Differential Effects of Diltiazem and Nitroglycerin on Cytosolic Ca\(^{2+}\) Concentration and on Force in the Bovine Ophthalmic Artery

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Purpose. To determine the mechanisms of inhibition by diltiazem (Dil) and nitroglycerin (NG) of the contraction induced by serotonin (5-HT) in the ophthalmic artery.

Methods. Using front-surface fluorometry of fura-2 and the medial strips of the bovine ophthalmic artery, [Ca\(^{2+}\)]\(_i\) and force were monitored simultaneously. Changes in the force at a constant [Ca\(^{2+}\)]\(_i\) were determined by use of receptor-coupled membrane permeabilization with α-toxin.

Results. In the presence of extracellular Ca\(^{2+}\), 5-HT (10\(^{-5}\) M) induced an initial transient and subsequently lower steady state elevation of [Ca\(^{2+}\)]\(_i\). The transient elevation of [Ca\(^{2+}\)]\(_i\) was dependent on both intracellular and extracellular [Ca\(^{2+}\)]\(_i\), whereas the steady state elevation was dependent on only extracellular Ca\(^{2+}\). For a given level of elevation of [Ca\(^{2+}\)]\(_i\), 5-HT produced a greater force than the depolarization with high external K\(^+\) (118 mM) solution. In the permeabilized ophthalmic artery smooth muscle, 5-HT enhanced the contractile response to constant cytosolic Ca\(^{2+}\) (pCa 6.5) in the presence of guanosine triphosphate (GTP, 10 \(^{−6}\) M), but not in its absence. Therefore, 5-HT induces [Ca\(^{2+}\)]\(_i\) elevation, depending on both extracellular (Ca\(^{2+}\) influx) and intracellular Ca\(^{2+}\) (Ca\(^{2+}\) release), and it potentiates the Ca\(^{2+}\) sensitivity of the contractile apparatus through the activation of G-proteins. 5-HT-induced release of Ca\(^{2+}\) from the store was inhibited by NG, but not by Dil, in a concentration-dependent manner. However, neither NG nor Dil inhibited caffeine (20 mM)-induced release of Ca\(^{2+}\) from the store. Dil (10 \(^{−6}\) M) and NG (10 \(^{−6}\) M) in the presence of extracellular Ca\(^{2+}\). Dil equally inhibited the steady state elevations of [Ca\(^{2+}\)]\(_i\) and force induced by 5-HT, whereas NG inhibited the force to a greater extent than expected from the reduction in [Ca\(^{2+}\)]\(_i\). In the permeabilized ophthalmic artery smooth muscle, NG (10 \(^{−6}\) M), but not Dil (10 \(^{−6}\) M), decreased the force development induced by GTP (10 \(^{−6}\) M) and 5-HT (10 \(^{−6}\) M) at constant [Ca\(^{2+}\)]\(_i\) (pCa 6.5). These results indicate that NG, but not Dil, decreases the Ca\(^{2+}\) sensitivity of contractile apparatus.

Conclusions. The authors found that 5-HT contracts the ophthalmic artery smooth muscle by the elevation of [Ca\(^{2+}\)]\(_i\) mediated by the release of intracellular Ca\(^{2+}\) and the influx of extracellular Ca\(^{2+}\), as well as by an increase in the Ca\(^{2+}\) sensitivity of the contractile apparatus through the activation of G-proteins, and that Dil relaxes 5-HT-mediated contraction of ophthalmic artery primarily by inhibiting the Ca\(^{2+}\) influx and, hence, by decreasing [Ca\(^{2+}\)]\(_i\) without having any effect on the Ca\(^{2+}\) sensitivity of the contractile apparatus. Nitroglycerin relaxes the ophthalmic artery not only by decreasing [Ca\(^{2+}\)]\(_i\) (inhibition of both the Ca\(^{2+}\) release and Ca\(^{2+}\) influx) but also by decreasing the Ca\(^{2+}\) sensitivity of the contractile apparatus.


Vasospastic events induced by agonists released from aggregated platelets or thrombus in the ophthalmic and retinal arteries have been suggested to play an important role in the pathophysiology of several ocular diseases, including amaurosis fugax,\(^{1,2}\) central retinal artery occlusion,\(^{3,4}\) and low-tension glaucoma.\(^{5,6}\) Ca\(^{2+}\) channel blockers and nitroglycerin (NG), which are used widely as antivasospastic agents for the treatment of cardiovascular disorders such as angina pectoris, have been reported to have therapeutic effects...
on these vasospastic ocular diseases. Although studies have suggested that Ca²⁺ channel blockers and NG relax the ophthalmic and retinal arteries and, thus, increase the blood flow of these arteries cellular mechanisms of the relaxations of the ocular arteries induced by these vasodilators have yet to be clarified.

Although changes in the cytosolic Ca²⁺ concentration ([Ca²⁺]i) play a central role in the regulation of smooth muscle tonus, the force does not correlate directly with [Ca²⁺]i in smooth muscle. Studies using Ca²⁺ indicators have shown that some types of receptor-activating agonists and vasodilators can modulate the relation between [Ca²⁺]i and force ([Ca²⁺]i-force relation). Our previous studies on the coronary artery showed that NG, but not the Ca²⁺ channel blocker diltiazem (Dil), produces more relaxation than that expected from the extent of reduction of [Ca²⁺]i, and that G-protein mediates decreases in Ca²⁺ sensitivity of the contractile apparatus induced by a vasodilating peptide, adrenomedullin, in permeabilized vascular smooth muscle. However, the changes in [Ca²⁺]i and the modulation of Ca²⁺ sensitivity of contractile apparatus, as induced by agonists and by vasodilators, as well as the role of G-proteins, have never been investigated in either ophthalmic or retinal arteries.

In the current study, we demonstrated the first successful recordings of [Ca²⁺]i and force in the intact ophthalmic arteries, and of contraction and relaxation at a constant [Ca²⁺]i in the membrane-permeabilized ophthalmic arteries. We clarified the differences in the effects between diltiazem and nitroglycerin on the changes in [Ca²⁺]i, in the modulation of [Ca²⁺]i-force relation, and in the Ca²⁺ sensitivity of contractile apparatus during contractions of the ophthalmic arteries induced by serotonin (5-HT), which causes vasospasm of the ocular arteries on the release of aggregating platelets. We demonstrated the existence of G-protein-mediated potentiation and NG-induced reduction of Ca²⁺ sensitivity of contractile apparatus during the contraction induced by 5-HT in the ophthalmic arteries.

METHODS

Tissue Preparation

Eyes of bovines of either sex were obtained from a local slaughterhouse immediately after the animals had been killed. All studies were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Eyes were placed in ice-cold saline solution and brought to the laboratory. Ophthalmic arteries (diameter, 1 to 1.5 mm) before the optic nerve was penetrated. Fat and adventitia were removed by dissection under a binocular microscope. Endothelial cells were removed by rubbing the intraluminal surface with a cotton swab, and complete removal of the endothelial cells was confirmed by the lack of response to bradykinin (100 nM). Medial preparations were cut into circular strips (approximately 1 x 3 mm, 0.2 mm thick). The total number of arterial strips used in this study was 110. Elevations of [Ca²⁺]i and force induced by 118 mM K⁺ depolarization were not affected by chemical denervation with 1.2 μM 6-hydroxydopamine, 3 μM tetradotoxin, 1 μM prazosin, 1 μM phentolamine, and 1 μM guanethidine, which indicates that there was no possible release of neurotransmitters from the nerve endings in the vascular strips used in the current study (data not shown).

Fura-2 Loading

Vascular strips thus obtained were loaded with [Ca²⁺]i indicator dye, fura-2, by incubation in oxygenated (a mixture of 95% O₂ and 5% CO₂) Dulbecco’s modified Eagle’s medium containing 25 μM fura-2-AM (an acetoxymethyl ester form of fura-2) and 5% fetal bovine serum for 3 to 4 hours at 37°C, as previously described. Subsequently, the strips were washed with normal physiological salt solution (PSS) containing 1.25 mM Ca²⁺ at 37°C to remove the dye from the extracellular space and then equilibrated in normal PSS for at least 1 hour before the initiation of the measurements.

Nonloaded strips showed no significant peak in the excitation spectrum when the fluorescence emission was monitored at 500 nm. In the fura-2-loaded strips, the intensity of the fluorescence markedly increased. The fura-2-loaded strips showed a fluorescence excitation spectrum with a peak at 350 nm (Fig. 1) and fluorescence emission spectra with a peak at 500 nm. These characteristics indicated that the fluorescence spectra obtained with the fura-2-loaded strips are specific for this dye.

Loading the vascular strips with fura-2 did not alter the time course or the maximum levels of the force development during the 118 mM K⁺ depolarization (data not shown), suggesting that no tissue damage occurred by possible acidification of the cells caused by formaldehyde release in AM-ester hydrolysis.

Measurement of Force

Strips were mounted vertically in a quartz organ bath, and a force-transducer (strain gauge TB-612T; Nihon Koden, Tokyo, Japan) was used. During a 1-hour equilibration period, the strips were stimulated with 118 mM K⁺ depolarization every 15 minutes, and the resting force was increased in a stepwise manner. After equilibration, the resting force was adjusted to 250 to 300 mg because the maximal response was obtained at a resting force of this range. The force development
was expressed as a percentage, assigning the values in normal (5.9 mM K+) and 118 mM K+ PSS to be 0% and 100%, respectively. The absolute values of 10 different measurements of [Ca2+]i at rest (0%) and during 118 mM K+-depolarization (100%) were 104 ± 6.5 nM and 713 ± 14.2 nM, respectively. The responses of the fluorescence ratio (100%) and force (100%) to 118 mM K+-depolarization were stable during the measurements, regardless of the wide variety of protocols, whereas the absolute values of [Ca2+]i obtained by Grynkiewicz's procedure at the end of measurements varied depending on the protocols. Therefore, the statistical analysis was performed using the fluorescence ratio (%) in this study.

Because the [Ca2+]i levels at the steady state of elevation induced by 5-HT were small, which made it difficult to compare directly the [Ca2+]i-force relation obtained by 5-HT by itself with that by K+-depolarization in the wide ranges of [Ca2+]i levels, the following protocol was performed to assess the effect of 5-HT on the Ca2+-sensitivity of the contractile apparatus: We first determined the basic [Ca2+]i-force relation15 and then examined the effect of 5-HT on this basic [Ca2+]i-force relation.20 Briefly, as shown in Figure 3A, after recording 0% resting levels and 100% responses to 118 mM K+ depolarization in fluorescence ratio and force in normal PSS, the vascular strips were incubated in Ca2+-free PSS containing 2 mM EGTA for 10 minutes, followed by a 5-minute exposure to Ca2+-free PSS without EGTA. The fluorescence ratio decreased gradually to reach a steady state; however, the force was maintained at approximately the resting level (0%). Next, the solution was changed to Ca2+-free 118 mM K+ PSS, and the cumulative applications of extracellular Ca2+ (0 to 5 mM) were initiated during 118 mM K+-depolarization. Both [Ca2+]i and force increased stepwise with cumulative and stepwise elevations of the extracellular Ca2+ concentration ([Ca2+]o). Thus, we obtained the basic [Ca2+]i-force relation of Ca2+-induced contraction (Ca2+-contraction), as shown in Figure 3C. To determine the effect of 5-HT on the basic [Ca2+]i-force relation, a similar protocol was performed in the presence of 5-HT.

**Membrane Permeabilization of Ophthalmic Arterial Rings**

Small rings (50 μm wide and 1 mm diameter) of ophthalmic artery smooth muscle were permeabilized with Staphylococcus aureus α-toxin, and isometric tension was measured as described.22 In brief, the rings were mounted in a well on a bubble plate, and the solution...
was changed by sliding the bubble plate to an adjacent well. The strips were incubated in normal relaxing solution (K+ methanesulphonate, 74.1 mM; Mg2+ methanesulphonate, 2 mM; MgATP, 4.5 mM; EGTA, 1 mM; creatine phosphate, 10 mM; PIPES, 30 mM) for 5 to 10 minutes and then permeabilized by 60 to 75 minutes of incubation at 22°C to 25°C in 5000 U/ml (based on rabbit red blood cell hemolysis) of S. aureus α-toxin. Permeabilized strips were treated with A23187 (10 μM) in the relaxing solution for at least 10 minutes to deplete the sarcoplasmic reticulum of Ca2+ without affecting the Ca2+-sensitivity of the contractile apparatus.22 In the activating solution (pCa 6.5), 10 mM EGTA was used, and a specified amount of Ca2+ methanesulphonate was added to give a desired concentration of free calcium ions. Ionic strength was kept constant at 0.2 M by adjusting the concentration of K+ methanesulphonate.

Drugs and Solutions

The physiological salt solution (PSS) was of the following composition: NaCl, 123 mM; KCl, 4.7 mM; NaHCO3, 15.5 mM; KH2PO4, 1.2 mM; MgCl2, 1.2 mM; CaCl2, 1.25 mM; and D-glucose, 11.5 mM. High K+(118 mM) PSS was identical with normal PSS, except for an equimolar substitution of KCl for NaCl (NaCl, 10.9 mM; KCl, 116.8 mM). Ca2+-free solution was identical with normal PSS, except for a substitution of 2 mM EGTA for 1.25 mM CaCl2. All solutions, except for the solutions used for the permeabilized strips, were gassed with a mixture of 95% O2 and 5% CO2 (pH adjusted to 7.4 at 37°C). 5-hydroxytryptamine hydrochloride (5-HT) and diltiazem hydrochloride was purchased from Sigma (St. Louis, MO). Fura-2-AM was purchased from Dojindo Laboratories (Kumamoto, Japan). Nitroglycerin (water-soluble form) was obtained from Nihon-Kayaku (Tokyo, Japan). S. aureus α-toxin was purchased from Calbiochem (La Jolla, CA). Guanosine triphosphate was purchased from Boehringer Mannheim (Mannheim, Germany). Piperazine-N,N’-bis (2-ethanesulfonic acid) (PIPES) was purchased from Katayama Chemical Industries (Osaka, Japan).

Data Analysis

Values are expressed as mean ± standard error. Student’s t-test was used to determine the statistical significance. Statistical analysis of the shift of the [Ca2+]i-force curves was carried out by analysis of covariance. P < 0.05 was considered significant.

RESULTS

Changes in [Ca2+]i and Force During Contraction Induced by 5-HT in the Presence and Absence of Extracellular Ca2+

To characterize the changes in [Ca2+]i and force induced by 5-HT, we used 5-HT at the minimum concentration (10-5 M) to induce the maximum contraction. When the vascular strips were exposed to 5-HT (10-5 M) in the presence of extracellular Ca2+ (1.25 mM), the [Ca2+]i and the force abruptly rose, reaching peaks at 1 to 2 minutes and at 3 to 6 minutes, respectively. Then they slowly declined to a lower, steady state level. Peak levels of [Ca2+]i and force were 98.7% ± 6.3% and 116.4% ± 8.4% of 118 mM K+-induced response (100%) (n = 5), respectively. The 5-HT-induced contraction was larger than the 118 mM K+-induced contraction, whereas 5-HT produced a much smaller [Ca2+]i elevation than did 118 mM K+:

Changes in [Ca2+]i and Force Induced by Cumulative Applications of Extracellular Ca2+ During 118 mM K+-Depolarization in the Absence and Presence of 5-HT

To evaluate the effect of 5-HT on [Ca2+]i, the force and the [Ca2+]i-force relationship of Ca2+-contraction. To examine the physiological role of the release of intracellular Ca2+ in the elevations of [Ca2+]i and force induced by 5-HT, 5-HT was applied in the absence of extracellular Ca2+. When the strips were exposed to Ca2+-free solution containing 2 mM EGTA, [Ca2+]i gradually decreased to reach a steady state level (at 15 minutes, [Ca2+]i = 15.9% ± 1.6%). Subsequent application of 10-5 M 5-HT caused transient increases in [Ca2+]i and force, which then declined and returned to the prestimulation levels (Fig. 2B). Peak levels of [Ca2+]i and force were 1% ± 1.8% and 50.4% ± 2.8% of 118 mM K+-induced response (100%) (n = 5). Thus, [Ca2+]i elevation was small, indicating that the contribution of the release of intracellular [Ca2+]i to the elevation of [Ca2+]i induced by 5-HT was small. In addition, the extent of contraction was much greater than that expected from the elevation in [Ca2+]i when compared to the relation between contraction and [Ca2+]i for high K+-depolarization.
was carried out in the presence of 5-HT. Figure 3B shows a representative recording of changes in [Ca$^{2+}$]$_i$ and force observed in the presence of $10^{-6}$ M 5-HT. Both [Ca$^{2+}$]$_i$ and force increased stepwise with elevations of [Ca$^{2+}$]$_o$. In the presence of 5-HT, the increase in [Ca$^{2+}$]$_i$ was approximately similar to that in the absence of 5-HT; however, the increase in force was much greater than that in the absence of 5-HT (Fig. 3B).

Figure 3C shows the averaged values of changes in [Ca$^{2+}$]$_i$ and force, and in the [Ca$^{2+}$]$_i$ (abscissa)-force (ordinate) relation, obtained from six different measurements determined according to the protocol shown in Figures 3A and 3B. In the presence of 5-HT (open circles), [Ca$^{2+}$]$_i$-force relation shifted up from the line of the basic [Ca$^{2+}$]$_i$-force relation of Ca$^{2+}$-contraction ($P < 0.05$ by an analysis of covariance).

**Effects of Dil and Nitroglycerin on Changes in [Ca$^{2+}$]$_i$, Force, and [Ca$^{2+}$]$_i$-Force Relation of Contractions Induced by 5-HT in the Presence of Extracellular Ca$^{2+}$**

Figure 4 shows the effects of various concentrations of Dil (Fig. 4A) and NG (Fig. 4B) on time courses of changes in [Ca$^{2+}$]$_i$ and force induced by 5-HT ($10^{-5}$ M) in normal PSS (1.25 mM Ca$^{2+}$). The strips were incubated with various concentrations ($10^{-7}$, $10^{-6}$, $10^{-5}$ M) of Dil and NG 10 minutes before and during the application of $10^{-5}$ M 5-HT. Dil inhibited in a concentration-dependent manner the initial transient elevation of [Ca$^{2+}$]$_i$ induced by 5-HT. Nitroglycerin ($\leq 10^{-6}$ M) did not inhibit this transient elevation of [Ca$^{2+}$]$_i$, and the maximum concentration ($10^{-5}$ M) of NG only inhibited it slightly. In contrast to the effects on the initial transient elevation of [Ca$^{2+}$]$_i$, both Dil and NG inhibited in a concentration-dependent manner the steady state elevation of [Ca$^{2+}$]$_i$ and force. Dil equally inhibited the steady state elevations of [Ca$^{2+}$]$_i$ and force. For a given reduction of [Ca$^{2+}$]$_i$, NG inhibited the force to a greater extent than Dil. Effects of Dil and NG on the [Ca$^{2+}$]$_i$ (abscissa)-force (ordinate) relation during contractions induced by 5-HT were examined 20 minutes after the initiation of contractions (Fig. 4C). Nitroglycerin ($10^{-7}$ to $10^{-5}$ M) caused down displacement of the [Ca$^{2+}$]$_i$-force relation from the line of the [Ca$^{2+}$]$_i$-force relation of 5-HT-induced contractions ($P < 0.05$ by analysis of covariance), resulting in apparent overlap with the line of the basic [Ca$^{2+}$]$_i$-force relation (open squares), whereas Dil ($10^{-7}$ to $10^{-5}$ M) did not shift the [Ca$^{2+}$]$_i$-force relation of contractions induced by 5-HT (open triangles).

**Effects of Dil and Nitroglycerin on Changes in [Ca$^{2+}$]$_i$ and Force Induced by Either 5-HT or Caffeine in the Absence of Extracellular Ca$^{2+}$**

Figure 5 shows the effects of various concentrations of Dil and NG on time courses of changes in [Ca$^{2+}$]$_i$.
FIGURE 3. (A) Representative recordings of the changes in fluorescence ratio ([Ca$^{2+}$]$_i$) and force induced by the cumulative applications of various concentrations (0 mM to 5 mM) of extracellular Ca$^{2+}$ during 118 mM K$^+$-depolarization. Extracellular Ca$^{2+}$ concentrations ([Ca$^{2+}$]$_o$) in mM are shown by closed triangles. (B) Representative recordings of the changes in fluorescence ratio ([Ca$^{2+}$]$_i$) and force induced by cumulative applications of various concentrations (0 mM to 2.5 mM) of extracellular Ca$^{2+}$ during 118 mM K$^+$-depolarization in the presence of 10$^{-5}$ M serotonin (5-HT). Extracellular Ca$^{2+}$ concentrations ([Ca$^{2+}$]$_o$) in mM are shown by closed triangles. (C) [Ca$^{2+}$]$_i$-force relation of the contractions induced by cumulative applications of Ca$^{2+}$ during 118 mM K$^+$-depolarization in the absence (• = control) and presence of 10$^{-5}$ M 5-HT (○). Data were based on repeated experiments carried out by the protocol shown in A and B ($n$ = 5 to 6). Some error bars were drawn within the size of symbols because of the small standard error values.

and force induced by 5-HT (10$^{-5}$ M) in the absence of extracellular Ca$^{2+}$. The strips were incubated with various concentrations of Dil and NG 10 minutes before and during the application of 5-HT in the absence of extracellular Ca$^{2+}$. Nitroglycerin (10$^{-7}$ to 10$^{-5}$ M), but not Dil (10$^{-7}$ to 10$^{-5}$ M), inhibited in a concentration-dependent manner transient elevations of [Ca$^{2+}$]$_i$ and force induced by 5-HT in Ca$^{2+}$-free solution (Fig. 5). Nitroglycerin (≥10$^{-6}$ M) significantly inhibited the peak elevations of [Ca$^{2+}$]$_i$ and force induced by 5-HT ($P < 0.05$ by Student's t-test).

Figure 6 shows the effects of Dil (10$^{-5}$ M) and NG (10$^{-5}$ M) on elevations of [Ca$^{2+}$]$_i$ and force induced by 20 mM caffeine in the absence of extracellular Ca$^{2+}$. After a 15-minute incubation in Ca$^{2+}$-free PSS, caffeine caused transient elevations of [Ca$^{2+}$]$_i$ and force with a peak ([Ca$^{2+}$]$_i$, 19.4% ± 3.9%; force, 12.9% ± 2.6%). Treatment with 10$^{-5}$ M Dil and 10$^{-5}$ M NG for 10 minutes before and during the application of caffeine in Ca$^{2+}$-free solution did not inhibit the peak elevations of [Ca$^{2+}$]$_i$ and force induced by 20 mM caffeine (Fig. 6).

Contraction Induced by 5-HT and Relaxation Induced by Dil and Nitroglycerin at a Constant [Ca$^{2+}$]$_i$ in the Permeabilized Smooth Muscle:

Analysis of the [Ca$^{2+}$]$_i$-force relation using a front-surface fluorometry of fura-2 indicated that the
[Ca$^{2+}$]i-force relation, obtained with the presence of 5-HT, located above that of the basic [Ca$^{2+}$]i-force relation (Fig. 3C) and that of NG, but not Dil, induced the downward displacement of the [Ca$^{2+}$]i-force relation during contractions induced by 5-HT (Fig. 4C). To clarify whether G-protein is involved in these 5-HT-induced potentiations and NG-induced reductions of Ca$^{2+}$ sensitivity of the contractile apparatus, we determined the effects of these substances on the contractility of receptor-coupled ophthalmic arterial strips permeabilized with S. aureus α-toxin (Fig. 7). To maintain a constant [Ca$^{2+}$]i without affecting the Ca$^{2+}$-sensitivity of the contractile apparatus, the sarcoplasmic reticulum was depleted of calcium by A23187, whereas Ca$^{2+}$ was buffered with 10 mM EGTA to maintain pCa 6.5. As shown in Figure 7A, in the presence of 10 μM GTP, which itself had no effect on force, 10 μM 5-HT enhanced the contractile response to constant cytosolic Ca$^{2+}$ (pCa 6.5). 5-HT (10 μM) alone, without GTP, had no effect on force (data not shown). These results suggest that 5-HT potentiates the Ca$^{2+}$-sensitivity of the contractile apparatus through the activation of G-protein. Nitroglycerin (10 μM), but not Dil (10 μM), decreased the force development induced by 10 μM GTP and 10 μM 5-HT at constant [Ca$^{2+}$]i (pCa 6.5) (Fig. 7). These results also indicate that NG, but not Dil, decreases the Ca$^{2+}$ sensitivity of the contractile apparatus in the ophthalmic artery.

**DISCUSSION**

This is the first study to show recordings of the simultaneous determinations of [Ca$^{2+}$]i and force in the oph-
thamic arteries. We also examined the contractility of the ophthalmic arteries following the receptor-coupled membrane permeabilization of smooth muscles. Using these methods, we characterized the effect of Dil and NG on the changes in $[\text{Ca}^{2+}]_i$, force, and the $[\text{Ca}^{2+}]_i$-force relation of the medial strips of the ophthalmic arteries during the contractions induced by the vasospastic agent 5-HT.2

As shown in Figure 2A, in the presence of extracellular Ca$^{2+}$, 5-HT induced an initial transient and a subsequently lower sustained elevation of $[\text{Ca}^{2+}]_i$. The removal of extracellular Ca$^{2+}$ mostly diminished the initial transient increase in $[\text{Ca}^{2+}]_i$ and completely abolished the sustained phase of $[\text{Ca}^{2+}]_i$ elevation (Fig. 2B). These results suggest that the initial transient phase was mediated primarily by the extracellular Ca$^{2+}$ influx and only partly by the intracellular Ca$^{2+}$ release, whereas the sustained phase was mediated exclusively by extracellular Ca$^{2+}$ influx. This notion is compatible with other results in the current study, namely that although NG, at the concentration ($10^{-6}$ M), inhibited Ca$^{2+}$ release induced by 5-HT (Fig. 5B), it had no significant effect on the initial transient elevation of $[\text{Ca}^{2+}]_i$ induced by 5-HT in the presence of extracellular Ca$^{2+}$ (Fig. 4B).

One of the novel findings of this study is that NG and Dil inhibited to different degrees the $[\text{Ca}^{2+}]_i$ transients induced by 5-HT in the ophthalmic arteries. As shown in Figure 4, NG and Dil, at their maximum concentration ($10^{-5}$ M), inhibited the steady state elevation of $[\text{Ca}^{2+}]_i$ induced by 5-HT to the same extent (15.5% and 16.4%, respectively), which indicates that NG and Dil inhibit the 5-HT-induced Ca$^{2+}$ influx with the same potency. In contrast, the two drugs affect the Ca$^{2+}$ release to different degrees. Nitroglycerin (up to $10^{-5}$ M), but not Dil, inhibited in a concentration-dependent manner the Ca$^{2+}$ release induced by 5-HT in Ca$^{2+}$-free PSS (Fig. 5). Because NG had no effect on the Ca$^{2+}$ release stimulated by caffeine, which directly affects the sarcoplasmic reticulum and releases the stored Ca$^{2+}$ (Fig. 6), it is unlikely that the inhibition of the Ca$^{2+}$ release by NG may be caused by the direct effect of NG on the Ca$^{2+}$ release channels of the sarcoplasmic reticulum. The precise mechanisms by which
NG inhibits the Ca\(^{2+}\) release induced by 5-HT were not clarified in the current study. However, it has been reported that sodium nitroprusside and 8-bromo cyclic guanosine monophosphate (cGMP) attenuated the formation of inositol 1,4,5-trisphosphate (IP\(_3\)) induced by adrenergic agonists in the rat aorta\(^{23,24}\). Therefore, it is likely that NG, a cyclic GMP-elevating agent\(^{25}\), may also inhibit the IP\(_3\) formation induced by 5-HT and thereby inhibit the Ca\(^{2+}\) release induced by 5-HT in the bovine ophthalmic artery. In addition, we previously observed that NG decreases the amount of Ca\(^{2+}\) stored in the histamine-sensitive storage site in the pig coronary artery\(^{13}\). Therefore, it is likely that NG may decrease the amount of Ca\(^{2+}\) stored in the 5-HT-sensitive storage site in the bovine ophthalmic artery.

In contrast to Dil, which equally inhibited the initial rise and the sustained components of [Ca\(^{2+}\)]\(_i\) elevation induced by 5-HT, NG preferably inhibited the sustained elevation of [Ca\(^{2+}\)]\(_i\) to the initial transient elevation of [Ca\(^{2+}\)]\(_i\) (Fig. 4). We have reported that, in contrast to the simple action of Dil as a Ca\(^{2+}\) channel blocker\(^{14}\), NG has multiple actions on Ca\(^{2+}\)-homeostasis\(^{13}\). It is possible that the inhibition of the second component of [Ca\(^{2+}\)]\(_i\) elevation by NG may be caused not only by the blockade of Ca\(^{2+}\) influx but also by

![Figure 6](image-url)

**Figure 6.** Effects of 10^{-6} M Dil (Δ) and 10^{-6} M nitroglycerin (□) on the elevations of [Ca\(^{2+}\)]\(_i\) (A) and force (B) induced by 20 mM caffeine in the absence of extracellular Ca\(^{2+}\). 20 mM caffeine (●, control) was applied after the 15-minute incubation in Ca\(^{2+}\)-free PSS (2 mM EGTA). Strips were incubated with Dil and nitroglycerin 10 minutes before and during the application of 20 mM caffeine. The abscissa scales indicate the time (in minutes) after the application of caffeine. Data are the mean ± standard error (shown as vertical bars; n = 4).

![Figure 7](image-url)

**Figure 7.** Vasorelaxing effects of 10^{-5} M Dil and 10^{-5} M nitroglycerin on the contractile response to 5-HT and guanosine triphosphate (GTP) at constant Ca\(^{2+}\) (pCa 6.5) in α-toxin-permeabilized ophthalmic arterial smooth muscle. The sarcoplasmic reticulum was depleted of calcium by treatment with 10 μM A23187 (see Methods for details). (A) Contractile response to 10 μM GTP and 10 μM 5-HT, superimposed on the contraction induced by highly buffered Ca\(^{2+}\) (pCa 6.5 with 10 mM EGTA). Note that 10 μM GTP, per se, had no contractile effect. (B) Effects of 10 μM Dil on the contraction induced by 10 μM GTP and 10 μM 5-HT at constant Ca\(^{2+}\) (pCa 6.5). (C) Effects of 10 μM nitroglycerin on the contraction induced by 10 μM GTP and 10 μM 5-HT at constant Ca\(^{2+}\) (pCa 6.5).
either Ca^{2+} uptake into the sarcoplasmic reticulum or Ca^{2+} extrusion into the extracellular space. In this regard, it is well known that in various types of cells, NG elevates cytosolic cyclic GMP levels and that cyclic GMP activates Ca^{2+} pump ATPase in the endoplasmic reticulum or sarcoplasmic reticulum and the plasma membrane, which results in enhancement of the Ca^{2+} uptake into the sarcoplasmic reticulum and the Ca^{2+} extrusion, respectively, thus reducing [Ca^{2+}]i.

In the current study, we demonstrated for the first time that 5-HT potentiates Ca^{2+} sensitivity of the contractile apparatus through the activation of G-proteins in the ophthalmic arteries. At first, we determined the basic [Ca^{2+}]i-force relation of Ca^{2+}-contraction by the cumulative applications of extracellular Ca^{2+} during 118 mM K+-depolarization (Fig. 3A). We then examined the effect of 5-HT on the [Ca^{2+}]i-force relation of Ca^{2+}-contraction by applying extracellular Ca^{2+} during 118 mM K+-depolarization in the presence of 5-HT (Fig. 3B). 5-HT shifted the [Ca^{2+}]i-force relation upward from the line of the basic [Ca^{2+}]i-force relation of Ca^{2+}-contraction (Fig. 3C). That is compatible with our previous observations in other types of vascular smooth muscles that agonists produce greater force than expected from the changes in [Ca^{2+}]i, shifting the [Ca^{2+}]i-force relation to the upper left from the basic [Ca^{2+}]i-force relation of Ca^{2+}-contraction and indicating an increase in the Ca^{2+} sensitivity of contractile apparatus.

In the current study, we found that NG, but not Dil, shifted the [Ca^{2+}]i-force relation obtained in the presence of 5-HT downward, seemingly to overlap with the basic [Ca^{2+}]i-force relation of Ca^{2+}-contraction (Fig. 4C) in fura-2 loaded ophthalmic arterial strips, and that it relaxed the contraction induced by 5-HT plus GTP at constant [Ca^{2+}]i in the permeabilized ophthalmic arteries (Fig. 7). These findings are the first direct demonstration that NG decreases Ca^{2+} sensitivity of the contractile apparatus of ophthalmic arteries, whereas Dil has no such effect. We previously observed similar differential effects of NG and Dil on [Ca^{2+}]i-force relation in the porcine coronary artery. In ophthalmic arteries, NG and Dil reduced the sustained elevation of [Ca^{2+}]i induced by 5-HT to the same extent (Fig. 4). However, NG induced a greater decrease in force than Dil because NG, in addition to its inhibition of the [Ca^{2+}]i elevations (Figs. 4C, 7), decreases Ca^{2+} sensitivity of the contractile apparatus.
of [Ca\textsuperscript{2+}]_i, mediated only partly by the release of intracellular Ca\textsuperscript{2+} and mostly by the influx of extracellular Ca\textsuperscript{2+}, but also by an increase in the Ca\textsuperscript{2+} sensitivity of the contractile apparatus through the activation of G-proteins. We demonstrated the differential effects of Dil and NG on the [Ca\textsuperscript{2+}]_i elevations and the increase in Ca\textsuperscript{2+} sensitivity of contractile apparatus induced by 5-HT. Dil inhibits Ca\textsuperscript{2+} influx without affecting Ca\textsuperscript{2+} release and Ca\textsuperscript{2+} sensitivity of the contractile apparatus, whereas NG inhibits not only Ca\textsuperscript{2+} influx and Ca\textsuperscript{2+} release, but also the increase in Ca\textsuperscript{2+} sensitivity of the contractile apparatus induced by 5-HT. Because of the simple effect of Dil as a Ca\textsuperscript{2+} channel blocker and the multiple effects of NG on the signal transduction systems, for a given reduction in [Ca\textsuperscript{2+}]_i, NG can induce greater inhibition of the 5-HT-induced contraction in the ophthalmic artery than Dil.

**Key Words**

Ca\textsuperscript{2+}, diltiazem, nitroglycerin, ophthalmic artery, serotonin

**Acknowledgment**

The authors thank Brian T. Quinn for critical reading of the manuscript.

**References**

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