGMP was not involved. Under conditions where outward currents had been largely eliminated, a small inward current sensitive to cadmium could be detected (Fig. 8*D*).

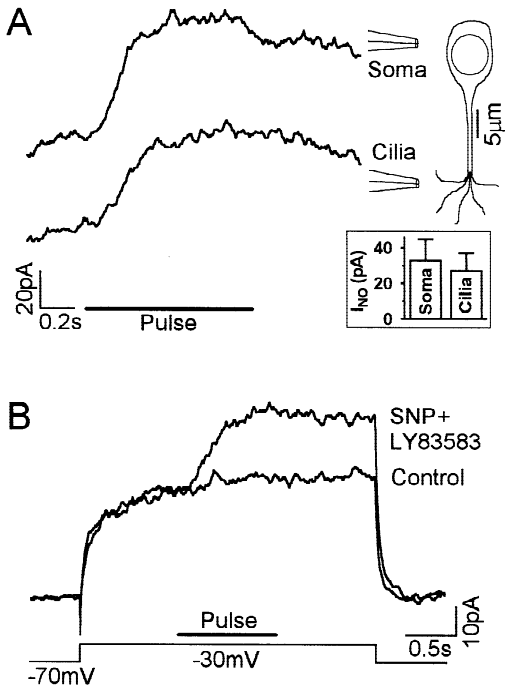

Fig. 7. (*A*) The NO-effect predominates in the soma. A fine ejection pipette (tip diameter ca. 1 μM) was used to apply SNP-pulses to the cilia and dendritic knob (1) and to the soma (2). *Inset:* Average peak current amplitudes induced by SNP-ejection onto the soma and the cilia from four cells at −30 mV. (*B*) The CNG channel-blocker LY83583 (20 μM in the bath) did not block the current induced by an SNP-puff. In other experiments, co-ejection of LY83583 with SNP was equally ineffective ($n = 5$).

Discussion

We recently reported that NO induces a K^+ current in dissociated ORNs from *Xenopus* and *Caudiverbera* (Schmachtenberg & Bacigalupo, 1999). Here we present a further analysis of this phenomenon, show that it is also present in the rat, and demonstrate its mediation by Ca^{2+}.

The Ca^{2+}-dependence of the NO effect, its current-voltage relationship and sensitivity to IbTX indicate the activation of a K_{Ca}-conductance. This conductance was sensitive to TEA but not to apamin (Fig. 2), suggesting an involvement of K_{Ca}-channels of large or intermediate, but not small conductance (Hille, 1992; Candia et al., 1992; Cai, Garneau & Sauve, 1998). A K_{Ca}-channel with a unitary conductance of 130 pS and strong Ca^{2+}-dependence has been described in mouse ORNs (Maue & Dionne, 1987).

Ca^{2+} involved in the activation of the NO-induced K^+ current may enter the cell through Ca^{2+}-permeant channels, or it may be released from internal stores. The observed reduction of the NO effect by cadmium, nifedipine and low external Ca^{2+} is consistent with the notion that NO caused Ca^{2+} influx, which in turn activated K_{Ca}-channels. Direct measurements of NO-induced inward currents and Ca^{2+}-imaging experiments supported that hypothesis.

Modulation of Ca^{2+} currents by NO has been reported for sympathetic neurons and heart muscle cells (Chen & Schofield, 1993; Campbell, Stamler & Strauss, 1996), but how Ca^{2+} influx in ORNs is caused by NO remains to be clarified. Our observations indicate that cyclic GMP is unlikely to be involved, since guanylyl cyclase-inhibitors did not prevent the effect (Schmachtenberg & Bacigalupo, 1999). Ca^{2+} entry is also a key step in olfactory transduction. This raises the question of why a NO-induced Ca^{2+} influx does not activate Ca^{2+}-dependent chloride channels, as occurs during odor responses (Lowe & Gold, 1993). One possibility is the existence of a spatial separation or chemical barrier between the two Ca^{2+}-entry pathways. Indeed, the experiments with focal stimulation indicated that the NO effect predominates in the soma, where Ca^{2+}-dependent chloride channels would not be expected. Two Ca^{2+} conductances have been described in ORNs: A somatic, voltage-dependent conductance (Schild, 1991; Delgado & Labarca, 1993) and the CNG conductance confined principally to the cilia. Our results support a Ca^{2+} entry via the somatic channels, because (i) the effect is present in cells without cilia, and in cells with cilia somatic stimulation yields larger currents than ciliary stimulation (Fig. 7*A*), although the density of the CNG channels is much lower in the soma. (ii) An activation of the CNG channels should cause a prominent inward current, as occurs during odor responses, but this was not the case (Fig. 5), and (iii) high doses of the CNG channel blocker LY83583 did not block the NO-induced current (Figs. 7*B* and 8*C*).

Broillet & Firestein (1996*a*, 1997) reported direct NO activation of native CNG channels from the tiger salamander and the rat as well as activation of recombinantly expressed rat α- and β-homomeric and α/β-heteromeric CNG channels by NO. The activation was only slowly reversible and could be mimicked by sulfhydryl modifying agents, suggesting that NO interacts with a cysteine residue leading to the oxidation of a free SH-group. However, regarding native rat olfactory CNG channels, another recent report (Lynch, 1998) found the opposite: a direct inhibition by NO.

Interestingly, two whole cell patch-clamp studies reporting the activation of inward currents in ORNs by NO also concluded an involvement of cGMP (Lischka & Schild, 1993; Inamura et al., 1998), proposing a NO-stimulation of soluble guanylyl cyclase and discarding a direct NO effect. In both studies the recordings were done at a holding potential of −70 mV, where the outward K^+ current described by us would be too close to its equilibrium potential to be detectable. Yet, in our experiments, neither short nor long pulses with the NO-donor SNP induced inward currents under normal ionic

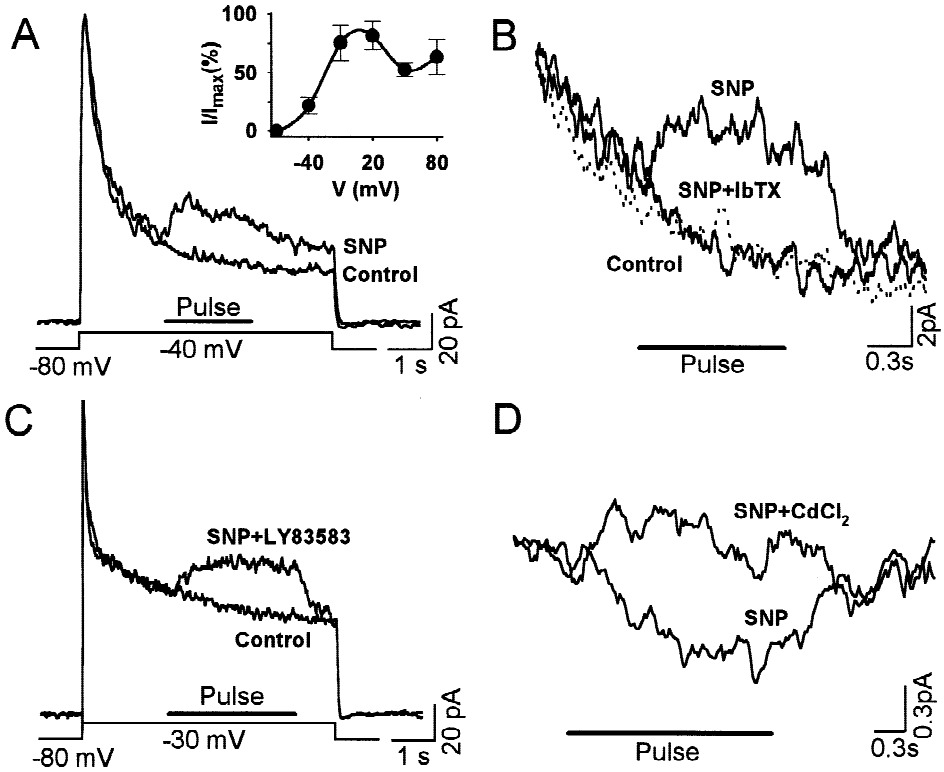

Fig. 8. NO elicits a similar effect in rat ORNs. (*A*) A 2 sec pulse of 10 mM SNP, applied during a depolarizating step to −40 mV, triggered a transient outward current. Control is without pulse. *Inset:* Current-voltage relation of the NO-induced current, averaged from five cells. Values (medium ± SE) were obtained by a series of depolarizing steps of 30 msec duration during a SNP-pulse. The voltage-gated currents were subtracted and the net currents were normalized to the peak values. (*B*) Co-ejection of 100 nM IbTX with SNP ($n = 7$). Recordings were obtained during 3 sec voltage steps to −30 mV. Control (dotted line) is without pulse. (*C*) Co-ejection of 10 μM LY83583. (*D*) With Cs^+ instead of K^+ in the internal solution and 10 mM TEA in the bath, SNP induced a small inward current at −30 mV, that could be blocked by co-ejection of 0.4 μM $CdCl_2$. Traces are averaged from 3 experiments in one cell.

conditions. Curiously, Lischka & Schild (1993) asserted an absolute requirement of the SNP-induced inward current for high (3 mM) GTP in the intracellular solution, which was not the case in Inamura et al. (1998), where no GTP was added. We used 0.1 mM GTP, but its omission did not make any difference. These contradictory results may reflect the versatility of NO reactions (*see* Stamler et al., 1997) or could be due to subtle experimental differences, such as the use of Ca^{2+}-free Ringer or proteolytic enzymes as opposed to the purely mechanical dissociation used by us. Unfortunately, the above mentioned whole-cell studies lack a pharmacological characterization of the observed current, and the hypothesis of a NO/cGMP-system is principally based on the comparison of SNP- with cGMP-effects.

The hypothesis of a NO-signaling system in the mature olfactory epithelium implies the necessity for the expression of NOS. Whereas several investigators failed to identify a putative NO source using antibodies against nNOS or mRNA *in situ* hybridization (reviewed in Broillet & Firestein, 1996*b*; Schild & Restrepo, 1998), attempts with the NADPH-diaphorase method yielded strong stainings in the olfactory epithelium of the rat (Kulkarni, Getchell & Getchell, 1994; Dellacorte et al., 1995). However, this histochemical method also marks the similar enzyme cytochrome P-450 reductase, which is present in the sustentacular cells of the olfactory epithelium (Kishimoto et al., 1993), thus preventing a conclusive interpretation of these results.

One possibility to reconcile the controversial data consists in the expression of a hitherto unidentified NOS isoform in the olfactory epithelium. Indeed, Arhold et al. (1997) found a transient expression of the inducible NOS isoform (iNOS) in developing ORNs from mouse embryos, whereas the other two isoforms were not detected. While this finding contrasts with previously identified nNOS expression in outgrowing ORNs from rat embryos with regard to NOS isoform and expression period (Bredt & Snyder, 1994; Roskams et al., 1994), all three reports suggest a role of NO in developmental processes like axonal pathfinding and synaptogenesis. This is of special interest since neurons are continuously generated in the olfactory epithelium, therefore NO might maintain a developmental role in the adult animal. Although NOS

expression in ORNs declined after birth, it could be reinduced by unilateral bulbectomy (Roskams et al., 1994), making the olfactory epithelium an excellent model for the study of NO function in neuronal outgrowth.

ORNs are not the only possible source of NO in the olfactory epithelium. nNOS has been found in extrinsic nerves innervating blood vessels and seromucous glands in the olfactory mucosa, where it may regulate blood flow and secretion (Hanazawa et al., 1994; Kulkarni et al., 1994). While it seems virtually impossible that NO from this distant source affects olfactory signaling in cilia and dendrites, it may reach more proximal targets like the somata or axonal parts of the ORNs. Alternatively, NO stimulation in vivo could occur in the olfactory bulb, where ORNs form synapses with mitral cells. NOS is abundant in the olfactory bulb and is produced by granule cells and other cell types (Kishimoto et al., 1993; Hopkins et al., 1994). The molecular target of ORN responsiveness to NO might be distributed all over the cell membrane, but actually be employed only in the olfactory bulb.

Finally, the high concentration of the CO-producing enzymes heme oxygenase-2 and cytochrome P-450 reductase in the olfactory epithelium led to the hypothesis that CO rather than NO might participate in olfactory signaling (Verma et al., 1993). Moreover, exogenous CO has been shown to activate the CNG channels of isolated ORNs from the tiger salamander (Leinders-Zufall, Shepherd & Zufall, 1995). The effect required GTP in the internal solution and could be blocked by the soluble guanylyl cyclase-inhibitor LY83583, indicating that it was mediated by cGMP. Based on these results, the authors suggested a CO/cGMP pathway in olfactory transduction and a possible switching between NO and CO during development.

Although the cGMP-dependent NO- and CO-stimulation of CNG channels appear comparable, the effect described here, involving neither cGMP nor the CNG channels, is clearly a separate phenomenon. Future research should clarify the putative functions of NO signaling in vertebrate ORNs.

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