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Nitric Oxide Regulates Cardiac Ca^{2+} Current

INVOLVEMENT OF cGMP-INHIBITED AND cGMP-STIMULATED PHOSPHODIESTERASES THROUGH GUANYLYL CYCLASE ACTIVATION*

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The effects of the nitric oxide (NO) donor 3-morpholino-sydnonimine (SIN-1) on the L-type Ca^{2+} current (I_{Ca}) were examined in frog ventricular myocytes under basal and phosphorylated conditions. SIN-1 was found to exert insignificant effects on basal I_{Ca} but to induce a biphasic action on stimulated I_{Ca}. Indeed, in the nanomolar range of concentrations (0.1–10 nM), SIN-1 induced a pronounced (≈40%) stimulation of I_{Ca} elevated by a non-maximal concentration of forskolin (0.3 μM). However, the stimulatory effects of SIN-1 on I_{Ca} were not additive with those of maximal concentrations (10 μM) of forskolin or intracellular cAMP. In contrast, at higher concentrations (100 nM to 1 mM), SIN-1 strongly reduced I_{Ca} (by up to 85%) which had been previously stimulated by cAMP, forskolin, or isoprenaline. All the effects of SIN-1 appeared to be mediated by the liberation of NO since they were suppressed by methylene blue and LY83583 and were not mimicked by SIN-1C, a metabolite of SIN-1. The stimulatory or inhibitory effects of SIN-1 were absent, respectively, in the presence of milrinone (10 μM) or when the hydrolysis-resistant cAMP analog 8-bromo-cAMP was used instead of cAMP to stimulate I_{Ca}. In addition to its effects on I_{Ca}, SIN-1 induced a dose-dependent stimulation of guanylyl cyclase activity in the cytosolic and membrane fractions of frog ventricle. The membrane form of guanylyl cyclase displayed a higher sensitivity to SIN-1 than the cytosolic form, which correlated with SIN-1 sensitivity of I_{Ca}. Our data suggest that the activatory and inhibitory effects of NO donors on I_{Ca} result from an inhibition of the cGMP-inhibited cAMP-phosphodiesterase and an activation of the cGMP-stimulated cAMP-phosphodiesterase, respectively, both linked to the activation of guanylyl cyclase, possibly a membrane form of the enzyme.

Cyclic AMP plays a dominant role in the neuro-hormonal regulation of cardiac contractility (Hartzell, 1988; Lindemann and Watanabe, 1989). The cAMP pathway depends on the activation of cAMP-dependent protein kinase, which phosphorylates a number of key proteins including the L-type calcium channel, responsible for the trans-sarcolemmal calcium current (I_{Ca}).[1] Cyclic AMP-dependent phosphorylation of the L-type calcium channel leads to an increase in I_{Ca} (Hartzell, 1988; Pelzer *et al.*, 1990). This pathway is involved in the sympathetomimetic effects of β-adrenergic agonists (Hartzell, 1988; Pelzer *et al.*, 1990; Hartzell and Fischmeister, 1992), as well as in the positive inotropic effects of histamine (Levi and Alloatti, 1988), glucagon (Méry *et al.*, 1990), prostaglandin I2 (Alloatti *et al.*, 1991), serotonin (Ouadid *et al.*, 1992), and parathyroid hormone (Wang *et al.*, 1991).

The physiological role of cGMP in the heart muscle is less well characterized. Early works suggested that cGMP was the key messenger involved in the parasympathetic negative modulation of cardiac contractility (Hartzell, 1988; Lindemann and Watanabe, 1989). Indeed, muscarinic agonists have been shown to enhance cGMP levels as well as the activity of the cGMP-dependent protein kinase in heart muscle (Goldberg *et al.*, 1975; Lincoln and Keely, 1981). In addition, intracardiac infusion of membrane permeant analogs of cGMP mimicks the negative inotropic effect of acetylcholine (ACh) (references in Fischmeister and Hartzell, 1987; Hartzell, 1988). However, the correlation between the negative inotropic effect of ACh and the induced enhancement of cGMP levels is still a matter of debate (Lindemann and Watanabe, 1989; Hongo *et al.*, 1993). Thus, at the single cell level, ACh appears to regulate cardiac L-type calcium channel current in an essentially cGMP-independent manner, via the reduction of adenylyl cyclase activity (Fischmeister and Hartzell, 1986; Hartzell, 1988).

A number of recent studies have focused on the modulation by cGMP of cardiac L-type calcium channel current. Three pathways for cGMP have been demonstrated: (i) activation of an endogenous cGMP-dependent protein kinase, which leads to the inhibition of I_{Ca} in mammalian cells (Levi *et al.*, 1989; Méry *et al.*, 1991; Lohmann *et al.*, 1991); (ii) activation of the cGMP-stimulated cAMP-phosphodiesterase (cGS-PDE), which, in frog ventricular myocytes, accounts for the inhibition of cAMP- and isoprenaline-stimulated I_{Ca} observed upon dialysis with cGMP (Hartzell and Fischmeister, 1986;

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[1] The abbreviations used are: I_{Ca}, L-type calcium current; ACh, acetylcholine; PDE, phosphodiesterase; cGS-PDE, cGMP-stimulated PDE; cGI-PDE, cGMP-inhibited, milrinone-sensitive PDE; SIN-1, 3-morpholino-sydnonymine; NO, nitric oxide; Iso, isoprenaline; SIN-1C, *N*-morpholino-imino-acetonitril; SNP, sodium nitroprusside; ANF, atrial natriuretic factor; Iso, isoprenaline; LY83583, 6-anilino-5,8-quinolinedione; EDRF, endothelium-derived-relaxing factor.

Fischmeister and Hartzell, 1987; Lohmann *et al.*, 1991); (iii) blockade of the cGMP-inhibited cAMP-phosphodiesterase (cGI-PDE), reported in mammalian and batracian heart cells (Fischmeister and Hartzell, 1990, 1991), which results in an increase of I_{Ca} in mammalian heart cells when the concentration of intracellular cAMP is not saturating (Ono and Trautwein, 1991). These observations argued for a role of cGMP as a second messenger in cardiac contractility which would involve guanylyl cyclase activation.

Two forms of guanylyl cyclase are present in cardiac myocytes. One is the monomeric transmembrane receptor for the atrial natriuretic factor (ANF) (Garbers, 1992). ANF enhances guanylyl cyclase activity and elevates cGMP concentration in heart myocytes (Rugg *et al.*, 1989; McCall and Fried, 1990). ANF and its brain counterpart (brain natriuretic peptide) have also been shown to regulate I_{Ca} (Gisbert and Fischmeister, 1988; Legrand *et al.*, 1992; Sorbera and Morad, 1990). The second form of cardiac guanylyl cyclase is a heterodimeric ($\alpha\beta$) soluble guanylyl cyclase (Ignarro, 1990) which is activated by endothelium-independent vasorelaxant compounds, such as glycerol trinitrate, sodium nitroprusside (SNP), and SIN-1, some of which have therapeutic applications. These compounds generate nitric oxide (NO) which enhances the catalytic activity of the soluble ($\alpha\beta$) guanylyl cyclase, presumably after binding to the heme moiety of the enzyme (Ignarro, 1990; Stamler *et al.*, 1992). However, studies using NO donors have led to conflicting results as to the involvement of NO in the spontaneous cardiac contractility or in its regulation by muscarinic agonists (Thelen *et al.*, 1992; for a review, see Lindemann and Watanabe, 1989). In addition, inhibition of NO synthase activity by arginine analogs produces no effect on spontaneous cardiac contractility (Garcia *et al.*, 1992; Klabunde *et al.*, 1992; Thelen *et al.*, 1992), although a reduction in cardiac cGMP content has been demonstrated (Klabunde *et al.*, 1992). Nevertheless, arginine analogs were found to stimulate (in rat; Yamazaki *et al.*, 1991) or reduce (in dog; White *et al.*, 1993) spontaneous beat rate, and to potentiate (Balligand *et al.*, 1993a) or reduce (Klabunde *et al.*, 1992) the positive inotropic effects of β-adrenergic agonists in rat heart. Moreover, arginine analogs were also found to reverse the negative chronotropic effects of muscarinic agonists in isolated rat cardiac myocytes (Balligand *et al.*, 1993a). It should be noted, however, that arginine analogs may not only act as NO synthase inhibitors since they have been demonstrated recently to act also as muscarinic receptor antagonists (Brixton *et al.*, 1993).

To get further insights into the cellular mechanisms of action of NO donors on cardiac muscle, we have examined in the present study the effects of SIN-1 on I_{Ca} recorded in frog isolated ventricular myocytes. This compound appeared to regulate I_{Ca} in a manner consistent with an elevation of intracellular cGMP concentration. Involvement of guanylyl cyclase in the effects of the NO donors was confirmed by the measurement of guanylyl cyclase activity in subcellular fractions of frog ventricle. An NO-sensitive guanylyl cyclase activity was found to be present in both the soluble and the membrane fractions. The data support the hypothesis that activation by NO donors of a membrane form of guanylyl cyclase modulates I_{Ca} via the regulation of two cAMP phosphodiesterases, the cGI- and the cGS-PDEs. A preliminary report of some of these results has appeared in abstract form (Méry *et al.*, 1993).

EXPERIMENTAL PROCEDURES

Materials—Sodium salt nucleotides and trypsin were purchased from Sigma (L'Isle d'Abeau Chesnes, France). Collagenase was from Boehringer-Manheim (Meylan, France). [α-^{32}P]GTP, [α-^{32}P]ATP, [8-^{3}H]cGMP, and [8-^{3}H]cAMP were from Amersham (Radiochemical Centre, United Kingdom). Rat atrial natriuretic factor (ANF) was obtained from Peninsula Laboratories Ltd. (Europe). ANF was prepared as a 100 μM stock solution in 50 mM HEPES, pH 7.4, containing 2.5% bovine serum albumin and stored at -20 °C. Dilutions were made in 50 mM HEPES, pH 7.4, containing 1% bovine serum albumin and used immediately. Milrinone was a generous gift from Starling-Winthrop (Rensselaer). SIN-1 and *N*-morpholino-imino-acetonitril (SIN-1C) were generous gifts from Dr. J. Winicki, Hoechst Laboratories (Puteaux, France). LY83583 (6-anilino-5,8-quinolinedione) was from Lilly. Tetrodotoxin was from Janssen Phamaceutica N.V. (Belgium) and all other drugs from Sigma. During the preparation of solutions, SIN-1 and SNP were protected from UV irradiation of natural light. They were solubilized in standard external solution less than 15 min prior to being applied onto the cell under investigation, *i.e.* only fresh NO donor-containing solutions were tested. However, on one occasion, the same stock solution (1 mM SIN-1) has been used on three consecutive cells, *i.e.* within the following 4 h with no significant change in the results. SIN-1C was used in the same conditions as SIN-1.

Electrophysiological Experiments—Ventricular cells were enzymatically dispersed from frog (*Rana esculenta*) heart, by a combination of collagenase and trypsin as previously described (Fischmeister and Hartzell, 1986). The isolated cells were stored in standard Ringer solution and kept at 4 °C until use (2–48 h following dissociation). The whole-cell configuration of the patch-clamp technique was used to record I_{Ca} on Ca^{2+}-tolerant cells. In the routine protocols, the cell was depolarized every 8 or 10 s from −80 mV holding potential to 0 mV for 400 ms. K^+ currents were blocked by replacing all K^+ ions with intracellular and extracellular Cs^+ (Fischmeister and Hartzell, 1986). The fast Na^+ current, which is much more sensitive to tetrodotoxin in frog than in mammals, was blocked by 0.3 μM tetrodotoxin. Under these conditions, the only time-dependent currents measured during a depolarization of the membrane potential could be blocked completely by 100 μM $CdCl_2$ (Fischmeister and Hartzell, 1986) and was attributed entirely to the L-type calcium channel (Argibay *et al.*, 1988). There was also a small, time-independent leak current, which in frog ventricular cells is not modified by activation of cAMP-dependent phosphorylation (Fischmeister and Hartzell, 1986; Hartzell and Fischmeister, 1986). All experiments were done at room temperature (21–24 °C).

Solutions—Control external solution consisted of: 107 mM NaCl, 10 mM HEPES, 20 mM CsCl, 4 mM $NaHCO_3$, 0.8 mM NaH_2PO_4, 1.8 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5 mM D-glucose, 5 mM sodium pyruvate, 0.3 μM tetrodotoxin, pH 7.4, adjusted with NaOH. Control or drug-containing solutions were applied to the exterior of the cell by placing the cell at the opening of 250-μm inner diameter capillary tubings through which solution flowed at a rate of ≈10 μl/min. Patch-electrodes (0.6–2.5 Mohms) were filled with control internal solution which consisted of: 119.8 mM CsCl, 5 mM EGTA (acid form), 4 mM $MgCl_2$, 5 mM creatine phosphate disodium salt, 3.1 mM ATP, 0.42 mM GTP, 62 μM $CaCl_2$ (pCa 8.5), 10 mM HEPES, pH 7.1 adjusted with KOH. Drug-containing solutions were then applied to the interior of the cell by a system that permitted perfusion of the patch electrode with different solutions (Fischmeister and Hartzell, 1987). Perfusion time depended on patch electrode resistance, access to the cell, and the molecular weight of the molecule tested. Typically, with cAMP (M_r = 351), the beginning of I_{Ca} stimulation occurred 1.5–3 min after the beginning of intracellular perfusion with this compound (*e.g.*, see Fig. 6).

Data Analysis—The maximal amplitude of whole-cell I_{Ca} was measured as previously described (Fischmeister and Hartzell, 1986). Currents were not compensated for capacitive and leak currents. The leak current (I_{400} or I_{200}) was routinely measured as the current amplitude at the end of the 400- or 200-ms duration pulse. On-line analysis of the recordings was made possible by programming a Compaq 386/25 Desk-pro computer in Pascal language to determine, for each membrane depolarization, peak and steady-state current values (Fischmeister and Hartzell, 1986). Current-voltage relationships for I_{Ca} and I_{200} (Fig. 3*A*) and I_{Ca} inactivation curve (Fig. 3*B*) were obtained from voltage clamp protocols previously described (Fischmeister and Hartzell, 1986; Argibay *et al.*, 1988).

The results are expressed as mean ± S.E. In the text, the "basal" condition refers to the absence of either isoprenaline, forskolin, cAMP or 8-Br-cAMP, *i.e.* to the current elicited by non-phosphorylated L-type calcium channels (Hartzell and Fischmeister, 1992). In the case of single applications, the effect of a compound is referred to as the percent variation over the basal level. Since it appeared that NO

donors have no effect on basal I_{Ca}, their effects are expressed as the percent change over the cAMP-dependent stimulation of I_{Ca}, *i.e.* 100 × (test I_{Ca} − reference I_{Ca})/(reference I_{Ca} − basal I_{Ca}).

Subcellular Fractionation of Frog Heart—All preparative procedures were carried out at 4 °C. Hearts were removed from four frogs and placed in 10 ml of 50 mM Tris-HCl, pH 7.5, containing 10 mM $MgCl_2$, 1 mM EDTA. Ventricles, freed of blood vessels, and connective tissue were then placed in 4 ml of fresh buffer, lacerated with scissors, and homogenized in a high speed mechanical homogenizer Ultraturrax. The homogenate was centrifuged at 3,000 × *g* for 5 min for sedimenting the "membrane" fraction. The 3,000 × *g* supernatant was then centrifuged at 10,000 × *g* for 15 min to obtain the 10,000 × *g* pellet. Finally, the 10,000 × *g* supernatant was centrifuged at 100,000 × *g* for 1 h to separate the 100,000 × *g* pellet and the 100,000 × *g* supernatant, referred to as the "supernatant" fraction. All fractions were aliquoted and stored up to 2 weeks in liquid nitrogen, without any loss of enzyme activities.

Guanylyl Cyclase Assay—Guanylyl cyclase activity was assayed as described previously (Brechler *et al.*, 1992) with minor modifications. The assay medium contained, in a final volume of 80 μl: 50 mM HEPES, pH 7.4, 4 mM $MnCl_2$, 0.1 mM EGTA, 1 mM [α-^{32}P]GTP (2 × 10^6 cpm), 0.5 mM [8-^{3}H]cGMP (20,000 cpm), 0.5 mM ATP, 1 mM methylisobutylxanthine, 3 mM dithiothreitol, 60 mM bacitracin, 0.3% bovine serum albumin, 25 mM creatine phosphate, 1 mg/ml creatine kinase. The incubation was initiated by the addition of 30–75 μg of fraction proteins and run at 37 °C. Results were obtained from triplicate determinations. It is noteworthy that when $MnCl_2$ was replaced for $MgCl_2$ in the assay medium for guanylyl cyclase activity, we could observe a 2–3-fold activation by ANF of the membrane guanylyl cyclase. However, the 30% activation by ANF observed in the presence of $MnCl_2$ gave better reproducibility, and thus only the results obtained with $MnCl_2$ are presented.

Adenylyl Cyclase Assay—Adenylyl cyclase was assayed as described previously (Méry *et al.*, 1990). The assay medium contained, in a final volume of 60 μl: 50 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM [α-^{32}P]ATP (10^6 cpm), 1 mM [8-^{3}H]cAMP (20,000 cpm), 50 μM GTP, 0.2 mM methylisobutylxanthine, 25 mM creatine phosphate, 1 mg/ml creatine kinase, and 10 μM forskolin. The incubation was initiated by the addition of 20–70 μg of fraction proteins and run at 37 °C. Results were obtained from triplicate determinations.

Aspartate Amino Transferase Assay—Aspartate amino transferase activity was assayed in the different frog heart fractions, using a kit from Roche. According to Pavé-Preux *et al.* (1988) the assays were optimized by adding 0.1 mM pyridoxal phosphate to the protein fraction 5 min before the incubation at 37 °C.

RESULTS

Effects of SIN-1 on Basal and Isoprenaline-stimulated I_{Ca}—I_{Ca} was recorded in frog ventricular cardiomyocytes using the whole-cell configuration of the patch-clamp technique. In Fig. 1, the maximal amplitude of I_{Ca} at 0 mV is plotted as a function of time in two different experiments. In Fig. 1*A*, the effect of two concentrations of SIN-1 (10 μM and 1 mM) were tested on basal I_{Ca}. It was found that SIN-1 had little effect on basal I_{Ca} amplitude. Also, SIN-1 did not modify significantly either the kinetics of I_{Ca} or the leak current amplitude as can be seen from the individual current traces shown on the upper part of Fig. 1*A*. Therefore, SIN-1, as well as its side products NO and SIN-1C possibly generated in the course of the experiment, appear to have negligible detergent-like effects. By contrast with the absence of effect on basal I_{Ca}, SIN-1 had dramatic effects on I_{Ca} stimulated by cAMP-dependent phosphorylation. In the experiment shown in Fig. 1*B*, the cardiomyocyte was rapidly exposed to 0.1 μM isoprenaline (Iso), a β-adrenergic agonist. I_{Ca} amplitude was increased nearly 9-fold by Iso. In the continuing presence of Iso, increasing concentrations of SIN-1 were successively applied to the cell. At 0.1 nM, SIN-1 had only a small and transient stimulatory effect, but larger concentrations (10 nM to 100 μM) produced a dose-dependent reduction of the β-adrenergic-stimulated I_{Ca}. In the presence of 100 μM SIN-1, the current almost returned to its basal level, observed prior to Iso stimulation. The inhibitory effect of SIN-1 on Iso-stimulated I_{Ca} occurred without any noticeable change in the kinetics of I_{Ca}, as shown by the individual current traces on the upper part of Fig. 1*B*.

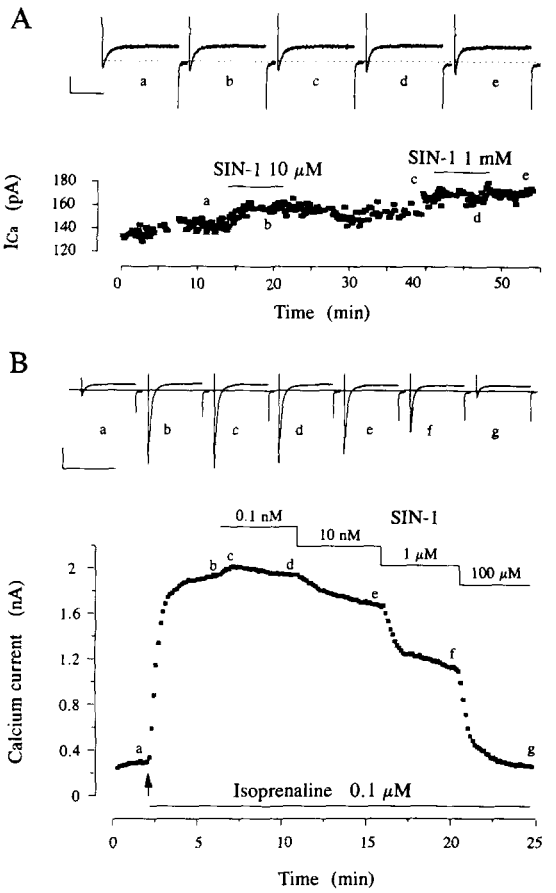


FIG. 1. **Effects of SIN-1 on I_{Ca}.** *A*, *lower part*: time course of the effects of SIN-1 on basal I_{Ca} in an isolated frog ventricular cardiomyocyte. Each symbol corresponds to a measure of I_{Ca} at 0 mV, obtained every 8 s (see "Experimental Procedures"). The cell was initially superfused with control Cs Ringer solution. During the periods indicated, the cell was successively exposed to 10 and 1 mM SIN-1. *A*, *upper part*: current traces were obtained at the times indicated by the corresponding letters on the *lower part*. *Vertical* and *horizontal bars* indicate 100 pA and 200 ms, respectively. The *dotted line* indicates the zero-current level. *B*, *lower part*: effect of SIN-1 on isoprenaline-stimulated I_{Ca}. The cell was initially superfused with control Cs Ringer solution. During the periods indicated, the cell was successively exposed to 0.1 μM isoprenaline, and to successively increasing concentrations of SIN-1 (0.1 nM, 10 nM, 1 μM, 100 μM). *B*, *upper part*: current traces were obtained at the times indicated by the corresponding letters on the *lower part*. *Vertical* and *horizontal bars* indicate 500 pA and 400 ms, respectively. The continuous line indicates the zero-current level.

Fig. 2 summarizes the results of a number of experiments similar to those of Fig. 1. In the micromolar range, and up to 1 mM, SIN-1 had no significant effect on basal I_{Ca} in frog ventricular cardiomyocytes (Fig. 2*A*). The effects of SIN-1 were then tested in the presence of three different concentrations of Iso (0.01, 0.1, and 1 μM). The effects of nanomolar concentrations of SIN-1 (0.1 and 10 nM, Fig. 2, *B* and *C*) were not significant, although a small potentiating effect was often seen with 0.1 nM SIN-1 (Fig. 2, *B* and *C*, see also Fig. 1*B*). When SIN-1 was used in the micromolar range of concentrations (1–100 μM), it had profond inhibitory effects on I_{Ca}, whether the current had been elevated by non-saturating concentrations of Iso (0.01 μM in Fig. 2*B*, 0.1 μM in Fig. 2*C*) or by a maximal concentration (1 μM, Fig. 2*D*). However, the

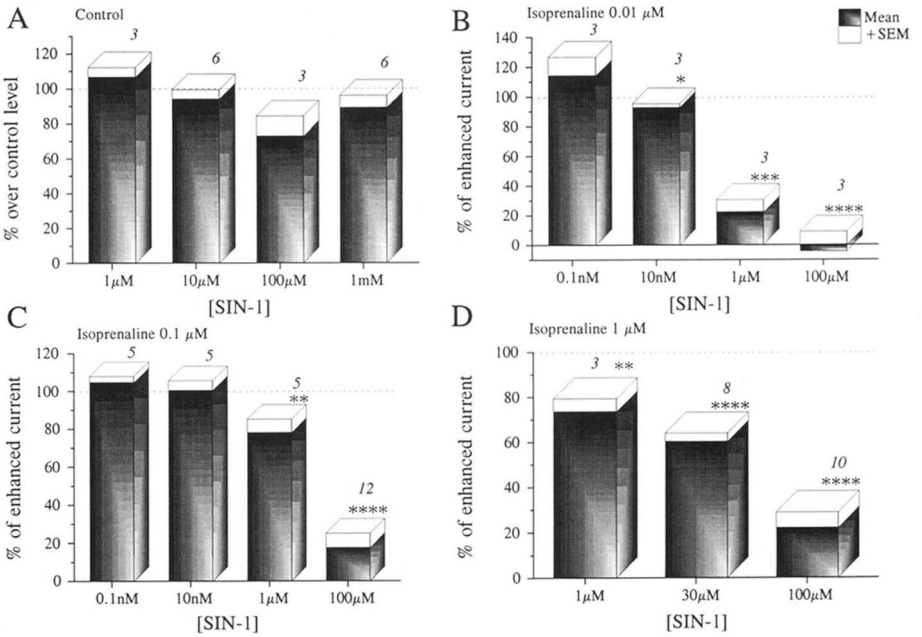


FIG. 2. **Summary of the effects of SIN-1 on basal and Iso-stimulated I_{Ca}.** *Part A* summarizes the effect of 1 μM to 1 mM SIN-1 on basal I_{Ca} in three to six different cells. *Filled bars* indicate the means and *open bars* the S.E. of the number of experiments indicated near the *bars*. In these experiments, the mean I_{Ca} amplitude was 237 ± 45 pA ($N = 10$). In *parts B–D*, a similar presentation is used to illustrate the effects of various concentrations of SIN-1 on I_{Ca} after prior stimulation with 10 nM (*B*), 0.1 μM (*C*), and 1 μM (*D*) Iso. In these experiments, the mean effect of 10 nM Iso on I_{Ca} was 311 ± 106% activation over basal I_{Ca} ($N = 5$). With 0.1 μM Iso activation reached 844 ± 94% ($N = 12$) and with 1 μM Iso it culminated at 994 ± 160% ($N = 16$). Significant statistical differences from Iso-stimulated level are indicated as $*p < 0.1$, $**p < 0.05$, $***p < 0.01$, and $****p < 0.005$.

inhibitory effect of a given concentration of SIN-1 (100 μM) was larger when I_{Ca} was not maximally stimulated by Iso (100 ± 9% inhibition in the presence of 10 nM Iso) than when a saturating concentration of the β-agonist was applied to the cell (78 ± 7% with 1 μM Iso). This may indicate that the inhibitory effect of SIN-1 on I_{Ca} competes with the stimulatory effect of cAMP.

Voltage Dependence—The effect of SIN-1 was then examined at different membrane potentials. The current-voltage relationship for I_{Ca} and I_{200} in the presence of 1 μM Iso are shown in Fig. 3*A*, in the absence and presence of 10 μM SIN-1. The experiment shown is representative of 11 similar experiments. Since both curves had a similar U-shape and their position on the voltage axis was not modified by SIN-1, one can conclude that the inhibitory effect of SIN-1 on I_{Ca} occurs in an essentially voltage-independent manner. SIN-1 had also negligible effects on the quasi-linear current-voltage relationship of the end-pulse current (I_{200}), which argues against significant toxic effects of NO or SIN-1 metabolites via a perturbation of membrane structure by lipid peroxydation (Stamler *et al.*, 1992). The quasi steady-state inactivation curve of I_{Ca} was measured in 11 cells by means of a double pulse protocol and is shown in Fig. 3*B* for the same cell and under the same experimental conditions as in Fig. 3*A*. SIN-1 was found to exert no significant effect on the shape and position of this curve, indicating that the large inhibitory effect of the compound on I_{Ca} amplitude cannot be explained by modifications of the inactivation properties of the L-type calcium channel. In that respect, SIN-1 or its released products NO and SIN-1C, behave in a completely different manner as L-type calcium channel-binding drugs, such as dihydropyridines.

Effect of SIN-1 on Forskolin-elevated I_{Ca}—Since SIN-1 inhibited Iso-stimulated and not basal I_{Ca}, we questioned whether these effects could be due to an action of SIN-1 on the β-adrenergic receptor itself or on the coupling mechanism between the β-adrenergic receptor and adenylyl cyclase. An alternative hypothesis was that, due to its redox properties, SIN-1 could induce a significant oxidation of Iso, thus inhibiting I_{Ca} by a reduction in the available amount of Iso. To test these different possibilities, we used forskolin instead of Iso to induce a cAMP-dependent stimulation of I_{Ca} (Hartzell and Fischmeister, 1987). Forskolin is much more resistant to oxidation than Iso, and it binds directly to the catalytic unit of adenylyl cyclase, bypassing, therefore, the β-adrenergic receptor and the G-protein. The results obtained with forskolin were somewhat different from those obtained with Iso. I_{Ca} amplitude was increased nearly 10-fold by saturating (10 μM) concentrations of forskolin (Hartzell and Fischmeister, 1987). A typical experiment with non-saturating concentrations of forskolin is shown in Fig. 4*A*. Forskolin (0.3 μM) induced a progressive stimulation of I_{Ca}. Additional application of 0.1 nM SIN-1 resulted in a further increase in I_{Ca} that developed over ≈5 min. With a higher concentration of SIN-1 (10 nM), the stimulation of I_{Ca} was less. Further increasing the concentration of SIN-1 to 1 and 100 μM induced a clear inhibition of I_{Ca}. At a 100 μM concentration, SIN-1 abolished half of the stimulatory effect of forskolin on I_{Ca}. The results of several similar experiments are summarized in Fig. 4*B*. Each data point represents one individual effect of SIN-1 at the indicated concentration on I_{Ca} stimulated by 0.3 μM forskolin. In all the experiments, SIN-1 was applied in successively increasing concentrations. As can be seen, some variability was observed between cells for the "crossover" concentration of SIN-1 yielding to inhibitory effects on I_{Ca}. This may reflect variations in enzymatic activities of the cells and/or in the degree of intracellular dialysis by the patch-electrode solution. The *bargraphs* indicate the mean values of the effects of SIN-1 when their absolute amplitudes exceeded 5% of the I_{Ca} amplitude in the presence of forskolin alone. At a 0.1 nM concentration, SIN-1 had a stimulatory effect on I_{Ca} in 9 out of 14 cells and an inhibitory effect in only two cells. The stimulatory effect of 0.1 nM SIN-1 was on average a 47 ± 13% increase over the forskolin-stimulated I_{Ca} amplitude. The threshold concentration for SIN-1 effect was 0.1 nM since lower doses of SIN-1 had no detectable effect (data not shown). The proportion of SIN-1 inhibitory effect over its stimulatory action increased progressively with SIN-1 concentration. At 100 μM, SIN-1 only reduced I_{Ca}. These results demonstrate that the inhibitory effect of SIN-1 takes place at a site which is downstream from adenylyl cyclase. In addition to its inhibitory effect, SIN-1 exerts a potentiating effect on I_{Ca} which appears more markedly when the current is stimulated by forskolin rather than by Iso.

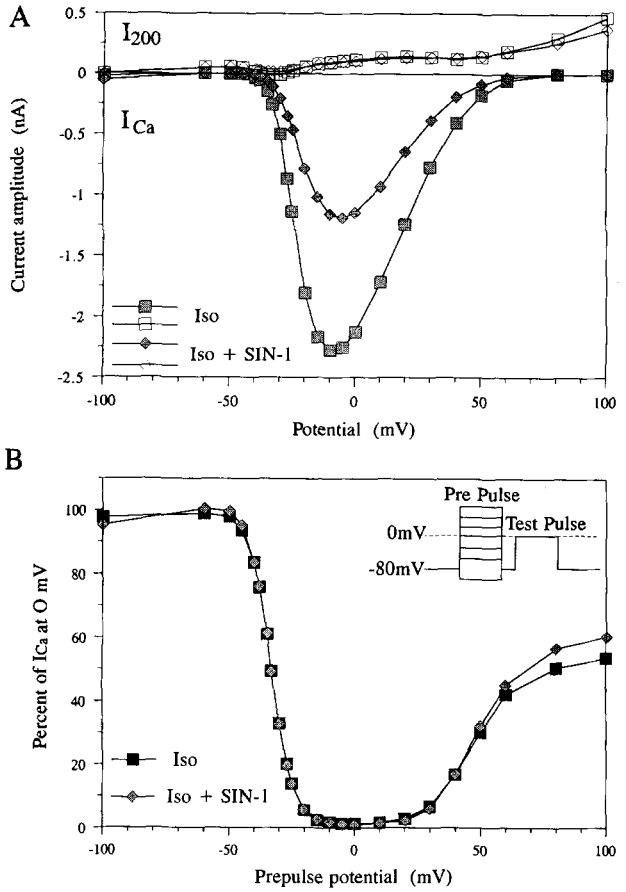


FIG. 3. **Voltage dependence of the inhibitory effects of SIN-1 on Iso-stimulated I_{Ca}.** *A*, current-voltage relationships of I_{Ca} (*filled symbols*) measured during 200 ms depolarizations to various potentials, and I_{200} (*open symbols*), the current measured at the end of the 200 ms pulse (see "Experimental Procedures"), in a frog ventricular cell extracellularly perfused with 1 μM Iso (*squares*), and 1 μM Iso plus 10 μM SIN-1 (*diamonds*). *B*, inactivation curves of I_{Ca}. A double step protocol consisting of a 200-ms duration prepulse to various potentials followed by a 200-ms test potential to 0 mV was used as indicated in the *inset*. I_{Ca} measured during a 200-ms test pulse at 0 mV is plotted as a function of prepulse potential and expressed as a percentage of I_{Ca} at 0 mV in the absence of prepulse. *Squares*, 1 μM Iso; *diamonds*, 1 μM Iso + 10 μM SIN-1.

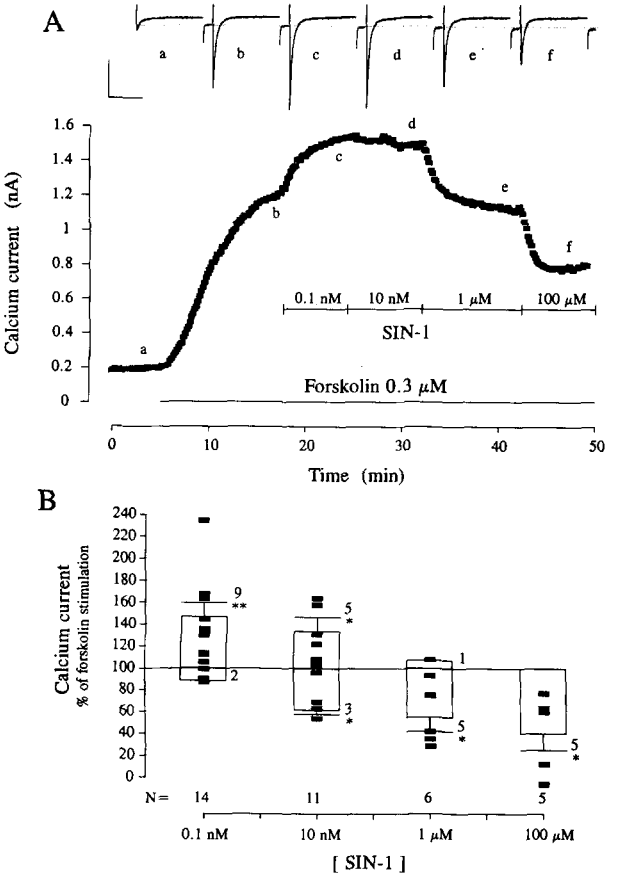


FIG. 4. **Effects of SIN-1 on forskolin-stimulated I_{Ca}.** *A, lower part:* the cell was initially superfused with control Cs Ringer solution. During the periods indicated, the cell was successively exposed to 0.3 μM forskolin, and to successively increasing concentrations of SIN-1 (0.1 nM, 10 nM, 1 μM, 100 μM). *A, upper part:* current traces were obtained at the times indicated by the corresponding letters on the lower part. *Vertical* and *horizontal bars* indicate 600 pA and 200 ms, respectively. The *dotted line* indicates the zero-current level. *B*, summary data of the stimulatory and inhibitory effects of SIN-1 on I_{Ca} stimulated by 0.3 μM forskolin. Each *square symbol* corresponds to a different experiment. The effects of four different concentrations of SIN-1 (0.1 nM, 10 nM, 1 μM, and 100 μM) were examined in the number of experiments *N* indicated above the concentrations. When the effects of SIN-1 on I_{Ca} exceeded a $\pm$5% variation with respect to the current level in forskolin alone, these effects were separated in stimulatory and inhibitory effects. Mean values and S.E. for these two categories of effects are indicated by the *open bars* together with the number of cells that met the criteria. Significant differences from forskolin-stimulated level are indicated as * ($p < 0.05$) and ** ($p < 0.001$).

Involvement of NO Liberation in the Effects of SIN-1 on I_{Ca}—The spontaneous liberation of NO from SIN-1 is accompanied by the release of SIN-1C, another metabolite of SIN-1 (Reden, 1990). Therefore, we examined whether the effects of SIN-1 could be due to SIN-1C liberation. Fig. 5*A* summarizes the effects of several concentrations of SIN-1C on 0.1 μM Iso-stimulated I_{Ca}. It appears that up to 1 μM, SIN-1C had no significant effect on I_{Ca}. However, at 100 μM, SIN-1C exerted a small but significant inhibition of Iso-stimulated I_{Ca} ($-13.7 \pm 4.6\%$). Similar results were obtained when forskolin (0.3 μM) was used instead of Iso to stimulate I_{Ca} (Fig. 5*B*). Note that, unlike SIN-1, nanomolar concentrations of SIN-1C did not induce any elevation of forskolin-stimulated I_{Ca}. These results suggest that at concentrations below 1 μM, both the stimulatory and inhibitory effects of SIN-1 on I_{Ca} cannot be explained by an effect of SIN-1C. In addition, the $\approx$10% inhibitory effect of 100 μM SIN-1C must be considered as an upper limit of the participation of this compound in the effects of 100 μM SIN-1 described above. Indeed, SIN-1 releases NO during as long as 16 h when dissolved in a physiological solution (Manzoni *et al.*, 1992). Since solutions containing SIN-1 were never used for more than 4 h in our experiments, it was most unlikely that a solution containing 100 μM SIN-1 at the beginning could have generated as much as 100 μM SIN-1C during the course of our experiments.

The above results suggest that NO, rather than SIN-1C, is the mediator of SIN-1 regulation of I_{Ca}. However, there remains the possibility that uncleaved SIN-1 may by itself be responsible for some of the effects described here. To examine this hypothesis, we performed two sets of additional experiments. First, we examined the effects of another NO donor, SNP. SNP was also found to decrease I_{Ca} stimulated by 1 μM Iso, although with a lower potency than SIN-1. Indeed, a 100 μM concentration of SNP reduced Iso (1 μM)-stimulated I_{Ca} by only 40 $\pm$ 10% ($N = 5$, $p < 0.01$), and a 10 mM SNP concentration was needed to induce an inhibition of 87 $\pm$ 11% ($N = 4$, $p < 0.005$; not shown). The inhibitory effects of SNP on I_{Ca} as those of SIN-1 were not accompanied by any change

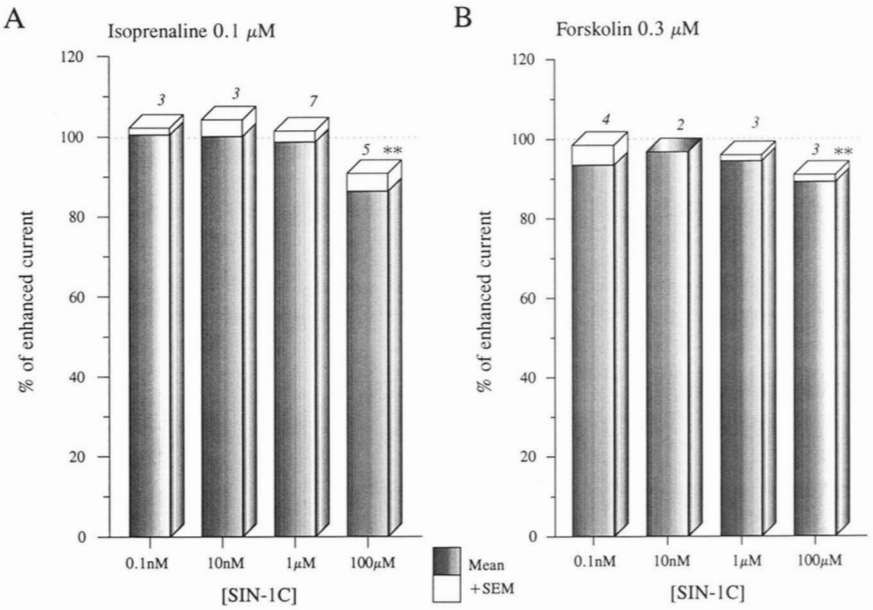


FIG. 5. **Effects of SIN-1C on isoprenaline- (*A*) and forskolin- (*B*) stimulated I_{Ca}.** The effects of different concentrations of SIN-1C on I_{Ca} stimulated either by 0.1 μM isoprenaline (*A*) or 0.3 μM forskolin (*B*) are summarized. *Filled* and *open bars* are, respectively, mean values and S.E. of the number of experiments indicated near the *bars*. Statistical analysis of data were done according to the *t* test (** $p < 0.05$).

in the shape and position of the I_{Ca} current-voltage and steady-state inactivation curves (not shown). Second, we investigated the effects of SIN-1 in the presence of methylene blue or LY83583. These compounds are commonly used as blockers of the NO-sensitive guanylyl cyclase. However, it has been recently shown that methylene blue (Marczin *et al.*, 1992) and LY83583 (Barbier and Lefebvre, 1992; Kontos and Wei, 1993) rather act as generators of superoxide ions, which are responsible for the degradation of NO in solutions. Methylene blue exerted effects on its own on I_{Ca}: on average, 50 μM methylene blue blocked 50 ± 5 % of I_{Ca} stimulated by 0.1 μM Iso ($N = 7$). Nevertheless, the presence of methylene blue completely abolished the effects of a further application of SIN-1. Indeed, in the presence of 50 μM methylene blue, SIN-1, used at 1, 10, and 100 μM concentrations, exerted only a −3 ± 7% ($N = 5$), −7 ± 7% ($N = 3$), and −17 ± 21% ($N = 4$) variation of I_{Ca}, respectively. Similarly, in the presence of 50 μM LY83583, 10 μM SIN-1 produced only a 13 ± 5% ($N = 3$) variation of I_{Ca}. Taken together, these experiments strongly suggest that NO is the mediator of both the stimulatory and inhibitory effects of SIN-1 (and SNP) on I_{Ca}.

Mechanisms Involved in the Inhibitory Effect of SIN-1 on I_{Ca}—So far, the inhibitory effects of SIN-1 on I_{Ca} resemble in some respects those of intracellular cGMP. Indeed, cGMP and SIN-1 have negligible effects on basal I_{Ca}, but inhibit I_{Ca} stimulated by Iso (Hartzell and Fischmeister, 1986; Fischmeister and Hartzell, 1987) or forskolin (Hartzell and Fischmeister, 1987). A possibility was that the effects of SIN-1 on I_{Ca} were mediated via a NO-induced stimulation of guanylyl cyclase activity.

It has been previously shown that intracellularly perfused cGMP in frog ventricular cells not only inhibited Iso- and forskolin-stimulated I_{Ca}, but also I_{Ca} directly stimulated by intracellular perfusion with cAMP (Hartzell and Fischmeister, 1986; Fischmeister and Hartzell, 1987). The effects of SIN-1 were thus examined in the presence of cAMP. In the experiment of Fig. 6*A*, I_{Ca} was stimulated by intracellular perfusion with 10 μM cAMP. In the continuing presence of intracellular cAMP, superfusion of the cell with 1 μM SIN-1 resulted in a rapid decrease of I_{Ca} to about 70% of its level in cAMP alone. Increasing the concentration of SIN-1 to 100 μM induced a further inhibition of I_{Ca}. The inhibitory effect of SIN-1 on cAMP-elevated I_{Ca} (as well as on Iso- and forskolin-stimulated I_{Ca}, data not shown) were only partially reversible. Thus, like cGMP, SIN-1 induces an inhibition of cAMP-elevated I_{Ca}. The inhibitory effect of cGMP on I_{Ca} in frog heart cells was shown to be mediated by the activation of the cGS-PDE and to be antagonized by the poorly hydrolyzable analog of cAMP, 8Br-cAMP (Hartzell and Fischmeister, 1986; Fischmeister and Hartzell, 1987). Therefore, the effect of SIN-1 was examined when I_{Ca} was stimulated by intracellular perfusion with 10 μM 8-Br-cAMP. As shown in Fig. 6*B*, superfusion of the cell with 1 or 100 μM SIN-1, in the continuing presence of intracellular 8-Br-cAMP, had only a negligible effect on I_{Ca}. The results of a number of similar experiments are summarized in Fig. 6*C*. When cAMP was used to enhanced I_{Ca}, SIN-1 produced dose-dependent inhibitions of I_{Ca}, even at the lowest concentration tested (10 nM). A 100 μM concentration of SIN-1 induced a 73 ± 5% inhibition of cAMP-elevated I_{Ca}. A similar result was found with 100 μM SNP, which inhibited cAMP (10 μM)-stimulated I_{Ca} by 66 ± 10% ($N = 4$, $p < 0.01$; not shown). In marked contrast, SIN-1 (1 and 100 μM) had no significant effect on I_{Ca} stimulated by 10 μM 8Br-cAMP. These results indicate that the inhibitory effect of SIN-1 is dependent on a PDE activity.

Mechanisms Involved in the Stimulatory Effect of SIN-1 on I_{Ca}—As shown above, nanomolar concentrations of SIN-1 were frequently found to produce a further stimulation of I_{Ca}. This was more consistently found in the presence of forskolin (Fig. 4), but could also be seen occasionally in the presence of 0.1 μM Iso (Fig. 1*B*) or of 2–10 μM intracellular cAMP (data not shown). In guinea pig ventricular cells, activation of I_{Ca} has been shown to be linked to the inhibition, by cGMP, of a cGI-PDE (Ono and Trautwein, 1991). Since a cGI-PDE has also been found to regulate I_{Ca} in frog ventricular cells (Fischmeister and Hartzell, 1990; Méry *et al.*, 1990), we designed the next series of experiments to define whether activation of I_{Ca} by nanomolar concentrations of SIN-1 could result from a limited activation of guanylyl cyclase leading to the inhibition of the cGI-PDE. Fig. 7 shows that upon superfusion of a frog cardiomyocyte with 0.3 μM forskolin I_{Ca} was increased about 4-fold. The addition of 0.1 nM SIN-1 produced a further and reversible increase (≈+12%) of I_{Ca}. After washout of SIN-1, the cell was exposed to a saturating concentration of

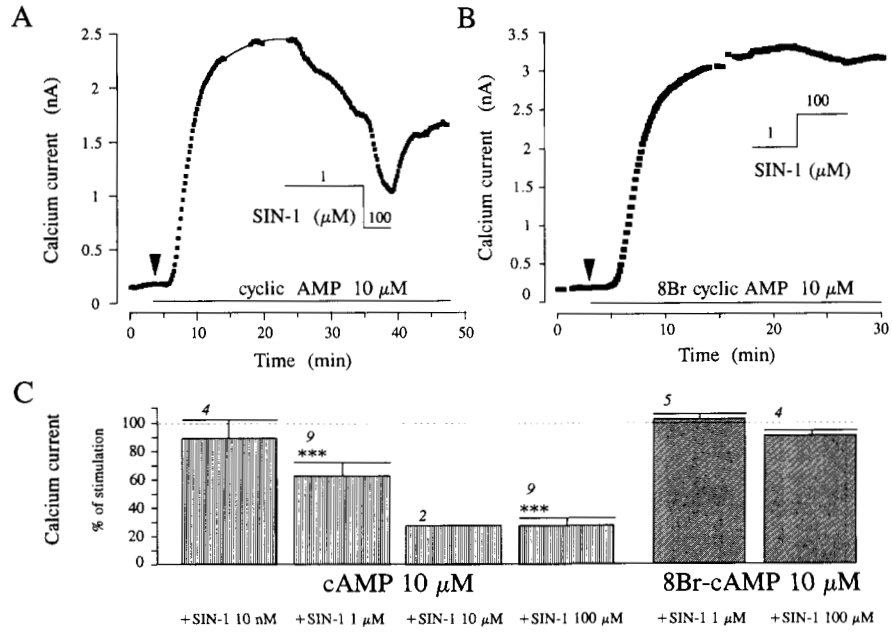


FIG. 6. **Effect of SIN-1 on cAMP- and 8-Br-cAMP-stimulated I_{Ca}.** In *A* and *B*, a frog ventricular cell was initially superfused with control Cs Ringer solution and internally dialyzed with control intracellular Cs solution. The *arrows* indicate the addition of 10 μM cAMP (*A*) or 8-Br-cAMP (*B*) to the intracellular solution which then perfused the cell throughout the rest of the experiment. During the periods indicated, the cells were superfused with 1 or 100 μM SIN-1. *Part C*, summary of the effects of 10 nM, 1, 10, and 100 μM SIN-1 on cAMP (10 μM)-stimulated I_{Ca} and of 1 and 100 μM SIN-1 on 8-Br-cAMP (10 μM)-stimulated I_{Ca}. The *bars* indicate the means and S.E. of the number of experiments indicated near the *bars*. (***, $p < 0.005$).

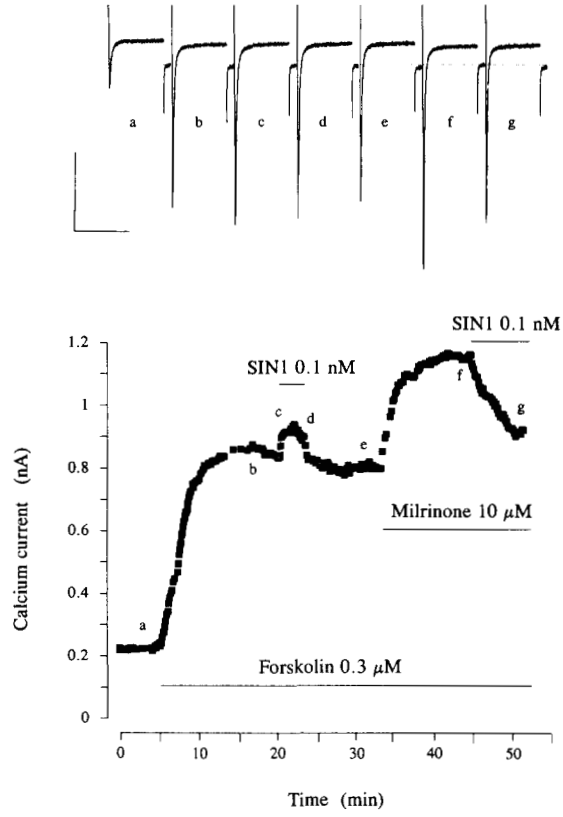


FIG. 7. **Comparison of the effects of SIN-1 in the absence and presence of milrinone.** *Lower part*, the cell was initially superfused with control Cs Ringer solution. During the periods indicated, the cell was successively exposed to 0.3 μM forskolin alone, and to 0.1 nM SIN-1, 10 μM milrinone, and 10 μM milrinone + 0.1 nM SIN-1 in the continuing presence of forskolin. *Upper part*, current traces were obtained at the times indicated by the corresponding letters on the lower part. *Vertical* and *horizontal bars* indicate 400 pA and 400 ms, respectively. The *dotted line* indicates the zero-current level.

milrinone (10 μM), a specific inhibitor of the cGI-PDE (Nicholson *et al.*, 1991). As already demonstrated (Fischmeister and Hartzell, 1990), milrinone potentiated I_{Ca} by ≈40%. When the same 0.1 nM concentration of SIN-1 was applied onto the cell in the continuing presence of milrinone, it now produced an inhibitory effect on I_{Ca}, reversing by ≈70% the stimulatory effect of milrinone. In five similar experiments, 0.1 nM SIN-1 produced a 28.4 ± 10.7% ($p < 0.05$) stimulation of forskolin-stimulated I_{Ca}. This effect was much smaller than the stimulation induced by 10 μM milrinone (65.0 ± 14.0%, $p < 0.01$). When SIN-1 was added in the presence of milrinone, I_{Ca} amplitude returned to a level which was only 25.4 ± 16.5% above the level in the presence of forskolin alone. Therefore, milrinone not only suppressed the potentiation of I_{Ca} by SIN-1, as expected if SIN-1 exerted its activatory effect on I_{Ca} through the cGI-PDE, but it also unmasked an inhibitory effect of nanomolar concentrations of the NO donor.

Characterization of the SIN-1-sensitive Guanylyl Cyclase—It is of general agreement that the soluble, αβ heterodimeric form of guanylyl cyclase is the one sensitive to NO (Garbers, 1992). However, several early reports have described the sensitivity to NO donors of membrane-bound guanylyl cyclases from various tissues (Lacombe and Eberentz-Lhomme, 1983; for a review, see Waldman and Murad, 1987). Since SIN-1 regulates transarcolemmal I_{Ca}, we posed the question as to the form, either membrane-bound or soluble, of guanylyl cyclase which may control L-type calcium channel activity. In that aim, we have examined the distribution of the SIN-1-sensitive guanylyl cyclase in frog ventricular fractions. It was compared with that of adenylyl cyclase and aspartate amino transferase referred to as membrane and cytosolic markers, respectively. The homogenate of frog heart ventricles was submitted to quantitative fractionation by differential centrifugation. Four fractions were obtained: a membrane fraction (3,000 × *g* pellet), 10,000 × *g*, and 100,000 × *g* pellets and a supernatant fraction (100,000 × *g* supernatant). Proteins, basal, SIN-1-, and ANF-stimulated guanylyl cyclase activities obtained in the different fractions are reported in Table I. The corresponding recoveries and distribution profiles are depicted in Table II, in parallel with those for adenylyl cyclase and aspartate amino transferase which each

TABLE I

Subcellular distribution of proteins, basal, ANF-, and SIN-1-stimulated guanylyl cyclase activities from frog heart

Proteins and guanylyl cyclase activity, assayed under basal conditions and in the presence of either 0.5 μM ANF or 100 μM SIN-1, were quantified in the different fractions of frog heart as described under "Experimental Procedures." Data are the means ± S.E. of four determinations. The activities relative to basal are given in parentheses.

Fraction	Protein	Guanylyl cyclase activity		
		Basal	+0.5 μM ANF	+100 μM SIN-1
	mg		*nmol/mg/10 min*	
Homogenate	40 ± 4	0.36 ± 0.07	0.49 ± 0.03 (1.4)	10.3 ± 0.5 (29)
Membrane	19 ± 2	0.28 ± 0.03	0.35 ± 0.03 (1.3)	2.0 ± 0.1 (7)
10,000 × *g* pellet	3 ± 0.5	0.69 ± 0.07	0.81 ± 0.01 (1.2)	1.7 ± 0.1 (3)
100,000 × *g* pellet	2 ± 0.5	1.03 ± 0.02	1.02 ± 0.0 (1)	3.6 ± 0.1 (4)
Supernatant	11 ± 1	0.75 ± 0.04	0.75 ± 0.02 (1)	22.4 ± 0.2 (30)

TABLE II

Distribution pattern of total proteins, adenylyl cyclase, guanylyl cyclase, and aminotransferase activities in subcellular fractions from frog heart

Membrane and supernatant represent the 3,000 × *g* pellet and the 100,000 × *g* supernatant fractions, respectively. The results are calculated from the data given in Table I as percentages of total proteins, total adenylyl cyclase, total basal guanylyl cyclase, and total aspartate aminotransferase activities, or percentages of total guanylyl cyclase activity over basal measured in the presence of either 0.5 μM ANF or 100 μM SIN-1, recovered in each fraction.

	Membrane	10,000 × *g* pellet	100,000 × *g* pellet	Supernatant
Proteins	54	9	6	31
Adenylyl cyclase	82	13	5	0
Guanylyl cyclase				
Basal	30	11	13	47
+ 0.5 μM ANF (activity over basal)	83	17	0	0
+ 100 μM SIN-1 (activity over basal)	12	1	2	85
Aspartate aminotransferase	<0.05	<0.5	3	97

gave a distinct pattern of distribution. Forskolin-stimulated adenylyl cyclase activity was present essentially in the membrane fraction (80% of the total activity) while it was not detected in the supernatant. In contrast, 97% and less than 0.05% of total aspartate amino transferase activity were recovered in the supernatant and the membrane fractions, respectively. The distributions of basal, ANF-, and SIN-1-stimulated guanylyl cyclase activities gave three clearly distinct patterns. Basal guanylyl cyclase activity could be detected in all fractions, but was mostly present, as expected, in the supernatant and the membrane fractions which displayed 47 and 30% of the total activity, respectively. ANF-sensitive guanylyl cyclase had a distribution relatively similar to that of adenylyl cyclase, 83% of the total activity, due to ANF, being recovered in the membrane fraction while no ANF-sensitive activity was detectable in the supernatant. Finally, SIN-1-sensitive guanylyl cyclase had a distribution relatively similar to that of aspartate aminotransferase. However, a significant 12% fraction of its activity was found in the membrane fraction, and this cannot be imputed to a contamination of the membrane fraction by the supernatant, since, considering the recovery of aspartate aminotransferase activity in the membrane fraction, it did not exceed 0.05%. These results suggest that SIN-1-sensitive guanylyl cyclases are present in both the membrane and the supernatant fractions.

The dose-dependent activation of the membrane and the supernatant guanylyl cyclase activities as a function of SIN-1 concentrations are shown in Fig. 8. Clearly, two features differentiate between the activities in both fractions. First, activation by SIN-1 of the guanylyl cyclase in the membrane fraction was significant with 3 μM SIN-1 and maximal at 10 μM SIN-1, while activation of the enzyme in the supernatant was not noticeable below 20 μM SIN-1 (Fig. 8, *left upper*). Second, while the degree of activation of the enzyme in the supernatant reached at least 120-fold activation with 100 μM SIN-1, that of the membrane-bound enzyme did not exceed

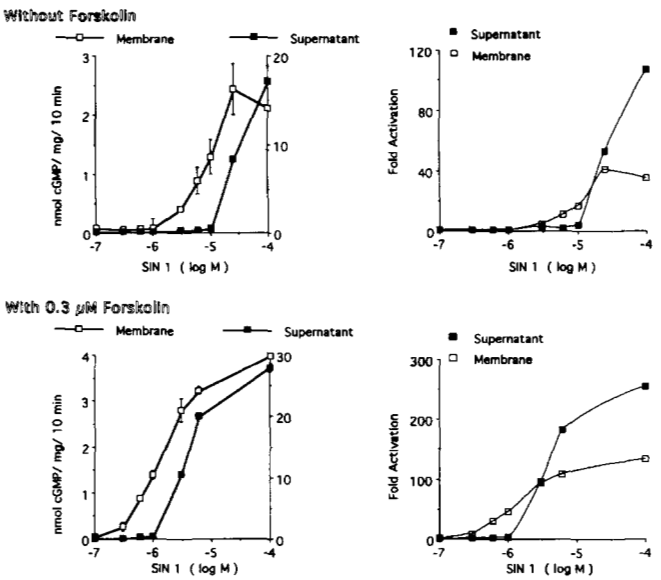


FIG. 8. **Activation by SIN-1 of the guanylyl cyclase activities in the membrane and cytosolic fractions of frog heart ventricle.** Guanylyl cyclase activity was measured with varying concentrations of SIN-1, in the membrane fraction (3,000 × *g* pellet, *open squares*) and in the supernatant fraction (100,000 × *g* supernatant, *closed squares*), in the absence (*top panels*) or in the presence of 0.3 μM forskolin (*bottom panels*) as described under "Experimental Procedures." Results are the means ± S.E. of six determinations obtained from two different experiments and are expressed in nmol cGMP formed/mg/10 min (*top* and *bottom left panels*) or as fold activation over basal activity (*top* and *bottom right panels*).

40-fold activation (Fig. 8, *right upper*). Since the effect of SIN-1 on I_{Ca} were studied in the presence of adenylyl cyclase activators (isoprenaline or forskolin), we examined the sensitivity to SIN-1 of guanylyl cyclases in the membrane and in the supernatant fractions with 0.3 μM forskolin added to the

assay medium. In these conditions, which were closed to those of the experiment shown in Fig. 4, an increase in guanylyl cyclase activation and a higher sensitivity to SIN-1 were observed in both fractions. Nevertheless, the two major differences between the two enzymes in the membrane and in the supernatant fractions still remained: (i) the membrane enzyme displayed a higher sensitivity to SIN-1 than the supernatant enzyme, and (ii) the degree of activation by SIN-1 of the supernatant enzyme was higher than that of the membrane enzyme (Fig. 8, *bottom*). Actually, the sensitivity toward SIN-1 of the guanylyl cyclase in the membrane fraction matches that of I_{Ca} inhibition. Thus, half-maximal inhibition of I_{Ca} and half-maximal activation of membrane guanylyl cyclase occur with 1 μM SIN-1, while, maximal inhibition and maximal activation, respectively, are observed at 100 μM of the NO donor. These observations support the proposal that activation of the membrane-bound guanylyl cyclase by SIN-1 drives the inhibition of L-type calcium channel current.

DISCUSSION

In the present study, we examined the effects of NO donors on I_{Ca} and guanylyl cyclase activity in frog ventricular cells. Several main conclusions can be drawn from our experiments: (i) SIN-1 had no effect on basal I_{Ca}, *i.e.* in the absence of cAMP-dependent stimulation of I_{Ca}; (ii) in the micromolar range of concentration, SIN-1 and SNP exerted profound inhibitory effects on I_{Ca} elevated by either Iso, forskolin, or intracellular cAMP; (iii) in the nanomolar range of concentrations, SIN-1 exerted a significant potentiation of the stimulatory effect of forskolin and, to a lesser extent, of Iso and cAMP on I_{Ca}; (iv) SIN-1 stimulated guanylyl cyclase activity in heart ventricle; (v) when examined in subcellular fractions of ventricular tissue, a SIN-1-sensitive guanylyl cyclase activity was found in the soluble as well as in the membrane-bound compartment; (vi) both stimulatory and inhibitory effects of SIN-1 on I_{Ca} were mediated by the liberation of NO and accumulation of intracellular cGMP; (vii) the stimulatory effect of SIN-1 on I_{Ca} was mediated by the inhibition of the cGI-PDE; (viii) the inhibitory effect of SIN-1 on I_{Ca} was mediated by the stimulation of the cGS-PDE.

Although the effects of SIN-1 and SNP on I_{Ca} are in accordance with the release of NO and the subsequent activation of guanylyl cyclase, other additional effects of NO could not be completely discounted. For instance, it has been found recently that NO donors strongly inhibit glyceraldehyde-3-phosphate dehydrogenase in the heart (Brüne and Lapetina, 1989; Snyder, 1992). In skeletal muscle, this enzyme has been found to bind to the α1-subunit of the L-type calcium channel (Kim *et al.*, 1990). Whether glyceraldehyde-3-phosphate dehydrogenase also binds to cardiac L-type calcium channel and affects its activity is unknown. Other works indicate that NO released by SIN-1 can induce *S*-nitrosylation of cysteine residues, scavenge tyrosyl residues, and possibly deaminate primary amines residues in proteins (Stamler *et al.*, 1992). Although the L-type calcium channel could be subject to such alterations, SIN-1 did not alter basal I_{Ca} and did not modify either the current-voltage or the inactivation curves of I_{Ca} which would argue against a significant functional modification of the L-type calcium channel protein by SIN-1.

A key finding of the present study was that an endogenous cGMP production controls the regulation of cardiac L-type calcium channel activity. Intracellular perfusion with cGMP was shown earlier to modulate cardiac L-type calcium channel activity in various species (Lohmann *et al.*, 1991). In frog ventricular myocytes, cGMP induces a strong inhibition of cAMP-enhanced I_{Ca} which is mediated by the activation of the cGS-PDE (Hartzell and Fischmeister, 1986; Fischmeister and Hartzell, 1987; Simmons and Hartzell, 1988). Micromolar concentrations of SIN-1 reproduce the inhibitory effect of cGMP on I_{Ca} suggesting that calcium channel activity is regulated by the endogenous cGMP. According to previous observations, and along with the present data, this would rely on the activation of the cGS-PDE. On the other hand, the cGI-PDE appears to be responsible for the stimulatory effect of nanomolar concentrations of SIN-1 on I_{Ca}. This enzyme is the receptor for bipyridines such as milrinone, amrinone, etc. (Nicholson *et al.*, 1991) and is functionally coupled to the L-type calcium channel in frog (Méry *et al.*, 1990; Fischmeister and Hartzell, 1990; Brechler *et al.*, 1992) and guinea-pig ventricular cells (Ono and Trautwein, 1991; Lohmann *et al.*, 1991). How the two cGS- and cGI-PDE enzymes contribute to the biphasic modulation of I_{Ca} in response to a progressive increase in guanylyl cyclase activity is yet unclear. Both enzymes hydrolyze cAMP and cGMP, but their kinetic properties differ in several aspects: 1) the cGI-PDE has a higher affinity for cAMP as substrate than the cGS-PDE (Beavo, 1988); 2) the cGI-PDE is more sensitive to cGMP than the cGS-PDE (Beavo, 1988; Nicholson *et al.*, 1991); 3) the cGS-PDE has a higher capacity to hydrolyze cAMP and cGMP than the cGI-PDE (Beavo, 1988; Nicholson *et al.*, 1991); 4) the cGI-PDE may be regulated by cAMP-dependent phosphorylation, while the cGS-PDE is not (Nicholson *et al.*, 1991; Beavo and Reifsnyder, 1990). Besides, in frog cells, the cGI-PDE may be less efficiently coupled to the L-type calcium channel than the cGS-PDE (Fischmeister and Hartzell, 1990, 1991). Based on these considerations, it is likely that guanylyl cyclase and cGMP participate in the fine tuning of cardiac cAMP concentration, by positive and negative controls via inhibition of the cGI-PDE and stimulation of the cGS-PDE, respectively. However, this hypothesis is questioned by the fact that exogenous cGMP, even in the nanomolar range of concentrations,[2] was never found to potentiate Iso-, forskolin- or cAMP-enhanced I_{Ca} (Hartzell and Fischmeister, 1986; Fischmeister and Hartzell, 1987). Here we routinely found a stimulatory effect of SIN-1 with forskolin, but this effect was only occasionally found in experiments where I_{Ca} had been elevated by prior applications of Iso or intracellular cAMP. Surprisingly, however, milrinone always stimulated I_{Ca} in all of the above experimental conditions. Further experiments are needed to clarify the differential effect on I_{Ca} of cGI-PDE inhibition induced by endogenous or exogenous cGMP, or by synthetic agents.

Increases in cardiac cGMP levels were found to occur in response to various exogenous compounds, including NO donors (Waldman and Murad, 1987; Hartzell, 1988; Lindemann and Watanabe, 1989). The NO-sensitive guanylyl cyclase is generally referred to as the soluble guanylyl cyclase (Waldman and Murad, 1987; Ignarro, 1990; Garbers, 1992). However, the present results show that a NO-sensitive guanylyl cyclase is also present in the membrane fraction of cardiac cells. This surprising localization could not be attributed to a contamination of the membrane fraction by entrapped soluble guanylyl cyclase (as suggested by Waldman and Murad, 1987). In fact, 12% of the total guanylyl cyclase activity is recovered in the membrane fraction, the contamination of which by a cytosolic marker was found not to exceed 0.05%. The membrane-bound NO-sensitive guanylyl cyclase is likely different from an ANF-like receptor since such receptors have never been found to be activated by NO or NO donors (Waldman and Murad, 1987; Ignarro, 1990; Garbers, 1992). An attractive

[2] R. Fischmeister, and P.-F. Méry, unpublished data.

proposal is that SIN-1 activates a membrane-bound heterodimeric form, $\alpha\beta$, of guanylyl cyclase since the cloning of the $\beta 2$ subunit has revealed that the COOH-terminal sequence of this protein has the potential to be prenylated, and thus to associate with membranes (Garbers, 1992). It is conceivable that a discrete membrane compartment may be involved in the local regulation of calcium channel activity. Such a proposal is supported by the additional finding that SIN-1 was severalfold less potent when dialyzed intracellularly than when applied outside the cell (data not shown).

The regulation of cardiac I_{Ca} by NO donors may have physiological relevance. Indeed, NO, which is an endothelium-derived-relaxing factor (EDRF; Ignarro, 1990), can also be synthetized by endocardial (Schulz *et al.*, 1991) and intrinsic neuronal cardiac cells (Klimaschewski *et al.*, 1992; Schmidt *et al.*, 1992). Besides, NO released by the endothelial cells of the intracoronary microvasculature is likely to diffuse to the neighboring cardiac myocytes (Brutsaert, 1993). Thus, both in periphery of the heart and in the most inner mass of the muscle (Weber, 1992), there is the physical possibility for released EDRF-NO to reach the cardiomyocytes. It is worth noting that removal of the cardiac endothelium (Ramciotti *et al.*, 1992) or endocardium (Smith *et al.*, 1992) has profound effects on cardiac muscle contractility, associated in part with the irreversible failure of some neurotransmitters to enhance cGMP levels (Smith *et al.*, 1992). Therefore, it is likely that EDRF-NO modulates the activity of cardiac muscle under physiological conditions. Besides, neurotransmitters which are responsible for a stimulation of the production of EDRF-NO in the heart (*e.g.* ACh; Lamontagne *et al.*, 1991; Balligand *et al.*, 1993a) are likely to affect cardiac contractility via this pathway. Finally, NO and NO synthase may play a role in certain cardiac pathologies. Indeed, endotoxins can induce the long term expression of the Ca^{2+}-independent inducible NO synthase in heart myocytes (Salter *et al.*, 1991; Schulz *et al.*, 1992; Debelder *et al.*, 1993). On the short term basis, cytokines accumulation was shown to decrease cardiac contractility through the stimulation of a NO synthase activity in the cardiomyocytes themselves (Finkel *et al.*, 1992; Balligand *et al.*, 1993b). This raises the interesting possibility that, depending on the state of the tissue, NO can serve as a short distance bidirectional messenger between non-myocytes and myocytes.

SIN-1 is a metabolic product of molsidomine, which is used in the treatment of several cardiovascular disorders (Reden, 1990). A number of studies have showed that SIN-1 relaxes arteries by mimicking the endothelium-dependent relaxation of smooth muscle cells. Circulating levels of SIN-1 during a therapeutical treatment with molsidomine may vary between 1 and 100 nM (Fach and Becker, 1984). Although further work is needed to examine whether the conclusions reached on frog cardiac cells can be applied to other species, the present study suggests that in this range of concentration SIN-1 is likely to act on cardiac performance.

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