Inositol Triphosphate Generation in Cultured Rat Retinal Pigment Epithelium

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Purpose. To evaluate the generation of the second messenger molecule inositol triphosphate (IP$_3$) in response to phagocytic challenge in cultures of retinal pigment epithelium (RPE) prepared from normal Long Evans or dystrophic Royal College of Surgeons (RCS) rats.

Methods. RPE cultures were phagocytically challenged with isolated rat rod outer segments (ROS) or polystyrene latex spheres (PSL). Carbachol was added to some cultures as a positive control to stimulate the IP$_3$ pathway. Inositol triphosphate levels in RPE cells were analyzed by high-pressure liquid chromatography and radioreceptor assay. Incorporation of $^3$H-myoinositol into phosphatidylinositol lipids was analyzed by thin layer chromatography and autoradiography.

Results. Long Evans RPE phagocytically challenged with ROS or exposed to carbachol showed a significant increase in IP$_3$ levels compared to unchallenged control cultures or cultures phagocytically challenged with PSL. RCS RPE cells did not show an increase in IP$_3$ levels upon phagocytic challenge with ROS or PSL. Carbachol treatment of RCS RPE produced an increase in IP$_3$ levels, demonstrating that the components of the IP$_3$ pathway are present and the pathway can be activated. Phagocytic challenge of Long Evans RPE with ROS was associated with a decrease in the IP$_3$ precursor, phosphatidylinositol bisphosphate (PIP$_2$). No decrease in PIP$_2$ was observed in RCS RPE incubated with ROS.

Conclusions. These results show that phagocytic challenge of Long Evans RPE with ROS is associated with PIP$_2$ hydrolysis and subsequent generation of IP$_3$ as a second messenger. In RCS RPE, ROS stimulation of the IP$_3$ pathway is absent. Invest Ophthalmol Vis Sci. 1994;35:409-416.

Of one of the most important functions of the RPE is the phagocytosis and degradation of outer segment membranes that are shed from the adjacent rod and cone photoreceptor cells. Removal and elimination of the shed outer segment material is an essential factor in the renewal process of the photoreceptor outer segment membranes and the maintenance of normal vision. When this process is disrupted, as in the dystrophic Royal College of Surgeons (RCS) rat, unphagocytized photoreceptor debris accumulates in the subretinal space, resulting in retinal degeneration and blindness. Several laboratories have studied ROS phagocytosis by RPE in vitro. Results from these studies suggest that an RPE cell surface protein acts as a receptor for binding ROS, and that the receptor is present on the surface of Long Evans and RCS RPE. Subsequent to ROS binding to the RPE surface, it has been proposed that phagocytosis is triggered by a transmembrane signal, probably involving the generation of second messenger molecules. Receptor-ligand interaction in many cell types is followed by hydrolysis of the membrane phospholipid phosphatidylinositol bisphosphate (PIP$_2$) to yield two second messengers, diacylglycerol and inositol triphosphate (IP$_3$). Diacylglycerol remains in the plasma membrane where it has been associated with the activation...
of protein kinase C.\textsuperscript{17} IP\textsubscript{3}, on the other hand, is liberated into the cytosol, where it is believed to release calcium from the endoplasmic reticulum, which in turn activates calcium-dependent kinases.\textsuperscript{16} Neither of these second messengers has previously been measured in phagocytically challenged RPE.

The extracellular interaction of RPE with ROS has been directly related to an increase in the activity of intracellular protein kinases and phosphatases in RPE.\textsuperscript{14,15} ROS-specific changes in the phosphorylation of 14 proteins were identified in Long Evans RPE.\textsuperscript{14} In RCS RPE, ROS-specific changes in protein phosphorylation were observed but none corresponded to those in Long Evans RPE.\textsuperscript{19} One possibility that might explain the abnormality observed in ROS-specific protein phosphorylation and the phagocytic deficit in RCS RPE is a defect in second messenger generation.

In these studies, IP\textsubscript{3} levels were compared between normal Long Evans and dystrophic RCS RPE cells under control conditions and after phagocytic challenge with ROS.

**MATERIALS AND METHODS**

**Preparation of RPE Cultures**

Normal pigmented Long Evans rats were obtained from Charles River Breeding Laboratories (Wilmington, MA). Dystrophic, pigmented RCS (RCS rdy+p+) rats were maintained in a breeding colony. Treatment of animals in this investigation conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the ARVO Resolution on the Use of Animals in Research. RPE cultures were prepared from 5- to 6-day-old Long Evans or age-matched RCS rats as previously described.\textsuperscript{18} Cultures were plated at equal density in 24 well plates and grown to confluency in RPMI + 20% fetal calf serum.

**Preparation of Phagocytic Challenge Particles**

ROS were isolated from adult Long Evans rats using sucrose step gradient centrifugation.\textsuperscript{22} After collection from the gradient, the ROS were fixed for 1 hour at 4°C in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4. Fixed ROS present a stable surface for receptor interaction, are ingested equally as well as fresh ROS,\textsuperscript{20} and do not add measurably to the IP\textsubscript{3} assay systems used in this study. ROS were centrifuged, and the pellet was resuspended in 0.1 M glycine to quench any residual free aldehyde groups that could result in nonspecific binding.\textsuperscript{23} The ROS were pelleted and resuspended in Krebs-Hensleit buffer (KHB, pH 7.4) + 20% fetal calf serum at a concentration of 2.5 × 10\textsuperscript{7} particles/ml and stored at 4°C until use.

For these studies of receptor-mediated phagocytosis, polystyrene latex (PSL) microspheres were used as a negative control because phagocytosis of PSL is probably not receptor mediated and would not be expected to generate second messengers.\textsuperscript{24} PSL particles (carboxylated polystyrene, 1.07 μm in diameter, Polysciences Inc., Warrington, PA) were prepared by washing three times in KHB + 20% fetal calf serum and were resuspended in the same buffer to a final concentration of 2.5 × 10\textsuperscript{7} particles/ml.

**Labeling of RPE Cultures With 3H Myoinositol**

Cultures were incubated for 72 hours with 20 μCi/well \textsuperscript{3}H-myoinositol (Specific Activity 47.7 Ci/mmol, carrier free, New England Nuclear, Boston, MA) in 225 μl KHB supplemented with 15 mM glucose, 1.8 mM calcium chloride, 1 mM cytidine, and 0.1 mM cold inositol.\textsuperscript{22} This effectively labels the phosphatidylinositol lipids, including PIP\textsubscript{2}, the precursor of IP\textsubscript{3}. IP\textsubscript{3} is a short-lived second messenger that is quickly broken down to inositol bisphosphate (IP\textsubscript{2}) and inositol monophosphate (IP). To be able to measure an increase in production of IP\textsubscript{3} and its breakdown products, cells were preincubated in KHB containing 50 mM LiCl for 20 minutes before initiation of the experiment to slow the hydrolysis of the inositol phosphates.\textsuperscript{25,26}

**Techniques for Inositol Triphosphate Measurements**

Two types of assays were used to measure IP\textsubscript{3}. The first involved prelabeling PIP\textsubscript{2} with tritiated myoinositol as described above. Radiolabeled IP\textsubscript{3}, IP\textsubscript{2}, and IP are generated from PIP\textsubscript{2} if receptor activation is linked to IP\textsubscript{3} as a second messenger. Measurement of the radioactivity in these soluble inositol phosphates was done using high-pressure liquid chromatography (HPLC) reflecting changes in the generation and hydrolysis of IP\textsubscript{3}.\textsuperscript{25-27} In addition, the concentration of IP\textsubscript{3} was directly measured in RPE sonicates using a commercially available radioreceptor assay kit (New England Nuclear-Du Pont, Boston, MA).

**Phagocytic Challenge and Analysis**

To test the effects of phagocytic challenge on IP\textsubscript{3} generation, cultures were incubated under one of four conditions. Control cultures received 25 μl cold medium to measure resting levels of inositol phosphates. Phagocytically challenged cultures received 25 μl rat ROS or PSL. Other cultures were incubated with carbacbol (1 mM final concentration), a muscarinic agonist used as a positive control to stimulate the IP\textsubscript{3} pathway.\textsuperscript{25,26} To initiate the experiment, the phagocytic challenge particles were settled onto the RPE surface by centrifugation of the culture plates at low speed (Sorvall GLC-2B, 3,000 rpm) for 5 minutes. Cultures were then processed immediately or incu-
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bated for 30 minutes at 37°C. Culture plates were placed on ice and cooled to 4°C. The 3H containing media was removed, and each well was extracted with 200 µl 10% perchloric acid. Four wells were pooled for analysis of isotope incorporation by HPLC. After brief sonication on ice, aliquots were taken for protein assay, and samples were centrifuged 30 minutes at 38K (Ti75 rotor, Beckman [Palo Alto, CA] L5–65 Ultracentrifuge). The supernatant was used for analysis of ³H-myoinositol incorporation into soluble inositol phosphates. HPLC separations of 600 µl samples were run on a Partisil 10-SAX column (Whatman, Clifton, NJ) and eluted using a linear ammonium phosphate/NaCl gradient.²²,²⁷ Radioactivity of the inositol phosphates was measured by a Berthold radioactivity monitor (Berthold Instruments, Nashua, NH). Radioactivity was normalized to total protein values for each sample.

For analysis of phosphatidylinositol lipids, Long Evans and RCS RPE membrane samples were normalized for protein (65 µg) before processing and analysis by thin layer chromatography. We are assuming that protein concentration is related to lipid content for these samples. Briefly, membranes were washed by resuspension in 2 M KC1 and processed for thin layer chromatography according to Rouser et al.²⁸ RPE membrane samples were extracted in acidified solvent (chloroform, methanol, HCl 248:130:2) 15 minutes on a multiple-sample vortexer at room temperature. The organic phase was collected, and the aqueous phase of each sample was reextracted for 5 minutes (chloroform, methanol, HCl 150:75:22). The combined organic extracts of each sample were evaporated under dry nitrogen gas in a heating block at 37°C to reduce the sample volume. Samples were applied as a band onto thin layer chromatography plates (0.2 mm silica gel 60, E. Merck Co., Darmstadt, Germany), and lipids were separated for 3½ hours in sealed chambers containing chloroform, methanol, ammonium hydroxide, and water (115:80:22:7). The thin layer chromatography sheets were dried, and ³H-myoinositol labeled lipids were visualized by autoradiography on Kodak AR-5 X-ray film (Eastman Kodak, Rochester, NY). PIP₂, PIP, PI, and myoinositol in separated RPE membrane samples were identified by comparison with the migration of pure phospholipid standards.

A radioreceptor assay was used to measure the amount of IP₃ in control and challenged cultures as described by the manufacturer. Briefly, RPE cultures were incubated and phagocytically challenged as described above. Each culture was immediately harvested in 200 µl 20% trichloroacetic acid. Samples were sonicated and centrifuged. The supernatant was extracted and the pH neutralized. Duplicate aliquots of the supernatants were assayed for IP₃ content and quantitated by scintillation counting. Values were determined by linear regression to a standard curve of purified IP₃ that was run simultaneously in the assay. Correlation coefficients of the curves were between 0.95 and 0.99.

RESULTS

Phagocytic challenge with ROS was associated with increased generation and hydrolysis of IP₃ in Long Evans RPE but not RCS RPE. The increase in IP₃, and especially in its degradation products IP₂ and IP, were evident in HPLC scans of Long Evans cultures incubated with ROS compared to control scans (Fig. 1A). RCS RPE challenged with ROS did not show a similar increase (Fig. 1B). HPLC scans reveal Long Evans and RCS RPE have comparable ³H-myoinositol incorporation into the inositol phosphates under control conditions. In both Long Evans and RCS strains, exposure to carbachol produced an increase in IP₃, IP₂, and IP, but in RCS relatively more IP₃ was evident. Phagocytic challenge with PSL spheres produced no detectable changes in HPLC levels of inositol phosphates compared to control in Long Evans or RCS RPE (not shown).

The steady state metabolism of inositol phosphates was not significantly different between Long Evans and RCS RPE. ³H-myoinositol incorporation into individual RPE samples, as measured by HPLC, revealed that the total area of combined inositol peaks averaged 161 ± 30 × 10⁶ dpm (mean ± SD), with a summed peak height of 3.3 ± 0.5 × 10⁶ dpm (mean ± SD). The distribution of ³H-myoinositol incorporation into the inositol phosphates of Long Evans and RCS RPE showed that the majority of radiolabel (89% in Long Evans; 86% in RCS) was incorporated into IP, with minor amounts in IP₂, IP₃, and IP₄ under control conditions (Fig. 2). As confirmed by radioreceptor assay, the average concentration of IP₃ in Long Evans RPE under control conditions (4.8 ± 2.3 pmol/100 µg protein SD), was not significantly different from that of RCS RPE under control conditions (5.01 ± 2.1 pmol/100 µg protein).

Because of the rapid hydrolysis of IP₃ and the ease in detecting the breakdown products IP₂ and IP, HPLC of total radiolabeled inositol phosphates was used to determine the effect of experimental conditions on IP₃ generation. Both ROS and carbachol produced increased appearance of ³H-myoinositol in the inositol phosphates of Long Evans RPE as analyzed by HPLC (Fig. 3), indicating an increase in the generation and hydrolysis of IP₃ compared to control. In RCS RPE, only carbachol treatment was associated with an increase in ³H-myoinositol incorporation, whereas PSL produced no effect in either Long Evans or RCS RPE (Fig. 3).
**FIGURE 1.** Representative HPLC scans of the radioactive inositol phosphates from Long Evans (A) and RCS (B) RPE cells. A preparation of purified standards is shown at the lower right to illustrate the 30-minute elution profile for myoinositol (myo), two isoforms of IP, IP2, IP3 (shown as a filled peak), and IP4 along the x-axis. The RPE traces are plotted on a reduced scale in the y-axis, with radioactivity levels ranging from 0 to 850 disintegrations per minute, to highlight the changes observed in IP3. No differences were observed between Long Evans and RCS inositol phosphates under control conditions. Both Long Evans and RCS showed increased levels of radioactivity in the inositol phosphates after carbachol treatment. Phagocytic challenge with ROS was associated with an increase in the areas and peak heights of inositol phosphates in Long Evans but not in RCS RPE.

Direct measurement of IP3 by radioreceptor assay supports the HPLC data, showing that ROS induce an increase in IP3 in Long Evans and that carbachol is associated with an increase in IP3 in both Long Evans and RCS (Fig. 4).

The possibility remained that the lack of IP3 generation in RCS RPE in response to ROS could be due to a deficiency of PIP2, the lipid precursor of IP3. Thin layer chromatography showed that under control conditions no differences were observed between Long Evans and RCS in 3H-myoinositol incorporation into the inositol-containing phospholipids (Fig. 5). In some Long Evans RPE samples, ROS challenge is associated with a decrease in PIP2 alone. In other Long Evans samples, such as the one shown, the reduction of all labeled phosphatidylinositol lipids is evident (Fig. 5). Analysis of RCS RPE shows that no decrease in levels of PIP2 or other phosphatidylinositol lipids was observed in cultures phagocytically challenged with ROS (Fig. 5).

**FIGURE 2.** Control levels of inositol phosphates in Long Evans and RCS RPE. No differences in the control levels of 3H-myoinositol incorporation into IP, IP2, IP3, and IP4 were observed between Long Evans (solid bars) and RCS (shaded bars). The incorporation into individual inositol phosphate species was calculated as a percentage of the total combined HPLC peak areas. The values for the total areas averaged 1.6 × 10^8 dpm per sample in these experiments. The bars represent the mean and standard deviation obtained from three experiments.

**DISCUSSION**

The results of these studies show that phagocytic challenge with ROS is associated with an increase in the second messenger, IP3, and a decrease in its precursor molecule, PIP2, in normal Long Evans RPE. IP3 has been shown to function as a second messenger after activation of a number of receptors17,18 and has been observed to increase during phagocytosis in neutrophils.29 Our current results suggest that in normal Long Evans RPE, an ROS-specific receptor at the apical surface of the RPE is coupled to the activation of a phospholipase that hydrolyses PIP2 in the RPE plasma membrane to generate diacylglycerol and IP3. Typically, diacylglycerol remains associated with the membrane and may be associated with protein kinase C activation. IP3 is liberated as a soluble message and may be responsible for mobilization of calcium into the cytosol to induce activation of calcium-dependent kinases. Although the identity of the ROS receptor is not known, we do know that this interaction leads to the ROS-specific activation of protein kinase C as well as other RPE kinases and phosphatases, leading to changes in the phosphorylation of specific proteins.15,16,21 One of these proteins is the 80 kD substrate of protein kinase C, providing indirect evidence that diacylglycerol production may be associated with ROS phagocytosis.21 It remains to be determined whether other ROS-specific changes in phosphorylation are related to activation of a calcium-dependent kinase through generation of IP3. The specific roles of diacylglycerol and IP3 as second messengers in ROS phagocytosis remain to be defined.

A significant increase in ROS-induced generation of IP3 was not observed in dystrophic RCS RPE. We examined whether a reduction of the PIP2 precursor was responsible for the lack of IP3 in RPE cells because RCS photoreceptor cells have been reported to have a reduced capacity for phosphatidylinositol synthesis.30,31 Autoradiograms from our experiments showed that the levels of PI, PIP, and PIP2 in RCS RPE were comparable to normal under control conditions. Our results agree with a previous study showing no differences between the level of phosphatidylinositol in
FIGURE 3. 3H myoinositol incorporation into total inositol phosphates. Inositol peak heights of HPLC traces were summed for control samples and for experimental samples. Data expressed as the ratio of experimental to control values ± SD shows an increase in total inositol phosphates is associated with ROS and carbachol treatment in Long Evans RPE (solid bars). Only carbachol treatment was effective in inducing an increase in inositol phosphates in RCS RPE (shaded bars).

Long Evans and RCS RPE membranes\textsuperscript{32} and suggest that phosphatidylinositol lipid metabolism is normal for the major species in RCS RPE. Some minor, additional radiolabeled lipids were observed in RCS samples, and the significance of these labeled species is unclear. They may reflect changes in lipid metabolism or labeling efficiency in RCS and require further study.

As would be expected from the lack of IP\textsubscript{3} generation in RCS RPE under phagocytic challenge, there

FIGURE 4. IP\textsubscript{3} concentration measured by radioreceptor assay in RPE cultures. A significant increase in production of IP\textsubscript{3} in Long Evans RPE was associated with incubation with ROS (P < 0.01) or with carbachol (P < 0.02), as analyzed using Student's t-test (asterisks). There was a significant increase in IP\textsubscript{3} generated in RCS RPE by treatment with carbachol (P < 0.01) (asterisk). No other condition produced significant changes compared to control values. The control level of IP\textsubscript{3} was assigned as 100% for each experiment, and the experimental conditions are expressed as a percentage of the control for this combined representation of four experiments.

FIGURE 5. ROS-induced changes in RPE phosphatidylinositol lipids. (A) Thin layer chromatography autoradiograms of phosphatidylinositol lipids from LE RPE show the relative positions of labeled myoinositol (myo), phosphatidylinositol (PI), phosphatidylinositol phosphate (PIP) and phosphatidylinositol bisphosphate (PIP\textsubscript{2}), the precursor of IP\textsubscript{3}. Phagocytic challenge of Long Evans RPE with PSL produces no changes in these phosphatidylinositol lipids. In Long Evans RPE, a decrease in PIP and PIP\textsubscript{2} is associated with phagocytic challenge with ROS compared to control values. (B) In some Long Evans RPE cultures (left), phagocytic challenge with ROS can be associated with a decrease in all phosphatidylinositol lipids. In RCS RPE (right), no decrease in levels of phosphatidylinositol lipids was observed in cultures challenged with ROS compared to cultures under control conditions.
was no decrease observed in the amount of precursor lipid PIP$_2$ after incubation with ROS. Although a change was never observed in RCS RPE, in Long Evans RPE the change in phosphatidylinositol lipids was variable. Most often, a reduction in PIP$_2$ alone was seen. In some RPE samples, a reduction was observed across all phosphatidylinositol lipids induced by ROS. This may indicate that as PIP$_2$ is hydrolyzed to IP$_3$, the cell tries to replace it through the addition of phosphates to the lower species, PIP and PI, therefore reducing the apparent amount of all three. Pulse-chase studies could clarify whether this pathway is used under conditions of very high PIP$_2$ hydrolysis.

RCS inositol phosphates were shown to elute at the same gradient location as purified standards and have the same distribution as in normal RPE. These results suggest that under control conditions, the synthesis and processing of inositol phosphates are normal in RCS RPE. An occasional sample did show some increase in IP$_3$ that is reflected in the larger standard deviation observed in the data from RCS RPE. This variability may be related to the occasional ingestion of bound ROS, which has been observed.

Whereas RPE cells are capable of engulfing a wide range of particles, there is no evidence that a cell surface receptor is involved in the phagocytosis of nonspecific particles such as PSL. Thus, as might be expected, the phagocytic challenge with PSL produced no change in IP$_3$ or PIP$_2$ in either Long Evans or RCS RPE. This useful negative control illustrates that nonspecific phagocytosis occurs without an increase in IP$_3$. Even though RCS RPE is able to ingest normal numbers of nonspecific particles, it remains a mystery as to why ROS are not ingested using this nonspecific mechanism. Perhaps because RCS RPE binds ROS normally, they may be held in a way that prevents ingestion via nonspecific mechanisms.

Muscarinic receptors in cultured human RPE have been identified, and treatment with the muscarinic agonist carbachol provides clear evidence that these receptors are coupled to phosphoinositide turnover. As might be expected from the typical IP$_3$ pathway, activation of muscarinic receptors results in the phagocytic defect in RCS RPE. One possible site is the cytoplasmic domain of the receptor molecule, which may have altered interaction with the phospholipase responsible for PIP$_2$ hydrolysis and generation of IP$_3$. Another possibility is the altered function of a G protein, if one plays a role, in transmembrane signaling during ROS phagocytosis. In RCS RPE, the generation of IP$_3$ in response to carbachol treatment shows that the muscarinic receptors on the RPE surface and the downstream enzyme cascade, including the phospholipase, are functional. However, because we do not know if the same phospholipase is involved in the transmembrane signaling associated with ROS phagocytosis, this enzyme remains another potential site for the defect.

**Key Words**

retinal pigment epithelium, Royal College of Surgeons rat, phagocytosis, rod outer segments, inositol triphosphate

**References**


