Biphasic Nature of Inotropic Action of Nitric Oxide Donor NOC7 in Guinea-Pig Ventricular Trabeculae

Mitsuyoshi OHBA and Hiroshi KAWATA

Department of Physiology, Faculty of Medicine, Fukuoka University, Fukuoka, 814-0180 Japan

Abstracts: The effects of nitric oxide (NO) donor on the contractility of guinea-pig ventricular trabeculae were explored to clarify whether NO affects the function of sarcoplasmic reticulum (SR) and the contractile elements. NO donor, 3-(2-hydroxy-1-methyl-2-nitroso-hydrazino)-N-methyl-1-propanamine (NOC7), increased monotonically the amplitude of twitch tension induced by electrical stimulation at a concentration of 20 μM. A higher concentration of NOC7 (200 μM) caused a biphasic response: transiently increased the amplitude of twitch and then decreased it. On wash-off of the higher concentration of NOC7, a rebound increase of the twitch amplitude was observed. An inhibitor of NO-sensitive guanylyl cyclase, 1H-[1,2,4]oxadiazolo-[4,3-a]quinoxalin-1-one (ODQ), abolished the monotonic increase and rebound increase in the amplitude of tension but did not affect the decrease in the amplitude of tension at the higher concentration of NOC7. Oscillatory contractions developed by β-escin–skinned muscle fibers were not changed by NOC7 at either concentration. Caffeine-induced tension transients indicating the Ca²⁺-accumulating and -releasing functions of intracellular Ca²⁺ stores were not affected by NOC7. NOC7 did not change the steady tension developed in 1.6 μM Ca²⁺ containing solution with and without ODQ. These results suggest that the biphasic inotropic effects by NOC7 were not caused by modifying the function of SR and the Ca²⁺ sensitivity of myofilaments of the guinea-pig ventricular trabecula, but at least the positive inotropic effect was mediated through cGMP-dependent mechanisms. [Japanese Journal of Physiology, 49, 389–394, 1999]

Key words: nitric oxide, contractility, β-escin, cardiac muscle.

NO is generated by a NO synthase in the heart and plays a significant role in cardiac function (for review, see Kelly et al. [1]). Although many investigations on the vasorelaxant effect of NO donors have been reported, the effects on myocardial contractility were still controversial. Negative inotropic effects by sodium nitroprusside (SNP) were reported in guinea-pig myocyte [2], rat atria [3], human myocardium [4] and rat ventricle [5]. A concentration-dependent biphasic contractile response was also reported; that is, a low dose of NO donors increased contractile response and a high dose decreased it [6, 7]. On the contrary, only a positive inotropic effect was reported in rat cardiomyocytes with the new NO donor, SPM3672 [8]. In addition, data showing that NO does not influence cardiac contractile function have been shown using different NO donors [9–11]. NO enhances soluble guanylyl cyclase activity and increases cGMP levels in cardiac muscle [1]. It has been suggested that the inotropic effects are partly induced by changing the L-type calcium current (I_Ca L) through modulating the cGMP-dependent phosphodiesterase [12, 13] but not by changing the ATP-sensitive K⁺ channel current [14]. Apart from the change of ion channel function by cGMP, a rapid release of Ca^{2+}\text{ from SR by the NO-donor, S-nitroscysteine [15], and a decrease in the myofilament responsiveness to Ca^{2+} by 8-bromo-cGMP [16] have been reported. These distinct results have been attributed to different myocardial tissues from different animals and to the type of NO donors employed.

Because NO is a gas and freely passes across cell
membranes, it may act on SR or on contractile elements either directly or indirectly through the well-known cGMP-dependent signaling pathway. In the present experiment, we explored the effects of NO on the contractility of guinea-pig ventricular trabeculae. We found that NO had biphasic effects on the twitch tension, however, it had no direct effects on the function of the SR and the Ca\(^{2+}\) sensitivity of myofilaments in the chemically skinned fiber.

**METHODS**

Guinea pigs of either sex, weighing about 300 g were sacrificed by being stunned and bled according to procedures approved by the Animal Experimentation Committee of the Animal Center, Fukuoka University. The heart was removed quickly and the fine trabecula (approximately 2×0.3 mm) was excised. Contractile tension was measured on both intact and chemically skinned fibers. For the experiments with chemically skinned fibers, thinner and shorter trabeculae (approximately 1.5×0.15 mm) were used. Details of the recording methods have been published previously [17]. Twitch tensions were obtained by electrically stimulated trabecula at 1 Hz. The temperature of the bath where either modified Tyrode solution or myoplasmic solutions were superfused was kept at 24°C and the bath contained 40 µl of solution. NO released into the superfused solution from NO donor would be rapidly oxidized when the concentration of oxygen is high. Since it is essential that the lowest O\(_2\) level that maintains tissue responsiveness be determined prior to experiments [18], in the present experiment we chose atmospheric air (20% O\(_2\)). Even in this hypoxic condition, we confirmed that muscle could contract more than 4 h without deterioration. Modified Tyrode solution contained (mM): 135 NaCl, 2.7 KCl, 1.0 MgCl\(_2\), 1.8 CaCl\(_2\), 5.6 glucose, 10 HEPES, and was titrated to pH 7.3 with NaOH.

Protocol and myoplasmic solutions to permeabilize the thin trabeculae were similar to a previous report [17] except that the chemical employed to permeabilize the cell membrane was β-escin instead of α-toxin. The myoplasmic solutions were made according to a computer program [19] and small changes from the previous constitution were made to correct the binding of GTP to Ca\(^{2+}\) and Mg\(^{2+}\) and to modify the pH to 7.3 and temperature to 24°C. Apparent stability constants for the complexes between GTP and Ca\(^{2+}\), and between GTP and Mg\(^{2+}\) used to calculate the concentration of myoplasmic solutions were 3,802 and 10,471 M\(^{-1}\), respectively. β-Escin of 100 µM in 0.25% dimethyl sulfoxide (DMSO) was used to perforate the cell membrane. If the trabecula was thin enough, then perforation was completed within 20 min showing that the steady tension in pCa 5.8 solution (1.6 µM free Ca\(^{2+}\), 3 mM EGTA) was achieved. The relaxing solution used with skinned fibers had the following composition (mM): 105 K methanesulfonate (KMS), 3.7 Na\(_2\)ATP, 10 Na\(_2\)-phosphocreatine (Na\(_2\)PCr), 4.4 MgCl\(_2\), 2 EGTA, and 30 N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES). KMS was used to adjust the ionic strength of myoplasmic solutions to 0.18 M. To assay the Ca\(^{2+}\) remaining in SR, 20 mM caffeine was contained in an assay solution (0.1 mM EGTA, 0.1 mM free Mg\(^{2+}\)), which elicits caffeine-induced tension transients.

NOC7 (Dojindo Laboratories, Japan) was dissolved in 10 mM NaOH (or KOH for chemically skinned fibers) to make 200 mM solution and stored at 5°C. Then it was diluted to either 20 or 200 µM of NOC7 just before use. The final concentration of NaOH (or KOH) of 10 µM did not affect the twitch contractions and the tension developed by the chemically skinned fibers. We did not measure the level of NO concentration directly, however, confirmed the presence of NO released from NOC7 using the Griess reaction that detects the oxidative metabolites of NO [20]. We also confirmed the generation of NO from NO donors using the same experimental procedure and chamber used in the present experiment; the guinea-pig aortic ring precontracted by 6 µM noradrenaline was completely relaxed by 114 mM NOC7.

ODQ (Tocris Cookson, UK) and KT5823 (Calbiochem-Novabiochem, USA) were dissolved in 50% DMSO as stock solutions of 10 and 0.2 mM, and then diluted to 50 and 0.8 µM, respectively. The peak tension was not affected by the 0.5% DMSO. Superoxide dismutase (SOD) was purchased from Wako Pure Chemicals (Japan). KMS was purchased from Tokyo Kasei (Japan); EGTA, BES, MgCl\(_2\), CaCl\(_2\) and Na\(_2\)ATP were from Fluka (Switzerland); HEPES was from Dojindo Laboratories; Na\(_2\)PCr was from Oriental Yeast (Japan). β-Escin, DMSO and all other chemicals were purchased from Sigma (USA).

**RESULTS**

**Effects of NOC7 on the tension development**

Characteristics of NO generation from NO donors differ among the agents. The experiments on chemically skinned muscle fiber have to be conducted at low temperature (24°C) to keep the skinned muscle from deteriorating. Since the rate of NO release can vary depending on the temperature of solutions [21], we first explored how the NOC7 acts on the twitch ten-
sion of the trabecula at 24°C and pH 7.3. In preliminary experiments, we examined the dose dependency of twitch tensions to NOC7 at 24°C. We found that only positive inotropic was observed at 10 to 100 μM of NOC7 and biphasic inotropic was seen at the concentration of more than 150 μM. Thus, we used two concentrations of NOC7, 20 and 200 μM, in the following experiments. NOC7 at 20 μM caused only a positive inotropic effect in the twitch tension (the amplitude increased by 26%, n=5) (Fig. 1A and C: left). The rate of rise and the rate of relaxation of the twitch were also increased (Fig. 2A). A higher concentration of NOC7 (200 μM) initially induced a transient increase in the amplitude of twitch tension (Fig. 1A and C: right, Fig. 3A and C) and increased the rate of rise and fall of twitch tension (data not shown) as at a lower concentration of NOC7. Following the initial increase, the twitch amplitude decreased by 39% (n=3) in 10 min. When the amplitude was reduced, the twitch duration was abbreviated (Fig. 2B). When 200 μM NOC7 was washed, a marked rebound increase in the amplitude of twitch tension was seen (Fig. 1A and C: right, Fig. 3A and C). Neither atropine (10^-7 M) nor propranolol (10^-8 M) changed both the biphasic inotropic response to, and the rebound increase on removal of 200 μM NOC7 (data not shown). ODQ, inhibitor of soluble guanylyl cyclase [22] blocked the positive inotropic effects of 20 μM NOC7 (Fig. 1B: left). It also suppressed the initial and rebound positive inotropic by 200 μM NOC7 (Fig. 1B: right), but did not alter the negative inotropic (Fig. 1B: right). Figure 3 shows that the NOC7-induced biphasic inotropic action was not changed by 1,000 U/ml SOD that stops NO being converted to peroxynitrite [13].

**Effects of NOC7 on the tension developed by chemically skinned muscle fibers**

To explore the effects of NOC7 on the function of SR and the contractile elements, the cell membrane was permeabilized by β-escin but internal membrane was kept intact [17]. Figure 4 shows the oscillatory contractions induced by a solution containing 0.1 mM EGTA, 0.5 mM free Mg²⁺ and about 0.5 μM free Ca²⁺ (oscillatory solution) in which the SR of cardiac muscle can induce spontaneous cyclic release of Ca²⁺. Neither the concentration of 20 (A) or 200 μM (B) NOC7 added to the oscillatory solution changed the frequency and amplitude of the oscillatory tensions. These results were not altered by 50 μM GTP added to the oscillatory solution to replenish the loss of endogenous GTP (C and D).

Figure 5 shows that the Ca²⁺-accumulating function of SR was not changed by NOC7. After depleting the SR of Ca²⁺ by 20 mM caffeine added to the relaxing solution and washing away caffeine with relaxing solution, the trabecula was incubated for 10 min in loading solution (0.3 μM free Ca²⁺, 0.5 mM EGTA and 1 mM free Mg²⁺). The loading solution allows the SR to take up Ca²⁺ [17]. To assay the Ca²⁺ accumulated

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**Fig. 1. Effects of NOC7 and ODQ on twitch tensions.** Either a concentration of 20 or 200 μM NOC7 was applied for 10 min as indicated by a solid line. A: control, B: in 50 μM ODQ containing solution (under dotted line) and C: washout.

**Fig. 2. Effects of NOC7 on the time course of twitch tension.** C shows control and N under NOC7 of 20 μM (A: 9 min after NOC7 application) and of 200 μM (B: 7 min after NOC7 application).

**Fig. 3. Effects of SOD on the tension changes induced by NOC7.** A: control, B: in 1,000 U/ml SOD containing solution (under dotted line) and C: washout. NOC7 of 200 μM was applied for 10 min as indicated by a solid line.
to SR after loading the Ca\(^{2+}\) with or without NOC7, the amplitude of caffeine-induced tension transients (NOC7 20 \(\mu\)M and NOC7 200 \(\mu\)M) was compared with the magnitude of the first (C1) and third (C2) transients. The magnitude of tension transient at NOC7 20 \(\mu\)M was 101±3\% (\(n=11\)) of the control (the mean amplitude of C1 and C2) and was 101±1\% (\(n=3\)) at NOC7 200 \(\mu\)M. Changes in the Ca\(^{2+}\)-releasing function of SR by NOC7 were also examined (Fig. 6). After loading the Ca\(^{2+}\) to SR, the trabecula was incubated in releasing solution (Mg\(^{2+}\) and Na\(_2\)ATP are omitted and 1.6 \(\mu\)M free Ca\(^{2+}\)) with or without NOC7 for 10 min. The releasing solution accelerates Ca\(^{2+}\)-induced Ca\(^{2+}\) release from, and avoids Ca\(^{2+}\) reuptake to SR. Then, the Ca\(^{2+}\) remaining in SR was assayed [17]. The amplitude of caffeine-induced tension transients at NOC7 20 and 200 \(\mu\)M were 97±7\% (\(n=14\)) and 102±11\% (\(n=8\)) of the control (the mean of C1 and C2).

Figure 7 shows the effect of NOC7 on the steady tension activated in pCa 5.8 solution (1.6 \(\mu\)M free Ca\(^{2+}\)). The change of pCa-tension curve during the application of NOC7 cannot be obtained since the generation of NO from the NO donor is time-depen-

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**Fig. 4. Effects of NOC7 on the spontaneous contracts induced by chemically skinned fiber.** A and B are obtained in control oscillatory solutions and C and D are in 50 \(\mu\)M GTP containing oscillatory solutions. The frequency of spontaneous contractions at 20 \(\mu\)M NOC7 (A) was 99±5\% (\(n=4\)) of the control frequency and 103±13\% (\(n=4\)) at 200 \(\mu\)M NOC7 (B). NOC7 was applied for 10 min as indicated by a solid line.

**Fig. 5. Effects of NOC7 on the Ca\(^{2+}\) accumulating function to SR in chemically skinned fibers.** C1 was the caffeine-induced tension transient obtained in assay solution before application of NOC7 and C2 was at 30 min after washing NOC7. Middle panel was the caffeine-induced tension transient after NOC7 of either a concentration of 20 or 200 \(\mu\)M was added only in the loading solution.

**Fig. 6. Effects of NOC7 on the Ca\(^{2+}\) releasing function from SR in chemically skinned fibers.** C1 was the caffeine-induced tension transient before application of NOC7 and C2 was at 40 min after washing NOC7. Middle panel was the caffeine-induced tension transient after NOC7 either of 20 or of 200 \(\mu\)M was added only in the releasing solution.

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with superoxide anion, elicited Ca$^{2+}$ release from rabbit skeletal SR vesicles [15]. Peroxynitrite was also able to stimulate $I_{\text{Ca-L}}$ directly through S-nitrosylation and/or oxidation of the channel subunit complex in ferret ventricular myocytes [13]. Thus, peroxynitrite may increase developed tension. However, in the present experiments, SOD, which prevents peroxynitrite formation [13], did not affect the biphasic nature of inotropic action by NOC7, indicating no contribution of peroxynitrite to the inotropism.

On the other hand, the increase of twitch tensions was blocked by ODQ, specific inhibitor of soluble guanylyl cyclase [22], suggesting that the positive inotropic effects of the low concentration of NOC7 are mediated by cGMP as mentioned above. A cGMP-mediated positive inotropic effect could be induced by the activation of cGMP-inhibited cAMP phosphodiesterase that increases $I_{\text{Ca-L}}$ [23]. The transient increase observed immediately after the application of the higher concentration of NOC7 was also blocked by ODQ, suggesting the same mechanism as that at the low concentration. This result seems to be due to the slow rate of generation of NO from NOC7 at 24°C. Although a half-life for NO release of NOC7 is 5 min at 37°C and pH 7.4, the half-lives of many NO donating compounds show unusual temperature dependence; that is, a 2- to 9-fold increase has been reported ongoing from 37 to 22°C [21]. In the previous experiments at 37°C and pH 7.4, the twitch contraction showed a biphasic response to the low concentration of NOC7 (20 µM); the twitch increased more quickly (within 5 to 7 min) than the present one [24].

NO has been reported to trigger Ca$^{2+}$ release from skeletal and cardiac SR [15]. However, the present study showed that NOC7 did not change the frequency and amplitude of spontaneous contractions of the chemically skinned fibers. Moreover, the Ca$^{2+}$ released from SR and the capacity of SR to accumulate Ca$^{2+}$ were not increased by the low concentration of NOC7. Therefore, the present results suggested the positive inotropic effect is not mediated through the changes in the SR function.

The higher concentration of NOC7 reduced the amplitude of twitch tension and shortened the twitch duration. This negative inotropic effect might be due to a large increase in cGMP produced by high doses of NO donors, mediated through the activation of cGMP-dependent protein kinase [6]. Moreover, 50 µM 8-bromo-cGMP has been reported to reduce myocyte twitch amplitude and time to peak shortening with reducing the Ca$^{2+}$ sensitivity of myofilaments [16]. However, we found that the higher concentration of NOC7 still reduced the twitch tensions under

**Fig. 7. Effects of NOC7 on the Ca$^{2+}$ sensitivity of myofilaments in chemically skinned fibers.** Each row shows the tension developed in pCa 5.8 solution. NOC7 was applied for 10 min as indicated by a solid line. A and B were conducted after the steady tension was achieved in control pCa 5.8 solution. C and D were taken after application of 50 µM ODQ for 30 min in the pCa 5.8 solution. Since data were digitized and recorded by high gain, small artifacts are seen.

**DISCUSSION**

An NO donor, NOC7, induced the positive and negative inotropic effects on the contractions of the guinea-pig ventricular trabecula depending on the concentration. Present results suggest that the positive inotropic effects of NOC7 are related to cGMP-dependent mechanisms. However, the inotropic effects might not be attributed to the change in the function of SR or to the change in the Ca$^{2+}$ sensitivity of myofilaments.

Although the present results showed that exogenous NO released by the low concentration of NOC7 increased the amplitude of twitch contractions monotonically mediated via the cGMP-dependent mechanism, the possibility that NO can affect cardiac function by non-cGMP-mediated action has been reported [1]. Peroxynitrite, which NO produces in combination...
KT5823, which selectively inhibits protein kinase activity [25], (our unpublished observation). Moreover, the present result that the negative inotropic effect by a higher concentration of NOC7 was not blocked by ODQ suggests NO-activated cGMP-independent inotropism. No direct actions of NOC7 on the Ca\(^{2+}\) sensitivity of myofilaments and on the function of SR were found in the chemically skinned fibers, suggesting another cGMP-independent mechanism.

In summary, NO donor–induced positive inotropic action was cGMP-dependent. However, NO donor–induced negative inotropic action was cGMP-independent. The biphasic inotropic action might not be caused by changes in the function of SR and the Ca\(^{2+}\) sensitivity of myofilaments.

REFERENCES