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Cyclic 3′, 5′-Guanosine Monophosphate Synthesis Induced by Atrial Natriuretic Peptide, C-Type Natriuretic Peptide, and Nitric Oxide in the Rat Retina

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Abstract: This study was undertaken to determine whether pathways exist in the rat retina for atrial natriuretic peptide (ANP)-, C-type natriuretic peptide (CNP)-, and nitric oxide (NO)- cyclic 3′, 5′-guanosine monophosphate (cGMP). Exposure of the retina to ANP (10^{-7} mol/L), CNP (10^{-7} mol/L), S-nitroso-N-acetylpenicillamine (10^{-5} mol/L, SNAP; a NO donor), A23187 (10^{-5} mol/L; a Ca^{2+} ionophore), and carbachol (10^{-3} mol/L) caused 1.45 $\sim$ 1.67-fold increases in cGMP content ($P < .01$). The increase in cGMP content induced by A23187 was blocked by 2-4-carboxyphenyl · 4455-tetramethyl imidazoline 1-oxyl 3-oxide (10^{-3} mol/L, carboxy-PTIO; a NO scavenger). Both carboxy-PTIO (10^{-3} mol/L) and N^G-nitro-L-arginine (10^{-3} mol/L, L-NNA; a NO synthase inhibitor) blocked the increase in cGMP content induced by carbachol. Atropine (10^{-5} mol/L; a muscarinic receptor antagonist) also blocked the cGMP increase induced by carbachol. These data demonstrate that ANP-, CNP-, and NO-cGMP pathways exist in the rat retina and that the NO-cGMP pathway may be linked to the activation of the muscarinic receptor. **Jpn J Ophthalmol 1998;42:269–274** © 1998 Japanese Ophthalmological Society

Key Words: Atrial natriuretic peptide (ANP), C-type natriuretic peptide (CNP), cyclic 3′, 5′-guanosine monophosphate (cGMP), nitric oxide (NO), rat retina, retinal cGMP pathways.

Introduction

Cyclic 3′, 5′-guanosine monophosphate (cGMP) is a common second messenger produced in most mammalian tissues. The spectrum of cGMP-regulated events includes renal and intestinal ion transport, platelet aggregation, contraction of cardiac and smooth muscles caused by calcium movements, and regulation of the central nervous system.[1] Cyclic 3′, 5′-guanosine monophosphate is synthesized by guanylyl cyclases (GCs), which are divided into two types based on their general structure and cellular distribution. One is the membrane-associated type called particulate GC, which contains subtypes: GC-A, GC-B, GC-C, and retinal GC (retGC). The other is the cytosolic type called soluble GC. Guanylyl cyclase-A is selectively activated by atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP), GC-B is activated by C-type natriuretic peptide (CNP), and GC-C is activated by heat-stable entrotoxins and guanylin. Retinal GC is found associated with the particulate fractions of mammalian retina. Retinal GC activity is regulated by retinal cyclase regulators in a Ca^{2+}-dependent manner.[2–4] Soluble GC is activated by nitric oxide (NO).[1,5–8]

Studies of the retina to date have concentrated on the regulation of cGMP production by retGC and the regulation of the Na^+ channel by cGMP in visual transduction, most of which occurs in the rod outer segment of photoreceptor cells.[2–4] On the other hand, it is well known that intracellular cGMP levels participate in the regulation of various functions in the central nervous system.[1,7,9–12] Especially, NO-cGMP pathways play major roles in long-term po-

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tentiation and depression, and in typical models of neuronal information processing, such as memory and learning, which occur in the hippocampus and cerebellum, respectively.[13,14]

There have been few reports concerning the cGMP production systems of other GCs in the retina. Therefore, this study was undertaken to determine whether ANP, CNP, and the reagents related to NO cause cGMP production and to confirm the existence of ANP-, CNP-, and NO-cGMP pathways in the rat retina, especially the NO-cGMP pathway that enhances cGMP production.

Materials and Methods

Animals

The animals used, male Wistar rats obtained from SLC Inc. (Shizuoka), were handled in accordance with the Guidelines for Animal Experiments in the Faculty of Medicine, Tottori University. They were housed individually in plastic cages, with free access to food and water, under room conditions of 23°C and 50% humidity with 12 hours of room light (average 500 lux; 7 AM–7 PM) alternating with 12 hours of darkness. The rats were sacrificed by decapitation at 8 weeks of age under room light after 3 to 4 hours of light cycle exposure.

Tissue Preparation, Incubation, and cGMP Assay

After enucleation, an incision was made at the margin of the ora serrata to remove the lens, vitreous, and ciliary body. The retina was then removed.

Each detached retina was incubated for 60 minutes at 37°C in 180 μL of a Krebs Ringer buffer (KRB, composition in mmol/L: NaCl 135, KCl 4.8, $NaHCO_3$ 25.0, glucose 10.0, and HEPES 12.5; pH 7.5) previously equilibrated with O_2/CO_2 (95:5). Each retina was further incubated for 0.5, 1, 2, or 5 minutes, in 180 μL of fresh KRB containing one of the following agonists: ANP (10^{-7} mol/L), CNP (10^{-7} mol/L), S-nitroso-N-acetylpenicillamine (10^{-5} mol/L, SNAP; an NO donor), A23187 (10^{-5} mol/L; a Ca^{2+} ionophore), N-methyl-D-aspartate (10^{-3} mol/L, NMDA), or carbachol (10^{-3} mol/L). After the tissues were stimulated with the agonists indicated, the reactions were terminated by mincing in 20 μL of trichloroacetic acid (final 5%).

In our inhibitory experiments to determine the increase in cGMP by the agonists related to NO, the tissues were preincubated with 2-4-carboxyphenyl · 4455-tetramethyl imidazoline 1-oxyl 3-oxide (10^{-3} mol/L, carboxy-PTIO; a NO scavenger), N^G-nitro-L-arginine (10^{-3} mol/L, L-NNA; a NO synthase inhibitor), and atropine (10^{-5} mol/L; a muscarinic receptor antagonist) for 10 minutes before the addition of the agonists. Cyclic 3′, 5′-guanosine-monophosphate levels in the tissues were determined in duplicate by using the Amersham International EIA Kit (Amersham, Arlington Heights, IL, USA).[15] The protein content was measured in duplicate by the method of Lowry and colleagues,[16] using bovine serum albumin as the standard.

Reagents

Cyclic 3′,5′-guanosine monophosphate EIA Kits[15] were purchased from Amersham. Atrial natriuretic peptide and CNP were obtained from Peptide Institute (Osaka). S-nitroso-N-acetylpenicillamine and carboxy-PTIO were purchased from Dojindo Laboratories (Kumamoto). N^G-nitro-L-arginine was obtained from Funakoshi (Tokyo). All other chemicals were from Wako Pure Chemicals (Osaka). The concentration of each reagent was determined according to previous studies,[5,7,10–12] to achieve a maximal reaction on the whole retina.

Statistical Analysis

All results were expressed as means ± standard error of the mean (SEM). For multiple comparison, an analysis of variance (ANOVA, Kruskal-Wallis test) technique was used and the data obtained were assessed by Scheffe's *F*-test. Values of $P < .05$ were considered significant.

Results

Effects of ANP, CNP, and SNAP on Increase in cGMP Content in Rat Retina

Figure 1 shows the time course of cGMP levels in the rat retina in response to ANP (10^{-7} mol/L), CNP (10^{-7} mol/L), and SNAP (10^{-5} mol/L). Exposure of the retina to ANP (10^{-7} mol/L) caused maximally a 1.45-fold increase in cGMP content ($P < .01$) after 2 minutes of incubation, compared with the basal value (5.74 ± 0.16 pmol/mg protein, $n = 5$). Thereafter, the cGMP level gradually decreased, but remained significantly increased ($P < .01$) for the rest of the 5-minute period of incubation. Exposure of the retina to CNP (10^{-7} mol/L) caused maximally a 1.59-fold increase in cGMP content ($P < .01$) after 2 minutes of incubation, compared with the base

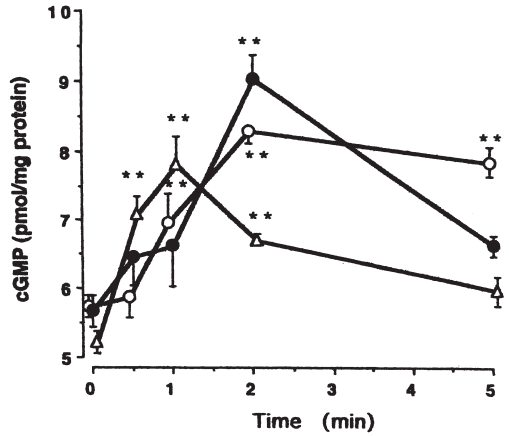

Figure 1. Time course of cGMP levels in response to ANP (10^{-7} mol/L), CNP (10^{-7} mol/L), and SNAP (10^{-5} mol/L) in rat retina. ○: ANP, ●: CNP, △: SNAP. Data are means ± SEM of separate determinations (n = 4–6). $^{**}P < .01$ (compared with each basal value at 0 minute).

value (5.68 ± 0.23 pmol/mg protein, n = 5). The cGMP level showed a gradual decrease for the rest of the 5-minute period of incubation without significant increase ($P > .05$). Exposure of the retina to SNAP (10^{-5} mol/L) caused maximally a 1.50-fold increase in cGMP content ($P > .01$) after 1 minute of incubation, compared with the basal value (5.23 ± 0.16 pmol/mg protein, n = 5). Thereafter, the cGMP level showed a gradual decrease, but remained significantly increased ($P < .01$) during the first 2-minute period of incubation. During the remainder of the 5-minute period of incubation, the cGMP level was not significantly increased.

Effects of A23187 (Ca^{2+} Ionophore) on Increase in cGMP Content in Rat Retina and its Involvement in NO Production

Figure 2 shows the time course of the cGMP level in the rat retina in response to A23187 (10^{-5} mol/L). It also shows the inhibitory effect of carboxy-PTIO (10^{-3} mol/L) on the increase in cGMP content induced by A23187 (exposure time to A23187: 0.5 minutes). Exposure of the retina to A23187 (10^{-5} mol/L) caused a 1.53-fold increase in cGMP content ($P < 0.01$) after 0.5 minutes of incubation, compared with the basal value (6.39 ± 0.38 pmol/mg protein, n = 5). Thereafter, the cGMP level showed a gradual decrease, but remained significantly increased ($P < .01$) for the rest of the 5-minute period of incubation. The increase in cGMP content at 0.5 minutes was significantly inhibited by carboxy-PTIO (10^{-3} mol/L; a NO scavenger) ($P < .01$).

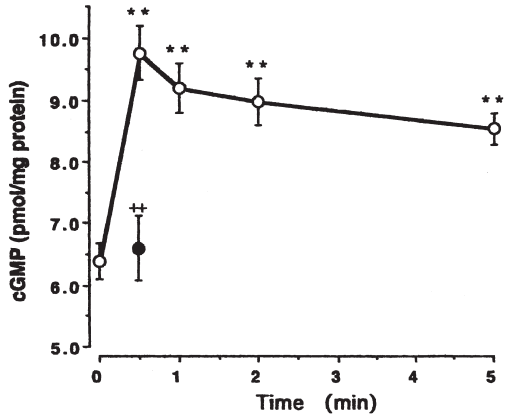

Figure 2. Time course of cGMP level in response to A23187 (10^{-5} mol/L) and inhibitory effect of carboxy-PTIO (10^{-3} mol/L) on increase in cGMP content induced by A23187 in rat retina. ○: A23187, ●: A23187 + carboxy-PTIO. Preincubation time with carboxy-PTIO: 10 minutes. Data are means ± SEM of separate determinations (n = 5). $^{**}P < .01$ (compared with basal value of A23187 at 0 minute). $^{++}P < .01$ (compared with response data to A23187 at 0.5 minutes).

Effects of NMDA and Carbachol on Increase in cGMP Content in Rat Retina

Figure 3 shows the time course of cGMP levels in the rat retina in response to NMDA (10^{-3} mol/L) and carbachol (10^{-3} mol/L). N-methyl-D-aspartate (10^{-3} mol/L) failed to increase the cGMP level, but carbachol (10^{-3} mol/L) stimulated cGMP production in a time-dependent manner. Exposure of the retina to carbachol (10^{-3} mol/L) caused maximally a 1.67-

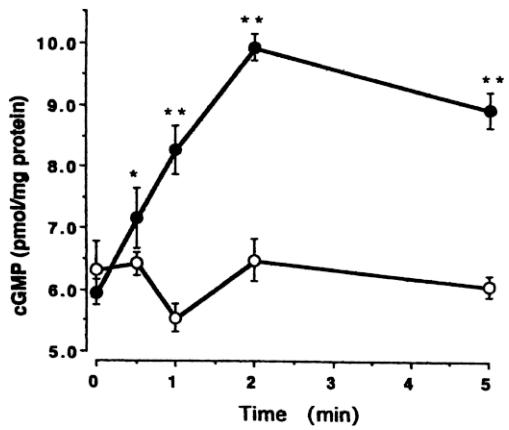

Figure 3. Time course of cGMP levels in response to NMDA (10^{-3} mol/L) and carbachol (10^{-3} mol/L) in rat retina. ○: NMDA, ●: carbachol. Data are means ± SEM of separate determinations (n = 5). $^{*}P < .05$, $^{**}P < .01$ (compared with basal value of carbachol at 0 minute).

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fold increase in cGMP content ($P < .01$) after 2 minutes of incubation, compared with the basal value (5.96 ± 0.20 pmol/mg protein, $n = 5$). Thereafter, the cGMP level showed a gradual decrease, but remained significantly increased ($P < .01$) for the rest of the 5-minute period of incubation.

Involvement of NO in Carbachol-Stimulated cGMP Increase in Rat Retina

Figure 4 shows the inhibitory effects of carboxy-PTIO (10^{-3} mol/L), L-NNA (10^{-3} mol/L), and atropine (10^{-5} mol/L) on the increase in cGMP content in the rat retina induced by a 2-minute exposure to carbachol (10^{-3} mol/L). Both carboxy-PTIO (10^{-3} mol/L) and L-NNA (10^{-3} mol/L; a NO synthase inhibitor) significantly blocked the increase in cGMP content induced by carbachol ($P < .01$). Atropine (10^{-5} mol/L; a muscarine receptor antagonist) also significantly blocked the cGMP increase induced by carbachol ($P < .01$).

Discussion

This study was undertaken to determine whether ANP, CNP, and the reagents related to NO cause cGMP production and to confirm the existence of ANP-, CNP-, and NO-cGMP pathways in the rat retina. First of all, we exposed the whole retina to ANP, CNP, and SNAP (a NO donor) and observed their effects on cGMP levels. Atrial natriuretic peptide (10^{-7} mol/L), CNP (10^{-7} mol/L), and SNAP (10^{-5} mol/L) produced increases in cGMP levels. These data demonstrate that ANP-, CNP-, and NO-cGMP pathways exist in the rat retina, in addition to the well-known retGC-cGMP pathway.[2–4] Combining our results with the other reports on GC,[1,4–8,17–19] we can suggest that the NO-cGMP pathway exists dominantly in the inner nuclear layer and the ANP-cGMP pathway exists dominantly in the rod photoreceptor in the rat retina. Because there has been no histological study about GC-B, the present results could not indicate the location of the CNP-cGMP pathway, although we proved the existence of the CNP-receptor (GC-B) in the rat retina.

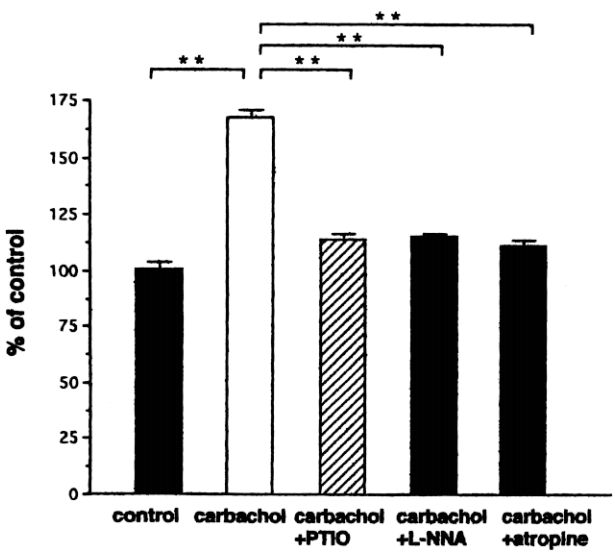

Figure 4. Inhibitory effects of carboxy-PTIO (10^{-3} mol/L), L-NNA (10^{-3} mol/L) and atropine (10^{-5} mol/L) on the increase in cGMP content induced by carbachol (10^{-3} mol/L) in rat retina. Exposure time to carbachol: 2 minutes. Preincubation time with carboxy-PTIO, L-NNA, and atropine: 10 minutes. Data are means ± SEM of separate determinations ($n = 5$). Control: cGMP content before inhibitory experiment. $^{**}P < .01$.

Nitric oxide can be produced in cells by the action of an enzyme, NO synthase. It plays multiple important roles, including the relaxation of smooth muscles, the inhibition of platelet aggregation, the inhibition of macrophage and leukocytic action, and neurotransmission. As regards the central nervous system, there have been many reports showing that in the rat cerebellum, glutamate stimulates the NMDA receptor in generator cells and the activation of the receptor results in the increase of intracellular Ca^{2+}, which activates constitutive NO synthase. NO synthase produces NO and L-citrulline from L-arginine, and NO diffuses freely to target cells, where it activates soluble GC, causing increased production of cGMP.[9–11]

There have been few reports about the production of NO in the retina, and what stimulation causes NO production has not yet been clarified. Therefore, referring to studies of NO in the rat cerebellum,[9–11] we researched the following: exposure of the rat retina to A23187 (10^{-5} mol/L), which produces a large influx of Ca^{2+}, caused significant increases in cGMP levels compared with the basal value. Carboxy-PTIO (10^{-3} mol/L) significantly blocked this increase in cGMP content induced by A23187. Our findings suggests that in the rat retina there is a NO-cGMP pathway where the increases in intracellular Ca^{2+} activate NO production and cause increased production of cGMP, as occurs in the rat cerebellum and other tissues.[9–11]

Furthermore, we investigated whether NMDA increases cGMP levels in the rat retina as it does in the rat cerebellum, but NMDA (10^{-3} mol/L) failed to increase cGMP levels in the rat retina. Then, after referring to the report that carbachol caused increases in cGMP levels in rat cortical primary cultures,[12] we

used carbachol to determine its effect on the increase in cGMP content in the rat retina. Exposure of the retina to carbachol (10^{-3} mol/L) caused significant increases in cGMP levels compared with the basal value. Additionally, both carboxy-PTIO (10^{-3} mol/L) and L-NNA (10^{-3} mol/L) significantly blocked the increase in cGMP content induced by carbachol. These results indicate that carbachol activates constitutive NO synthase, causing NO production, which increases the cGMP level. Atropine (10^{-5} mol/L) also significantly blocked the cGMP increase induced by carbachol. These data demonstrate that the NO-cGMP pathway may be linked to the activation of the muscarine receptor.

To consider which cells in the retina relate to the NO-cGMP pathway, we referred to other reports. In 1985, Sandell[20] reported that the distribution of an enzyme, nicotinamide adenine dinucleotide phosphate (NADPH) diaphorase, was examined histochemically in the rat retina. Nicotinamide adenine dinucleotide phosphate diaphorase was found in a sparse population of cells at the inner margin of the inner nuclear layer. Using immunohistochemistry, Yamamoto and colleagues[21] reported in 1993 that NO synthase was localized in amacrine cells in the rat retina. It was also reported that glycinergic amacrine cells in the frog or rabbit retina possessed muscarinic receptors.[22,23] Studies reported previously,[22,23] show that the NO-cGMP pathway may be linked to the activation of the muscarinic receptor in the frog or rabbit retina, suggesting that the activation of the muscarinic receptor on the amacrine cells enhances NO production in the retina. It was also reported that by stimulating the rabbit retina with flickering light, acetylcholine is released and light stimulation depolarizes NADPH diaphorase (NO synthase)-reactive amacrine cells.[24] It may be that the light stimulates the release of acetylcholine, which activates the muscarinic receptor on the amacrine cells[24–26] and enhances NO production in the same cells.

Histochemical and molecular cloning studies have revealed that ANP-receptor (GC-A) and NO-cGMP exist in the retina of various species, including man as well as the rat.[4,8,20,27–29] The presence of the CNP-receptor (GC-B) has been proved by molecular cloning and expression studies,[19] although its existence has not been reported in the rat retina in these studies. Our results suggest that cGMP levels are controlled by ANP, BNP, CNP, and NO release form, and that they may interact in the regulation of photosignal processing in the rat retina.

Based upon these findings, we can conclude that ANP-, CNP-, and NO-cGMP pathways exist in the rat retina, in addition to the already known retGC-cGMP pathway[2–4], and that the NO-cGMP pathway may be linked to the activation of the muscarinic receptor on the amacrine cells. The existence of these cGMP systems suggests that processing of the photosignals may be regulated by intracellular cGMP levels in the various layers of the retina, in addition to visual transduction in photoreceptor cells.

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