Different Responsiveness to Nitric Oxide–Cyclic Guanosine Monophosphate Pathway in Cholinergic and Tachykinergic Contractions of the Rabbit Iris Sphincter Muscle

Hideki Chuman,* Tomomi Chuman,* Nobuhisa Nao-i,* Atsushi Sawada,* Ryuichi Yamamoto,† Hideyuki Kobayashi,† and Akihiko Wada†

**Purpose.** In the rabbit iris sphincter muscle, sodium nitroprusside (SNP), a nitric oxide (NO) donor, inhibits cholinergic contraction but does not affect tachykinergic contraction in vitro. The objectives of the current study were to clarify the mechanism for the different responsiveness to NO in cholinergic and tachykinergic muscular contractions, and to examine whether the mechanism for NO-induced inhibition of cholinergic muscular contraction is operative in vivo.

**Methods.** Iris sphincter muscle was dissected from the rabbit eye pretreated with or without endotoxin (lipopolysaccharide, LPS) in vivo. Cyclic guanosine monophosphate (cGMP) content in the iris sphincter muscle was determined by radioimmunoassay. The motor activity of the ring-shaped iris sphincter muscle was measured isometrically. Sodium nitroprusside, carboxy-2-phenyl-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide (C-PTIO, a scavenger of NO radicals), and 8-bromo cGMP (a permeable cGMP analogue) were administered between the first and second administrations of carbachol and neurokinin A, both of which had caused sustained contraction in the iris sphincter muscle.

**Results.** Sodium nitroprusside inhibited the contraction of the iris sphincter muscle caused by carbachol but had no effect on the contraction caused by neurokinin A. Application of C-PTIO significantly reduced SNP-induced cGMP accumulation in the muscle, as well as the SNP-induced inhibition of muscular contraction caused by carbachol. Neither carbachol nor neurokinin A influenced SNP-induced cGMP accumulation in the muscle. Induction of 8-bromo-cGMP significantly diminished the muscular contraction caused by carbachol but not that caused by neurokinin A. In vivo pretreatment of the eye with LPS increased, in a time-dependent manner, the cGMP accumulation in the iris sphincter muscle, which was significantly inhibited by pretreatment of N5-nitro-L-arginine methyl ester (an inhibitor of NO synthesis) in vivo.

**Conclusions.** These results demonstrate that in rabbits the increase in cGMP accumulation induced by NO in the iris sphincter muscle is involved in the cholinergic contraction but not in the tachykinergic contraction, suggesting that different sensitivities to cGMP are essential for the different responsiveness to NO. Furthermore, the results of this study showed that the NO–cGMP pathway is operative in vivo and regulates iris sphincter muscle tone, at least when the eyes are infected with bacteria. Invest Ophthalmol Vis Sci. 1997;38:1719–1725.

The iris sphincter muscle, a bundle of circularly arranged smooth muscle cells, is innervated by cholinergic nerves, the activation of which induces muscular contraction. In the rabbit iris sphincter muscle, tachykinergic contractile responses to sensory nerve stimuli have been reported.1 Nitric oxide (NO), identified as the endothelium-derived relaxing factor,2–5 is now recognized as a ubiquitous modulator for a variety of functions6–10 and probably acts through accumulation of cyclic guanosine monophosphate (cGMP) in the tissue.5,7,11 We recently reported that sodium nitroprusside (SNP, an NO donor12) inhibited cholinergic contraction of isolated rabbit iris sphincter muscle caused by carbachol but did not affect the tachykinergic contraction caused by neurokinin A, indicating...
that the cholinergic contraction is NO sensitive, whereas the tachykinergic contraction is NO insensitive. Thus, we speculated that in rabbits the cholinergic and tachykinergic responses have distinct features for the fine adjustment of iris sphincter muscle tone. However, the mechanism(s) for the different responsiveness to NO in cholinergic and tachykinergic muscular contractions has yet to be determined. Although we observed that SNP increased accumulation of cGMP in rabbit iris sphincter muscle in vitro, there was no direct evidence linking this increased accumulation to the inhibitory effect of SNP on cholinergic contraction of that muscle.

In the current study, to clarify the mechanism(s) for the different responsiveness to NO in cholinergic and tachykinergic contractions of the rabbit iris sphincter muscle, we measured the accumulation of cGMP in the muscle after treatments of NO-related agents and examined the effects of these agents and 8-bromo cGMP on the motor activity of the muscle caused by either carbachol or neurokinin A in vitro. In addition, to determine whether the mechanism for NO-induced inhibition of cholinergic muscular contraction is operative in vivo, we measured the accumulation of cGMP in iris sphincter muscle dissected from rabbit eye pretreated with endotoxin (lipopolysaccharide, LPS) in vivo.

MATERIALS AND METHODS

Assay of Cyclic Guanosine Monophosphate Content in the Iris Sphincter Muscle

Eyes of adult albino rabbits of either sex (2.5 to 3.4 kg) were enucleated under anesthesia with sodium pentobarbital. Another group of adult rabbits (2.5 to 3.2 kg) were pretreated with or without N^6-nitro-L-arginine methyl ester (L-NNAME; 50 mg/kg, intraperitoneally) repeatedly administered 1 hour before and 0.5, 1, 3, 6, 12 hours after the intravitreal injection of LPS or control solution. The intravitreal injection of LPS (20 ng/20 µl) or vehicle (0.9% NaCl, 20 µl) was carried out under topical anesthesia with lidocaine hydrochloride. As described previously, the sphincter muscles were dissected from the pupillary margin under microscopic observation. To measure cGMP content in the muscle, half of the iris sphincter muscle was incubated in an organ bath containing 5 ml of Krebs' solution, continuously aerated with 95% O_2 plus 5% CO_2, for 10 minutes at 37°C in the presence of absence of SNP, carbachol, or neurokinin A. Sodium nitroprusside-C-PTIO, SNP-carbachol, or SNP-neurokinin A were simultaneously administered. After reaction, the tissues were rapidly homogenized in 1 ml of 0.1 N HCl by Polytron (PT 10-35, Kinematica, Littay, Switzerland). The homogenates were centrifuged at 12,000g for 15 minutes, and 500 µl of the supernatants were used for measurement of cGMP, using the Yamasa cGMP assay kit (Yamasa Shoyu, Chiba, Japan). Proteins in homogenates were measured in the method described, using and bovine serum albumin as a standard.

Measurement of Motor Activity of the Rabbit Iris Sphincter Muscle

Eyes of adult albino rabbits (2.2 to 3.1 kg) of either sex were enucleated under anesthesia with sodium pentobarbital. The sphincter muscle was dissected from the pupillary margin. A dissected ring-shaped sphincter muscle of the iris (1 to 1.5 mm wide) was suspended vertically in an organ bath containing 10 ml of Krebs' solution continuously aerated with 95% O_2 plus 5% CO_2 at 37°C. The composition of the Krebs' solution was as follows (concentrations in millimoles per liter): NaCl 120.7, KCl 5.9, MgCl_2 1.2, CaCl_2 2.5, NaH_2PO_4 1.2, NaH_2CO_3 15.5, and glucose 11.5.1 In light of reports that prostaglandins did not induce the contraction of the rabbit iris sphincter muscle but increased inositol triphosphate accumulation and phosphatidic acid formation, we added indomethacin (3 × 10^{-6} mol/l) to Krebs' solution to inhibit the interaction of prostaglandins. Isometric force was measured continuously using a force-displacement transducer (UFER UM-203, Kishimoto, Kyoto, Japan) and recorded by a pen recorder (HORIZ-8K, San-ei, Tokyo, Japan). A resting tension of 150 mg was applied to the preparation and maintained throughout the experiment. Once the maximum contraction after the first administration of carbachol or neurokinin A had been obtained, the preparation was washed with fresh Krebs' solution twice, and a gradual return to a steady state level was observed. We then made the second administration of carbachol or neurokinin A. All isometric force measurements are given as relative values to the maximum contraction induced by the first administration of carbachol or neurokinin A. Thus, the effects of pretreatment of SNP, C-PTIO, and 8-bromo-cGMP on the second contractions were expressed as percentages of the first maximum contraction caused by carbachol or neurokinin A. A treatment of SBP, C-PTIO, and 8-bromo-cGMP was given 10 minutes before the second administration of carbachol or neurokinin A.

These experiments were carried out in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Drugs

8-bromo-cyclic guanosine monophosphate sodium salt (8-bromo-cGMP), carbamylcholine chloride (carba-
cGMP and Rabbit Iris Sphincter Muscle

Chol), indomethacin, lipopolysaccharide (LPS), N\textsuperscript{\textcircled{6}}-nitro-L-arginine methyl ester (L-NAME), and sodium nitroprusside (SNP) were purchased from Sigma (St. Louis, MO); neurokinin A was purchased from Protein Research Foundation (Osaka, Japan); and carboxy-2-phenyl-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide (C-PTIO) was purchased from Dojindo Laboratories (Kumamoto, Japan).

Statistical Analysis

Values are expressed as means ± standard error of the mean (SEM). For statistical analysis, we used one-way analysis of variance (ANOVA) with post hoc mean comparison with the Newman-Keuls multiple range test. Student’s t-test was used for comparisons of two group means. Findings of $P < 0.05$ were taken to indicate statistical significance.

RESULTS

Cyclic Guanosine Monophosphate Accumulation in the Iris Sphincter Muscle

The cGMP content in rabbit iris sphincter muscle that was untreated with drugs was 2.82 ± 0.48 pmol/mg protein ($n = 5$). SNP at $10^{-5}$ mol/l increased the accumulation of cGMP in the muscle 860% (Fig. 1). To clarify that this effect was related to the NO liberated from SNP, we examined the effect of C-PTIO on SNP-induced cGMP accumulation in the muscle. C-PTIO decreased, in a concentration-dependent manner, the cGMP accumulation induced by SNP (10$^{-5}$ mol/l) to 78%, 44%, and 35% at 3 $\times$ 10$^{-5}$, 10$^{-4}$, and 3 $\times$ 10$^{-4}$ mol/l, respectively (Fig. 1). These results indicate that NO liberated from SNP increases the cGMP accumulation in the muscle.

To determine whether the intracellular signal transduction pathway stimulated by carbachol or neurokinin A influences the SNP-induced NO formation, the amount of cGMP in the muscle was measured after treatment with carbachol (10$^{-6}$ mol/l) or neurokinin A (10$^{-7}$ mol/l) in the presence or absence of SNP (10$^{-5}$ mol/l). Neither carbachol (Fig. 2A) nor neurokinin A (Fig. 2B) influenced the basal cGMP content or the SNP-induced accumulation of cGMP in the muscle, suggesting that carbachol and neurokinin A do not have properties that enhance the stability of NO radicals.

To determine whether the mechanism for NO-induced inhibition of cholinergeric muscular contraction is operative in vivo, the accumulation of cGMP in iris sphincter muscle was measured after pretreatment of the eye with LPS in vivo. After treatment of eyes with LPS for 9, 12, 15, and 24 hours, the cGMP accumulation in the muscle was increased in a time-dependent manner (Fig. 3). L-NAME (50 mg/kg, intraperitoneally) had no discernible effect on basal cGMP content in iris sphincter muscle (data not shown) but significantly reduced the LPS-elicited cGMP accumulation at 15 hours to 17% (Fig. 3). These results suggest that the NO-cGMP pathway is operative in vivo.

Motor Activity of the Rabbit Iris Sphincter Muscle

Carbachol and neurokinin A caused sustained contractions of the iris sphincter muscle. The maximum control contractions caused by the first administrations of carbachol at 10$^{-6}$ mol/l and neurokinin A at 10$^{-7}$ mol/l were 256 ± 32 mg (100%, $n = 5$) and 2890 ± 38 mg (100%, $n = 5$), respectively. Sodium nitroprusside significantly decreased the muscular contraction caused by carbachol in a concentration-dependent manner, whereas the muscular contraction caused by neurokinin A was not affected by SNP even at high concentration (Fig. 4). In the presence of C-PTIO at 10$^{-4}$ mol/l, the inhibitory effects of SNP at 10$^{-5}$ and 10$^{-4}$ mol/l on carbachol-induced muscular...
FIGURE 2. Effects of carbachol (A) and neurokinin A (B) on sodium nitroprusside-induced accumulation of cyclic guanosine monophosphate in isolated rabbit iris sphincter muscle. The preparation was treated with or without carbachol (10^-6 mol/l), neurokinin A (10^-7 mol/l), and sodium nitroprusside (10^-5 mol/l), or both, for 10 minutes. Mean ± SEM (n = 4). *P < 0.05 indicates a significant difference from the cyclic guanosine monophosphate accumulation in the muscle not treated with drug, indicated by (None). n.s. = non-significant difference from the cyclic guanosine monophosphate accumulation by sodium nitroprusside alone. CCh = carbachol; NKA = neurokinin A; SNP = sodium nitroprusside.

contraction were significantly diminished from 67% to 95% and from 63% to 88%, respectively (Fig. 4). These findings confirm that cholinergic contraction of the iris sphincter muscle is NO-sensitive, whereas tachykinergic contraction is NO-insensitive.

To examine effects of increased cGMP accumulation in iris sphincter muscle on cholinergic and tachykinergic muscular contractions, we treated the muscle with 8-bromo-cGMP between the first and second administrations of carbachol (10^-5 mol/l) or neurokinin A (10^-7 mol/l). As shown in Figure 5, 8-bromo-cGMP at 10^-5 and 10^-4 mol/l significantly inhibited the carbachol-induced muscular contraction 86% and 70%, respectively, but had no effect on the muscular contraction caused by neurokinin A. These results show that the increased accumulation of cGMP in the iris sphincter muscle induces depression of cholinergic muscular contraction but has no effect on tachykinergic muscular contraction.

DISCUSSION

The primary findings of the current study were that in rabbits the increased accumulation of cGMP induced by SNP through the formation of NO in the iris sphincter muscle inhibits cholinergic muscular contraction but does not affect tachykinergic muscular contraction. These results suggest that different sensitivities to cGMP are essential for the different responsiveness to NO in cholinergic and tachykinergic muscular contractions. Furthermore, the current results show that this NO-cGMP pathway is operative in vivo for the modulation of iris sphincter muscle tone, at least when the eyes are infected with bacteria.

Intracellular signal transduction by NO is thought to be triggered by activation of soluble guanylate cyclase, resulting in the accumulation of cGMP and a series of physiological responses. In the current results, SNP increased cGMP accumulation in the iris sphincter muscle and C-PTIO, a scavenger of NO radicals, significantly reduced the SNP-induced inhibition of the muscular contraction caused by carbachol. These results are in agreement with those in our previous report. In addition, C-PTIO also significantly reduced the SNP-induced cGMP accumulation in the muscle. Our results therefore indicate that the effects of SNP on cholinergic muscular contraction and the accumulation of cGMP in the muscle are caused by NO formation and are not a nonspecific side effect of the drug.

Sodium nitroprusside did not suppress the tachy-
kinergic contraction of iris sphincter muscle. One possible explanation for this is that the tachykinergic stimulus causes an excess formation of endogenous NO; therefore, subsequent administration of exogenous NO no longer inhibits the tachykinergic muscular contraction. This idea has been negated, however, by results of our previous study in which L-NAME, an inhibitor of NO formation from L-arginine, did not affect tachykinergic muscular contraction. 13 Another possibility is that the tachykinergic stimulus prevents the NO-induced accumulation of cGMP in the muscle by inhibition of soluble guanylate cyclase or induction of NO radical scavenger. However, in the current results, neither neurokinin A nor carbachol affected the basal cGMP content or the SNP-induced increase in cGMP content in the muscle. Our results imply that the intracellular signal transduction pathway for excitation–contraction coupling in the tachykinergic response is unaffected by the increased cGMP accumulation in the muscle, whereas that in the cholinergic response is suppressed. In fact, we observed that 8-bromo cGMP, a permeable cGMP analogue, significantly diminished the muscular contraction caused by carbachol but not that caused by neurokinin A. These results suggest that different sensitivities to cGMP are essential for the different responsiveness to NO in cholinergic and tachykinergic muscular contractions. It is possible that cholinergic and tachykinergic responses have distinct signaling pathways, downstream from phosphatidylinositol 4,5-bisphosphate (PIP2) hydrolysis, that play a role in excitation–contraction coupling in the rabbit iris sphincter muscle. Carbachol and neurokinin A induce the activation of phospholipase Cβ through a guanine–nucleotide-binding regulatory protein (G protein),19,20 termed Gq11, thereby stimulating phosphatidylinositol 4,5-bisphosphate hydrolysis.21–23 Thus, the cholinergic pathway appears to be more sensitive to cGMP than the tachykinergic pathway.

Our next objective was to clarify whether the NO–cGMP pathway operates in vivo to regulate iris sphincter muscle tone. Several isoforms of NO synthase (NOS) have been reported. These can be subclassified into two categories: constitutive NOS (cNOS) and inducible NOS (iNOS).5 Constitutively expressed in endothelial cells, forebrain, platelets, nonadrenergic noncholinergic nerves, and adrenal medullary cells,

FIGURE 4. Effects of SNP on carbachol- and neurokinin A-induced contractions of isolated rabbit iris sphincter muscle in the absence and presence of C-PTIO. Various concentrations of SNP (10^{-5} – 10^{-4} mol/l) or C-PTIO (10^{-4} mol/l), or both, were added to the organ bath between the first and second administrations of carbachol (10^{-6} mol/l) or neurokinin A (10^{-7} mol/l). Mean ± SEM (n = 5). A value of 100% corresponds to the contraction caused by the first administration of carbachol and neurokinin A. O = carbachol; * = C-PTIO + carbachol; □ = neurokinin A. *P < 0.05 indicates a significant difference from the contraction caused by the second administration of carbachol without SNP, indicated by (None). SNP = sodium nitroprusside; C-PTIO = carboxy-2-phenyl-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide.

FIGURE 5. Effect of 8-bromo-cyclic guanosine monophosphate on carbachol-and neurokinin A-induced contractions of isolated rabbit iris sphincter muscle. Various concentrations of 8-bromo cyclic guanosine monophosphate (10^{-5} – 10^{-4} mol/l) were added to the organ bath between the first and second administrations of carbachol (10^{-6} mol/l) or neurokinin A (10^{-7} mol/l). Mean ± SEM (n = 5). A value of 100% corresponds to the contraction caused by the first administration of carbachol and neurokinin A. O = carbachol; □ = neurokinin A. *P < 0.05 indicates a significant difference from the contraction caused by the second administration of carbachol without 8-bromo cyclic guanosine monophosphate, indicated by (None).
cNOS provides the physiological control of a variety of functions\(^\text{9,84}\); iNOS is induced by the stimulation of macrophages, Kupffer cells, hepatocytes, vascular smooth muscle cells, or fibroblasts by various signals, including LPS and cytokines, and immediately catalyzes excess formation of NO for cytotoxic and cytostatic effects.\(^\text{5,24}\) Recently, Mandai et al\(^\text{25}\) studied the changes in NOS activity associated with endotoxin-induced uveitis in rats. They speculated that the induction of iNOS plays a key role in the ocular inflammation elicited by LPS, because an inhibitor of NO synthesis reduces the inflammatory response. In addition, more recently, Jacquemin et al\(^\text{26}\) confirmed that LPS markedly induces iNOS mRNA expression in the epithelial cells of the iris–ciliary body in rats with endotoxin-induced uveitis. In our experimental system, it is difficult to estimate cNOS-induced NO formation. Thus, we measured accumulation of cGMP in iris sphincter muscle dissected from rabbit eye in which iNOS was induced by intravitreal injection of LPS in vivo. The LPS increased the accumulation of cGMP in the muscle, which was significantly inhibited by in vivo pretreatment of L-NAME. These results suggest that the NO–cGMP pathway is operative in the iris sphincter muscle in vivo, at least when iNOS catalyzes excess formation of NO. Finally, the NO–cGMP pathway appears to be pathophysiologically important to the regulation of the cholinergic contractility of the iris sphincter muscle that accompanies ocular inflammations that include uveitis and various postoperative conditions.

**Key Words**
cholinergic and tachykinergic contractions, cyclic guanosine monophosphate, nitric oxide, rabbit iris sphincter muscle, sodium nitroprusside

**Acknowledgments**
The authors thank Dr. Yujiro Asada for helpful suggestions.

**References**


