Isoproterenol Inhibits Rod Outer Segment Phagocytosis by Both cAMP-Dependent and Independent Pathways

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Purpose. The authors studied the involvement of cAMP-dependent second messenger systems in the inhibition of rod outer segment (ROS) phagocytosis by isoproterenol (ISO) and forskolin (FSK) using two membrane-permeant analogs of cyclic adenosine monophosphate (cAMP), the Rp and Sp diastereoisomers of cyclic adenosine 3',5' monophosphothioate (cAMPS). Rp-cAMPS is a potent competitive inhibitor of cAMP-dependent protein kinase I and II (PKA I and II), whereas Sp-cAMPS is a potent activator of these enzymes.

Methods. ROS phagocytosis was quantitated in cultured rat RPE cells using a previously described double immunofluorescence assay.

Results. Sp-cAMPS showed a dose-dependent inhibition of ROS phagocytosis, whereas 100 nM Rp-cAMPS had no effect on this process. Rp-cAMPS fully prevented the inhibitory effect of Sp-cAMPS and FSK but was able to prevent only partially the inhibition of ROS phagocytosis induced by ISO. Isoproterenol plus FSK showed an additive effect on the inhibition of phagocytosis, suggesting that they act at two independent sites. However, ISO plus Sp-cAMPS or FSK plus Sp-cAMPS showed no additivity.

Conclusions. Results suggest that FSK inhibits ROS phagocytosis by RPE cells through a cAMP-dependent pathway, whereas ISO inhibits ROS phagocytosis by RPE cells through cAMP-dependent and cAMP-independent pathways. Invest Ophthalmol Vis Sci. 1995; 36:730-736.
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8-bromo-cAMP and dibutyryl-cAMP,15-17 thus allowing their use on intact cells.

MATERIALS AND METHODS

Reagents

Unless otherwise noted in the text, all chemicals were purchased from Sigma Chemical, St Louis, MO. Sp-cAMPS and Rp-cAMPS were purchased from BIOLOG, La Jolla, CA.

Tissue Culture of Retinal Pigment Epithelial Cells

All procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals in Ophthalmic and Vision Research, and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Retinal pigment epithelial cells were isolated from 10-day-old Long Evans rats maintained on a 12-hour dark/12-hour light cycle.1 The cells were seeded on 18-mm diameter glass coverslips at a density of 30,000 cells/100 µl growth medium (minimum essential medium containing 10% fetal bovine serum, 40 µg/ml gentamycin [Whittaker Bioproducts, Walkersville, MD], 40 µg/ml kanamycin [Flow Laboratories, McLean, VA] and 2 mM glutamine). The cells were used when they reached confluence after 6 to 7 days of growth at 37°C in 95% air, 5% CO₂.

Incubation With Drugs

Stock (1 mM) solutions of Rp- and Sp-cAMPS were made in sterile distilled water and stored at −80°C. Coverslips on which RPE cells were growing were placed in 1 ml of fresh growth medium in 12-well tissue culture plates. An aliquot of Rp- or Sp-cAMPS was added to the final desired concentration, and the cells were incubated with the drug for varying periods of time, as indicated in the figure legends. When the effect of ISO or FSK was to be examined, aliquots from freshly made stock solutions of these drugs were added directly to the wells to achieve the final desired concentration. At the end of the preincubation period, ROS were added directly to the wells, and incubation was continued for 1 hour in the presence of the drug(s).

Isolation and Feeding of Rod Outer Segments

Rod outer segments were isolated from Long Evans rats older than 30 days of age.18 The final pellet was suspended in growth medium to the required concentration of ROS, usually 10⁸/ml; 0.1 ml of the ROS suspension, which was warmed to 37°C, was added to each well, and incubation was carried out for 1 hour at 37°C.

RESULTS

Effects of Sp-cAMPS and Rp-cAMPS on ROS Phagocytosis by RPE Cells

Initial experiments tested a range of Sp-cAMPS and Rp-cAMPS concentrations from 10⁻⁵ to 10⁻² µM. The activator of PKA, Sp-cAMPS, inhibited ROS phagocytosis by RPE cells in a dose-dependent manner (Fig. 1).
Rod outer segment ingestion was decreased to 50% of control by 10 μM Sp-cAMPS (IC₅₀). The inhibitor of PKA, Rp-cAMPS, had no effect on ROS phagocytosis (Fig. 1), demonstrating that at concentrations of up to 100 μM, the drug has no nonspecific toxic effect on the cells. Neither isomer had any inhibitory effect on the binding of ROS.

**Competitive Effects of Rp-cAMPS and Either Sp-cAMPS, ISO, or FSK on ROS Phagocytosis**

Rp-cAMPS antagonized the inhibitory effect of Sp-cAMPS on ROS phagocytosis in a dose-dependent manner (Fig. 2A). The inhibition of ROS ingestion by 10 μM Sp-cAMPS (IC₅₀) was completely blocked by a 15-minute preincubation with 100 μM Rp-cAMPS. This indicates that sufficient Rp-cAMPS diffuses into the cell during the preincubation period to inactivate PKA I and II, such that they are no longer responsive to activation by Sp-cAMPS.

However, when the above experiment was repeated using IC₅₀ concentrations of ISO or FSK (100 nM), instead of Sp-cAMPS, a 15-minute preincubation with Rp-cAMPS was unable to block the inhibitory effect of these drugs. Although Rp-cAMPS has been reported to diffuse rapidly into cells, it seemed possible that a 15-minute preincubation with this drug would not be sufficient time for this inhibitor to reach a sufficiently high concentration in the cell to block the activity of PKA completely. Thus, we preincubated cells with 100 μM Rp-cAMPS at times from 15 minutes to 60 minutes, after which they were incubated for 15 minutes with 100 nM FSK or ISO. Rod outer segments were then fed for 1 hour in the presence of both drugs. As seen in Figure 2B, a 60-minute preincubation with Rp-cAMPS was able to prevent completely the inhibitory effect of FSK. However, these same incubation conditions resulted in only a 40% recovery of the inhibition of ROS ingestion induced by 100 nM ISO.

Because 100 μM Rp-cAMPS partially reversed the
inhibitory effect of 100 nM ISO, we conducted a dose-response experiment in which the concentration of ISO varied from 3.3 nM to 100 nM, whereas the concentration of Rp-cAMPS remained at 100 µM. As shown in Figure 2C, even at the lowest concentration (3.3 nM) of ISO used, 100 µM Rp-cAMPS was only partially able to reverse the inhibitory effect of ISO. In fact, the absolute increase caused by 100 µM Rp-cAMPS on the number of ROS ingested is essentially constant over the whole range of ISO concentrations examined. The dose ratio of antagonist/agonist (Rp-cAMPS/ISO) varied by a factor of 30, with no change in the increase of the number of ROS ingested, suggesting independent mechanisms of action.

Additive Effects on ROS Phagocytosis of ISO, FSK, and Sp-cAMPS
ISO (10 nM), FSK (10 nM), and Sp-cAMPS (1 µM) separately showed approximately a 30% to 40% inhibition of ROS ingestion (Fig. 3A). When cells were treated with ISO (10 nM) + FSK (10 nM) together, the inhibition of ROS ingestion increased to 60%. However, when cells were incubated with ISO (10 nM) + Sp-cAMPS (1 µM) or FSK (10 nM) + Sp-cAMPS (1 µM), the inhibition of ROS ingestion was not increased above that seen on treatment with each drug alone. Furthermore, there was also no significant difference in the inhibition of ROS ingestion at any concentration of ISO up to 10 µM alone or with the same doses of ISO + 1 µM Sp-cAMPS (Fig. 3B).

Effect of 1,9-dideoxy-forskolin on ROS Phagocytosis
To demonstrate a possible adenylyl cyclase-independent effect of FSK on ROS phagocytosis, we used 1,9-dideoxyforskolin (ddFSK); this analog of FSK is unable to stimulate adenylyl cyclase or raise cAMP levels. However, ddFSK showed no effect on ROS phagocytosis at a range of concentrations (10⁻⁸ to 10⁻⁴ M) tested (data not shown).

DISCUSSION
We have previously shown that ISO and FSK are able to inhibit the phagocytosis of ROS by RPE cells at concentrations of agonist that do not measurably raise the intracellular cAMP concentration. To investigate further this puzzling observation, we have used two membrane-permeant cAMP analogs, Rp-cAMPS and Sp-cAMPS, to determine whether cAMP production and PKA activation play a role in the inhibition of ROS phagocytosis elicited by ISO and FSK. Rp-cAMPS is a potent competitive inhibitor of cAMP-dependent protein kinase I and II, preventing the dissociation of the catalytic and regulatory subunits, whereas Sp-cAMPS is a potent activator of these enzymes. Thus,
if a cAMP-dependent activation of PKA is responsible for the inhibition of ROS phagocytosis by ISO and FSK, Sp-cAMPS should be able to mimic the inhibitory action of these drugs, whereas Rp-cAMPS should be able to inhibit the action of these drugs. Our results show that Sp-cAMPS does in fact mimic these reagents and dose dependently inhibits ROS phagocytosis by RPE cells (Fig. 1), whereas Rp-cAMPS alone has no effect on ROS phagocytosis. The inhibitory effect of Sp-cAMPS was dose dependently antagonized by Rp-cAMPS (Fig. 2A). Thus, the Sp- and Rp- isomers of cAMPS behave as agonist and antagonist with respect to the inhibition of phagocytosis of ROS by RPE cells. Taken together, these results provide evidence that the activation of PKA is involved in at least one mechanism for the inhibition of ROS phagocytosis.

In preliminary experiments, we preincubated cells with Rp-cAMPS for 15 minutes before applying any of the agonist drugs. Although this preincubation time completely antagonized the inhibitory effect of subsequently applied Sp-cAMPS (Fig. 2A), it did not antagonize the inhibitory effects of either FSK or ISO. We thus studied the effect of increasing the preincubation time with Rp-cAMPS on the ability of this antagonist to block the inhibitory actions of FSK or ISO. As shown in Figure 2B, a 15-minute preincubation with Rp-cAMPS had little or no effect on the inhibitory activity of either FSK or ISO; however, increasing the preincubation time with Rp-cAMPS to 60 minutes completely blocked the inhibitory activity of FSK and partially blocked the inhibitory activity of ISO.

These results suggest that Sp- and Rp-cAMPS diffuse into the cell at a relatively slow rate compared to the intracellular accumulation of cAMP caused by stimulation with ISO or FSK. Thus, a 15-minute preincubation with Rp-cAMPS allows a sufficient amount of the antagonist to accumulate in the cell to block the subsequent activation of PKA by extracellularly applied Sp-cAMPS, which diffuses equally slowly into the cell. FSK and ISO, by contrast, cause a rapid intracellular induction of cAMP production. When these agonists are applied after only a 15-minute incubation with Rp-cAMPS, the rapid production of cAMP causes activation of PKA before sufficient Sp-cAMPS has accumulated in the cell to inhibit completely all the available PKA. It is also likely that the kinetic of cAMP for PKA is much less than that of Sp-cAMPS for this enzyme. Much lower intracellular concentrations of the native second messenger, which are below the limits of detection by the radioimmunoassay used, would be required to achieve the IC50 level of PKA activation. Similarly, the affinity of PKA for cAMP is in all probability much greater than the affinity of the enzyme for the Rp-analog, thus requiring much higher intracellular concentrations of Rp-cAMPS to block the effect of endogenously generated cAMP. In this regard, it should be noted that 10 μM Sp-cAMPS (extracellular) is required to achieve a 50% inhibition of phagocytosis (Figs. 1, 2A), whereas approximately 100 nM ISO achieves the same level of inhibition (Figs. 2C, 3B).

We thus evaluated the antagonist isomer, Rp-cAMPS, in combination with previously studied activators of the cAMP pathway in RPE cells using a 60-minute preincubation. Our previous data demonstrated that 100 nM of either ISO or FSK inhibited ROS ingestion by about 50% in RPE cells without causing a measurable increase in the level of total intracellular cAMP. If this inhibition involves the activation of PKA by very low levels of cAMP, Rp-cAMPS should block this effect. Therefore, we investigated whether Rp-cAMPS could prevent the inhibition of ROS phagocytosis caused by ISO or FSK. In the present experiments, both ISO and FSK at 100 nM inhibited ROS phagocytosis by approximately 40% (Fig. 2B), in agreement with our previous results. A 1-hour preincubation with 100 μM Rp-cAMPS was able to prevent completely the inhibitory effect of 100 nM FSK (Fig. 2B). This indicates that FSK exerts its inhibitory effect solely through the cAMP-mediated activation of PKA and that this effect is prevented by the inhibition of PKA by Rp-cAMPS. However, the response to 100 nM ISO alone was only partially (40%) reversed by 100 μM Rp-cAMPS (Fig. 2B). To explore further whether there is an involvement of PKA in the inhibition of ROS phagocytosis by ISO, we compared the dose-dependent inhibition of phagocytosis by ISO in the presence of Rp-cAMPS (100 μM). This concentration of Rp-cAMPS completely reverses the inhibitory action of 100 nM FSK. However, the inhibitory effect of 3.3 nM ISO, which reduced ROS phagocytosis by only 20%, could be reversed only partially by 100 μM Rp-cAMPS (Fig. 2C). If there was a direct relationship between the amount of cAMP produced in response to ISO and the ability of Rp-cAMPS to antagonize the inhibitory effect of ISO, then Rp-cAMPS would exert a greater effect when lower doses of ISO are applied (FSK and ISO result in an equally low production of cAMP at doses below 100 nM). However, no greater reversal of ISO inhibition by Rp-cAMPS was found with 3.3 nM ISO than with 100 nM ISO (Fig. 2C). It thus appears that the inhibitory effect of ISO is not mediated solely through its ability to activate adenyl cyclase, followed by the cAMP-mediated activation of PKA. We conclude, therefore, that activation of PKA is not the primary pathway involved in the inhibition of ROS phagocytosis by ISO.

We have also investigated the interactions between ISO, FSK, and the activator isomer, Sp-cAMPS, on the inhibition of ROS ingestion. ISO (10 nM), FSK (10 nM), and Sp-cAMPS (1 μM) separately showed an approximately 40% inhibition of ROS ingestion (Fig. 3A). When cells were treated with ISO + FSK together,
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the inhibition of ROS ingestion increased to 60%, showing that ISO and FSK in combination exert an additive effect on this process. However, when cells were incubated with ISO + Sp-cAMPS or FSK + Sp-cAMPS, the inhibition of ROS ingestion was not increased above that seen on treatment with each drug alone, demonstrating that Sp-cAMPS did not act additively with these drugs.

Furthermore, there was no significant difference in the inhibition of ROS ingestion between increasing concentrations of ISO alone or increasing concentrations of ISO + 1 μM Sp-cAMPS (Fig. 3B). These data suggest that ISO and FSK act at different, noninteracting sites that give an additive response. These results support the hypothesis that FSK inhibits ROS phagocytosis solely through its role in increasing intracellular cAMP, whereas ISO acts by increasing cAMP as well as through a cAMP-independent pathway. Sp-cAMPS appears to interact with ISO or FSK in a mutually exclusive way, with a combined response that is less than the sum of their separate activities (Fig. 3). Because the only reported action of Sp-cAMPS is to activate PKA, cAMP produced in response to either ISO or FSK competes with the action of this drug.

Several recent studies have challenged the concept that PKA is the sole mediator of cAMP-dependent pathways, especially in the relaxation of vascular smooth muscle. Although ISO and FSK are known to raise intracellular cAMP by stimulating adenylyl cyclase, each of these drugs may affect cellular function through pathways not linked to cAMP production. A direct activation of Ca2+ and K+ channels by activated G proteins has been demonstrated in cardiac muscle cells and neuronal cells, respectively. By analogy, the cAMP-independent effect of ISO on ROS phagocytosis could be explained by beta-adrenergic receptor activation of G proteins and their subsequent interaction with unknown targets.

To demonstrate a possible adenylyl cyclase-independent effect of FSK on ROS phagocytosis, we used ddFSK; this analog of FSK is unable to stimulate adenylyl cyclase or raise cAMP levels, but it does alter K+ channels indistinguishably from FSK. However, ddFSK showed no effect on ROS phagocytosis over a range of concentrations from 0.01 μM to 10 μM (data not shown). This result further supports the suggestion that FSK inhibits ROS phagocytosis solely through the cAMP-mediated activation of PKA.

We previously have demonstrated that dose–response curves that were generated to correlate the inhibition of ROS phagocytosis with the increase in the gross cellular level of cAMP by ISO and FSK showed no apparent direct correlation between these two parameters. In this article, we clarify this puzzling observation by demonstrating that FSK does indeed exert its inhibitory effect through a cAMP-mediated activation of PKA because this effect can be completely blocked by Rp-cAMPS. Furthermore, ddFSK shows no inhibition of ROS phagocytosis. In light of these findings, our previous results can only be explained by the hypothesis that at low doses, FSK causes a localized intracellular rise in cAMP too small, too transient, or both to be detected by the radioimmunoassay of total cAMP levels in cultured cells that was used. This low level of cAMP must, however, be sufficient to activate PKA. We further demonstrate that the inhibition of ROS phagocytosis by ISO is partly dependent (40%) and partly independent (60%) of the cAMP-stimulated activation of PKA. Our data strongly suggest that ISO inhibits ROS phagocytosis in RPE cells through a cAMP-dependent mechanism, as well as through a second pathway that is totally cAMP independent. The cAMP-dependent action of ISO can be blocked by Rp-cAMPS, whereas the cAMP-independent pathway is unaffected by this inhibitor of PKA. This cAMP-independent pathway must be truly independent of any action of cAMP, not just independent of PKA. If this were not the case, FSK would also show the same component of Rp-cAMPS-independent inhibition of ROS phagocytosis as is shown by ISO. In other words, if cAMP inhibited ROS phagocytosis by activating PKA as well as by a PKA-independent mechanism that could not be blocked by Rp-cAMPS, then FSK and ISO would show identical responses to Rp-cAMPS. Because this was not observed, we conclude that part of the inhibitory effect of ISO must be due to an action that does not involve cAMP.

It has been reported recently that during ROS phagocytosis by RPE cells, the second messenger, inositol triphosphate (IP3), is generated. Additionally, it has been shown that the stimulation of RPE cells from the Royal College of Surgeons rat by carbachol, which causes an increase in IP3, increases the number of ROS ingested by these cells. These results suggest that the ROS phagocytosis receptor may be linked to G protein of the Gq class, which stimulates the phospholipase C-mediated hydrolysis of phosphatidyl inositol bisphosphate to generate IP3. These data suggest that activation and inhibition of phagocytosis may be regulated by separate IP3 and cAMP pathways. Alternatively, it is possible that the cAMP-independent inhibitory action of ISO may be linked to “cross-talk” between the Gs and Gq pathways. Thus, when the beta-adrenergic receptor is stimulated by ISO, it could activate adenylyl cyclase and generate cAMP. This part of the pathway would be inhibited by Rp-cAMPS. It may also, directly or indirectly, result in the inactivation of Gq to which the phagocytosis receptor may be linked. If this postulate is valid, it may be found that the Gq to which the phagocytosis receptor is linked is a unique member of this family of G proteins because
the genetic defect in the Royal College of Surgeons rat is expressed only in the RPE cell.

**Key Words**

retinal pigment epithelium, phagocytosis, cAMP, adenylyl cyclase, protein kinase A, G-proteins

**References**