Communication

Regulation of the Phosphorylation State of Rhodopsin by Dopamine*

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G protein-coupled receptors (GPCRs) are regulated by kinases and phosphatases that control their phosphorylation state. Here, the possibility that the state of GPCR phosphorylation could be affected by paracrine input was explored. We show that dopamine increased the rate of dephosphorylation of rhodopsin, the light receptor, in intact frog retinas. Further, we found that rod outer segments from dopamine-treated retinas contained increased rhodopsin phosphatase activity, indicating that this effect of dopamine on rhodopsin was mediated by stimulation of rhodopsin phosphatase. Dopamine is a ubiquitous neuromodulator and, in the retina, is released from the inner cell layers. Thus, our results identify a pathway for feedback regulation of rhodopsin from the inner retina and illustrate the involvement of dopamine in paracrine regulation of the sensitivity of a GPCR.

G protein-coupled receptors (GPCRs)[1] represent a widespread family of proteins that transduce a large variety of signals, such as light, odorants, hormones, and neurotransmitters. They have common structural elements, including seven transmembrane domains, and are regulated by many homologous mechanisms. Understanding these regulatory mechanisms is therefore a central question in signal transduction. Deactivation of a GPCR involves phosphorylation of the receptor, and its subsequent resensitization requires dephosphorylation. Accordingly, the light receptor, rhodopsin, undergoes light-dependent phosphorylation and must be subsequently dephosphorylated (1). The phosphorylation state of GPCRs is regulated typically by GPCR kinases (GRKs) and second messenger-regulated kinases, and, on the other hand, by phosphatases. GRKs preferentially phosphorylate agonist-occupied or activated GPCRs, whereas the second messenger-dependent kinases (cAMP-dependent protein kinase and protein kinase C) may phosphorylate nonactivated receptors (2, 3). Phosphatases that regulate GPCRs belong to the phosphatase 2A family or

are dependent on Ca^{2+} (4–8).

The kinases and phosphatases that affect the phosphorylation of GPCRs may in turn be regulated. Most obviously, second messenger-regulated kinases may mediate input from different signal transduction pathways (9–11). GRKs can also be regulated by other pathways (12). Rhodopsin kinase (GRK1) activity, for example, is inhibited by the Ca^{2+}-binding protein, recoverin, when Ca^{2+} levels are high (13). β-Adrenergic receptor kinase (GRK2) and GRK5 are both phosphorylated by the second messenger kinase, protein kinase C, resulting in their activation (14, 15) and inactivation (16), respectively. Less is known about the dephosphorylation of GPCRs and the regulation of their phosphatases, although rhodopsin dephosphorylation appears to be affected by Ca^{2+} levels. Bovine rhodopsin can be dephosphorylated by a phosphatase 2A (5, 6) and by a Ca^{2+}-sensitive phosphatase (7), both of which are present in photoreceptor outer segments. In *Drosophila*, rhodopsin is dephosphorylated by the *rdgC* protein, which possesses a putative Ca^{2+}-binding domain in addition to a phosphatase catalytic domain (8, 17).

Regulation of kinases and phosphatases thus provides upstream mechanisms for modulating GPCRs. The focus of the present study was on whether input from the inner retina could affect the phosphorylation state of rhodopsin. Such input could potentially originate from general light- or dark-adaptive signals or from a circadian oscillator. The most likely candidate for an intercellular messenger is the major catecholamine in retina, dopamine. Amacrine and interplexiform cells in the inner retina release dopamine in response to light and under the control of a circadian clock (18, 19). Photoreceptor cells possess dopamine receptors (20–22), and dopamine has been shown to influence retinomotor movements and phototransductive membrane shedding (23–26). We demonstrate here that dopamine feedback to the photoreceptor cells affects the kinetics of rhodopsin dephosphorylation in intact frog retinas, indicating that the light receptor can be regulated by paracrine input.

EXPERIMENTAL PROCEDURES

Materials—Dopamine hydrochloride, R(+)-SCH-23390 hydrochloride, and spiperone hydrochloride were purchased from Research Biochemicals International, Inc. [^{32}P]Orthophosphoric acid (~9,000 Ci/mmol) was from NEN Life Science Products. All other chemicals were reagent grade. Northern grass frogs (*Rana pipiens*) weighing 20–30 g were purchased from Carolina Biological Supply Co. and treated according to NIH and University of California at San Diego animal care guidelines.

Incubation of Frog Retinas and Analysis of Rhodopsin Phosphorylation—The procedure for incubation of retinas and analysis of rhodopsin phosphorylation followed that described previously (27). Retinas were removed from dark-adapted animals. Each intact retina was incubated under dim red light in 1 ml of amphibian culture medium (35 mM NaHCO$_3$, 75 mM NaCl, 3 mM KCl, 2 mM CaCl$_2$, 1 mM MgSO$_4$, 10 mM Na-HEPES, pH 7.3, 10 μg/ml phenol red, 1 mg/ml casamino acids, 10 mM β-D(+)-glucose, 0.1 mg/ml Na-L-ascorbate, and 20 μCi/ml [^{32}P]orthophosphate) for 2 h and then for 10 min with or without receptor ligands. One retina from each frog served as the experimental, and the other served as the control. Retinas were illuminated by a calibrated flash of light that photoexcited 6 ± 3 or 80 ± 6% of the rhodopsin (27) and then incubated up to 1 h under dim red light. All incubations were carried out at 22–23 °C. The specific ^{32}P incorporation into rhodopsin was determined after SDS-PAGE (12% acrylamide) by densitometry of Coomassie Blue-stained and radioactive (PhosphorImager) bands and expressed in relative units per constant amount of rhodopsin. Three methods were used for preparation of rhodopsin sam-

[1] The abbreviations used are: GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; ROS, rod outer segment; PAGE, polyacrylamide gel electrophoresis.
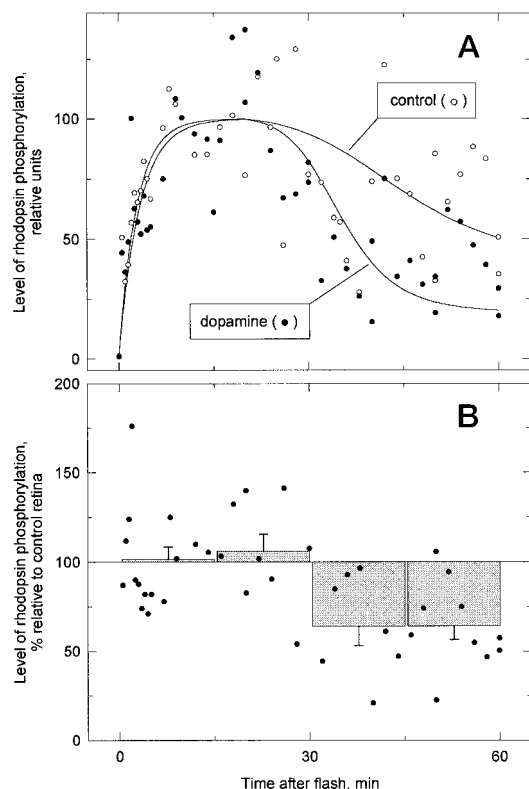
FIG. 1. **The effect of dopamine on the phosphorylation and dephosphorylation of rhodopsin after a flash of light.** Retinas were incubated with (*closed circles*) or without (*open circles*) 100 μM dopamine added to the medium. Crude ROSs were prepared from each retina (Method 1) at the indicated time after the light flash. The intensity of the light flash was such that it photoexcited 80% of the rhodopsin. Each point represents a single retina. A single frog provided one control retina and one dopamine-treated retina. Data were obtained from 12 separate experiments. *A*, the amount of ^{32}P incorporated per constant amount of rhodopsin, expressed relative to the maximal level of the control retina curve (which was found at 20 min after the flash). Curves are a result of exponential (0–20 min) and logistic (20–60 min) fitting of the data. There was considerable variation in the amount of ^{32}P incorporated among different animals and among different experiments following the initial ~10 min after the flash. Such variation is also evident in published profiles of rhodopsin phosphorylation in intact frog retinas by others (*e.g.* Fig. 1 in Ref. 44). *B*, different plot of the same data. Here, the amount of incorporated ^{32}P (per mg rhodopsin) in each dopamine-treated retina is expressed relative to that in the control retina from the same animal; the rhodopsin phosphorylation level in each control retina was normalized to 100%. The superimposed histograms indicate the mean of the relative ^{32}P incorporation in each 15-min interval after the flash. *Error bars* indicate ± S.E. *p* < 0.01 between dopamine-treated retinas and control retinas in the 30–45 min interval and in the 45–60 min interval.

ples: Method 1, at the end of the incubation period, 250 μl of buffer A (20 mM sodium phosphate, 50 mM Tris-Cl, 10 mM EDTA, 10 mM EGTA, pH 7.3) was added to each retina. Rod outer segments (ROSs) were detached from the rest of the retina by vortexing for 10 s. After allowing the retina remnants to settle for 2 min on ice, the ROS-enriched fraction was removed and centrifuged for 30 s at 16,000 × *g*. The pellet (crude ROSs) was suspended in 100 μl of 1% SDS at room temperature and centrifuged (20,000 × *g*, 20 min, room temperature) to remove debris, before adding SDS-PAGE sample buffer; Method 2, the crude ROSs from Method 1 were purified further by sucrose gradient centrifugation (28); and Method 3, reactions of rhodopsin phosphorylation in intact retinas were stopped by adding 250 μl of 50% trichloroacetic acid, and the total retinal proteins were subjected to SDS-PAGE. In all three methods, each rhodopsin fraction from an individual retina in SDS-PAGE sample buffer was divided into two portions. One was heated at 100 °C for 15 min, and the other was kept at room temperature. Heating in SDS promotes the oligomerization of rhodopsin, so that rhodopsin no longer migrates with an apparent molecular mass of ~36 kDa in the gel. The heated sample was therefore used to measure protein and
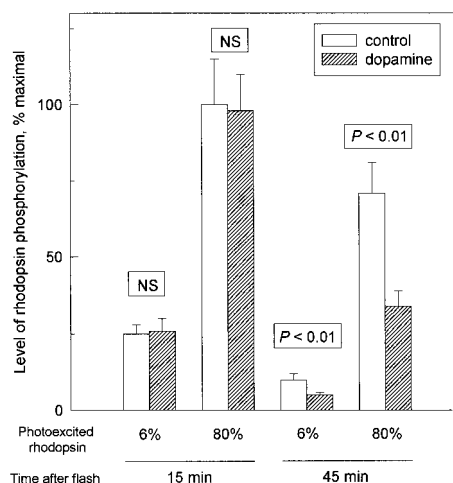

FIG. 2. **Dopamine has the same effect on the phosphorylation and dephosphorylation of rhodopsin at two levels of flash intensity.** Retinas were exposed to a flash of light that excited either 6 or 80% of rhodopsin. The state of rhodopsin phosphorylation was determined from crude ROSs (Method 1). There was no significant difference (*NS*) between 1 μM dopamine-treated and control samples 15 min after a flash of either intensity. However, 45 min after either flash, rhodopsin in dopamine-treated retinas contained ~50% less phosphate. Data represent the means ± S.E. and were pooled from six retinas from four different experiments.

radioactivity that was not from rhodopsin in this area (~36 kDa) of the gel. This background was subtracted from the data obtained from lanes with samples that were not heated. Peripherin/rds, for example, is a photoreceptor outer segment phosphoprotein (29) that has a similar apparent molecular mass. We confirmed by Western blot analysis that bovine peripherin/rds does not oligomerize under the conditions used in the present experiments.

Two-dimensional TLC of Nucleotides—Purified ROSs (Method 2, above) from dopamine-treated and control retinas were collected from a sucrose gradient (50 μl), and 10-μl aliquots were suspended in 90 μl of buffer B (10 mM Tris-Cl, pH 7.3, 1 mM EDTA, 1 mM EGTA, 0.05% digitonin). The samples were precipitated with MeOH (90%, 30 min, ice) and centrifuged (20,000 × *g*, 30 min, 4 °C). Each supernatant was lyophilized and then resuspended in 10 μl of water. A 1-μl aliquot was loaded on to a Cellulose polyethyleneimine TLC plate (8 × 8 cm). Stepwise chromatography was run in the first direction with 0.2 (1 min), 1 (3 min), and 1.6 M LiCl (10 min). The LiCl was removed by washing the plates in MeOH (15 min). Dry plates were used for stepwise chromatography in the second direction with 0.5 (0.5 min), 2 (1 min), and 4 M sodium formate (8 min) (pH 3.4) (30). Radioactivity was quantified using a PhosphorImager.

Assay of Phosphatase Activity in ROSs—^{32}P phosphorylation of rhodopsin was performed in intact retinas as above. Rod outer segments from dopamine-treated and control retinas were purified as described (Method 2, above), and their cytosolic fractions (0.6 mg/ml) were used to assay phosphatase activity (1 nM rhodopsin; 22 °C for 30 min, during which time ^{32}P release was linear) (7, 31). Radioactive products were separated by SDS-PAGE and analyzed by a PhosphorImager.

Statistical Analyses—Paired Student's *t* tests were performed to determine the probability (*p*) of no significant difference.

RESULTS AND DISCUSSION

After retinas were exposed to a flash of light, rhodopsin was phosphorylated, reaching maximal phosphorylation level after 10 min (27). The rate of phosphorylation in control retinas and in retinas exposed to 100 μM exogenous dopamine was similar (Fig. 1). After 30 min, the level of rhodopsin phosphorylation decreased, with rhodopsin in the dopamine-treated retinas dephosphorylated at a faster rate. By 45 min after the light flash, the level of phosphorylation of rhodopsin in dopamine-treated retinas was only 50% that in control retinas (Fig. 1). A similar result was obtained by three different procedures of sample preparation, as described under "Experimental Procedures"; results from Method 1 are illustrated in Fig. 1. Moreover, this
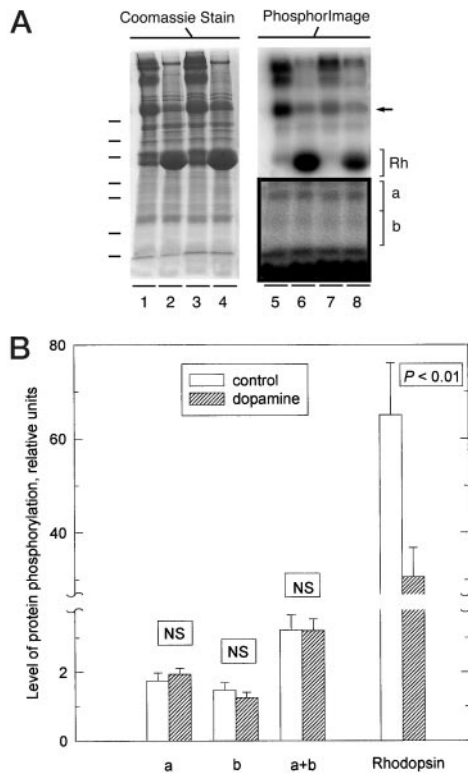
FIG. 3. **Effect of dopamine on the state of phosphorylation of other ROS proteins.** Purified ROSs (Method 2) were prepared from retinas 45 min after a flash effecting photoexcitation of 80% rhodopsin. To test whether dopamine (100 μM) affected the phosphorylation state of proteins other than rhodopsin, radioactivity was measured from two regions of the gel below the position of the rhodopsin monomer. These regions (*a* and *b*) are free of rhodopsin, and each contained a weak radioactive protein band (regions above the position of the rhodopsin monomer may be contaminated by rhodopsin and its oligomers). *A*, example of Coomassie Blue-stained gel and PhosphorImager analysis of the gel. *Lanes 1*, *2*, *5*, and *6*, control retinas. *Lanes 3*, *4*, *7*, and *8*, dopamine-treated retinas. *Lanes 1*, *3*, *5*, and *7*, ROSs that were heated in SDS sample buffer to promote oligomerization of rhodopsin. *Lanes 2*, *4*, *6*, and *8*, samples that were not heated. The PhosphorImager of the *lower part* of the gel (containing regions *a* and *b*, indicated on the *right*) is shown at a greater exposure than the *upper part*, so that the radioactive band in *a* is evident. The positions of molecular mass standards (66, 45, 36, 29, 24, 20, and 14.2 kDa) are shown on the *left*. *Rh*, position of rhodopsin monomer. The *arrow* indicates the position of rhodopsin dimer. *B*, measurements of radioactivity from regions *a* and *b* (*panel A*) and from rhodopsin. There was no significant difference (*NS*) in radioactivity of regions *a* and *b* between control and dopamine-treated retinas. Data represent the means ± S.E. of 12 retinas.

result was found irrespective of whether 80 or 6% of the rhodopsin was photoexcited by the flash (Fig. 2).

To test whether or not the effect of dopamine resulted in a general effect on ROS protein phosphorylation, we carried out two tests. First, the amount of [^{32}P]ATP was measured in ROSs following incubation of retinas for 45 min in the presence or the absence of dopamine. PhosphorImager analysis of two-dimensional TLC plates showed that in dopamine-treated retinas the amount of ROS [^{32}P]ATP was similar to that in control retinas (102 ± 7%; n = 12; p = 0.94). Second, we observed that the radioactivity of minor phosphoproteins was unaffected by dopamine (Fig. 3). These results are consistent with dopamine having a specific effect on the phosphorylation state of rhodopsin.

Further analysis of the reduction in the level of rhodopsin phosphorylation 45 min after the flash showed that it was effected by nanomolar concentrations of exogenous dopamine (Fig. 4). These concentrations are in the range of reported dissociation constants (K_d) for dopamine receptors in the high
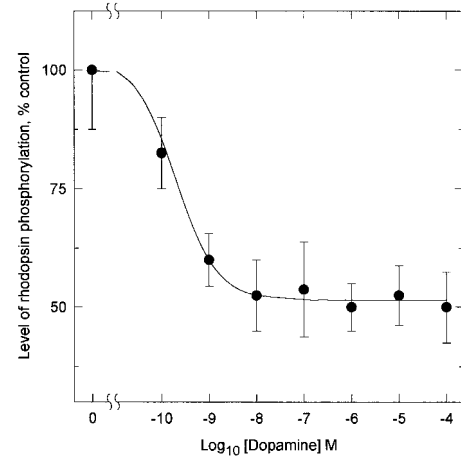

FIG. 4. **Rhodopsin dephosphorylation is regulated by nanomolar dopamine.** Retinas, in the presence of different concentrations of dopamine, were illuminated by a flash and incubated for an additional 45 min. Crude ROSs were prepared from each retina (Method 1). The data shown (mean ± S.E.) represent the amount of rhodopsin phosphorylation relative to that in control retinas (no exogenous dopamine), which was normalized to 100%. In each experiment, one retina was used for each concentration, and the experiment was repeated six times. The *curve* is a result of nonlinear regression analysis.

affinity state (32).

Dopamine receptors fall into two general classes, D1-like and D2-like. D1 receptors act by activating adenylate cyclase. D2 receptors typically act by inhibiting adenylate cyclase (32–34). To test which class of receptor might be involved in mediating the dopamine effect on rhodopsin phosphorylation, we tested whether antagonists selective for D1-like or D2-like receptors would counter the lowered phosphorylation level found 45 min after the light flash. As illustrated in Fig. 5, SCH-23390, a selective D1 antagonist, did not interfere with the dopamine effect. However, spiperone, a selective D2 antagonist, did; it resulted in a higher level of phosphorylation. This finding is consistent with previous reports identifying D2-like receptors on rod photoreceptors (20, 35–37).

These results indicate that exposure to dopamine and activation of D2 receptors on photoreceptor cells alters the kinetics of rhodopsin dephosphorylation. One explanation is that dopamine leads to activation of rhodopsin phosphatases. Alternatively, dopamine could lead to preferential phosphorylation at a site that is dephosphorylated more rapidly. Protein kinase C phosphorylates a domain that is not a primary phosphorylation site for rhodopsin kinase (38, 39), and stimulation of protein kinase C phosphorylation of rhodopsin results in faster dephosphorylation (27). However, altering the relative activities of protein kinase C and rhodopsin kinase results in a different rate of phosphorylation (27), which was not evident in dopamine-treated samples (Fig. 1).

In experiments to test whether ROSs from dopamine-treated retinas contained greater phosphatase activity, purified ROS membranes containing ^{32}P-phosphorylated rhodopsin were incubated with ROS cytosol from control or dopamine-treated retinas (7, 31). Fig. 6 illustrates that dopamine-treated ROS cytosol contained significantly more rhodopsin phosphatase activity. Previous work has shown that dopamine may regulate phosphatase-1 via D1 receptors and cyclic AMP-dependent kinase phosphorylation of DARPP-32 (dopamine and cAMP-regulated phosphoprotein), a phosphatase-1 inhibitor (40). However, this is the first report suggesting an effect of dopamine on other phosphatases and on phosphatase activity via D2 receptors.

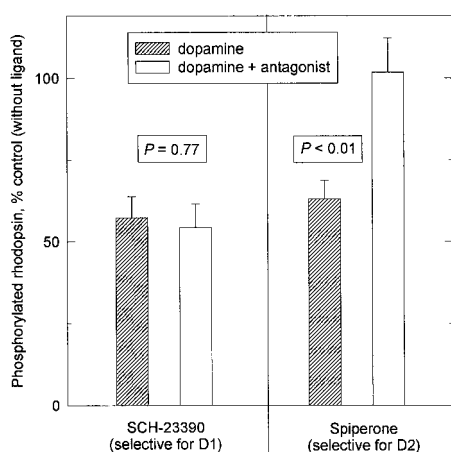Dephosphorylation of GPCRs has received less attention

FIG. 5. **Identification of the dopamine receptor subtype responsible for mediating the effect of dopamine on rhodopsin dephosphorylation.** Retinas were incubated for 45 min after the flash of light. They were incubated with 1 nM dopamine ± SCH-23390 (100 nM), a selective D1-like receptor antagonist, or ± spiperone (100 nM), a selective D2-like receptor antagonist. One retina from each frog was treated with dopamine, and the other was treated with dopamine plus antagonist. Spiperone but not SCH-23390 inhibited the effect of dopamine; in these retinas the level of rhodopsin phosphorylation was higher. Each value shown is the mean ± S.E. of seven retinas from four separate experiments, expressed as a percentage of the rhodopsin phosphorylation level in control retinas (no exogenous dopamine, no antagonists) at the same time.
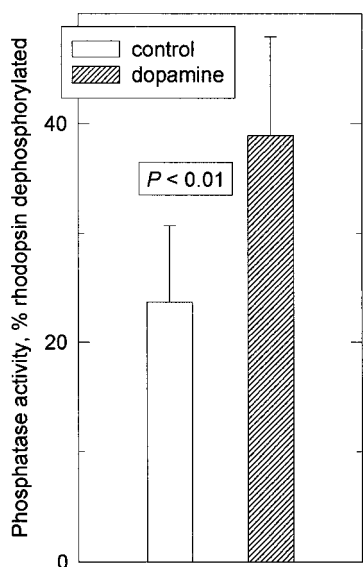

FIG. 6. **The effect of dopamine on rhodopsin phosphatase activity in rod photoreceptor outer segments.** Intact frog retinas were treated with dopamine (1 nM) or spiperone (100 nM, control) and incubated for 45 min after the flash. The cytosolic fraction from purified rod outer segments of these retinas was then used to dephosphorylate frog rhodopsin that had been previously ^{32}P-phosphorylated in intact retinas. Phosphatase activity was determined by the release of ^{32}P from rhodopsin (7, 31).

than their phosphorylation. In comparison to our knowledge of kinases that phosphorylate GPCRs, less is known about the phosphatases that dephosphorylate them, and, in particular, how these phosphatases are regulated. However, dephosphorylation of rhodopsin is necessary to complete the rhodopsin cycle following light activation and then deactivation by phos-

phorylation and arrestin binding (41–43). The importance of rhodopsin dephosphorylation is emphasized by *Drosophila rdgC* mutants. In the absence of rhodopsin phosphatase (the product of the *rdgC* gene), the phosphorylation state of rhodopsin is abnormally high, termination of the light response is defective, and the photoreceptor cells degenerate (8). The present results demonstrate a role for dopamine in the regulation of rhodopsin dephosphorylation and indeed suggest that it effects stimulation of rhodopsin phosphatase. Because dopamine is normally released by cells in the inner retina, these results identify the potential for a novel means of regulation of rhodopsin: from the inner retina back to the light receptor.

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