

## Full Paper

# Characterization of Forskolin-Induced $\text{Ca}^{2+}$ Signals in Rat Olfactory Receptor Neurons

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Received November 29, 2004; Accepted February 15, 2005

**Abstract.** Forskolin-induced  $\text{Ca}^{2+}$  signals were examined in isolated rat olfactory receptor neurons (ORNs) using a  $\text{Ca}^{2+}$  indicator, fura-2. In the soma of the ORNs, forskolin caused an increase in the intracellular  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_i$ ) that was enhanced by a phosphodiesterase (PDE) 1 inhibitor, 8-methoxymethyl-3-isobutyl-1-methyl-xanthine, but not a PDE4 inhibitor, rolipram. Forskolin-induced  $\text{Ca}^{2+}$  signals were abolished with the removal of extracellular  $\text{Ca}^{2+}$  and un-affected by treatment with thapsigargin or caffeine plus ryanodine. Niflumic acid, a  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  channel inhibitor, or nifedipine, an L-type  $\text{Ca}^{2+}$  channel inhibitor, slowed the initial rate of the increase in  $[\text{Ca}^{2+}]_i$  in response to forskolin. Nifedipine did not affect the increase in  $[\text{Ca}^{2+}]_i$  that was slowed by niflumic acid. In  $\text{Ca}^{2+}$  measurements with a confocal microscope and a calcium indicator, Fluo-4, the onset of the response to forskolin in the knob region occurred simultaneously or earlier, but not later, than that in the soma. It is suggested that the forskolin-induced  $\text{Ca}^{2+}$  signals are due to  $\text{Ca}^{2+}$  influx, but not the release of  $\text{Ca}^{2+}$  from  $\text{Ca}^{2+}$  stores, and that the initial rapid increase in  $[\text{Ca}^{2+}]_i$  is associated with the activation of the voltage-dependent  $\text{Ca}^{2+}$  channels in rat ORNs.

**Keywords:** olfactory receptor neuron, forskolin,  $\text{Ca}^{2+}$  signal, phosphodiesterase,  $\text{Ca}^{2+}$  channel

## Introduction

Olfactory receptor neurons (ORNs) present in the olfactory epithelium detect chemical stimuli, that is, odors. Two intracellular messengers, cAMP and inositol-1,4,5-trisphosphate ( $\text{IP}_3$ ), are believed to mediate membrane depolarization in ORNs (1–3). However, it has been reported that cAMP signal transduction is a solo pathway for olfaction in mammals because knock-out mice with a disrupted cAMP signaling cascade do not exhibit detectable responses to various odorants (4–6).

Odorants bind to their receptors on the ciliary membrane of ORNs and activate adenylyl cyclase, resulting in an increase in the intracellular cAMP (2, 7). The cAMP activates nucleotide-gated channels, which cause an increase in the intracellular  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_i$ ). The number of mouse olfactory receptor genes

is about 1000 and each ORN expresses only a single receptor gene (7). 3-Isobutyl-1-methyl-xanthine (IBMX), a non-selective phosphodiesterase (PDE) inhibitor, has been widely used to elicit  $\text{Ca}^{2+}$  response via cAMP-dependent pathways in amphibian ORNs (8–11). In rat and human ORNs, however, IBMX fails to elicit any  $\text{Ca}^{2+}$  response (12). Therefore, forskolin, an activator of adenylyl cyclase, has been used as a control agonist to stimulate mammalian ORNs when the responses to odorants are examined (13–16). However, the  $\text{Ca}^{2+}$  signals caused by forskolin have not been fully investigated in mammalian ORNs. As forskolin activates adenylyl cyclase in a concentration-dependent manner (17), it is possible to characterize the  $\text{Ca}^{2+}$  signals via cAMP-dependent pathways quantitatively. Immunohistochemical experiments have shown that several subtypes of PDE are expressed in rodent ORNs (18, 19). PDE1 and 2 are expressed in the same ORNs with a different subcellular distribution, but PDE4 is expressed in a subset of ORNs. It is not clear which subtypes of

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PDE play a role in Ca<sup>2+</sup> signals in response to forskolin in mammalian ORNs.

Forskolin increases the intracellular cAMP. The cAMP in the cilia of ORNs opens cyclic nucleotide-gated channels permeable to Ca<sup>2+</sup>. The Ca<sup>2+</sup> passing through these channels in turn activates Ca<sup>2+</sup>-activated Cl<sup>-</sup> channels, which potentiates the depolarization induced by cyclic nucleotide-gated channels in the cilia and knob (20, 21). However, the relative contribution of the depolarization induced by Cl<sup>-</sup> channel activation to the Ca<sup>2+</sup> response to forskolin remains obscure in ORNs. On the other hand, diltiazem and nifedipine, L-type Ca<sup>2+</sup> channel inhibitors, at relatively high concentrations strongly suppress the Ca<sup>2+</sup> signals caused by odorants in rat and toad ORNs, respectively (14, 22). However, such high concentrations of Ca<sup>2+</sup> channel inhibitors inhibit cyclic nucleotide-gated channels (23, 24). Moreover, in cultured rat ORNs, nifedipine inhibited but did not abolish the voltage-dependent Ca<sup>2+</sup> currents (25), suggesting the existence of different types of voltage-dependent Ca<sup>2+</sup> channels. It is worth examining the inhibitory effects of inhibitors of Ca<sup>2+</sup> channel subtypes on the Ca<sup>2+</sup> signals in ORNs. In salamander ORNs, furthermore, it has been reported that the release of Ca<sup>2+</sup> from intracellular Ca<sup>2+</sup> stores is necessary for the spread of a Ca<sup>2+</sup> wave from the cilia to the soma (11). It is still unclear whether or not the Ca<sup>2+</sup> stores contribute to the Ca<sup>2+</sup> signals in mammalian ORNs.

The aim of the present experiments was to quantitatively characterize the Ca<sup>2+</sup> signals dependent on cAMP in rat ORNs. For this purpose, we examined the effects of various inhibitors of PDE subtypes, Ca<sup>2+</sup>-activated Cl<sup>-</sup> channels, voltage-dependent Ca<sup>2+</sup> channels, and K<sup>+</sup> channels on Ca<sup>2+</sup> response to forskolin. The effects of inhibitors of Ca<sup>2+</sup> channel subtype on the Ca<sup>2+</sup> signals induced by high KCl were also examined. Furthermore, we examined whether or not the release of Ca<sup>2+</sup> from the Ca<sup>2+</sup> stores was involved in the Ca<sup>2+</sup> signals in rat ORNs.

## Materials and Methods

### *Cell preparation*

All experiments were carried out in compliance with the regulations of the Animal Research Committee of the Graduate School of Veterinary Medicine, Hokkaido University. Isolated ORNs were prepared according to the protocol described by Wetzel et al. (26) with some modifications. Adult male rats (Wistar) purchased from Clea Japan (Tokyo) were killed with 100% CO<sub>2</sub>, and the olfactory epithelium was dissected out. The tissue was cut into small pieces and digested with divalent cation-free solution containing 10–15 units/ml papain, 2 mM cysteine, and 2 mM EDTA for 10–15 min at room

temperature. Then the tissue was transferred to low Ca<sup>2+</sup> solution and gently triturated with fire-polished Pasteur pipettes. The cell suspension was filtered through a nylon mesh, and aliquots were placed on concanavalin A-coated cover slips. The cells having a dendrite and knob were used.

### *Intracellular Ca<sup>2+</sup> measurements*

The ORNs were incubated with a fluorescent indicator, fura-2 acetoxymethyl ester (fura-2/AM, 5  $\mu$ M), in normal buffer solution for 90 min at room temperature. Then they were stored in a moist chamber at 4°C until used. Drugs were applied through a micropipette placed close to the cell. The fura-2 fluorescence at 500 nm with excitation at 340 and 380 nm was detected using an inverted microscope (Diaphot 300; Nikon, Tokyo) equipped with a CCD camera (C6790; Hamamatsu Photonics, Hamamatsu). Each image was recorded at 2-s intervals and analyzed with an image processor (AQUACOSMOS, Hamamatsu Photonics). The Ca<sup>2+</sup> signal in the soma was expressed as the ratio of fluorescent intensity (F340/F380).

Ca<sup>2+</sup> signals in the knob and soma were measured with a laser scanning confocal microscope (Fluoview FV500; Olympus, Tokyo). The ORNs were incubated with 5  $\mu$ M fluo-4/AM in normal buffer solution for 1 h at room temperature. Then, they were rinsed once and resuspended in fresh normal buffer solution. A cover slip coated with Cell-Tak (Becton Dickinson Labware, Bedford, MA, USA) was attached to the bottom of a hand-made chamber (volume 100  $\mu$ l) with dental-wax and an aliquot of the suspension was transferred into the chamber. The chambers were then centrifuged (500 rpm, 5 min) to facilitate proper attachment of the slender dendrites of the isolated ORNs. They were stored on ice until used. During the experiments, the cells were superfused with normal or stimulant-containing solution at a rate of 1 ml/min using a peristaltic pump (15-s dead time). The cells were irradiated with a visible argon laser beam (488 nm) and the emitted fluorescence (>505 nm) was guided through a 40x water immersion objective to a pinhole diaphragm. Images were recorded at 2–5-s intervals. The Ca<sup>2+</sup> signal was expressed as arbitrary fluorescent intensity. All experiments were performed at room temperature.

### *Solutions*

Normal buffer solution consisted of 138 mM NaCl, 5 mM KCl, 2 mM CaCl<sub>2</sub>, 1.5 mM MgCl<sub>2</sub>, 10 mM HEPES, 10 mM glucose, and 1 mM Na-pyruvate (pH = 7.4). In low Ca<sup>2+</sup> solution, the Ca<sup>2+</sup> concentration was decreased to 0.5 mM. In Ca<sup>2+</sup>-free solution, CaCl<sub>2</sub> was removed and 0.5 mM EGTA was added. In high

KCl solution (40 or 50 mM KCl), the  $\text{Na}^+$  concentration was decreased correspondingly. Divalent cation-free solution consisted of 138 mM NaCl, 10 mM HEPES, 10 mM glucose, and 1 mM Na-pyruvate, (pH = 7.4).

### Materials

Apamin, charybdotoxin, forskolin, 3-isobutyl-1-methyl-xanthine (IBMX), 8-methoxymethyl-IBMX (8-MM-IBMX), rolipram, and niflumic acid were purchased from Sigma Chemical Co. (St. Louis, MO, USA). *Erythro*-9-(2-hydroxy-3-nonyl) adenosine (EHNA) was from BIOMOL Research Laboratories (Plymouth Meeting, PA, USA).  $\omega$ -Conotoxin GIVA and  $\omega$ -agatoxin IVA were from Peptide Institute (Osaka). Caffeine monohydrate, nifedipine, and thapsigargin were from Wako Pure Chemicals (Osaka). Ryanodine was from AgriSystem (Wind Gap, PA, USA). Tetraethylammonium (TEA) chloride was from Nacalai Tesque (Kyoto). Forskolin, IBMX, 8-MM-IBMX, rolipram, niflumic acid, nifedipine, ryanodine, and thapsigargin were prepared from stock solutions in dimethyl sulfoxide (DMSO) giving final DMSO concentrations less than 0.3%.

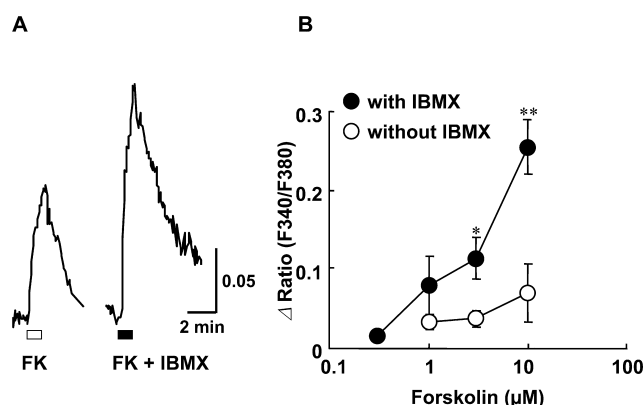
### Statistical analyses

The rate of  $[\text{Ca}^{2+}]_i$  increase induced by forskolin was expressed as the time taken to reach half of the maximal response during the application of forskolin ( $T_{1/2\text{max}}$ ). Results are expressed as means  $\pm$  S.E.M. Statistical differences were evaluated by Student's *t*-test or Dunnett's test for multiple comparison. A *P* value of less than 0.05 was considered significant.

## Results

### Forskolin-induced $\text{Ca}^{2+}$ signals in soma of rat ORNs

Forskolin was applied for 1 min at intervals of at least 5 min. Stimulation of ORNs with forskolin (10  $\mu\text{M}$ ) caused an increase in  $[\text{Ca}^{2+}]_i$  in the soma. The forskolin-induced increase in  $[\text{Ca}^{2+}]_i$  was expressed as  $\Delta\text{ratio}$  (F340/F380), which was the maximal ratio during the application of forskolin minus the basal ratio. IBMX (100  $\mu\text{M}$ ) itself, a non-selective PDE inhibitor, did not cause any detectable change in  $[\text{Ca}^{2+}]_i$  (data not shown). On the other hand, the forskolin-induced increase in  $[\text{Ca}^{2+}]_i$  was significantly enhanced by simultaneous application of IBMX (100  $\mu\text{M}$ ) (Fig. 1A). The concentration-response curves for forskolin in the presence and absence of IBMX were constructed in two separate groups of cells (Fig. 1B). A mixture of forskolin (10  $\mu\text{M}$ ) and IBMX (100  $\mu\text{M}$ ) was used to check the responsiveness of the cells, and there were no significant differences in  $\text{Ca}^{2+}$  signals between these two groups



**Fig. 1.** Forskolin-induced  $[\text{Ca}^{2+}]_i$  increase in the soma of rat ORNs. A: Forskolin (10  $\mu\text{M}$ , FK) or forskolin plus IBMX (100  $\mu\text{M}$ ) were applied for 1 min to the same cells at an interval of 10 min. Vertical scale bar: ratio (F340/F380). B: Concentration-dependent increase in  $[\text{Ca}^{2+}]_i$  caused by forskolin with or without IBMX (100  $\mu\text{M}$ ). Values are the mean  $\pm$  S.E.M. ( $n = 8 - 10$ ). \* $P < 0.05$ , \*\* $P < 0.01$  vs without IBMX (unpaired Student's *t*-test).

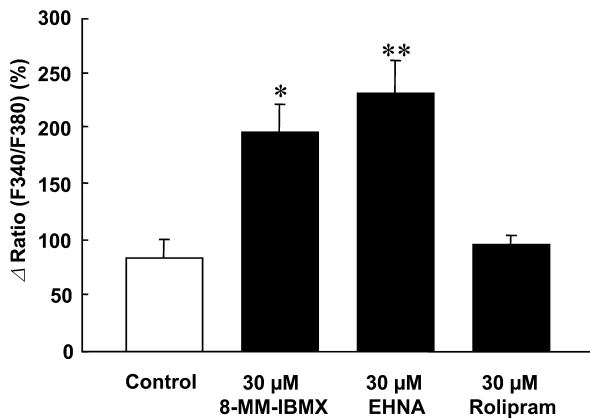
(data not shown). The basal ratio of fura-2 fluorescence (F340/F380) was relatively constant during experiments at  $0.53 \pm 0.04$  to  $0.52 \pm 0.03$  ( $n = 27$ ). Regardless of the presence or absence of IBMX (100  $\mu\text{M}$ ), forskolin increased  $[\text{Ca}^{2+}]_i$  (Fig. 1B). The increase in  $[\text{Ca}^{2+}]_i$  was detectable at a concentration of 1  $\mu\text{M}$  forskolin in the presence of IBMX and at 10  $\mu\text{M}$  in the absence of IBMX. The difference of the increase in  $[\text{Ca}^{2+}]_i$  in response to forskolin (3 and 10  $\mu\text{M}$ ) in the presence and absence of IBMX was significant.

### Effects of PDE inhibitors

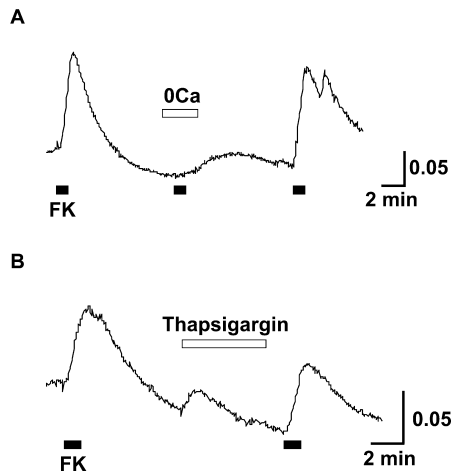
The effects of several PDE inhibitors on the increase in  $[\text{Ca}^{2+}]_i$  of the soma in response to forskolin were examined. Rolipram (30  $\mu\text{M}$ ), a PDE4 inhibitor, caused a small increase in basal  $[\text{Ca}^{2+}]_i$  ( $0.02 \pm 0.01$ ,  $n = 8$ ). 8-MM-IBMX (30  $\mu\text{M}$ ), a PDE1 inhibitor, and EHNA (30  $\mu\text{M}$ ), a PDE2 inhibitor, caused little, if any, increase in  $[\text{Ca}^{2+}]_i$ . The forskolin (10  $\mu\text{M}$ )-induced  $\text{Ca}^{2+}$  signal was significantly enhanced by simultaneous application of 8-MM-IBMX (30  $\mu\text{M}$ ) or EHNA (30  $\mu\text{M}$ ) (Fig. 2). In contrast, the application of rolipram (30  $\mu\text{M}$ ) with forskolin caused no enhancement.

### Dependency of extracellular $\text{Ca}^{2+}$

Forskolin (10  $\mu\text{M}$ ) markedly increased  $[\text{Ca}^{2+}]_i$  of the soma in the presence of extracellular  $\text{Ca}^{2+}$ . In the absence of extracellular  $\text{Ca}^{2+}$ , it failed to evoke any change in  $[\text{Ca}^{2+}]_i$  (Fig. 3A). After the reintroduction of extracellular  $\text{Ca}^{2+}$ , a slight increase in  $[\text{Ca}^{2+}]_i$  occurred and the response to forskolin was restored. Similar results were obtained in 3 other cells.

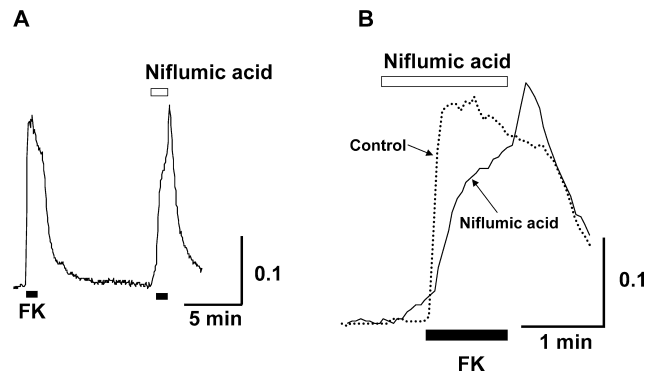


**Fig. 2.** Effects of PDE inhibitors on forskolin-induced Ca<sup>2+</sup> signals in the soma of rat ORNs. Cells were stimulated with forskolin (10 μM) at intervals of at least 5 min. The second application of forskolin was done in the absence (control) or the presence of 8-MM-IBMX (30 μM), EHNA (30 μM), or rolipram (30 μM). Columns show the mean ± S.E.M. (n = 7–10) of the Δratio (F340/F380) as a percentage of that caused by the first application of forskolin in each cell. \**P* < 0.05, \*\**P* < 0.01 vs control (Dunnett's test).



**Fig. 3.** Extracellular Ca<sup>2+</sup> dependency of forskolin-induced Ca<sup>2+</sup> signals in the soma of rat ORNs. A: Effects of the removal of extracellular Ca<sup>2+</sup> on forskolin-induced Ca<sup>2+</sup> signals. Cells were stimulated with forskolin (10 μM, FK) before and during exposure to Ca<sup>2+</sup>-free solution containing 0.5 mM EGTA (0Ca) and after reintroduction of Ca<sup>2+</sup>. B: Effects of thapsigargin on forskolin-induced Ca<sup>2+</sup> signals. Cells were stimulated with forskolin (10 μM, FK) before and after treatment with thapsigargin (1 μM) for 5 min. Vertical scale bar: ratio (F340/F380).

To explore whether or not the release of Ca<sup>2+</sup> from intracellular Ca<sup>2+</sup> stores is involved in the forskolin-induced increase in [Ca<sup>2+</sup>]<sub>i</sub> of the soma in rat ORNs, we first examined the effects of thapsigargin, an irreversible inhibitor of sarcoplasmic-endoplasmic reticulum Ca<sup>2+</sup>-ATPases, on the Ca<sup>2+</sup> signals induced by forskolin. As shown in Fig. 3B, thapsigargin (1 μM) by itself caused



**Fig. 4.** Effects of niflumic acid on forskolin-induced Ca<sup>2+</sup> signals in the soma of rat ORNs. A: Cells were stimulated with forskolin (10 μM, FK) in the presence or absence of niflumic acid (300 μM). B: The time course of the increase in [Ca<sup>2+</sup>]<sub>i</sub> caused by forskolin in the presence or the absence (control) of niflumic acid. Vertical scale bar: ratio (F340/F380).

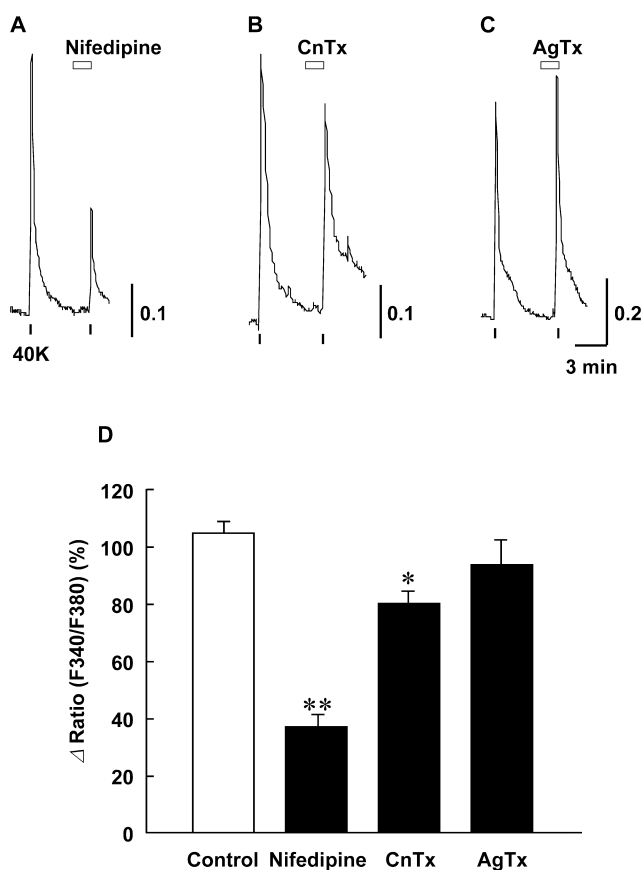
an increase in [Ca<sup>2+</sup>]<sub>i</sub> in 3 out of 5 cells. Thapsigargin had no significant effect on the forskolin-induced Ca<sup>2+</sup> signals (control: 0.13 ± 0.04, n = 5; thapsigargin: 0.15 ± 0.04, n = 5). We then examined the effect of caffeine in combination with ryanodine, which keeps ryanodine receptor channels in an open state. Treatment with caffeine (30 mM) plus ryanodine (100 μM) for 1 min had no effect on the forskolin-induced Ca<sup>2+</sup> signals (control: 0.13 ± 0.03, n = 3; caffeine plus ryanodine: 0.15 ± 0.02, n = 3).

#### Effects of a Ca<sup>2+</sup>-activated Cl<sup>-</sup> channel inhibitor

To investigate the contribution of Ca<sup>2+</sup>-activated Cl<sup>-</sup> channels to the forskolin-induced Ca<sup>2+</sup> signals of the soma, niflumic acid was applied before and during the first (n = 4) or the second (n = 4) application of forskolin. As shown in Fig. 4, niflumic acid (300 μM) caused a small but perceptible increase in [Ca<sup>2+</sup>]<sub>i</sub> in a few cells. Niflumic acid (300 μM) slightly but significantly reduced the peak increase in [Ca<sup>2+</sup>]<sub>i</sub> in response to forskolin (control: 0.19 ± 0.04 vs niflumic acid: 0.14 ± 0.02, n = 8, *P* < 0.05). After the removal of niflumic acid, a slight increase in [Ca<sup>2+</sup>]<sub>i</sub> occurred in 5 out of 8 cells. In addition, niflumic acid significantly decreased the rate of increase in [Ca<sup>2+</sup>]<sub>i</sub> caused by forskolin. The time taken to reach half of the peak level (*T*<sub>1/2max</sub>) was 10.6 ± 2.8 s (n = 8) for the control cells and 21.0 ± 3.6 s (n = 8, *P* < 0.05 vs control) for cells treated with niflumic acid.

#### Effects of voltage-dependent Ca<sup>2+</sup> channel inhibitors

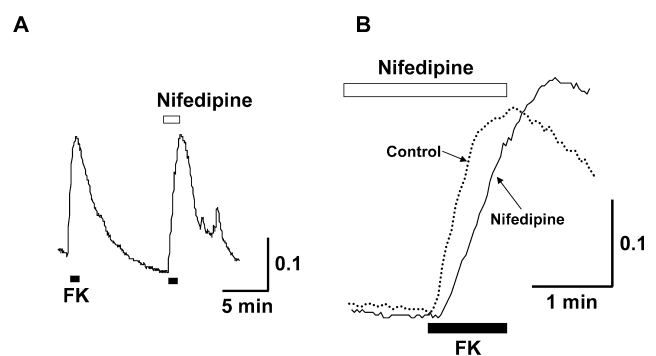
The effects of inhibitors of various Ca<sup>2+</sup> channel subtypes on the increase in [Ca<sup>2+</sup>]<sub>i</sub> caused by high KCl (40 mM) in the soma were examined (Fig. 5: A–D). High KCl was applied to the cells for 10 s at intervals of



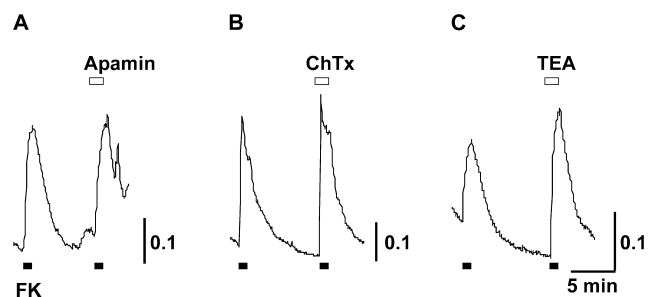
**Fig. 5.** Effects of  $\text{Ca}^{2+}$  channel inhibitors on high KCl-induced  $\text{Ca}^{2+}$  signals in the soma of rat ORNs. A–C: Cells were stimulated with 40 mM KCl (40K) at intervals of at least 5 min. The second application of high KCl was done in the absence (control) or the presence of 3  $\mu\text{M}$  nifedipine (A), 1  $\mu\text{M}$   $\omega$ -conotoxin GVIA (CnTx, B), or 0.1  $\mu\text{M}$   $\omega$ -agatoxin IVA (AgTx, C). Vertical scale bar: ratio (F340/F380). D: Columns show the mean  $\pm$  S.E.M. ( $n=6$ ) of the  $\Delta$ ratio (F340/F380) as a percentage of that caused by the first application of high KCl in each cell. \* $P<0.05$ , \*\* $P<0.01$  vs control (Dunnett's test).

5 min or more. The application of high KCl caused a reproducible increase in  $[\text{Ca}^{2+}]_i$  (first:  $0.42 \pm 0.03$ ,  $n=6$ ; second:  $0.43 \pm 0.02$ ,  $n=6$ ). Nifedipine (3  $\mu\text{M}$ ) significantly reduced the high KCl-induced  $[\text{Ca}^{2+}]_i$  increase by about 60%. An N-type  $\text{Ca}^{2+}$  channel inhibitor,  $\omega$ -conotoxin GVIA (1  $\mu\text{M}$ ), also significantly decreased it by about 20%. A P/Q type  $\text{Ca}^{2+}$  channel inhibitor,  $\omega$ -agatoxin IVA (0.1  $\mu\text{M}$ ), did not show significant inhibition. These results suggest that L-type  $\text{Ca}^{2+}$  channels mainly and N-type  $\text{Ca}^{2+}$  channels partially contribute to the  $\text{Ca}^{2+}$  signals in response to high KCl-induced depolarization in rat ORNs.

Nifedipine (3  $\mu\text{M}$ ) was applied before and during the first ( $n=3$ ) or second ( $n=3$ ) application of forskolin to examine the effect of nifedipine on the forskolin-induced  $\text{Ca}^{2+}$  signals (Fig. 6). There was no significant



**Fig. 6.** Effects of nifedipine on forskolin-induced  $\text{Ca}^{2+}$  signals in the soma of rat ORNs. A: Cells were stimulated with forskolin (10  $\mu\text{M}$ , FK) in the presence or the absence of nifedipine (3  $\mu\text{M}$ ). B: Time course of the increase in  $[\text{Ca}^{2+}]_i$  caused by forskolin in the presence or the absence (control) of nifedipine. Vertical scale bar: ratio (F340/F380).

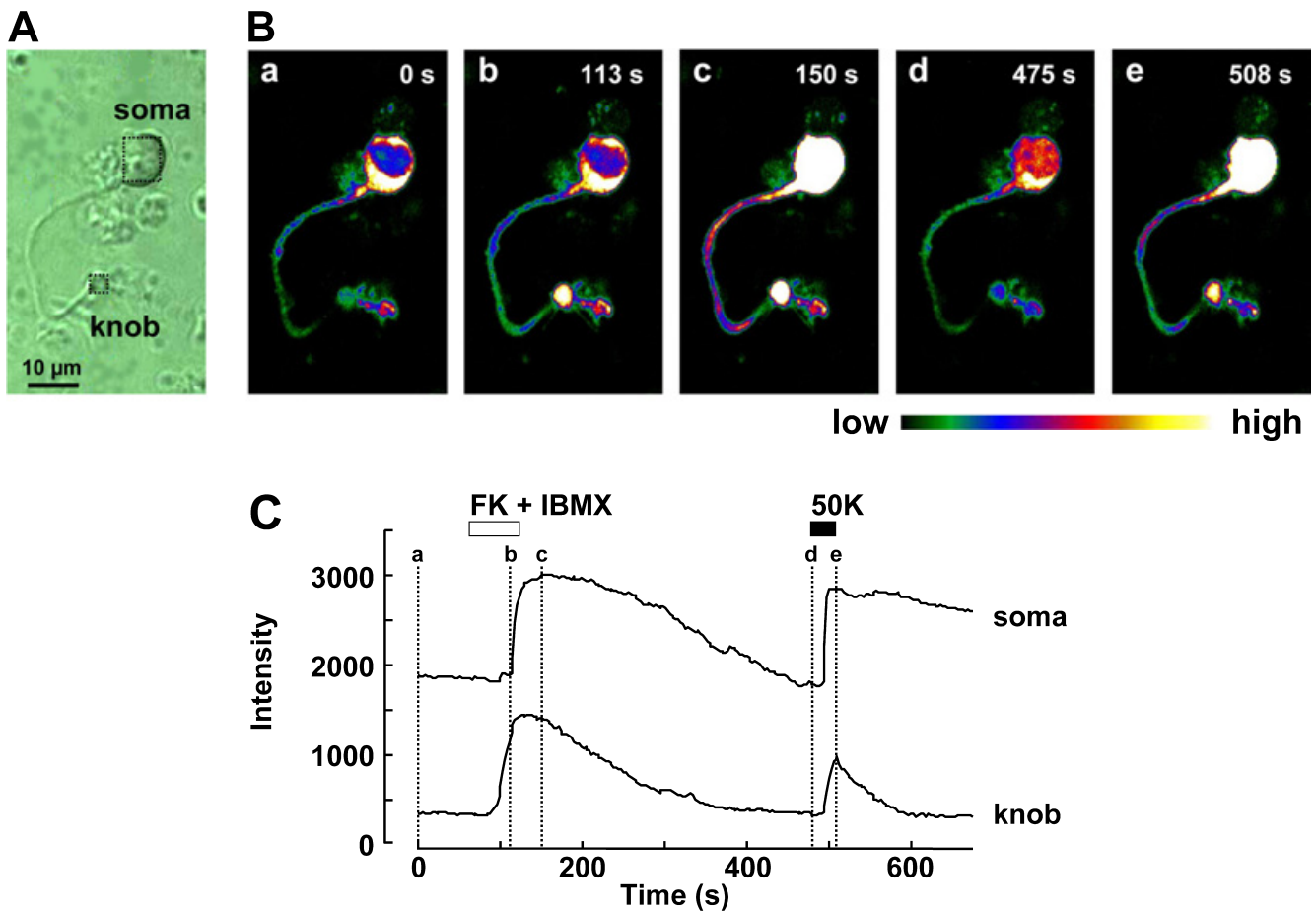


**Fig. 7.** Effects of  $\text{K}^{+}$  channel inhibitors on forskolin-induced  $\text{Ca}^{2+}$  signals in the soma of rat ORNs. A–C: Cells were stimulated with forskolin (10  $\mu\text{M}$ , FK) at intervals of at least 5 min. The second application of forskolin was done in the absence or the presence of 1  $\mu\text{M}$  apamin (A), 0.1  $\mu\text{M}$  charybdotoxin (ChTx, B), or 10 mM TEA (C). Vertical scale bar: ratio (F340/F380).

difference in the forskolin-induced  $[\text{Ca}^{2+}]_i$  increase in the presence ( $0.11 \pm 0.02$ ,  $n=6$ ) and absence ( $0.13 \pm 0.02$ ,  $n=6$ ) of nifedipine. Similarly to the effect of niflumic acid, nifedipine also significantly decreased the rate of  $[\text{Ca}^{2+}]_i$  increase induced by forskolin (control:  $T_{1/2\text{max}} = 15.7 \pm 3.1$  s vs nifedipine:  $T_{1/2\text{max}} = 22.5 \pm 1.9$  s,  $n=6$ ,  $P<0.05$ ). In the presence of niflumic acid (300  $\mu\text{M}$ ), however, nifedipine did not cause any significant inhibition of the forskolin-induced  $\text{Ca}^{2+}$  signals (niflumic acid:  $\Delta$ ratio =  $0.05 \pm 0.01$ ,  $T_{1/2\text{max}} = 19.2 \pm 1.6$  s vs niflumic acid plus nifedipine:  $\Delta$ ratio =  $0.05 \pm 0.01$ ,  $T_{1/2\text{max}} = 20.7 \pm 2.0$ ,  $n=4$ ).

#### Effects of $\text{K}^{+}$ channel inhibitors

Next, we examined the effects of  $\text{K}^{+}$  channel inhibitors on the forskolin-induced  $\text{Ca}^{2+}$  signals of the soma (Fig. 7). Two different types of  $\text{Ca}^{2+}$ -activated  $\text{K}^{+}$



**Fig. 8.** Spatiotemporal dynamics of forskolin-induced Ca<sup>2+</sup> signal in an isolated rat ORN. **A:** Translucent image of an isolated ORN in which the regions selected for temporal analysis are shown by rectangles. **B:** Pseudocolor images of the same cell at the time points (a–e) indicated in panel C. **C:** The temporal changes of [Ca<sup>2+</sup>]<sub>i</sub> in both the soma and knob regions as depicted in panel A. Forskolin (10 µM, FK) plus IBMX (100 µM), and 50 mM KCl (50K) were applied to the cell for 60 and 30 s, respectively. Images were recorded at 2.5-s intervals.

channel inhibitors, apamin (1 µM) and charybdotoxin (0.1 µM), did not have any significant effect on the forskolin-induced [Ca<sup>2+</sup>]<sub>i</sub> increase (control:  $0.12 \pm 0.05$ , apamin:  $0.12 \pm 0.04$ ,  $n = 4$ , and control:  $0.21 \pm 0.04$ , charybdotoxin:  $0.23 \pm 0.04$ ,  $n = 10$ ). On the other hand, TEA (10 mM) significantly enhanced the forskolin-induced Ca<sup>2+</sup> signal (control:  $0.14 \pm 0.04$  vs TEA:  $0.25 \pm 0.05$ ,  $n = 6$ ,  $P < 0.05$ ).

#### Ca<sup>2+</sup> imaging in knob and soma

Both the knob and soma showed an increase in [Ca<sup>2+</sup>]<sub>i</sub> when an ORN was stimulated with either forskolin (10 µM) or a mixture of forskolin and IBMX (100 µM). The onset of response in the knob and soma was not always simultaneous. In 8 out of 20 cells responding to forskolin and 4 out of 14 cells responding to the mixture of forskolin and IBMX, the onset of the response in the knob region occurred earlier than that in the soma, as

shown in Fig. 8. The onset of the response in the soma never preceded that in the knob. The onset of the response to 50 mM KCl was simultaneous in the knob and the soma of all cells.

#### Discussion

The present experiments demonstrated that forskolin, which increases the intracellular cAMP concentration by activating adenylyl cyclase, evoked an increase in [Ca<sup>2+</sup>]<sub>i</sub> of rat ORNs. The response to forskolin was enhanced by some PDE inhibitors. PDE inhibitors evoked increases in [Ca<sup>2+</sup>]<sub>i</sub> in some cells that had been stimulated by forskolin even when [Ca<sup>2+</sup>]<sub>i</sub> had already returned to the basal level (data not shown). This is probably due to a slight and continuous activation of adenylyl cyclase in the cell. Although three types of PDE (i.e., PDE1, 2, and 4) were reported to exist in rodent ORNs (19, 27), in our

studies, the PDE1 inhibitor 8-MM-IBMX and PDE2 inhibitor EHNA enhanced the forskolin-induced  $\text{Ca}^{2+}$  signals, but the PDE4 inhibitor rolipram did not. These results suggest that PDE1 and 2 contribute to the breakdown of cAMP produced by forskolin in rat ORNs. A large number of ORNs express both PDE1 and 4. PDE1 is localized exclusively to the cilia, but PDE4 is not found in the cilia (19). Forskolin may activate adenylyl cyclase in the cilia. On the other hand, it has been reported that PDE1 is a  $\text{Ca}^{2+}$ -calmodulin-dependent PDE but PDE4 is  $\text{Ca}^{2+}$ -independent (18). This may be the reason why 8-MM-IBMX, but not rolipram, potentiates the  $\text{Ca}^{2+}$  signals induced by forskolin. It has been also reported that cGMP-stimulated PDE2 and guanylyl cyclase are expressed in a subset of the rat ORNs (19). If so, the ORNs used in our experiments might be PDE2-positive cells, although they would be a small fraction of the total number of ORNs. As EHNA inhibits adenosine deaminase (28), further studies are needed to investigate the effect of EHNA in olfactory signal transduction.

In amphibians, IBMX by itself is reported to cause an increase of  $[\text{Ca}^{2+}]_i$  (8–11). However, in our study, IBMX did not cause any detectable change in  $[\text{Ca}^{2+}]_i$ . Similar results have been reported in rat and human ORNs (12). It has been reported that mammalian ORNs express adenylyl cyclase type 3, which has a much lower level of basal activity than adenylyl cyclase type 1 (29). It seems likely that the adenylyl cyclase of amphibian ORNs has already been activated in part under resting conditions.

Cyclic AMP opens cyclic nucleotide-gated channels in the cilia, producing depolarization. The  $\text{Ca}^{2+}$  passing through these channels in turn activates  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  channels, which amplifies depolarization in ORNs (21), because  $\text{Cl}^-$  equilibrium potential is about  $-15$  mV (30) and thus the excitatory  $\text{Cl}^-$  current occurs in isolated rat ORNs (20). In our studies, niflumic acid, a  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  channel inhibitor, suppressed the  $\text{Ca}^{2+}$  signals caused by forskolin, suggesting that the depolarization enhanced by the  $\text{Cl}^-$  channels plays a crucial role in the  $\text{Ca}^{2+}$  signals. In *Xenopus* ORNs, voltage-dependent  $\text{Ca}^{2+}$  channels have been shown to be primarily present on the soma and the proximal dendrite (31). It has been reported that a high concentration of nifedipine ( $20 \mu\text{M}$ ) inhibited the  $\text{Ca}^{2+}$  signals in response to odorants (22). However, several  $\text{Ca}^{2+}$  channel inhibitors including nifedipine at high concentrations are reported to inhibit nucleotide-gated channels as well (23, 24). In order to avoid the non-specific effects of the  $\text{Ca}^{2+}$  channel inhibitors, we examined the effect of  $\text{Ca}^{2+}$  channel inhibitors on the  $\text{Ca}^{2+}$  signals in response to high KCl. High KCl-induced  $\text{Ca}^{2+}$  signals were suppressed by not only nifedipine but also  $\omega$ -conotoxin GIVA. Based

on the pharmacological analysis, it is suggested that high KCl increased  $[\text{Ca}^{2+}]_i$  through L-type  $\text{Ca}^{2+}$  channels by 60% and N-type  $\text{Ca}^{2+}$  channels by 20% in rat ORNs. T-type  $\text{Ca}^{2+}$  channels may be involved in the remaining response to high KCl because these channels are reported to exist in amphibian ORNs (32, 33). Nifedipine decreased the rate of the increase in  $[\text{Ca}^{2+}]_i$  caused by forskolin. However, nifedipine did not cause a further inhibition of the forskolin-induced  $\text{Ca}^{2+}$  signals in the presence of niflumic acid. These results suggest that the depolarization induced by the activation of  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  channels is needed to activate the voltage-dependent L-type  $\text{Ca}^{2+}$  channels, which mainly contribute to the initial phase of the  $\text{Ca}^{2+}$  signals in response to forskolin. The slight increase in  $[\text{Ca}^{2+}]_i$  after removal of niflumic acid may be due to relief from suppression of the depolarization. The peak amplitude of the  $\text{Ca}^{2+}$  signal was significantly decreased by niflumic acid but not by nifedipine. The depolarization amplified by  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  channels might activate  $\text{Ca}^{2+}$  channel subtypes other than the L-type.

Several types of  $\text{K}^+$  channels have been reported in ORNs (3). In newts,  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels have been suggested to regulate the excitability of amphibian ORNs (34). In cultured rat ORNs, however, TEA-sensitive large delayed-rectifier  $\text{K}^+$  currents but not  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  currents were observed with the whole-cell voltage-clamp technique (25). In the present experiments, the forskolin-induced  $\text{Ca}^{2+}$  signals were not significantly affected by  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channel inhibitors, apamin and charybdotoxin. On the other hand, TEA enhanced the response to forskolin, suggesting that the delayed-rectifier  $\text{K}^+$  channels regulate the excitability of rat ORNs.

The present study demonstrated that the  $\text{Ca}^{2+}$  signals induced by forskolin depended on the extracellular  $\text{Ca}^{2+}$ . In salamander ORNs, it has been reported that thapsigargin- and caffeine-sensitive intracellular  $\text{Ca}^{2+}$  stores are present in the soma, dendrite, and knob, but not in the cilia, and that these  $\text{Ca}^{2+}$  stores serve to amplify the  $\text{Ca}^{2+}$  signals through  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release. (11). Although thapsigargin itself caused an increase in  $[\text{Ca}^{2+}]_i$  in the present study, the forskolin-induced  $\text{Ca}^{2+}$  signals were not affected by treatment with thapsigargin or caffeine plus ryanodine. It is suggested that  $\text{Ca}^{2+}$  stores do not play a role in the forskolin-induced  $\text{Ca}^{2+}$  signals of rat ORNs.

Nifedipine and niflumic acid failed to completely block the forskolin-induced  $[\text{Ca}^{2+}]_i$  increase. It is unlikely that forskolin directly causes an increase in  $[\text{Ca}^{2+}]_i$  in the soma independently of that in the cilia and knob because the onset of the response to forskolin at the knob occurred simultaneously or earlier, but not later, than

that in the soma. The increase of [Ca<sup>2+</sup>]<sub>i</sub> in the knob is a prerequisite for the increase of [Ca<sup>2+</sup>]<sub>i</sub> in the soma. Further experiments are needed to define the remaining Ca<sup>2+</sup> influx pathways and examine the propagation of Ca<sup>2+</sup> from the cilia to the soma with high-time resolution.

The elevation of [Ca<sup>2+</sup>]<sub>i</sub> may mediate various cellular functions in ORNs. Odor adaptation is thought to be one of the most important roles of Ca<sup>2+</sup>. There are a number of reports about the desensitization mediated by Ca<sup>2+</sup> in olfactory transduction (35). It has been reported that the [Ca<sup>2+</sup>]<sub>i</sub> increase induced by cAMP activates CaM kinase II, which in turn inhibits adenylyl cyclase 3 (36). A knowledge of the role of Ca<sup>2+</sup> signals would help us to understand the complex mechanism of olfactory transduction.

### Acknowledgments

This work was supported by grants from the Akiyama Foundation and MAFF Research Program "Behavioral Mechanisms in Domestic Animals and Insects".

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