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cAMP Production via the Adenylyl Cyclase Pathway is Reduced in RCS Rat RPE

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cAMP production was investigated in retinal pigment epithelium (RPE) cells isolated from normal rats and from rats with an inherited retinal dystrophy (Rdy/p$^+$). In normal RPE cells, 5'-[*N*-Ethylcarboxamido]-adenosine (A2 receptors) produced a fivefold increase in the level of cyclic adenosine monophosphate (cAMP) over basal levels. However, only a onefold increase in cAMP was observed in dystrophic cells. cAMP production by prostaglandins E1 and E2 (prostaglandin receptors) in dystrophic RPE cells was only 29–38% of the level observed in normal cells. Direct stimulation of adenylyl cyclase by 10 μmol/l forskolin increased cAMP levels in normal RPE cells by 90 fold over basal, but only by sixfold in the dystrophic cells. These data suggest there may be a defect in the adenylyl cyclase signaling pathway in dystrophic RPE cells. Invest Ophthalmol Vis Sci 33:3121–3124, 1992

The Royal College of Surgeons (RCS) strain of rat exhibits an autosomal recessive mutation manifested as a degeneration of the retina.[1] One of the functions of the retinal pigment epithelium (RPE) is to phagocytize shed photoreceptor outer segments (OS).[2] However, in the diseased eye, OS fail to be ingested and a large accumulation of shed OS debris is observed.[3,4] Chimeric rat studies showed that the genetic lesion was expressed in the RPE,[5] and later it was demonstrated that the ingestion phase of OS phagocytosis was defective.[6] Kinetic studies in normal RPE supported the hypothesis that OS phagocytosis is mediated by specific cell surface receptors[7] that are probably glycoprotein in nature.[8] Because the binding of OS to the surface of dystrophic RPE is normal,[6] it has been suggested there could be a transmembrane signaling defect in the RPE of dystrophic rats,[7] in the ROS receptor itself or in the second messenger system coupled to the receptor.[8] We are evaluating the cyclic adenosine monophosphate (cAMP) intracellular signaling pathway as a potential regulator of OS phagocytosis because there is evidence that elevated cAMP levels inhibit OS phagocytosis.[9,10] In the present study, we investigated the stimulation of cAMP production, via the stimulatory guanine nucleotide binding protein (Gs) linked to adenylyl cyclase (AC), and by direct stimulation of the enzyme.

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Materials and Methods

All experiments conformed to the ARVO Resolution on the Use of Animals in Research. RPE cells from 9 to 11-day-old normal (Long Evans) and dystrophic RCS rats (Rdy/p$^+$)[11] were isolated and cultured according to published methods.[12] Primary cultures of RPE cells were seeded onto 18 mm glass discs in growth medium (Earle's minimal essential medium [MEM] containing 10% fetal bovine serum, 40 μg/ml^{-1} kanamycin, 40 μg/ml gentamycin, and 2 mmol/l L-glutamine) and used when confluent after 7 days.

All drugs and chemicals were obtained from Sigma (St. Louis, MO) unless otherwise noted. Dose-response or time-response analyses for the production of cAMP were carried out with the following drugs: 5'-[*N*-Ethylcarboxamido]-adenosine (NECA), prostaglandins E1 (PGE1) and E2 (PGE2), and forskolin (FSK). NECA was dissolved in dimethyl sulfoxide and the prostaglandins and FSK were dissolved in 100% ethanol, before serial dilutions of these drugs were made in MEM to the appropriate concentrations. Control discs contained the same concentration of solvent.

After drug treatment, the RPE cells were washed 2 × 5 sec in phosphate-buffered saline (10 mmol/l sodium phosphate and 145 mmol/l NaCl, pH 7.2) containing 1.27 mmol/l $CaCl_2$ and 0.81 mmol/l $MgSO_4$. The cells then were scraped off the discs into microfuge homogenization tubes (Kontes, Vineland, NJ), which contained 100 μl of ice-cold 0.1 N HCl. Samples were homogenized for 10 sec at 3500 rpm, and

duplicate 10 μl aliquots were taken for protein assay (BCA protein assay kit; Pierce, Rockford, IL). Cellular debris was pelleted and the supernatant was removed to fresh tubes for cAMP determinations using the Rianen cAMP[^{125}I] radioimmunoassay kit (NEN-DuPont, Billerica, MA), according to the acetylated protocol kit instructions.

Results

To investigate receptor-mediated activation of AC in RPE cells, we studied a number of receptors known to be coupled to this enzyme via Gs. Activation of A_2 adenosine receptors in RPE cells by 10 μmol/l NECA is known to stimulate AC.[13] Figure 1 shows the time-response curve for cAMP stimulation by NECA over 30 min. In normal RPE cells, the cAMP levels were raised fivefold within the first 5 min of incubation of the cells with the drug. After 15 min of incubation, cAMP levels were reduced to a level that was 50% of the maximum response. This remained constant to the 30 min time point. It appeared that after the initial stimulation of cAMP production, there was no further receptor-stimulated activation of AC to maximal levels, despite the constant presence of the drug. We also show, in Figure 1, that there was only a small cAMP increase (onefold) by the dystrophic RPE cells in response to 10 μmol/l NECA over the same time period. This striking difference suggests there may be

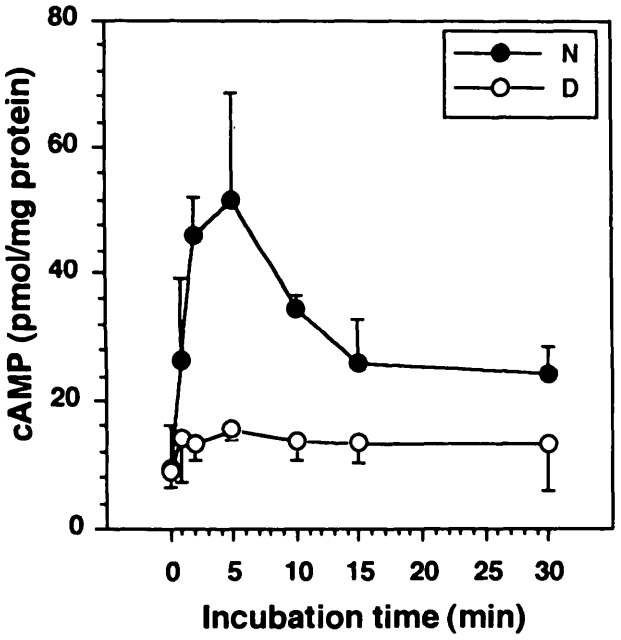

Fig. 1. Time course of cAMP production in normal (N) and dystrophic (D) RPE cells in the presence of 10 μmol/l 5'-[*N*-ethylcarboxamido]-adenosine. RPE cells isolated from age-matched rats were cultured for 7 days. cAMP was measured by radioimmunoassay. Each point is the mean of four determinations ± standard deviation.

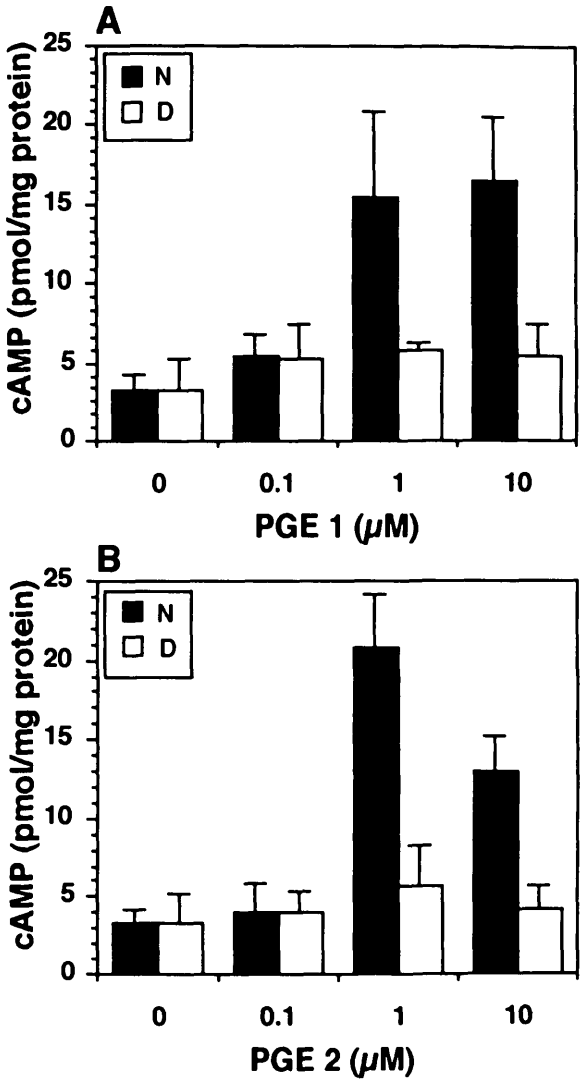

Fig. 2. cAMP production in normal (N) and dystrophic (D) RPE cells stimulated by PGE1 and PGE2. Cultured RPE cells isolated from age-matched rats were incubated with increasing concentrations of prostaglandins for 5 min. cAMP was measured using a radioimmunoassay protocol. Bars represent the mean of four determinations ± SD.

a defect in the pathway associated with cAMP production when stimulated by NECA.

Because we did not know whether the reduced response to NECA in dystrophic RPE cells was the result of a deficiency of A_2 receptors themselves or whether it was the result of a defect in the intracellular signaling pathway, we tried to repeat the above observation using another receptor known to be present in RPE cells. It recently was shown that human RPE cells possess prostaglandin receptors coupled to AC via Gs.[14] Thus, we carried out dose-response curves to PGE1 and PGE2 in normal and dystrophic RPE cells (Fig. 2). After a 5 min incubation of RPE cells with 0.1, 1.0, or 10 μmol/l PGE1 or PGE2, we observed no significant increase in the level of cAMP in dystrophic RPE cells, whereas cAMP levels in normal RPE cells

increased three to five times above basal levels when stimulated by 1 or 10 μmol/l PGE1 and PGE2 (Fig. 2). This experiment confirmed that the lack of response of the dystrophic RPE cells to NECA probably was not the result of a specific A_2 receptor-associated defect. Rather, the lack of cAMP production probably was the result of a defect in an intracellular step in the pathway.

We evaluated the integrity of the AC enzyme by direct stimulation using FSK. Normal and dystrophic RPE cells were incubated for 30 min with 10 μmol/l FSK, after which cAMP levels in the cells were determined (Table 1). In each experiment, the levels of cAMP in the FSK-treated dystrophic RPE cells ranged between 5 and 8% of the levels in FSK-treated normal cells. The dose-response curve to FSK after a 30 min incubation shows that in normal RPE cells, the AC is very active in the presence of 10 μmol/l FSK (Fig. 3). We observed a 90-fold increase in cAMP over the resting level in normal RPE cells. However, in the RCS cells, we observed only a sixfold increase in cAMP levels. With concentrations of FSK up to 1 μmol/l, cAMP production in normal and dystrophic RPE cells did not differ significantly (inset of Fig. 3). We did observe a small increase in the cAMP level using 1 μmol/l FSK in normal RPE cells, at which concentration there was already less production of cAMP in the dystrophic cells (inset of Fig. 3). The increase in cAMP production by 10 μmol/l FSK in the dystrophic RPE cells was only 8% of that observed in the normal cells. Because we observed a stimulation of cAMP in the dystrophic RPE cells, some of the AC enzyme must be present or partially active.

Discussion

It has been postulated that the phenotype of dystrophic rat RPE cells occurs because bound OS do not initiate a transmembrane signal, thereby preventing active ingestion of OS and subsequent digestion by RPE cells.[7] This could be the result of a defect in the receptor to which the OS bind or the result of a defect in the second messenger system coupled to the receptor.[8] Recently, the phospholipase C-phosphatidyl inositol (PI) intracellular signaling pathway was investigated as a potential activator of OS ingestion via protein kinase C (PKC).[15] The authors found that activation of PKC, and increased intracellular calcium, rapidly turned off ingestion, indicating that receptors for OS probably were not linked to the PI pathway.

Table 1. Stimulation of cAMP production in normal and dystrophic RPE cells by 10 μmol/l forskolin (FSK)

Experiment no.	*cAMP (N) (pmol/mg protein)*	*cAMP (D) (pmol/mg protein)*	*D/N ratio (%)*
1	1936 ± 392	124 ± 0.6	7%
2	2600 ± 278	124 ± 2.9	5%
3	742 ± 310	58 ± 21	8%
Average*	1759 ± 941	108 ± 43	6.6% ± 1.5

Cultured RPE cells from age-matched rats were incubated with 10 μmol/l FSK for 30 min. cAMP was measured by radioimmunoassay. Each experimental group consisted of 2 discs of RPE cells. Duplicate cAMP measurements were made for each disc, totaling four determinations ± standard deviation. * The mean of the three experimental groups representing a total of 12 cAMP determinations ± SD. N, normal RPE cells. D, dystrophic RPE cells.

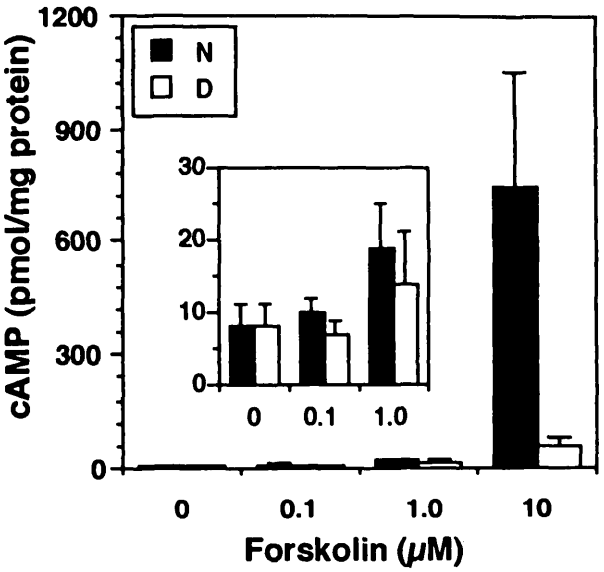

Fig. 3. Stimulation of cAMP production in normal (N) and dystrophic (D) RPE cells by forskolin, (FSK). Cultured RPE cells from age-matched rats were incubated with increasing concentrations of FSK for 30 min. cAMP levels were determined by radioimmunoassay. Bars represent the mean of six determinations ± SD. Inset shows an enlarged view of the response to low concentrations of FSK.

The goal of the present study was to determine the integrity of the AC signaling pathway in dystrophic RPE cells. Studies of cAMP production by NECA through A_2 adenosine receptors showed that dystrophic RPE cells resisted stimulation by NECA. This result could be explained by a number of possibilities:

1. A reduced number or a defect in the A_2 receptors in the dystrophic RPE. Our results do not support this possibility because we observed the same differential cAMP production between normal and dystrophic RPE cells, when cells were stimulated by PGE1 and PGE2 through prostaglandin receptors. This supports the view that decreased cAMP production in dystrophic RPE cells is not due to a specific A_2 receptor defect, but may be common to all receptors coupled to AC via Gs.
2. An overactive cAMP-phosphodiesterase (PDE) that would result in an apparently decreased level of cAMP upon activation of cAMP production. Although our studies did not address this possibil-

ity, the cAMP-PDE in RCS rat RPE has been studied in depth.[16] The authors of that study found there was no difference in the kinetic parameters (K_m) of cAMP-PDE in dystrophic RPE cells compared to normals.

3. An abnormality in Gs coupling to AC. Our FSK data could support this possibility. Maximal stimulation of AC in intact cells by FSK requires the coupling of Gs to the catalytic subunit of the enzyme.[17] In cyc^- S49 lymphoma cells, in which the α_s subunit of Gs is defective, only a twofold stimulation of cAMP is observed with FSK, compared to a 50-fold response in wild type cells.[18] We could conclude that Gs was defective in dystrophic RPE only if we were certain that the AC was active and present at normal levels in these cells.
4. There may be reduced levels of AC or a defect in AC. Our results with FSK clearly show that we are able to activate AC to a limited extent in dystrophic RPE. However, our results do not indicate whether this is due to a decrease in the level of AC or whether there is some loss of integrity of the enzyme. To conclude that AC is defective in dystrophic RPE cells requires confirmation that the Gs coupling is normal. A defect in AC has been reported in the *Drosophila* mutant *rutabaga*,[19] in which there is a lower V_{max} for the enzyme.

We are investigating the nature of this defect in dystrophic RPE cells at the level of Gs and adenylyl cyclase. Although the physiologic defect in the RPE has been known for a number of years, we believe this is the first time that a biochemical defect has been shown for these dystrophic RPE cells. Whether this defect in the adenylyl cyclase pathway is responsible for the etiology of retinal degeneration in the RCS rat awaits further investigation.

Key words: adenylyl cyclase, cAMP, receptors, retinal degeneration, retinal pigment epithelium

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