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Calcium-Dependent Phosphorylation of a Protein in the Frog Olfactory Cilia[1]

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In the cilia of vertebrate olfactory sensory neurons, cytoplasmic Ca^{2+} concentration increases in response to odorant stimulation, and this increase has been implicated to have important roles in the regulation of olfactory responses. Since protein phosphorylation is often a regulatory mechanism of biological reactions, we explored the effect of Ca^{2+} on phosphorylation reactions in the frog olfactory cilia. First, we found that a 45-kDa phosphoprotein (p45) is predominantly phosphorylated *in vitro* in the isolated cilia in a Ca^{2+}-dependent manner. However, later studies showed that the phosphorylation level of p45 is controlled by a dynamic equilibrium between phosphorylation and dephosphorylation. Although both activities are enhanced at high Ca^{2+} concentrations ($K_{1/2}$ = ~2 μM in both reactions), the enhancement of dephosphorylation is relatively greater than that of phosphorylation. As a result, the steady phosphorylation level of p45 is lower at high than at low Ca^{2+} concentration. The phosphorylation/dephosphorylation equilibrium was founed to involve protein kinases sensitive to zinc and heparin, and an unknown phosphatase(s). The present result suggests the presence of a novel Ca^{2+}-signaling pathway that involves phosphorylation of p45 in the olfactory cilia.

Key words: Ca^{2+}-signaling, dephosphorylation, dynamic equilibrium, olfactory cilia, phosphorylation.

In vertebrates, olfactory transduction takes place at the cilia of olfactory receptor cells. Odorant binding to a specific receptor in the cilia membrane leads to activation of a G-protein (Golf) that in turn activates adenylate cyclase type III (AC3) to increase the cAMP concentration. The elevation of cAMP concentration induces the opening of a cyclic nucleotide-gated cation channel (CNG channel) to depolarize the cell (*1, 2*).

Since Ca^{2+} as well as Na^+ carries the inward current (*2–4*), the cytoplasmic Ca^{2+} concentration increases on odorant stimulation (*5*). This increase in the Ca^{2+} concentration has been shown to have important roles in the modulation of olfactory responses (*6, 7*). For example, Ca^{2+} elevation in the cilia has been proposed to desensitize the olfactory cell through reduction of the CNG channel sensitivity to cAMP (*8, 9*) and rapid hydrolysis of cAMP by activation of phosphodiesterase (*10*). These effects are thought to be mediated by direct action of a Ca^{2+}/calmodulin (CaM) complex on the CNG channel or the phosphodiesterase.

Protein phosphorylation has also been suggested to be involved in the modulation of the olfactory response (*11–16*). Phosphorylation by cAMP-dependent protein kinase (PKA) (*11*), protein kinase C (PKC) (*11*) or G protein-coupled receptor kinase 3 (GRK3) (*12–14*) has been implicated to be involved in a rapid termination of the second-messenger signaling. Since Ca^{2+} often regulates kinase reactions, it is possible that Ca^{2+} also regulates the kinases in the olfactory cell. In fact, some of the kinases have been suggested to be regulated by Ca^{2+} (*15, 16*). However, since these studies were done to see the effect Ca^{2+} on a specific kinase, it was not possible to examine the effect of Ca^{2+} on phosphorylation reactions in general in the olfactory cilia.

In the present study, we searched for Ca^{2+}-dependent phosphorylations in the isolated olfactory cilia and found a 45-kDa major phosphoprotein (p45) whose phosphorylation level is affected by Ca^{2+} concentration. This protein is not only phosphorylated but also dephosphorylated in a Ca^{2+}-dependent manner. It was suggested that p45 is involved in a novel Ca^{2+}-signaling pathway in the olfactory cilia upon odorant stimulation.

EXPERIMENTAL PROCEDURES

Preparation of the Frog Olfactory Cilia—Partially purified cilia were prepared from bullfrog (*Rana catesbeiana*) olfactory epithelium with the calcium-shock method described by Chen *et al.* (*17*). Briefly, the olfactory epithelia were dissected from frog nasal cavity and rinsed in an ice-chilled Tris-buffered saline (TBS; 0.9% NaCl, 100 mM Tris-

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Abbreviations: AC3, adenylate cyclase type III; βARK, β-adrenergic receptor kinase; CaM, calmodulin; CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; CBB, Coomassie Brilliant Blue; CKII, casein kinase II; CNG channel, cyclic nucleotide-gated cation channel; GRK, G protein–coupled receptor kinase; MAPK, mitogen-activated protein kinase; PDE, phosphodiesterase; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; PP, protein phosphatase; TBS, Tris-buffered saline; TCA, trichloroacetic acid.

HCl, pH 7.5). The epithelia were precipitated by centrifugation at 1,500 ×*g* for 5 min and resuspended in TBS. The cilia were detached from the epithelia by raising the Ca^{2+} concentration to 10 mM. After agitation for 20 min on ice, the deciliated epithelia were removed by centrifugation at 1,500 ×*g* for 5 min. The supernatant was centrifuged at 12,000 ×*g* for 15 min, and the resultant pellet containing the cilia was washed twice with a cilia stock buffer (10 mM Tris-HCl, 3 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.2 mM EGTA, pH 8.0). The washed pellet was resuspended in a small volume of the cilia stock buffer (cilia stock solution) and stored at 4°C until use. Protein concentration of the cilia stock solution was determined by BCA Protein Assay Kit (Pierce) and adjusted to 10 μg/μl. All solutions used above contained protease inhibitors: phenylmethylsulfonylfluoride (100 μg/ml), leupeptin (10 μg/ml), pepstatin (5 μg/ml), and benzamidine (10 μg/ml).

Phosphorylation Measurements—A small volume of the cilia stock solution (3 μl) was mixed with 47 μl of a MOPS buffer (50 mM MOPS, 2.5 mM $MgCl_2$, 0.05% sodium cholate, 0.1 mM $CaCl_2$, 0.2 mM EGTA, pH 7.5) so that each test-tube contained 30 μg of proteins. This mixture was then pre-incubated in a block-bath incubator at 20°C for 15 min and then used as a cilia suspension. The phosphorylation reaction was started by adding 50 μl of a pre-warmed (20°C) ATP solution containing 2 mM [γ-^{32}P]ATP (0.78 MBq/μmole) and 0.1 mM GTP to 50 μl of the cilia suspension. In some of the measurements, we examined the effects of odorants on the phosphorylation. For direct comparison of these measurements with other measurements, we always added 0.1 mM GTP in the present study. However, in a separate experiment, GTP did not affect the phosphorylation, which indicated that the phosphorylation does not depend on GTP.

In all experiments, vanadate (100 μM at final) was added to the above reaction mixture to inhibit the intrinsic ATPase activity (*18*). In the absence of vanadate, the concentration of ATP (see below) decreased significantly during 10 min of incubation, but not in its presence. Vanadate did not significantly affect the phosphorylation or dephosphorylation reaction. The Ca^{2+} concentration of the reaction mixture was controlled by a Ca^{2+}/EGTA buffering system. The free Ca^{2+} concentration was calibrated with fluo-3 (Dojin, Kumamoto) using Ca^{2+}-standard buffers (Calbuf-2, World Precision Instruments). After incubation for an indicated period at 20°C, the reaction was quenched by thorough mixing with 200 μl of ice-chilled 10% (v/v) trichloroacetic acid (TCA). The sample was then centrifuged at 15,000 ×*g* for 15 min. The precipitate was washed once with ice-chilled TBS and dissolved in the SDS sample buffer. The sample was subjected to SDS-PAGE using a 9% separation gel. The gel was stained with CBB and dried. The radioactivity of ^{32}P incorporated into cilia proteins was visualized by autoradiography and quantitated by use of an Imaging Analyzer BAS2000 (Fuji Film).

Dephosphorylation Measurements—After a phosphorylation reaction had continued for 10 min, one portion of the reaction mixture was mixed with an equal volume of a cold (*i.e.*, non-radioactive) ATP solution (20°C). This solution had basically the same composition as the ATP solution described above except that it contained only non-radioactive ATP. The mixed solution was further incubated for an indicated period of time at 20°C for the chase of ^{32}P incorporated into the protein. At the same time, as a control, another portion of the reaction mixture was incubated in parallel without addition of the cold ATP solution. The both reactions were terminated by addition of ice-chilled 10% (v/v) TCA. The radioactivity remaining in the cilia proteins was analyzed as described above. The phosphorylation level after the chase was expressed as the relative value to that in the control experiment without chase.

Inhibition of the Kinase and the Phosphatase Activities—In our attempt to identify the types of kinases and phosphatases involved, the effects of various kinase and phosphatase inhibitors were examined. Kinase inhibitors were pre-incubated in the cilia suspension at 20°C for 15 min before addition of [γ-^{32}P]ATP. The phosphorylation reaction was terminated at 5 sec after the addition of [γ-^{32}P]ATP. Phosphatase inhibitors were added to the cold ATP solution, and the reaction was terminated at 150 sec after the start of the chase. Walsh inhibitor was purchased from Sigma; Cyclosporin A from Biomol Research Laboratories; heparin sodium salt and zinc chloride from Nacalai; bisindolylmaleimide I, hypericin, Ca^{2+}/CaM kinase II inhibitor (281–309) and okadaic acid from Calbiochem.

RESULTS

Ca^{2+}-Dependent Protein Phosphorylation in the Olfactory Cilia—When isolated olfactory cilia were incubated with [γ-^{32}P]ATP for a short period of time (5 s), ^{32}P was predominantly incorporated into a single protein band of 45 kDa (p45) (Fig. 1A, arrowhead in the right panel). The corresponding protein band was not visible in the CBB-staining (Fig. 1A, left panel). Incorporation of ^{32}P was higher at a high Ca^{2+} concentration (H in Fig. 1A, 20 μM) than at a low Ca^{2+} concentration (L, 10 nM). Incorporation of ^{32}P during the initial 5 s was taken as a measure of the kinase activity, and its activity was measured at various Ca^{2+} concentrations. The result (Fig. 1B) demonstrated that the kinase activity on p45 increased by ~3-fold with a $K_{1/2}$ = ~2 μM as the Ca^{2+} concentration increased, suggesting that the phosphorylation of p45 is regulated by Ca^{2+} concentration. We examined the effects of odorants, but did not see any significant effects on the phosphorylation of p45 (not shown). It should be noted that we used cilia membranes. Our result, therefore, suggested that the phosphorylation on p45 increases through the rise in the Ca^{2+} concentration that is induced by odorant stimulation but not through direct interaction with the odorant receptor molecules.

Time Course of Incorporation of ^{32}P into p45—When ^{32}P incorporation was measured for up to 20 min, the major phosphorylated product was still p45 and no other prominent ^{32}P incorporations were observed. Incorporation of ^{32}P into p45, however, was somewhat unexpected: the time course was different at different Ca^{2+} concentrations (Fig. 2).

Up to 20 s, ^{32}P incorporation into p45 (Fig. 2A) was higher at a high (filled circles in Fig. 2B) than at a low Ca^{2+} concentration (open circles), which was consistent with the result of the activity measurement in Fig. 1. However, at around this time point, the ^{32}P incorporation curves crossed over (Fig. 2B, arrow), and the relation of the phosphorylation level reversed: it was higher at low than high Ca^{2+} concentration (Fig. 2C). Both curves reached their maximums at about 2.5 min of incubation and remained at those levels

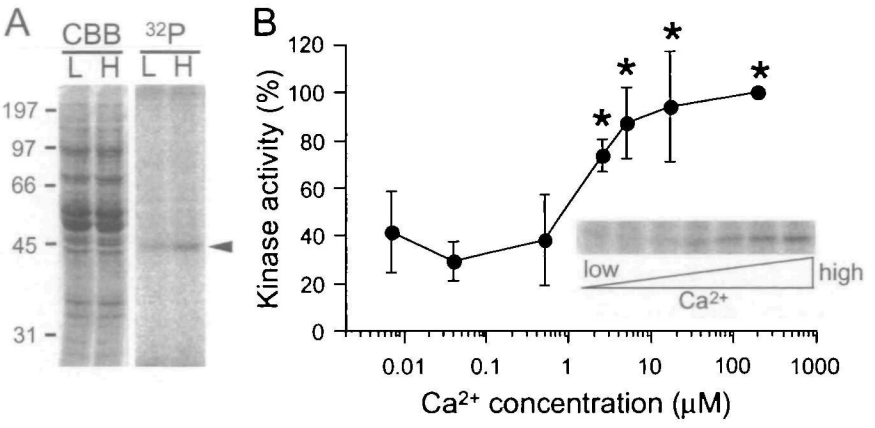

Fig. 1. **Calcium-dependent protein phosphorylation in the isolated frog olfactory cilia.** (A) CBB-staining of the frog cilia proteins (CBB) and autoradiograph of ^{32}P incorporated into them (^{32}P) during incubation for 5 s at a low (L, 10 nM) and a high (H, 20 μM) concentration of Ca^{2+}. A single band of 45 kDa was predominantly labeled (arrowhead). Numbers at the left indicate molecular masses of marker proteins (kDa). (B) Calcium-dependency of the kinase activity on p45. Incorporation of ^{32}P into p45 band in the initial 5 s at each concentration of Ca^{2+} was quantified and plotted. The data are shown as the percentages of the maximum activity at the highest concentration of Ca^{2+} (200 μM). Data are the means of independent experiments ± SD (n = 5). Asterisks indicate that these data are significantly different from the value at the lowest concentration of Ca^{2+} ($p < 0.05$, t-test). Inset represents an original autoradiograph of p45 bands. Each lane corresponds to the Ca^{2+} concentration shown in the graph.

throughout the measurement (Fig. 2C). These steady states seemed to represent an equilibrium rather than exhaustion of [γ-^{32}P]ATP, because most of the ^{32}P-ATP remained in the reaction mixture at 10 min after addition of ATP (not shown).

One mechanism that would account for the above result involves a dephosphorylation reaction in the labeling of p45 by ^{32}P and a change in the phosphorylation/dephosphorylation ratio with changing Ca^{2+} concentration. To confirm this idea, we examined directly whether the phosphate incorporated into p45 was removed in the cilia preparation.

Dephosphorylation of p45—To detect dephosphorylation reactions, we first labeled p45 in the cilia with ^{32}P, then added non-radioactive ATP at the same concentration to chase the ^{32}P incorporated into p45. This addition reduced the specific activity of [γ-^{32}P]ATP to a half of that at the beginning of the experiment. If phosphorylated p45 undergoes dephosphorylation and an equilibrium is established between phosphorylation and dephosphorylation, ^{32}P incorporated into p45 should be partially (~50% at the maximum) replaced with the non-radioactive one after the chase.

As shown in Fig. 3A, the level of ^{32}P incorporated into p45 declined after the chase at both a high (20 μM, filled circles) and a low (10 nM, open circles) Ca^{2+} concentration. The result indicated that the phosphate group incorporated into p45 was subjected to dephosphorylation. Furthermore, at the high Ca^{2+} concentration (filled circles), the dephosphorylation was faster (compare the initial rates of the decline of the ^{32}P level at the high and the low Ca^{2+} concentration), and reached ~50% of the original steady level at ~2 min after the beginning of the chase. At the low Ca^{2+} concentration (open circles), the dephosphorylation was slow.

By changing the Ca^{2+} concentration, we measured the initial rate of the decline of the ^{32}P level and calculated the phosphatase activity as a function of Ca^{2+} concentration. As shown in Fig. 3B, the phosphatase activity increased in a Ca^{2+}-dependent manner with an apparent $K_{1/2}$ = ~2 μM. All these results indicated that a dynamic equilibrium holds between phosphorylation and dephosphorylation of p45, and that Ca^{2+} promotes both reactions. Because the balance between phosphorylation and dephosphorylation determines the steady level of ^{32}P in p45, the higher level of the steady state at low Ca^{2+} concentration (Fig. 2C) indicates that the phosphorylation/dephosphorylation ratio is higher at low Ca^{2+} concentration.

Kinases Responsible for Phosphorylation of p45—Many kinds of serine/threonine kinases have been suggested to be expressed in the olfactory cilia (*11–14, 19–21*), but no phosphatases other than 2A-type (PP2A) (*22*) have been reported. To characterize the molecular mechanisms underlying the Ca^{2+}-dependent phosphorylation/dephosphorylation of p45, we tried to identify the types of the protein kinase and the phosphatase involved in the equilibrium.

First, we examined the effects of some of the known inhibitors on the kinase activity on p45 (Fig. 4). Kinases hitherto reported to stimulate phosphorylation of the olfactory cilia proteins are PKA (*11*), PKC (*11*), β-adrenergic receptor kinase 2 (βARK2/GRK3) (*13*), and CaMKII (*15*). Therefore, we examined the effects of the inhibitors of these kinases to see whether they contribute to the Ca^{2+}-dependent phosphorylation of p45.

A specific inhibitor of PKA (PKA-I in Fig. 4A), known as Walsh inhibitor, has been demonstrated to inhibit the odorant-induced PKA effect in the olfactory cilia at the concentration of 0.38 μM (*21*). However, in the present study, the kinase activity on p45 was not affected at the concentration of 1 μM (Fig. 4A). Similarly, a PKC inhibitor (PKC-I; 100 nM bisindolylmaleimide I, IC_{50} = 10 nM; Ref. *23*) and an inhibitory peptide of CaMKII (CaMKII-I; 1 μM, IC_{50} = 80 nM; Ref. *24*) had no effects on the reaction, although the Ca^{2+}-dependent increase in the kinase activity was always observed (Fig. 4A).

In contrast to the PKA, PKC, and CaMKII inhibitors, heparin, a known inhibitor of G protein-coupled receptor kinases (GRKs) (*25, 26*), remarkably blocked the kinase activity on p45 (Fig. 4A). We further examined the effect of zinc, which has an inhibitory effect on GRKs generally (*27, 28*). The result (Fig. 4A) showed that zinc blocked the kinase activity as effectively as heparin. Half-maximum inhibition was attained at ~400 nM heparin (Fig. 4B) and ~10 μM zinc (Fig. 4C). These values are within a range of the reported half-effective concentrations of the inhibitors for mammalian GRKs: for heparin, IC_{50} = 200 μM to GRK1 (*25*), 0.15–1.4 μM to GRK2, and 1–15 nM to GRK 5/6 (*26*);

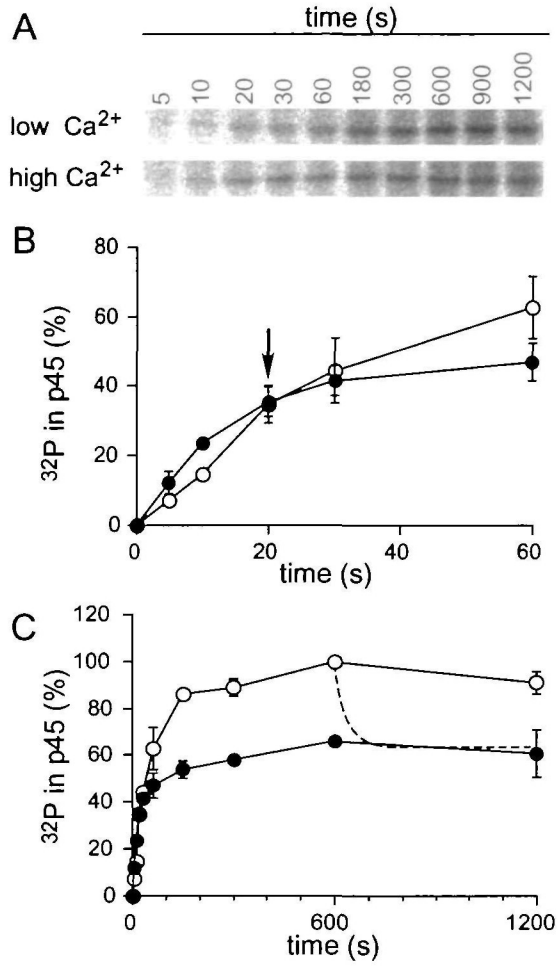

Fig. 2. **Time course of calcium-dependent phosphorylation of p45.** (A) Autoradiographs showing time courses of the incorporation of ^{32}P into p45 at a low (10 nM) and a high (20 μM) Ca^{2+} concentration. Incubation times are indicated at the top. (B) and (C) Incorporation of ^{32}P into p45 was plotted as a function of time. (B) is an expanded form of the first 60 s of (C). Open circles, measurements at a low Ca^{2+} concentration (10 nM); filled circles, at a high Ca^{2+} concentration (20 μM).The data are normalized as the percentages of the maximum incorporation at the low Ca^{2+} concentration (10 min). Data are the means of independent experiments ± SD (n = 3). Both curves crossed over at around 20 s (arrow). Dashed line in (C) represents the predicted time course of the phosphorylation level of p45 after a rapid increase of Ca^{2+} concentration from 10 nM to 20 μM (see text).

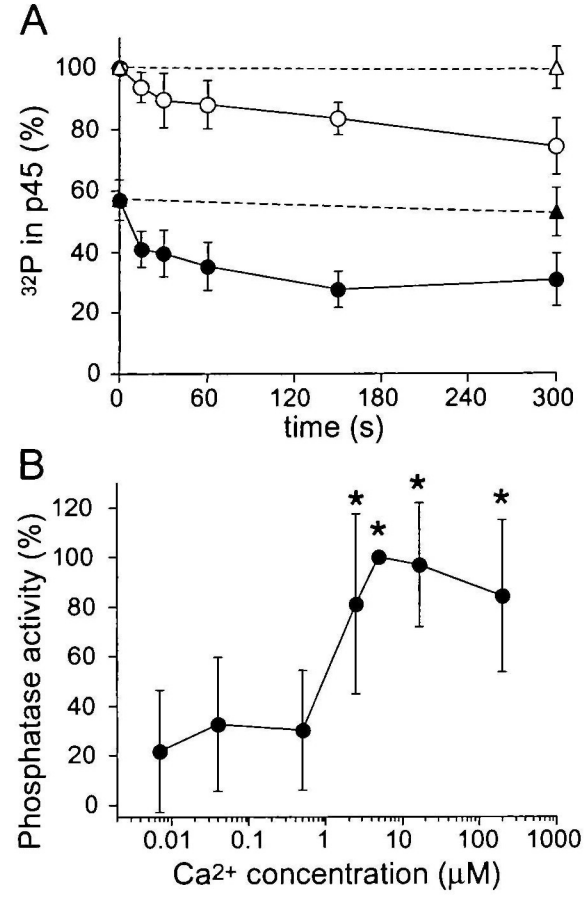

Fig. 3. **Calcium-dependent dephosphorylation of p45.** (A) Dephosphorylation time course. The change in the level of ^{32}P in p45 after the chase was plotted. Open circles, low Ca^{2+} (10 nM); filled circles, high Ca^{2+} (20 μM). The data are presented as relative values to the level at the low Ca^{2+} concentration before the chase. Dashed lines indicate the control ^{32}P levels without the chase. Data are the means of independent experiments ± SD (n = 4). (B) Calcium-dependency of the phosphatase activity on p45. The chase experiments were conducted at various concentrations of Ca^{2+}. The activity was measured as the reduction of ^{32}P in p45 after the chase for 30 s. Data are the means ± SD ($n \geq 5$). Asterisks indicate a significant difference from the value at the lowest concentration of Ca^{2+} ($p < 0.05$, t-test).

for zinc, IC_{50} = ~50 μM to GRK2 (*28*). Judging from the measured sensitivities to the inhibitors, the results suggested that the kinase responsible for the phosphorylation of p45 has GRK-like properties. If these inhibitors individually act on the distinct types of enzymes, the inhibitory effects should be additive. However, the inhibitory effects of heparin and zinc were not additive at their saturating concentrations (not shown), which suggests that the kinase is probably a single species.

Heparin is also known to inhibit casein kinase II (CKII) (*29*). However, hypericin, a strong inhibitor of both CKII (IC_{50} = 6 nM) and mitogen-activated protein kinases (MAPK; IC_{50} = 4 nM) (*30*), did not affect the phosphorylation (Fig. 4A). The result, therefore, ruled out the involvement of these two kinases in the phosphorylation of p45.

Phosphatases Responsible for Dephosphorylation of p45—To identify the phosphatase(s) responsible for dephosphorylation of p45, we examined the effects of some of the known phosphatase inhibitors on the dephosphorylation of p45. For this purpose, as in the experiment shown in Fig. 3, we did the chase experiment in the presence and absence of the phosphatase inhibitors. Fig. 5 shows the ^{32}P levels remaining in p45 after the chase.

When cold ATP was not added (Fig. 5, no chase), the steady phosphorylation level was higher at the low Ca^{2+} concentration. After the chase, the ^{32}P level decreased to ~50% of the original level at the high Ca^{2+} concentration (control), but the decrease was small at the low Ca^{2+} concentration. These results are consistent with those shown in Figs. 2 and 3.

Okadaic acid (OA in Fig. 5) is a powerful inhibitor of protein phosphatase-1 (PP1) and 2A (PP2A), but a weak inhib-

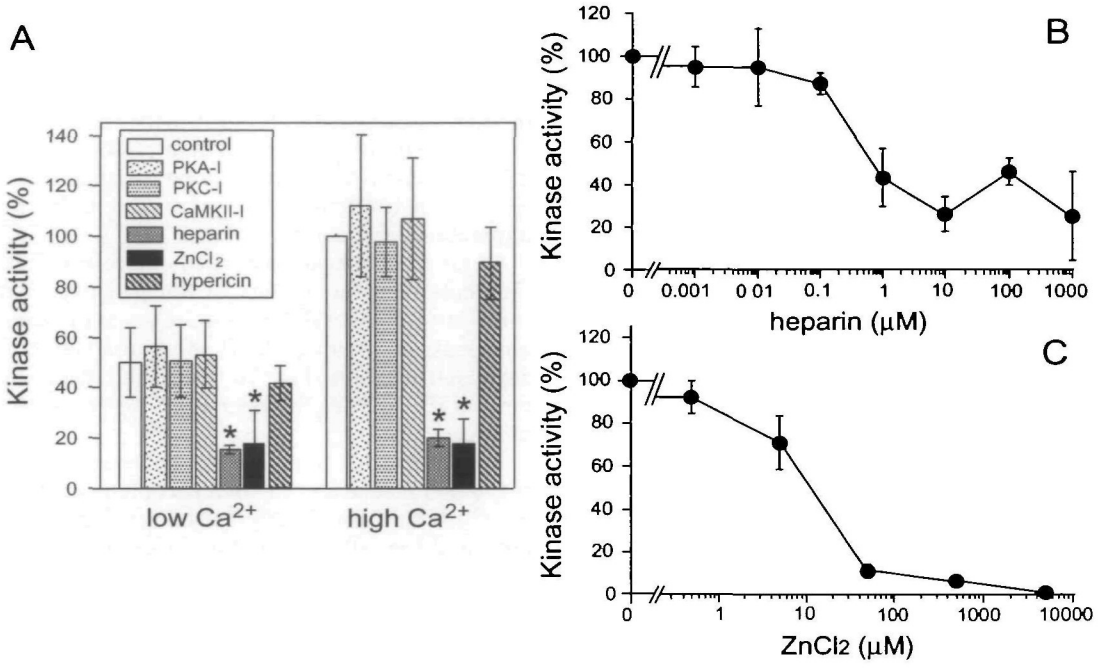

Fig. 4. **Effects of inhibitors on the kinase activity.** (A) The kinase activity was measured as indicated in the text in the absence (control) or presence of the kinase inhibitors. The inhibitors used were 1 μM Walsh inhibitor (PKA-I), 100 nM bisindolylmaleimide I (PKC-I), 1 μM CaMKII inhibitor (CaMKII-I), 1 mM heparin, 50 μM $ZnCl_2$ and 50 nM hypericin. The activity is presented as the relative value to the level in the high Ca^{2+} control. Data are the means ± SD ($n \geq 5$). Asterisks indicate a significant difference from the value of the control ($p < 0.05$, *t*-test). Low Ca^{2+}, 10 nM; high Ca^{2+}, 20 μM. (B and C) Dose-dependent effects of heparin (B) and zinc (C) on the kinase activity at a high concentration of Ca^{2+} (20 μM). The activity is presented as the relative value to the level in the high Ca^{2+} control in both (B) and (C). Data are the means ± SD (B, $n = 7$; C, $n = 5$).

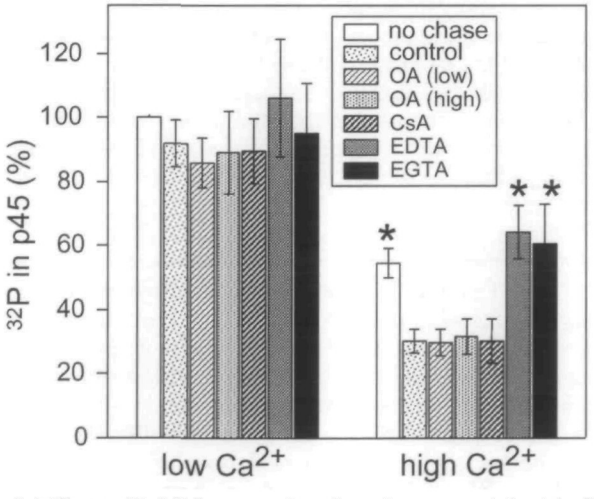

Fig. 5. **Effects of inhibitors on the phosphatase activity.** The ^{32}P level in p45 was monitored by the chase method (see text) in the absence (control) or presence of phosphatase inhibitors. Low Ca^{2+}, 10 nM; high Ca^{2+}, 20 μM. The inhibitors used were 100 nM [OA (low)] or 10 mM [OA (high)] okadaic acid, 1 μM cycrosporinA (CsA), 10 mM EDTA, and 1 mM EGTA. Each bar represents the level of ^{32}P remaining in p45 after the chase for 150 s. The data are presented as the percentages of the level without the chase (no chase) at the low Ca^{2+} concentration. The inhibitors did not affect the ^{32}P levels at the low Ca^{2+} concentration. Data are the means ± SD ($n \geq 5$). Asterisks indicate a significant difference from the value of the control ($p < 0.05$, *t*-test).

itor of the 2B-type (PP2B). PP1 and PP2A show a high sensitivity to okadaic acid with IC_{50} of 0.1–15 nM, but PP2B a relatively lower sensitivity with IC_{50} of 5 μM (*31*). These subtypes of phosphatases, therefore, can be distinguished by their sensitivities to okadaic acid. However, the ^{32}P level was unaffected by either a low [100 nM, OA (low) in Fig. 5] or a high [10 μM, OA (high)] concentration of okadaic acid, indicating that the dephosphorylation reaction is not affected by okadaic acid. Cyclosporin A (CsA in Fig. 5), another strong inhibitor for PP2B (*32*), also had no effects on the dephosphorylation. The results indicate that neither PP1, PP2A, nor PP2B is involved in the dephosphorylation of p45.

Another type of phosphatase, phosphatase 2C (PP2C), is known to be okadaic acid-insensitive but Mg^{2+}-sensitive (*31*). Unfortunately no specific inhibitors of PP2C are known. To evaluate the contribution of PP2C, therefore, we examined the effects of EDTA and EGTA. EDTA completely blocked the decrease of ^{32}P in p45 after the chase at the high Ca^{2+} concentration, and EGTA also blocked the dephosphorylation completely (Fig. 5). The concentration of EDTA used (10 mM) was enough to chelate both Mg^{2+} and Ca^{2+}, while the concentration of EGTA used (1.0 mM) was not enough to chelate Mg^{2+} (2.5 mM). Therefore, free Mg^{2+} remained in the presence of 1 mM EGTA, but the phosphatase activity was completely blocked. We concluded therefore that Ca^{2+} is crucial for expression of the phosphatase activity, and that Mg^{2+}-sensitive proteins, like PP2C, are not involved in the dephosphorylation of p45.

DISCUSSION

Protein phosphorylations in the olfactory cilia have been studied in many laboratories (*11–17*), but the effects of Ca^{2+} on these reactions have not been examined in detail, despite the fact that the intraciliary Ca^{2+} concentration rises on odorant stimulation. To date, only membrane-bound adenylate cyclase type III (AC3) has been reported to undergo Ca^{2+}-dependent phosphorylation in the cilia (*15*). In the present study, for the first time, we identified a novel phosphoprotein, p45, whose phosphorylation level is regulated in a Ca^{2+}-dependent manner by a dynamic equilibrium between phosphorylation and dephosphorylation (Figs. 1–3). The equilibrium is attained by a protein kinase sensitive to zinc and heparin (Fig. 4) and an unknown protein phosphatase(s) (Fig. 5).

Protein Phosphorylation in the Frog Olfactory Cilia—In the previous studies on protein phosphorylation in the olfactory cilia, many phosphoproteins, but not p45, were detected (*17, 19*). However, we found only p45 in this study. The ATP concentration we used (1 mM) was higher than those used previously (80 μM), and for this reason, the specific activity of [γ-^{32}P]ATP was lower than those used in previous studies. In practice, detection of protein phosphorylation depends on the specific activity of ^{32}P-ATP: the higher the specific activity is, the more phosphoproteins can be detected. Therefore, in the previous studies using ATP of high specific activity, many phosphoproteins were found (*17, 19*), while only p45 was detected in the present study. However, by use of ATP of low concentration and high specific activity, as in the previous studies, it may be impossible to detect phosphorylation that needs a high concentration of ATP. Our results, therefore, suggest that the phosphorylation of p45 requires mM levels of ATP. Since the intracellular ATP concentration is generally in the order of mM levels, it is highly possible that the phosphorylation and the dephosphorylation of p45 actually take place *in vivo*.

We did not detect Ca^{2+}-dependent phosphorylation of AC3. Its phosphorylation was demonstrated by an antibody that specifically recognizes a phosphorylated form of AC3 (*15*). Therefore, even if AC3 was phosphorylated, its level might have been too low to be detected in the present study. Since the phosphorylation of p45 is the most prominent phosphorylation reaction at physiological ATP concentrations, this reaction probably has some important roles in the olfactory cilia.

Enzymes Possibly Involved in the Phosphorylation and Dephosphorylation of p45—In the present study, we demonstrated that the phosphorylation level of p45 is controlled by kinase (p45 kinase) and phosphatase (p45 phosphatase) reactions that are both Ca^{2+}-dependent (Figs. 1 and 3).

The inhibitors of PKA, PKC, CKII, and MAPK had no effects on the phosphorylation, but heparin and zinc inhibited the p45 kinase activity (Fig. 4A), which suggested that the p45 kinase is a member of a family of general GRKs. Unlike other general GRKs, which are often inhibited at high Ca^{2+} concentrations (*34–36*), the p45 kinase activity is enhanced by Ca^{2+}.

Among the GRK subtypes, βARK2/GRK3 has been reported to be specifically enriched in mammal olfactory cilia (*12*). In frog also, we found an olfactory ciliary protein immunoreactive to an anti-βARK2 antiserum (*33*), and preliminarily observed its ciliary localization by immunohistochemistry. In addition, by RT-PCR, we have detected the mRNA that encodes a βARK2 homolog in frog olfactory epithelium (Katanosaka, K., Miwa, N., and Kawamura, S., unpublished observations). These results also support the idea that the p45 kinase may be βARK2/GRK3. However, we must be cautious on this point since so far we have only indirect evidence for it.

As for the dephosphorylation, our results indicated that Ca^{2+} enhances the activity of the p45 phosphatase. However, Ca^{2+}-sensitive PP2B does not seem to serve as the p45 phosphatase, because PP2B inhibitors did not affect the dephosphorylation (Fig. 5). Our result demonstrated that none of PP1, PP2A, PP2B, or PP2C serves as the p45 phosphatase.

At present, due to the lack of molecular characterization of the p45 kinase and the p45 phosphatase, the mechanism by which Ca^{2+} contributes to the activation of the p45 kinase and the p45 phosphatase is unknown. However, it is possible that a Ca^{2+}-binding protein is involved in the regulation of these enzymes, as in the case of some classes of GRKs (*34–36*). Previously, in exploring the Ca^{2+}-binding proteins that function in the frog olfactory cilia, we isolated a novel Ca^{2+}-binding protein, p26olf (*37, 38*). Since p26olf is highly enriched in the cilia, it has been speculated that it has a major role in the olfactory signaling (*33, 37*), and possibly in the p45 phosphorylation. Actually, p26olf binds to a βARK2-like protein in a Ca^{2+}-dependent manner *in vitro* (*33*), which suggested the presence of a Ca^{2+}-dependent control of the βARK2-like protein on phosphorylation reactions by p26olf. However, so far, we have obtained only inconclusive evidence concerning the effect of p26olf on the p45 phosphorylation/dephosphorylation. It is possible that another Ca^{2+}-binding protein is involved, or that both the p45-kinase and the p45 phosphatase bind Ca^{2+}.

We did not use purified enzymes in the present study, and it is therefore possible that the observed effects of zinc and heparin on the p45 kinase are also induced indirectly through other enzyme reactions. This point should be tested in future study.

Predicted Phosphorylation Level of p45—The phosphorylation and dephosphorylation of p45 are both enhanced by Ca^{2+} (Figs. 1 and 3). However, at the steady state, the phosphorylation level of p45 is higher at low than high Ca^{2+} concentration (Fig. 2C). Based on the results obtained in this study, we can predict the time course of the phosphorylation level of p45 after odorant stimulation.

At the resting state, the Ca^{2+} concentration in the cilia would be low and the phosphorylation level of p45, therefore, high. When odorants bind to their receptors, CNG channels open, leading to an increase in the Ca^{2+} concentration. As a result, the p45 kinase and the p45 phosphatase activities both increase. According to our measurements, the kinase and the phosphatase activity at 10 nM Ca^{2+} were 1.2 and 0.4%/s, respectively, and those at 20 μM Ca^{2+} were 2 and 1%/s, respectively (Figs. 1A and 3A). Based on these values, we calculated the predicted time course of the phosphorylation level of p45 after the Ca^{2+} concentration increases from 10 nM to 20 μM (dashed line in Fig. 2C). Because the enhancement of the phosphatase activity (1%/0.4%) is more than that of the p45 kinase activity (2%/1.2%), the phosphorylation level of p45 decreases with a

predicted time constant of ~30 s.

Possible Roles of Phosphorylation of p45 in the Olfactory Cilia—In the present study, p45 was detected as a protein labeled by ^{32}P but not as a protein band (Fig. 1A). So far, our attempt to purify p45 has not been successful. Because the identification of p45 at the molecular level was not possible in the present study, the identity of p45 is currently unknown.

Many G-protein–coupled receptors, such as rhodopsin and β-adrenergic receptor, are often phosphorylated and quenched after their specific stimulation (*39*). In the rat olfactory cilia, a receptor-like protein (50 kDa) has been reported to be transiently phosphorylated by βARK2 just after odorant stimulation (*12*). However, frog p45 of the present study would not be categorized in such a receptor family for the following reasons. First, the phosphorylation level of p45 is expected to decrease in response to odorant stimulation (see above discussion). Second, in rhodopsin and β-adrenergic receptor, ligand-binding is prerequisite for the phosphorylation of these receptor molecules, but phosphorylation of frog p45 did not depend on the odorant (see "RESULTS"). Instead, only the Ca^{2+} concentration is crucial for the regulation of its phosphorylation level (Figs. 1–3).

The fact that phosphorylation of p45 is controlled by a Ca^{2+}-dependent equilibrium may be related to the function of p45. One possible explanation is that p45 might be a protein whose function is closely associated with turnover of the phosphate group. In other words, a phosphorylation/dephosphorylation cycle itself could be essential for the function of p45, as in the case of myosin light chain: rapid turnover of phosphate is observed during smooth muscle contraction (*40*). Obviously, further studies are required to identify the physiological role of p45. However, our findings suggested for the first time the presence of a novel Ca^{2+}-signaling pathway that involves Ca^{2+}-dependent phosphorylation/dephosphorylation on p45 in the olfactory cilia.

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