Peroxynitrite is a positive inotropic agent in atrial and ventricular fibres of the frog heart

Jean-Michel Chesnais, Rodolphe Fischmeister, Pierre-François Méry

INSERM U-446, Laboratoire de Cardiologie Cellulaire et Moléculaire, Université Paris-Sud, Faculté de Pharmacie, Châtenay-Malabry, France

1. We report opposite inotropic effects of NO donors in frog cardiac fibres. The negative effect, elicited by either 3-morpholino-sydnonimine (SIN-1) or *S*-nitroso-*N*-acetyl-penicillamine (SNAP), involved cyclic GMP (cGMP) production. However, SIN-1, unlike SNAP, could elicit a positive effect, in a superoxide dismutase (SOD)-sensitive manner. SIN-1, unlike SNAP, can release both NO and superoxide anion, the precursors of peroxynitrite ($OONO^-$). The role of these messengers was examined.

2. Catalase did not reduce the positive inotropic effect of SIN-1. Thus, a conversion of superoxide anion into hydrogen peroxide was not involved in this effect. In addition, catalase did not modify the negative effects of SIN-1 plus SOD, or SNAP plus SOD.

3. LY 83583, a superoxide anion generator, elicited a positive inotropic effect, like SIN-1. The effect of LY 83583 was additive to the negative effects of SIN-1 or SNAP, and to the positive effect of SIN-1. Thus, superoxide anion generation, *per se*, did not account for the positive effect of SIN-1.

4. Authentic peroxynitrite ($OONO^-$), but not mock-$OONO^-$ (negative control plus decomposed $OONO^-$), exerted a dramatic positive inotropic effect in cardiac fibres. The effect of $OONO^-$ was larger in atrial fibres, as compared with ventricular fibres.

5. The positive effect of $OONO^-$ was not additive with that of SIN-1, suggesting a common mechanism of action. In contrast, the effects of either $OONO^-$ or SIN-1 were additive with the negative inotropic effect of SNAP. Furthermore, the effect of $OONO^-$, like that of SIN-1, was not antagonized by 1H-[1,2,4]xidiazolo[4,3-a]quinoxaline-1-one (ODQ; 10 μM), the guanylyl cyclase inhibitor.

6. The positive inotropic effects of SIN-1 and $OONO^-$ were not modified by hydroxyl radical scavengers, such as dimethyl-thio-urea (DMTU; 10 mM).

7. The positive inotropic effect of SIN-1 (100 μM) was abolished in sodium-free solutions, a treatment that eliminates the activity of the sodium–calcium exchanger. In contrast, the effect of SIN-1 was unchanged by a potassium channel inhibitor (tetraethyl-ammonium, 20 mM), or a sodium–potassium pump inhibitor (ouabain 10 μM).

8. We conclude that $OONO^-$ is a positive inotropic agent in frog cardiac fibres. The generation of $OONO^-$ accounts for the positive inotropic effect of SIN-1. $OONO^-$ itself was responsible for the positive inotropic effect, and appeared to modulate the activity of the sodium–calcium exchanger.

It has been suggested that nitric oxide (NO) participates in cardiac function (Shah, 1996; Wolin *et al.* 1997; Kojda & Kottenberg, 1999). *In vitro* and *in vivo* studies illustrated the multiple, and sometimes contradictory, effects of NO on cardiac contractility. Indeed, NO was found to elicit positive chronotropic effects (Musialek *et al.* 1997; Kojda *et al.* 1997), positive or negative inotropic effects (Mohan *et al.* 1996; Shah,

1996; Kojda & Kottenberg, 1999), and positive lusitropic effects (Brutsaert & Andries, 1992; Shah, 1996; Flesch *et al.* 1997). Furthermore, NO was involved in the staircase of the force–frequency relationship (reviewed in Shah, 1996). In apparent contrast, other investigators found that authentic NO, NO donors or endogenous NO production had no significant effects on the shortening of isolated myocytes,

on the contractility of isolated papillary muscles, and on the inotropism or chronotropism of whole hearts (Kennedy *et al.* 1994; Weyrich *et al.* 1994; MacDonell *et al.* 1995; Nawrath *et al.* 1995; Wyeth *et al.* 1996; Crystal & Gurevicius, 1996; MacDonell & Diamond, 1997).

We recently reported opposite inotropic effects of NO donors in fibres isolated from the atria or the ventricle of the frog heart (Chesnais *et al.* 1999). Indeed, SNAP, a nitrosothiol, and SNP, a cyanoferrate, always induced strong and similar negative inotropic effects in frog cardiac fibres. In contrast, SIN-1, a sydnonymine, elicited either a negative inotropic effect (typically in two thirds of the atrial fibres), or a positive inotropic effect (in most ventricular fibres, and in one third of the atrial fibres). The negative inotropic effect of NO donors was consistently antagonised by ODQ, an inhibitor of the heterodimeric guanylyl cyclase (Schrammel *et al.* 1996), suggesting the involvement of cGMP production. The guanylyl cyclase is indeed considered as an important target for NO in the heart (Shah, 1996; Kojda & Kottenberg, 1999). In cardiac myocytes, the increase in cGMP levels can co-ordinate the activity of various proteins, including the cGMP-dependent protein kinase, the cGMP-regulated phosphodiesterases (PDE2 and PDE3), and the cationic 'f' current (Iino *et al.* 1997; Méry *et al.* 1997; Musialek *et al.* 1997).

In frog fibres, the positive inotropic effect of SIN-1 was unaffected by ODQ (Chesnais *et al.* 1999). Previous studies had shown that NO might regulate cardiac contractility in a cGMP-independent manner (Stamler, 1994; Crow & Beckman, 1995; Beckman & Koppenol, 1996). Thio-nitrosylation by NO was involved in the modulation of the sarcolemmal L-type calcium channel (Campbell *et al.* 1996), the stimulation of the calcium channel-ryanodine receptor of the sarcoplasmic reticulum (Xu *et al.* 1998), and the inhibition of creatine kinase (Gross *et al.* 1996). NO also regulates mitochondrial respiration in a cGMP-independent manner, and this effect might involve the binding of NO on cytochrome oxidase (see Wolin *et al.* 1997).

Interestingly, the positive inotropic effect of SIN-1 was eliminated in the presence of superoxide dismutase (SOD) in frog fibres, suggesting the involvement of superoxide anion (Chesnais *et al.* 1999). In addition, LY 83583, a superoxide anion generator, mimicked the positive inotropic effect of SIN-1, in a SOD-sensitive manner. *In vitro* experiments have illustrated that SIN-1 can release both NO and superoxide anion, the combination of which gives rise to peroxynitrite, $OONO^-$ (Feelisch *et al.* 1989; Beckman & Koppenol, 1996; Mayer *et al.* 1998). In the present study, we investigated the mechanism of action of SIN-1, and the consequence of the simultaneous generation of NO and superoxide anion. Our aim was to identify the intermediate(s) involved in the positive inotropic effect of SIN-1.

A preliminary report of part of this work has appeared in an abstract form (Chesnais *et al.* 1997).

METHODS

Abbreviations

NO, nitric oxide; NOS, nitric oxide synthase; cGMP, cyclic GMP; PDE2, type 2 phosphodiesterase; PDE3, type 3 phosphodiesterase; SIN-1, 3-morpholino-sydnonimine; SNP, sodium nitroprusside; SNAP, *S*-nitroso-*N*-acetyl-penicillamine; SOD, superoxide dismutase; DMTU, dimethyl-thio-urea; LY 83583, 6-anilino-5,8-quinolinedione; ODQ, 1H-[1,2,4]xidiazolo[4,3-a]quinoxaline-1-one; DMSO, dimethylsulfoxide.

Contraction recordings

Frogs (*Rana esculenta*) were killed by decapitation and double pithed. Fibres (150–300 μm in diameter; 2–4 mm length) were dissected out from the endocardial surface of the hearts (Chesnais *et al.* 1999). Following dissection, the fibres were pinned at one end to the floor of the perfusion chamber, and hooked, at the other end, with a fine silk thread, to the force transducer (type 801, SensoNor, Horten, Norway). Before each experiment, the resting length was set by releasing the muscle until there was no resting tension and then increasing the length by 20%. Equilibration was processed as described (Chesnais *et al.* 1999). Field stimulation was applied with platinum electrodes at a constant frequency of 0·2 Hz, its amplitude being adjusted to 10% above the threshold value. In sodium-free solutions, the frequency of stimulation was reduced to 0·1 Hz.

This investigation conformed with the European Community and French national guidelines in the care and use of animals.

Solutions for contraction recordings

Control Ringer solution contained (mM): 88 NaCl; 0·513 NaH_2PO_4; 2·5 KCl; 1·8 $MgCl_2$; 1·8 $CaCl_2$; 2·4 $NaHCO_3$; 5 glucose; 5 sodium pyruvate; 5 creatine; 10 Hepes; and adjusted to pH 7·4 with NaOH. When indicated, 10 mM NaCl was substituted for 20 mM mannitol, or 88 mM NaCl was substituted for 88 mM LiCl. The solutions were perfused by gravity into the chamber, at a rate > 3 ml min^{-1}. The volume of the perfusion chamber (150 μl) was kept constant in order to avoid artefactual modifications of the tension due to fluid level variations. Experiments were performed at room temperature (19–26 °C). Some recordings lasted more than 2 h, and temperature did not change by more than 2 °C in a given experiment.

Aliquots of $OONO^-$ (kept in 0·3 M NaOH, at −20 °C) were used once, just before being applied onto the fibres. According to manufacturer's instructions, the concentration of $OONO^-$ in the stock solution was periodically measured (as an increase in absorbance at 302 nm). The negative control solution supplied by the manufacturer, as well as decomposed $OONO^-$ were used as mock-$OONO^-$. Stock solution of either $OONO^-$ or mock-$OONO^-$ were infused with a Hamilton syringe to the distal tip of the perfusion catheter using a single-syringe pump (KDS 100, Bioblock, Meudon, France). Different concentrations of $OONO^-$ (or of mock-$OONO^-$) were obtained by varying the pump rate according to the perfusion rate. The 'concentration' of mock-$OONO^-$ refers to the concentration of $OONO^-$ infused under identical conditions. ODQ (100 mM) was dissolved in dimethylsulfoxide (DMSO). DMSO, up to 0·1 μl ml^{-1}, had no effect on the contraction of frog cardiac fibres (data not shown). All other agents were diluted in Ringer solution at the most 10 min before superfusion onto the fibres, i.e. only fresh solutions were tested. Solutions were protected from natural light.

Data analysis

During the experiments, recordings were displayed on an oscilloscope and on a chart recorder. Isometric active tension,

passive tension, time to peak (T_{peak}), and half-time for relaxation $(T_{1/2})$ were measured on-line (sampling frequency, 0·5–2 kHz) and stored on a PC-compatible computer programed in Pascal language (Microsoft). In the experiments of Figs 1–3 and 5–7, each symbol is the amplitude of the active tension, measured every 5 s.

The results are expressed as the mean ± s.e.m. Differences between means were tested for statistical significance by Student's paired t test. The effect of the compounds used, alone or in combination, is referred to as the percentage variation over the basal level. The resting tension was not modified by the compounds investigated.

Drugs

SIN-1 was a generous gift from Dr J. Winicki (Hoechst Houdé Laboratories, Paris-la-Défense, France). LY 83583 was from Calbiochem (La Jolla, CA, USA) or from E. Lilly Pharmaceuticals (Indianapolis, IN, USA). SNAP was from Calbiochem, or Tocris-Cookson (Bristol, UK), or Alexis (San Diego, CA, USA). ODQ was from Tocris-Cookson. Superoxide dismutase (SOD, from erythrocytes) and catalase (from bovine liver) were from Sigma Chemical Co. (St Louis, MO, USA). Authentic $OONO^-$ and its negative control were from Alexis. Other drugs were from Sigma Chemical Co.

RESULTS

Effect of catalase on the positive inotropic effect of SIN-1

SIN-1 can exert both positive and negative inotropic effects in frog heart (Chesnais *et al.* 1999), suggesting the involvement of either superoxide anion itself, or its derivative, hydrogen peroxide (Mao *et al.* 1993; Beckman & Koppenol, 1996). To distinguish between these two possibilities, we examined the effect of catalase, a hydrogen peroxide scavenger. In the experiment of Fig. 1*A*, superfusion of a frog ventricular fibre with SIN-1 (100 µM) elicited a positive inotropic effect (+14% over basal). After washout of the NO donor, the addition of catalase (1000 U ml^{-1}), a hydrogen peroxide scavenger, induced a slight negative inotropic effect. In the continuing presence of catalase, addition of SIN-1 again increased the active tension (+18% over basal), and this effect was similar to that observed in the absence of catalase. The effects of the compounds were reversible.
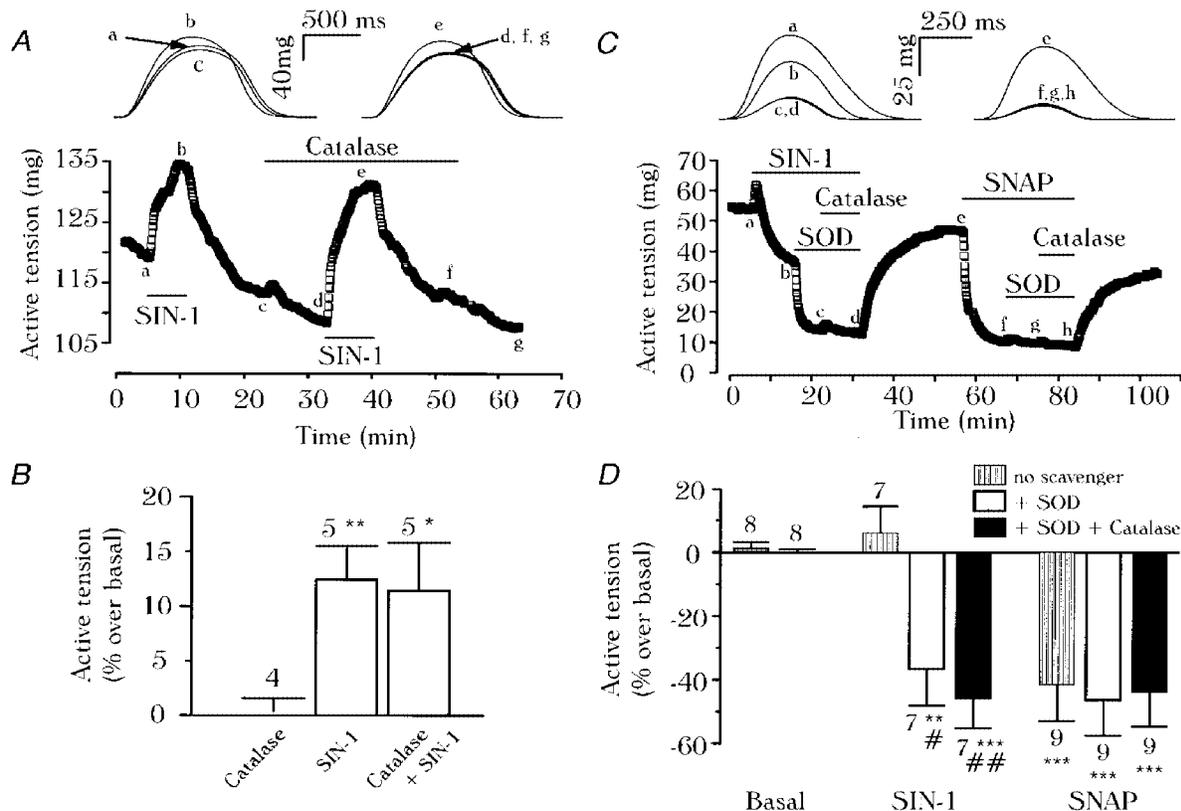
Figure 1. Effect of catalase in the presence of NO donors in frog cardiac fibres
A ventricular (*A*) or an atrial (*C*) fibre was initially superfused with control solution. In *A*, SIN-1 (100 µM) was applied in the absence or presence of catalase (1000 U ml^{-1}), as indicated by the lines. In *C*, SOD (50 U ml^{-1}) and catalase (200 U ml^{-1}) were added in the presence of SIN-1 (100 µM) or SNAP (100 µM). Top, traces were recorded at the times indicated by the corresponding letters on the main graphs. *B*, summary of the effects of catalase (1000 U ml^{-1}) and SIN-1 (100 µM), alone or in combination, in ventricular fibres where SIN-1 had induced positive effects. *D*, summary of the effects of SNAP (100 µM) or SIN-1 (100 µM) in the presence of SOD (50–200 U ml^{-1}), with or without catalase (200–1000 U ml^{-1}). In *B* and *D*, the active tension in the presence of agents was normalised to its value in the absence of agents. Bars and lines are the mean ± s.e.m. of the number of experiments indicated. Statistical differences from the basal level (*) or from SIN-1 (#) are indicated as * # $P < 0·05$; ** ## $P < 0·01$; *** $P < 0·005$.

On average, catalase (1000 U ml^{-1}), used either alone or in the presence of SIN-1 (100 μM), had no significant effects on the amplitude of the active tension in frog ventricular fibres (Fig. 1B). Thus, hydrogen peroxide does not appear to be involved in the positive inotropic effect of SIN-1.

Effect of catalase on the negative inotropic effect of SIN-1

When SIN-1 elicits a negative inotropic effect in frog cardiac fibres, SOD is able to strengthen this effect (Chesnais et al. 1999). In these experiments, the level of hydrogen peroxide might be raised as a result of SOD activity (Mao et al. 1993; Beckman & Koppenol, 1996). Since hydrogen peroxide can dramatically modify the effects of NO (Farias-Eisner et al. 1996), we examined the effect of catalase in the presence of NO donors and SOD. In the atrial fibre shown in Fig. 1C, SIN-1 (100 μM) induced a negative inotropic effect (−30% from basal), that was reinforced by the addition of SOD (50 U ml^{-1}) in the continuing presence of SIN-1 (−73% from basal). The negative inotropic effect of SIN-1 occurred together with a reduction in the time to peak of the contraction, T_{peak} (−8% from basal) and in the half-

time for relaxation, $T_{\frac{1}{2}}$ (−23% from basal). These effects were also potentiated by SOD (to, respectively, −10 and −41% from basal). The robust negative inotropic effect of SIN-1 plus SOD was not modified by the further addition of 200 U ml^{-1} catalase (−74% from basal). After washout of the compounds, application of SNAP (100 μM) elicited a large negative inotropic effect (−78% from basal), with an amplitude comparable to that observed in the presence of SIN-1 plus SOD. The nitrosothiol also reduced T_{peak} (−13% from basal) and $T_{\frac{1}{2}}$ (−41% from basal). The negative effect of SNAP was unchanged by the addition of SOD (50 U ml^{-1}) alone, or in the presence of catalase (200 U ml^{-1}).

As shown in Fig. 1D, SOD (50–200 U ml^{-1}) had no significant effects on frog cardiac contraction, either in the absence or in the presence of catalase (200–1000 U ml^{-1}). Furthermore, catalase did not modify the negative inotropic effect of SIN-1 (100 μM) in the presence of SOD (50–200 U ml^{-1}). The alterations in the kinetics of the contraction induced by SIN-1 plus SOD (−9·5 ± 1·6% from basal T_{peak}, $P < 0·005$; −21·3 ± 5·6% from basal $T_{\frac{1}{2}}$, $P < 0·01$) were not changed in the presence of catalase
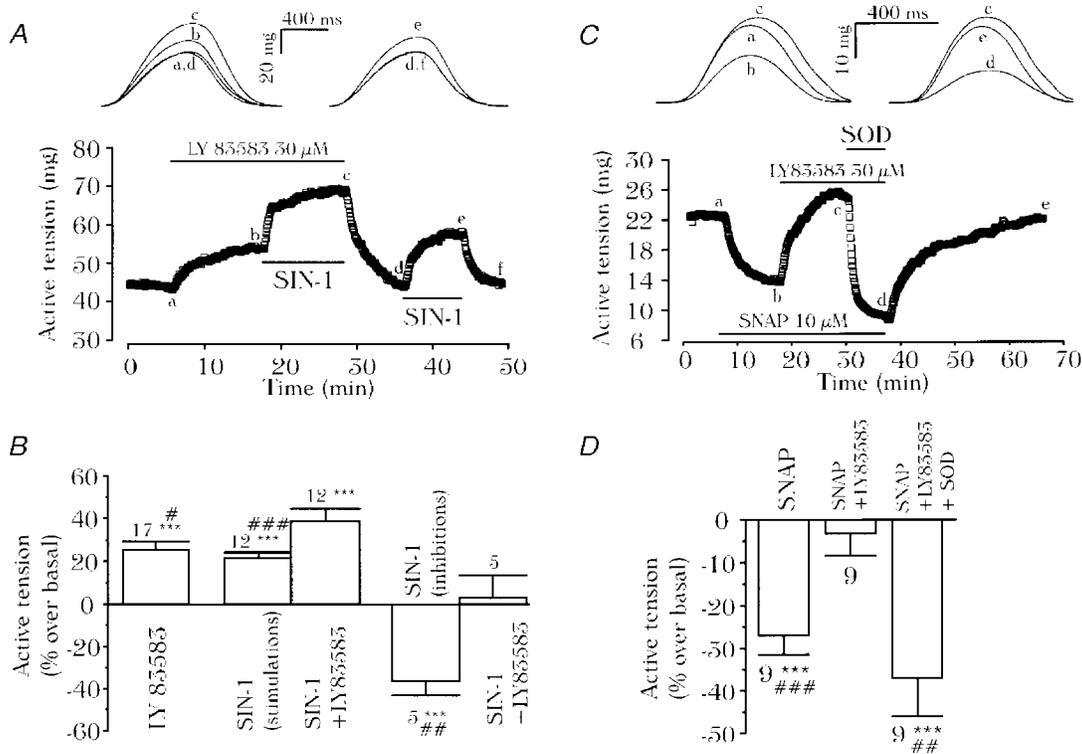
Figure 2. Effects of LY 83583 and NO donors in frog cardiac fibres

The control solution was superfused onto a ventricular (A) or an atrial (C) fibre. In A, SIN-1 (100 μM) and LY 83583 (30 μM) were added alone or in combination, as indicated by the lines. In C, SNAP (10 μM), LY 83583 (30 μM), and SOD (370 U ml^{-1}) were successively added to the solution. Top, traces were recorded at the times indicated by the corresponding letters on the main graphs. B summarises the inotropic effects of LY 83583 (10 μM) and SIN-1 (100 μM). Positive (11 ventricular plus 1 atrial fibre) and negative (5 atrial fibres) inotropic effects of SIN-1 were separated. D summarises the inotropic effects of SNAP (5–20 μM), alone, or in the presence of LY 83583 (30 μM), with or without SOD (200–370 U ml^{-1}). The active tension in the presence of agents was normalised to its value in the absence of agents. Bars and lines are the mean ± s.e.m. of the number of experiments indicated. Statistical differences from the basal level are indicated as * $P < 0·05$; ** $P < 0·01$; *** $P < 0·005$; and differences from SIN-1 plus LY 83583 (in B), or SNAP plus LY 83583 (in D) are indicated as # $P < 0·05$; ## $P < 0·01$; ### $P < 0·005$.

$(-9.7 \pm 2.3\%$ from basal T_{peak}; $-24.2 \pm 6.4\%$ from basal $T_{1/2}$, both $P < 0.01$). SIN-1 alone had exerted a positive effect in 5 of these 7 experiments. Catalase also did not modify the negative inotropic effects of SNAP (100 μM) in the presence of SOD (50–100 U ml^{-1}). Thus the conversion from superoxide anion to hydrogen peroxide does not play a significant role in the negative inotropic effects of NO donors. These experiments demonstrate that superoxide anion *per se* is involved in the positive inotropic effect of SIN-1.

Effects of NO donors in the presence of LY83583

Although SIN-1 releases both NO and superoxide anion, the precursors of peroxynitrite (Feelisch *et al.* 1989), one may question whether superoxide anion or peroxynitrite mediates its positive inotropic effect. To discriminate between these two possibilities, we compared the effects of SIN-1 with those of LY 83583, a superoxide anion generator which does not release NO (see Abi Gerges *et al.* 1997; Chesnais *et al.* 1999, and references therein). In the experiment of Fig. 2*A*, a ventricular fibre was first exposed to LY 83583 (30 μM), resulting in a 23% increase in the active tension over basal. Addition of SIN-1 (100 μM) to the solution induced an additional positive inotropic effect (to 55% over basal). These effects were reversible upon washout of the two compounds. When SIN-1 was applied

alone, it induced a positive inotropic effect (28% over basal), which was similar in amplitude to that observed in the presence of LY 83583.

The results of several similar experiments are summarised in Fig. 2*B*. LY 83583 (10 μM) always exerted positive inotropic effects, without significant changes in the kinetics of the contraction (data not shown). SIN-1 exerted either positive (12 out of 17 experiments) or negative (5 out of 17 experiments) inotropic effects in cardiac fibres. In both cases, LY 83583 produced an additional stimulatory effect. Thus SIN-1 and LY 83583 are unlikely to act by the same mechanism.

Although superoxide anion generation accounts for the positive inotropic effect of LY 83583 when applied alone (Chesnais *et al.* 1999), the mechanism of action of LY 83583 might be modified in the presence of a NO donor. This possibility was examined in the following experiments. In the experiment illustrated in Fig. 2*C*, LY 83583 (30 μM) was applied in the presence of SNAP (10 μM) in a frog atrial fibre. The negative inotropic effect of the nitrosothiol (a 39% decrease in the active tension) was antagonised by LY 83583. The active tension was even slightly enhanced by the combination of SNAP plus LY 83583, when compared with the basal level (11% over basal). Further addition of
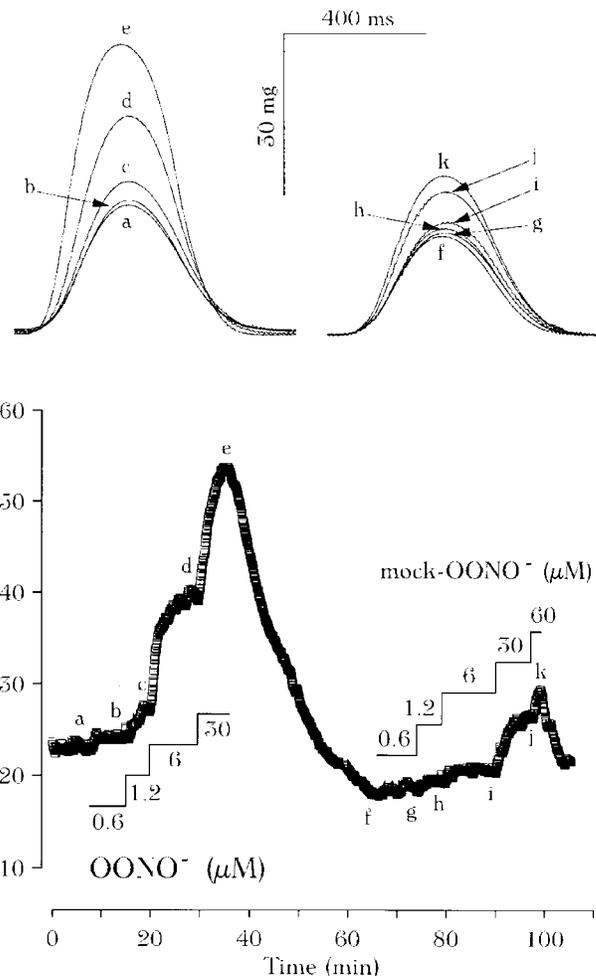
Figure 3. OONO⁻ is a positive inotropic agent in frog cardiac fibres

An atrial fibre was initially superfused with the control solution. Superfusion with authentic OONO⁻ (from 0·6 to 30 μM) or with mock-OONO⁻ (from 0·6 to 60 μM) is indicated by the lines. Top, individual contractions recorded at the times indicating by the corresponding letters on the main graph.

SOD (370 U ml^{-1}) restored the negative inotropic effect of SNAP (−54% from basal). The effects of these agents were reversible.

The results of several similar experiments are summarised in Fig. 2D. In a total of five atrial and four ventricular fibres where SNAP (5–20 μM) exerted a negative inotropic effect, LY 83583 (30 μM) was able to antagonise the effect of the nitrosothiol. In the presence of SOD (200–370 U ml^{-1}), the inhibitory effect of SNAP was restored. Therefore, super-oxide anion generation alone accounted for the positive effect of LY 83583, in the presence of the NO donor SNAP. Since the positive inotropic effects of SIN-1 and LY 83583 were additive, we conclude that these compounds do not share the same mechanism of action.

Contractile effects of OONO⁻ in frog cardiac fibres

The above findings suggested that the positive inotropic effect of SIN-1 might involve both NO and superoxide anion, the combination of which gives rise to peroxynitrite, OONO⁻ (Beckman & Koppenol, 1996). The typical experiment shown in Fig. 3 illustrates the contractile effects of authentic peroxynitrite (OONO⁻) on frog atrial fibres. Superfusion of cumulative concentrations of OONO⁻ (from 0·6 to 30 μM) induced a dose-dependent positive inotropic effect in the atrial fibre. This increase in the active tension induced by

OONO⁻ (up to 159% over basal at 30 μM), was fully reversible, and was not accompanied by any change in the kinetics of contraction (see individual recordings). The effects of mock-OONO⁻, the negative control of OONO⁻, are also illustrated in this experiment, in the 0·6 to 60 μM range. A significant positive inotropic effect of mock-OONO⁻ was observed at the highest concentrations used (6–60 μM). The increase in active tension induced by mock-OONO⁻ (37% over basal at 30 μM) was much weaker than that induced by OONO⁻. Therefore, neither the solvent used (NaOH, see Methods), nor the by-products of OONO⁻ can account for the positive inotropic effect of OONO⁻ in frog fibres.

The results of several similar experiments are summarised in Fig. 4. Individual results were pooled according to the range of concentrations of OONO⁻ or of mock-OONO⁻ used on atrial (Fig. 4A) and ventricular (Fig. 4B) fibres. In both preparations, the positive inotropic effect of OONO⁻ occurred in a dose-dependent manner, in the micromolar range of concentrations. The mean maximal effect of OONO⁻ was about 3 times larger in atrial (104 ± 20% over basal, at 10–30 μM) than in ventricular fibres (30 ± 5% over basal, at 10–30 μM). The kinetics of the contraction, i.e. T_{peak} and $T_{\frac{1}{2}}$, were only marginally affected by OONO⁻ in frog cardiac fibres (Table 1). On average, mock-OONO⁻ did
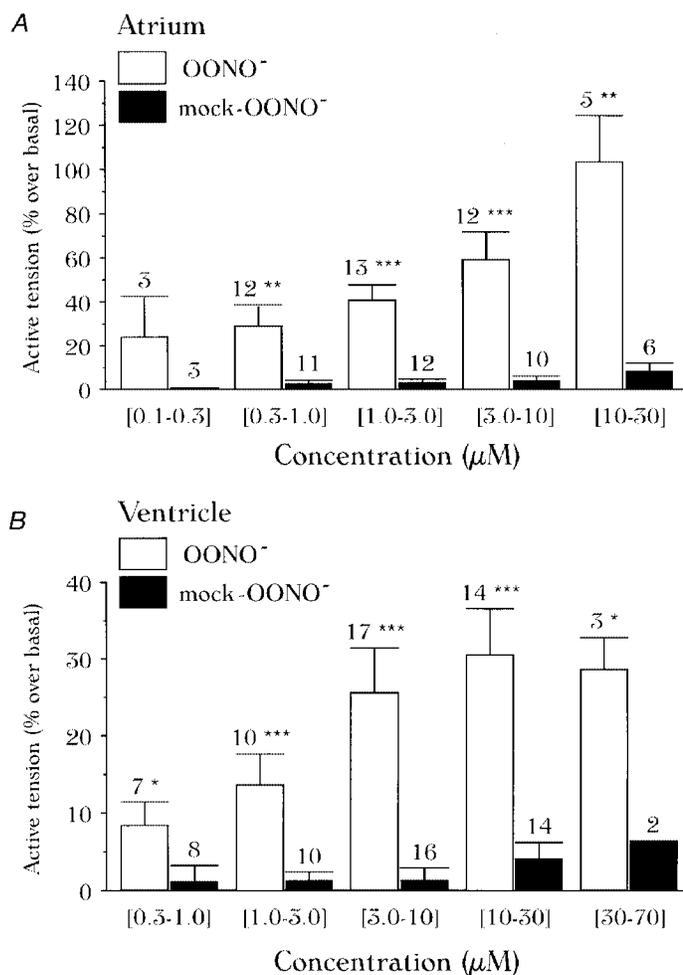
Figure 4. Summary of the inotropic effects of OONO⁻ in frog cardiac fibres

A and B, mean effects of OONO⁻ and of mock-OONO⁻ in ventricular and atrial fibres, respectively. Individual results were pooled and averaged within the ranges of concentrations indicated. The active tension in the presence of agents was normalised to its value in the absence of agent. Bars and lines are the mean ± s.e.m. of the number of experiments indicated. Statistical differences from the basal level are indicated as * $P < 0·05$; ** $P < 0·01$; *** $P < 0·005$.

Table 1. Effects of OONO⁻ and mock-OONO⁻ on the kinetics of the contraction in frog cardiac fibres

	Atrial fibres (% over basal)			Ventricular fibres (% over basal)		
	N	T_{peak}	$T_{½}$	N	T_{peak}	$T_{½}$
[OONO⁻] (μM)						
0·1–0·3	3	−0·8 ± 2·0	−3·7 ± 1·7	—	—	—
0·3–1·0	12	−0·4 ± 0·7	−0·3 ± 1·1	7	0·3 ± 0·7	4·0 ± 1·9
1·0–3·0	13	−0·5 ± 0·8	0·4 ± 1·8	10	0·1 ± 0·8	4·5 ± 2·1
3·0–10	12	−1·2 ± 1·6	−0·3 ± 2·7	17	4·2 ± 5·3	4·9 ± 2·0**
10–30	5	−0·3 ± 2·6	−0·6 ± 7·1	14	−0·2 ± 1·2	8·2 ± 2·7*
30–70	—	—	—	3	−1·4 ± 0·8	7·0 ± 3·9
[Mock-OONO⁻] (μM)						
0·1–0·3	3	−0·6 ± 0·5	−0·8 ± 0·8	—	—	—
0·3–1·0	11	1·5 ± 0·9	2·6 ± 1·0	8	1·7 ± 1·9	−8·7 ± 11·7
1·0–3·0	12	1·2 ± 0·9	1·8 ± 1·0	10	1·8 ± 1·2	1·3 ± 1·4
3·0–10	10	0·4 ± 1·8	1·8 ± 0·9	16	0·4 ± 0·7	−0·1 ± 0·8
10–30	6	4·7 ± 2·3	−1·2 ± 2·0	14	0·5 ± 0·7	2·3 ± 1·0
30–70	—	—	—	2	1·7	1·3

OONO⁻, peroxynitrite; T_{peak}, time to peak of the contraction; $T_{½}$, half-relaxation time; N, number of observations. Statistical difference from control is indicated by *$P < 0·05$; **$P < 0·01$.
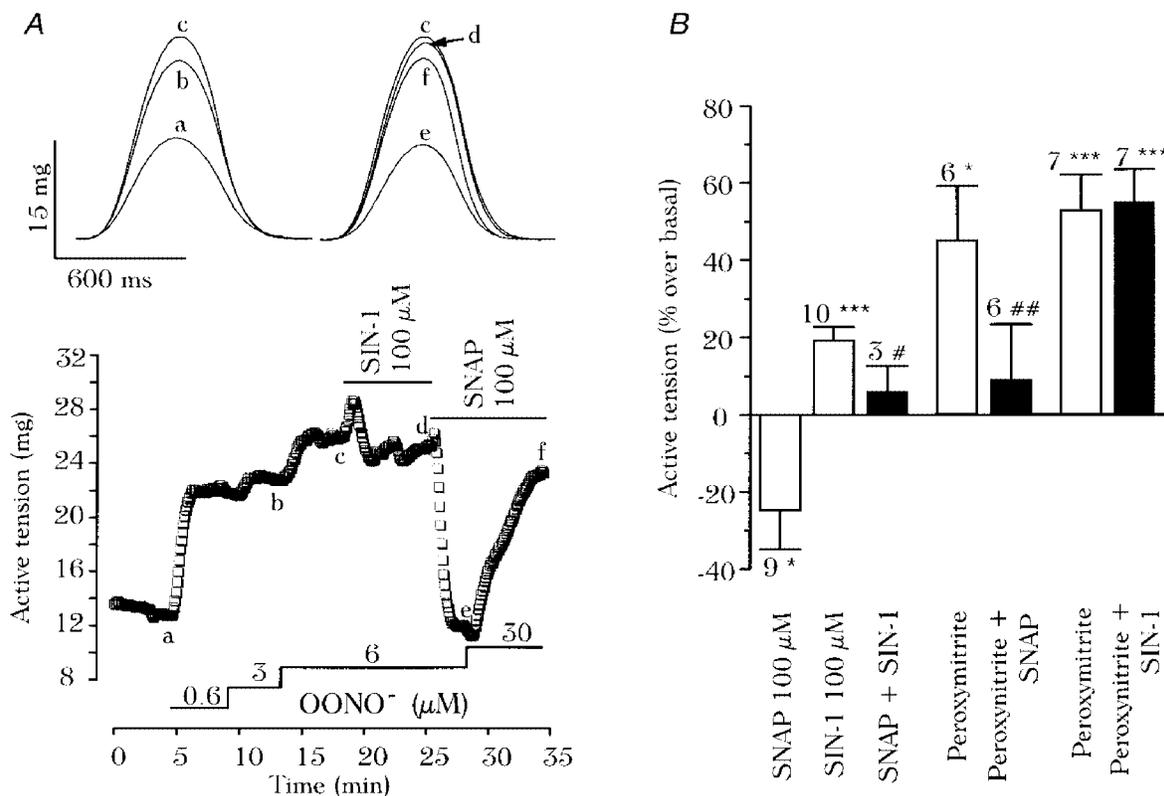
Figure 5. Interaction between OONO⁻ and NO donors in frog cardiac fibres

A, an atrial fibre was first superfused with the control solution. Superfusion with authentic OONO⁻ (0·6–30 μM), SIN-1 (100 μM) and SNAP (100 μM) are indicated by the lines. Top, contractions were recorded at the times indicated by the corresponding letters on the main graphs. B, summary of the effects of OONO⁻ (6–30 μM), SIN-1 (100 μM) and SNAP (100 μM), alone or in combination, on the active tension of cardiac fibres. The active tension in the presence of agents was normalised to its value in the absence of agents. Bars and lines are the mean ± s.e.m. of the number of experiments indicated. Statistical differences from the basal level (*) or from SNAP (#) are indicated as *# $P < 0·05$; ## $P < 0·01$; *** $P < 0·005$.

not mimic the effects of authentic $OONO^-$ (Fig. 4), although it could significantly increase the active tension in the high range of concentrations. Mock-$OONO^-$ did not modify the kinetics of the contraction (Table 1). Thus, $OONO^-$ is a potent positive inotropic agent, which probably mediates the positive effect of SIN-1.

Effects of NO donors in the presence of $OONO^-$

If the positive inotropic effects of SIN-1 and $OONO^-$ occur via the same mechanism, these effects should not be additive. In the experiment of Fig. 5*A*, an atrial fibre was superfused with increasing concentrations of $OONO^-$, from 0·6 to 6 μM. On top of the positive inotropic effect of 6 μM $OONO^-$ (121% over basal), SIN-1 (100 μM) was added. Although SIN-1 did increase the active tension when applied alone in this experiment (17% over basal, not shown), it tended to lower the inotropic effect of $OONO^-$. For a comparison, when SNAP (100 μM) was applied in the presence of $OONO^-$, a strong negative inotropic effect was observed. Interestingly, in the continuing presence of SNAP, increasing the concentration of $OONO^-$ from 6 to 30 μM restored the positive inotropic effect of this compound (+114% over basal).

The results of similar experiments are summarised in Fig. 5*B*. In these experiments, SNAP exerted a negative inotropic

effect when applied alone, while SIN-1 always increased the active tension of the fibres. The positive inotropic effect of SIN-1 was about 2 times weaker than that of $OONO^-$, and SIN-1 failed to significantly modified the active tension in the presence of its congener. In contrast, the positive inotropic effect of $OONO^-$ was balanced by the negative inotropic effect of SNAP. The nitrosothiol was also able to counteract the positive inotropic effect of SIN-1. None of these treatments significantly modified the kinetics of the contraction (data not shown). These results demonstrate that the effects of SIN-1 and $OONO^-$ were qualitatively similar, and were not additive. In contrast, both SIN-1 and $OONO^-$ counteracted the negative inotropic effect of SNAP, which involved the NO–cGMP pathway (Chesnais *et al.* 1999).

Effects of $OONO^-$ in the presence of ODQ, the guanylyl cyclase inhibitor

Unlike the negative inotropic effects of NO donors, the positive inotropic effect of SIN-1 was not modified by ODQ, a selective inhibitor of the heterodimeric guanylyl cyclase (Chesnais *et al.* 1999). Here, we also investigated the effects of $OONO^-$ in the presence of ODQ. In four frog fibres, $OONO^-$ (2–10 μM) elicited a $31·4 \pm 6·0$% increase in the active tension ($P < 0·005$), without significant changes in the kinetics of the contractions (not shown).
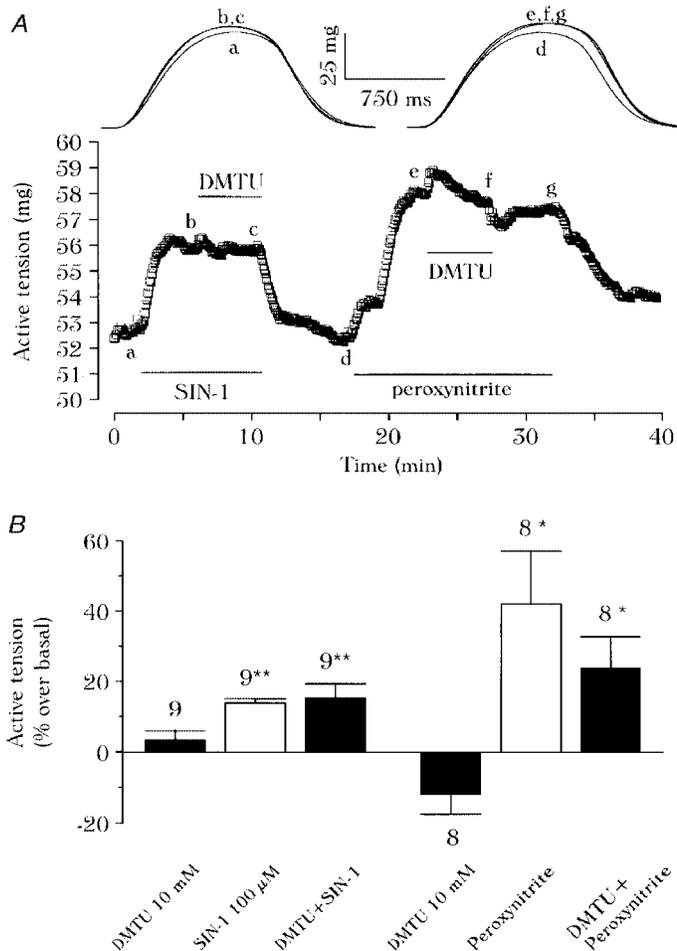
Figure 6. Effect of DMTU in the presence of SIN-1 or $OONO^-$ in frog cardiac fibres

In *A* a ventricular fibre was initially superfused with the control solution. SIN-1 (100 μM), DMTU (10 mM) and $OONO^-$ (14 μM) were applied as indicated by the lines. *B*, summary of the effects on the active tension of SIN-1 (100 μM) and $OONO^-$ (0·6–14 μM) in the absence or presence of DMTU (10 mM). The active tension in the presence of agents was normalised to its value in the absence of agents. Bars and lines are the mean $\pm$ s.e.m. of the number of experiments indicated. Statistical differences from the control level are indicated as * $P < 0·05$; ** $P < 0·01$.

This positive inotropic effect tended to be strengthened in the presence of 10 μM ODQ (to $55\cdot9 \pm 12\cdot4\%$ over basal, $P < 0\cdot005$), but the kinetics of the contraction were unchanged by the combination of $OONO^-$ plus ODQ (not shown). As already reported, 10 μM ODQ alone had no effect on the active tension ($-2\cdot1 \pm 1\cdot7\%$ over basal), and on T_{peak} and $T_{\frac{1}{2}}$ (respectively, $-0\cdot9 \pm 1\cdot6$ and $0\cdot2 \pm 1\cdot8\%$ over basal) in these experiments. Thus, the activation of the NO-sensitive guanylyl cyclase does not account for the positive inotropic effects of both SIN-1 and $OONO^-$. On the contrary, these effects might be somewhat weakened by an ODQ-sensitive negative inotropic effect that probably results from the conversion of $OONO^-$ into NO, as suggested earlier (Mayer *et al.* 1998).

Effects of hydroxyl radical scavengers

Peroxynitrite is a potential source of hydroxyl radicals, the generation of which might account for the positive inotropic effects reported above (Crow & Beckman, 1995; Beckman & Koppenol, 1996). The role of hydroxyl radicals was examined in the following experiments.

First, the positive inotropic effect of $OONO^-$ (like that of SIN-1) was unchanged by catalase, demonstrating that $OONO^-$ did not strengthen the hydrogen peroxide– hydroxyl radical pathway (data not shown).

Second, we studied the effects of dimethylthiourea (DMTU), which was shown to counteract the severe oxidative stress induced by hydroxyl radicals in cardiac myocytes (Nakamura *et al.* 1993). In Fig. 6A, the active tension of a ventricular fibre was enhanced by the superfusion of 100 μM SIN-1 (6% over basal). Addition of DMTU (10 mM) had no effect on the positive inotropic effect of SIN-1 (6% over basal). After the washout of these compounds, $OONO^-$ also enhanced the active tension (14% over basal). This positive inotropic effect was only slightly modified by the addition of 10 mM DMTU. In this fibre, DMTU (10 mM) alone had no effect on the cardiac contraction (not shown).

On average, the positive inotropic effect of SIN-1 (100 μM, Fig. 6B) was identical in the presence or absence of DMTU (10 mM). In this series of experiments, DMTU (10 mM, $n = 9$) had no effect on the active tension in frog fibres ($3\cdot6 \pm 2\cdot3\%$ over basal), as well as on T_{peak} ($-2\cdot3 \pm 1\cdot6\%$ from basal) or $T_{\frac{1}{2}}$ ($1\cdot0 \pm 1\cdot6\%$ over basal). In the other set of experiments, the effects of DMTU were studied in the presence of $OONO^-$. In these fibres, DMTU (10 mM) exerted a mean negative inotropic effect, although not significant, without any change in the kinetics of the contraction. The difference in the mean active tensions measured with $OONO^-$ alone or in the presence of DMTU was not significantly different.

Figure 7. Effect of SIN-1 in sodium-free solution in frog cardiac fibres

In *A*, the amplitude of active (□) and resting (×) tensions of a ventricular fibre are shown. The fibre was superfused with SIN-1 ($0\cdot1$ and/or 100 μM) in the presence and absence of sodium ions, as indicated by the lines. Top, contractions were recorded at the times indicated by the corresponding letters on the main graphs. *B*, summary of the effects of SIN-1 (100 μM) on the active tension in control and sodium-free solutions, elicited at $0\cdot2$ or $0\cdot1$ Hz, as indicated. Active tensions in the different conditions were normalised to the amplitude of the active tension elicited with the routine protocol (control solution, $0\cdot2$ Hz stimulation). Bars and lines are the mean $\pm$ s.e.m. of the number of experiments indicated. Statistical differences from the basal level are indicated as * $P < 0\cdot005$.
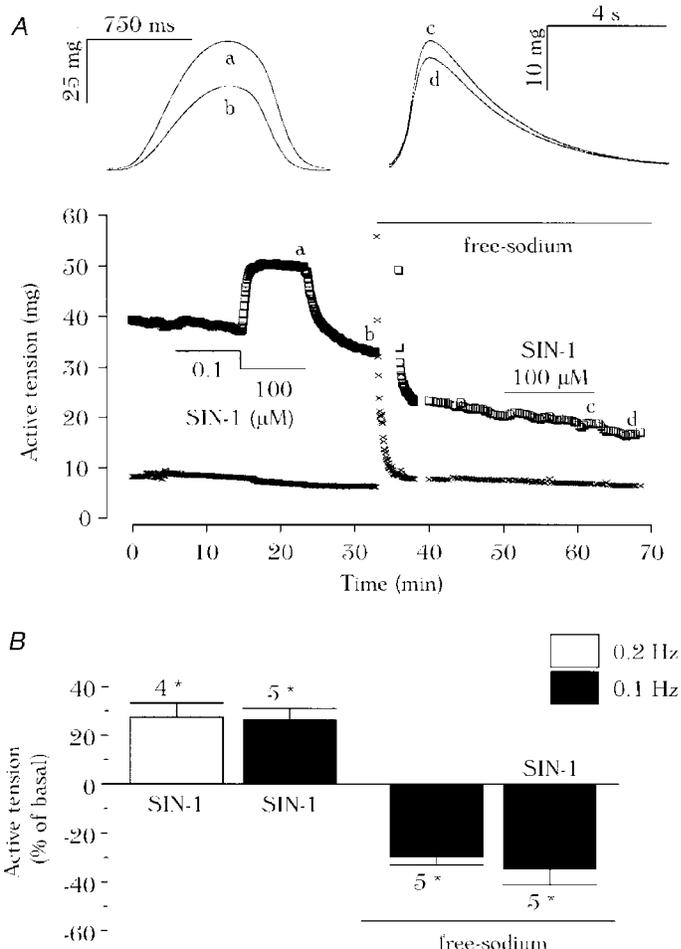
Third, mannitol was reported to scavenge hydroxyl radicals generated by SIN-1 *in vitro* (Hogg *et al.* 1992). In frog ventricular fibres, 20 mM mannitol had no effect on active tension ($2.9 \pm 5.4\%$ over basal, $n = 7$), T_{peak} ($-7.6 \pm 5.4\%$ from basal), and $T_{\frac{1}{2}}$ ($-2.0 \pm 1.4\%$ from basal). In these seven fibres, the positive inotropic effect of 100 μM SIN-1 ($27.8 \pm 2.4\%$ over basal) was not modified in the presence of mannitol ($28.7 \pm 7.3\%$ over basal). Altogether, these findings suggest that hydroxyl radical generation did not account for the positive inotropic effects of SIN-1 and OONO⁻.

The sodium–calcium exchanger is involved in the positive inotropic effect of SIN-1

While SIN-1/OONO⁻ might be expected to modify the activity of several proteins in frog cardiac fibres, we attempted to identify a target of SIN-1/OONO⁻ that could account for the positive inotropic effect. Among the different conditions examined (see below and Discussion), we found that the removal of external sodium ions totally antagonised the effect of SIN-1 in frog fibres. As shown in the typical experiment of Fig. 7A, the effect of SIN-1 was investigated in a frog ventricular fibre successively dialysed with the control solution, and with a sodium-free solution, where sodium had been substituted with lithium ions. In the control solution, 0.1 μM SIN-1 had no effect in this experiment, but raising the concentration to 100 μM SIN-1 induced a strong positive inotropic effect. After washout of SIN-1, the fibre was superfused with the sodium-free medium, inducing a transient rise in active and resting tensions, as already reported (Chapman, 1974). Since this treatment also induced a dramatic lengthening of the twitch, the frequency of stimulation was lowered from 0.2 to 0.1 Hz. Under this condition, the application of SIN-1 (100 μM) failed to modify the contraction of the frog fibre. Similar experiments were summarised in the graph of Fig. 7B. The positive inotropic effect of SIN-1 (100 μM) was not modified by the change in the frequency of stimulation (0.2 and 0.1 Hz) in the control solution. Removal of sodium ions from the perfusing solution induced a significant negative inotropic effect, associated with an increase in T_{peak} ($82.6 \pm 22.0\%$ over basal, $P < 0.01$) and $T_{\frac{1}{2}}$ ($763 \pm 160\%$ over basal, $P < 0.005$). Under these conditions, SIN-1 exerted no significant effects on either the active tension of the fibres (Fig. 7B), T_{peak} ($87.9 \pm 24.3\%$ over basal) or $T_{\frac{1}{2}}$ ($762 \pm 148\%$ over basal).

Since the sodium–calcium exchanger plays an important role in the contraction of the frog heart (Vassort & Rougier, 1972; Horackova & Vassort, 1979), these results suggested that a stimulation of the exchanger by SIN-1/OONO⁻ accounted for their positive inotropic effects. However, the lack of effect of SIN-1 in sodium-free solution might be secondary to the change in the repolarisation of the sarco-lemma induced by the inhibition of the sodium–calcium exchanger. Thus we specifically investigated the role of potassium channels and of the sodium–potassium pump, which participate in the repolarisation of the cardiac muscle.

The effect of SIN-1 was studied in the presence of tetra-ethyl-ammonium (TEA, 20 mM), an inhibitor of potassium channels. On average, TEA elicited a large increase in the active tension of frog cardiac fibres ($81.8 \pm 7.6\%$ over basal, $n = 4$, $P < 0.0001$), associated with a lengthening of the relaxation ($2.4 \pm 1.2\%$ over basal T_{peak}, and $58.1 \pm 17.9\%$ over basal $T_{\frac{1}{2}}$, $P < 0.01$). In these fibres, the positive inotropic effect of 100 μM SIN-1 ($33.6 \pm 4.2\%$ over basal, $n = 4$, $P < 0.0005$) was not antagonised by TEA, since, in the simultaneous presence of the compounds, the active tension was increased by $99.1 \pm 8.2\%$ over basal level ($n = 4$, $P < 0.0001$). Thus, potassium channel inhibition does not inhibit the positive inotropic of SIN-1, suggesting that these channels do not play a primary role in the effect of the OONO⁻ generator.

The effect of SIN-1 was also investigated in the presence of ouabain (10 μM), the inhibitor of the sodium–potassium pump. In five experiments where SIN-1 had induced positive inotropic effects ($22.6 \pm 3.6\%$ over basal, $P < 0.0005$), ouabain also elicited an increase in the active tension ($21.9 \pm 3.8\%$ over basal, $P < 0.0005$), associated with increases in T_{peak} ($3.9 \pm 0.9\%$ over basal, $P < 0.005$) and $T_{\frac{1}{2}}$ ($19.3 \pm 4.2\%$ over basal, $P < 0.005$). The positive inotropic effects of the agents were additive, since in the simultaneous presence of SIN-1 plus ouabain, the active tension was raised by $36.2 \pm 5.8\%$ over the basal level ($n = 5$, $P < 0.0005$). Accordingly, since ouabain did not antagonise the positive inotropic effect of SIN-1, a modulation of the sodium–potassium pump is unlikely to account for the effect of SIN-1. Altogether, these results strongly suggest that the sodium–calcium exchanger might be viewed as a specific target of SIN-1/OONO⁻ in frog cardiac fibres.

DISCUSSION

The present study examined the mechanism accounting for the positive inotropic effect of the NO donor SIN-1 in frog cardiac muscle. We found that OONO⁻ was the likely intermediate involved in this effect. Indeed, NO alone, superoxide anion alone, hydrogen peroxide or hydroxyl radicals were successively discarded as plausible candidates. In addition, authentic OONO⁻ elicited a positive inotropic effect, at a low range of concentrations. Interestingly, our results suggest that the sodium–calcium exchanger might be viewed as the locus of action of SIN-1/OONO⁻. These results are summarised in Fig. 8, and discussed below.

We have previously reported that NO donors could either increase or decrease the active tension in frog cardiac fibres (Chesnais *et al.* 1999). SNAP, SNP and SIN-1 elicited negative inotropic effects in both atrial and ventricular preparations. However, unlike SNAP and SNP, SIN-1 could also elicit a positive inotropic effect, which appeared predominant in ventricular fibres. Unlike the negative inotropic effects of the NO donors, the positive effect of SIN-1 was not antagonised by ODQ (Chesnais *et al.* 1999),

arguing against the involvement of cGMP production in this effect (see Schrammel *et al.* 1996). Interestingly, SOD eliminated the positive effect of SIN-1, and potentiated the negative inotropic effect of SIN-1. In contrast, SOD had no effect on the basal contraction, and on the negative inotropic effect of SNAP or SNP. These findings led us to conclude that superoxide anion was probably involved in the inotropic effects of SIN-1. We report now that catalase did not modify the potentiation by SOD of the negative inotropic effect of SIN-1. Hence, removal of superoxide anion rather than accumulation of hydrogen peroxide accounted for the effect of SOD. In addition, the positive inotropic effect of SIN-1 was mimicked by LY 83583. This compound behaved as a pure superoxide anion generator in frog fibres since SOD fully eliminated the positive inotropic effect of LY 83583 either in the absence or in the presence of a NO donor (Chesnais *et al.* 1999; the present study). However, we found that the positive effects of SIN-1 and LY 83583 were additive. Since the concentration of LY 83583 used in the present study is a saturating concentration in frog fibres, SIN-1 and LY 83583 are unlikely to act by the same mechanism. Accordingly, superoxide anion *per se* cannot account for the effect of SIN-1. An indirect participation of superoxide anion in the effect of SIN-1, by increasing the levels of hydrogen peroxide and/or hydroxyl radicals was also discarded (Feelisch *et al.* 1989; Beckman & Koppenol, 1996; Mayer *et al.* 1998). Indeed, catalase, a hydrogen peroxide scavenger, as well as mannitol and DMTU, hydroxyl radical scavengers, did not

modify the positive inotropic effect of SIN-1 (see Hogg *et al.* 1992; Nakamura *et al.* 1993). These observations strongly suggested that the effect of SIN-1 involved the concomitant generation of NO and superoxide anion. This possibility was strengthened by studying the effects of OONO⁻, the major product of NO and superoxide anion (Feelisch *et al.* 1989; Beckman & Koppenol, 1996; Mayer *et al.* 1998).

Authentic OONO⁻ appeared to be a strong positive inotropic agent in frog cardiac fibres. The inotropic effect of OONO⁻ was dose dependent and reversible. Mock-OONO⁻ did not mimic the effects of authentic OONO⁻, although it could induce a slight positive inotropic effect. Therefore, the solvent of OONO⁻ (NaOH) as well as the end-products of OONO⁻ did not account for the effects of authentic OONO⁻. OONO⁻ is known to generate significant amounts of hydroxyl radicals, which can contribute to its toxicity (Beckman & Koppenol, 1996). However, OONO⁻ as well as SIN-1 elicited a similar positive inotropic effect in the presence of either catalase or DMTU, demonstrating that hydroxyl radical generation was not involved in these inotropic effects.

Recent advances into the biological chemistry of OONO⁻ illustrated that this compound can behave as a source of nitrosothiol, and thereby activate the heterodimeric guanylyl cyclase (Mayer *et al.* 1998). However, in the frog heart, the positive inotropic effects of SIN-1 or OONO⁻ were not antagonised by ODQ, a selective inhibitor of the guanylyl cyclase (Chesnais *et al.* 1999, and the present
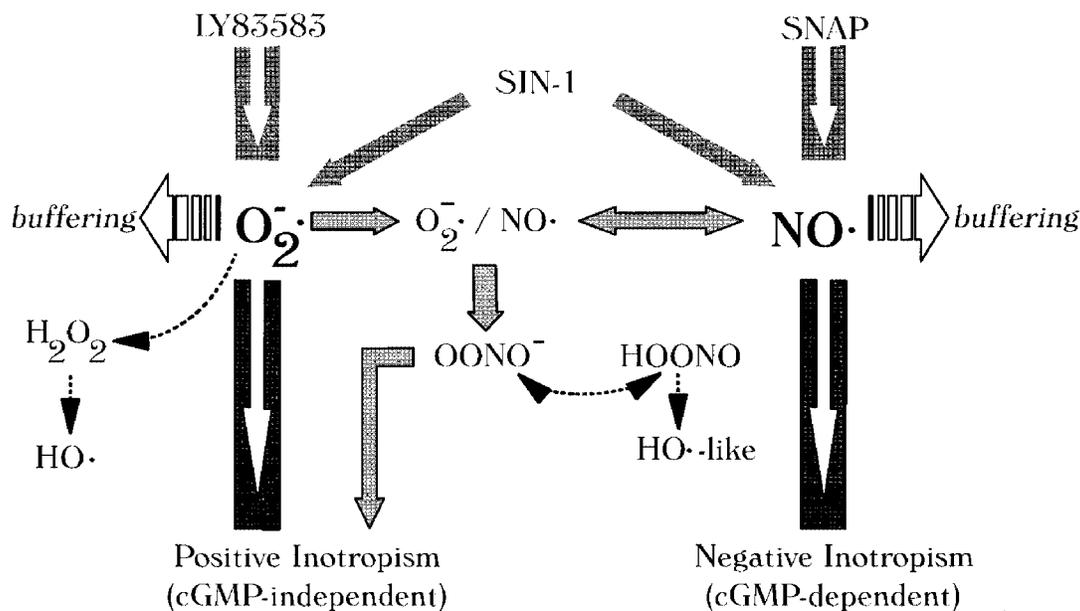
Figure 8. A schematic diagram of the regulation of cardiac contractility by NO in frog heart
This illustration is discussed in detail in the Discussion section. Filled and open arrows represent the pathways that have received experimental evidence in frog cardiac fibres, dotted arrows represent reactions and pathways that have been discarded, based on the present study. NO, nitric oxide; $O_2^{-}\cdot$, superoxide anion; H_2O_2, hydrogen peroxide; HO$\cdot$, hydroxyl radical; OONO⁻, peroxynitrite (adapted from Beckman & Koppenol, 1996; Mayer *et al.* 1998).

study). In contrast, ODQ tended to potentiate the positive inotropic effect of OONO⁻ (the present study), as well as that of SIN-1 (Chesnais et al. 1999). Thus, a bioconversion of OONO⁻ into nitrosothiol could weaken, rather than account for, the positive inotropic effect of this nitrogen derivative.

Although the chemistry of the positive inotropic effect of OONO⁻ remains to be established in detail, our study provides insights into the mechanism of action of OONO⁻ in frog cardiac fibres. Given the short half-life of OONO⁻ in physiological solutions, we hypothesised that OONO⁻ might act on a membrane component of the excitation–contraction coupling, rather than in an intracellular compartment. In support of this hypothesis, SIN-1 had no significant effects on the contraction of skinned myofibrils of the frog heart (data not shown). In addition, the positive inotropic effect of SIN-1 was totally abolished in sodium-free solutions, suggesting the involvement of the sodium–calcium exchanger in the positive inotropic effect of this compound. Potassium channel inhibition (with TEA) or sodium–potassium pump inhibition (with ouabain) did not mimic this blunting effect of sodium-free solutions. Thus, an alteration in the kinetics of relaxation, and/or a change in the repolarisation of the sarcolemma, per se, cannot account for the lack of effect of SIN-1 in sodium-free solutions. Altogether, these results support the view that the modulation of the sodium–calcium exchanger accounts for the positive inotropic effect of SIN-1/OONO⁻ in frog cardiac fibres.

In the heart, several effects of NO donors have been attributed to the stimulation of cGMP production, on the basis of their sensitivity towards guanylyl cyclase inhibitors such as Methylene Blue and/or LY 83583 (see Mohan et al. 1996; Méry et al. 1997). Our present findings suggest that some effects of NO donors must be attributed to the generation of OONO⁻. Consequently, the endogenous production of superoxide anion is likely to modify the effect of exogenous or endogenous NO, as previously suggested (Stamler, 1994; Beckman & Koppenol, 1996). Hence, depending on the level of superoxide anion and on the rate of OONO⁻ generation in vivo, the inotropic effects of NO might vary and/or switch from a cGMP-dependent to a cGMP-independent phenomenon. Our results suggest that SOD and ODQ might be reliable tools in investigating the relative participation of these mechanisms. For instance, a positive chronotropic effect of a NO donor was found to be insensitive to ODQ in the rat heart (Kojda et al. 1997). In addition, SIN-1 elicited an ODQ-resistant effect in guinea-pig atria (Musialek et al. 1997; see also Kennedy et al. 1994). Nevertheless, OONO⁻ generation is not an obligatory step in the positive inotropic effects of NO donors. In the rat heart, the positive inotropic effect of NO donors was inhibited by ODQ (Kojda et al. 1997). This effect appeared to involve the cGMP-dependent inhibition of PDE3, a phospho-diesterase tightly involved in the regulation of calcium influx in cardiac myocytes (see Méry et al. 1997; Kojda & Kottenberg, 1999). Interestingly, the plateau of the action

potential is very brief in the rat ventricle, and this unique characteristic might weaken the role of a modulation of the sodium–calcium exchanger by OONO⁻ in this species. In contrast, we recently found that OONO⁻ could elicit positive inotropic effects in human atrial fibres (authors' unpublished observations), and ongoing experiments in our laboratory are aimed at determining the role of the sodium–calcium exchanger in this effect.

We have observed that frog atrial muscle was more sensitive than the ventricular muscle to the negative inotropic effects of SNAP, SNP, and the combination SIN-1 plus SOD (Chesnais et al. 1999). Interestingly, the amplitude of the positive inotropic effect of OONO⁻ was also larger in the atria than in the ventricle. However, surprisingly, the positive inotropic effect of SIN-1 is more frequent in the ventricle than in the atria. This suggests that intrinsic factors may govern the sensitivity of the cardiac muscle towards NO derivatives. For example, the heterodimeric guanylyl cyclase and/or cGMP-regulated proteins, may be more efficiently coupled to the inotropism in the atria, as compared with the ventricle. In addition, the cardiac autonomous system may play a permissive (or a suppressive) role on the effect of NO (Schwarz et al. 1995), the extent of which may be different in the atria and the ventricle (Hartzell, 1988). These differences may result from the differential expression of the targets of the nitrogen species in the heart. However, we can also speculate that differences in the redox properties of the different cardiac structures might modulate the access of NO or nitrogen derivatives to their respective targets (see Lin et al. 1990; Ishibashi et al. 1993).

Altogether, our data suggest that the heterogeneity of the cardiac effects of NO might be explained, at least in part, by the generation of distinct nitrogen species. In addition, the heart muscle is not uniformly sensitive to these derivatives, and this property might account for some differences in the effects of NO in the different cardiac structures.

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Corresponding author

J.-M. Chesnais: INSERM U-446, Laboratoire de Cardiologie Cellulaire et Moléculaire, Université de Paris-Sud, Faculté de Pharmacie, 5 rue Jean-Baptiste Clément, F-92296 Châtenay-Malabry Cedex, France.

Email: michel.chesnais@cep.u-psud.fr