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Role of cGMP-inhibited Phosphodiesterase and Sarcoplasmic Calcium in Mediating the Increase in Basal Heart Rate with Nitric Oxide Donors

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P. Musialek, L. Rigg, D. A. Terrar, D. J. Paterson and B. Casadei. Role of cGMP-inhibited Phosphodiesterase and Sarcoplasmic Calcium in Mediating the Increase in Basal Heart Rate with Nitric Oxide Donors. *Journal of Molecular and Cellular Cardiology* (2000) **32**, 1831–1840. Nitric oxide (NO) donors increase heart rate (HR) through a guanylyl cyclase-dependent stimulation of the pacemaker current I_f, without affecting basal $I_{Ca\text{-}L}$. The activity of I_f is known to be enhanced by cyclic nucleotides and by an increase in cytosolic Ca^{2+}. We examined the role of cGMP-dependent signaling pathways and intracelluar Ca^{2+} stores in mediating the positive chronotropic effect of NO donors. In isolated guinea pig atria, the increase in HR in response to 1–100 μmol/l 3-morpholino-sydnonimine (SIN-1; with superoxide dismutase, $n=6$) or diethylamine-NO (DEA-NO, $n=8$) was significantly attenuated by blockers of the cGMP-inhibited phosphodiesterase (PDE3; trequinsin, milrinone or Ro-13-6438, $n=22$). In addition, the rate response to DEA-NO or sodium nitroprusside (SNP) was significantly reduced following inhibition of PKA (KT5720 or H-89, $n=15$) but not PKG (KT5728 or Rp-8-pCPT-cGMPs, $n=16$). Suppression of sarcoplasmic (SR) Ca^{2+} release by pretreatment of isolated atria with ryanodine or cyclopiazonic acid (2 μmol/l and 60 μmol/l, $n=16$) significantly reduced the chronotropic response to 1–100 μmol/l SIN-1 or DEA-NO. Moreover, in isolated guinea pig sinoatrial node cells 5 μmol/l SNP significantly increased diastolic and peak Ca^{2+} fluorescence ($+13\pm1\%$ and $+28\pm1\%$, $n=6$, $P<0.05$). Our findings are consistent with a functionally significant role of cAMP/PKA signaling (via cGMP inhibition of PDE3) and SR Ca^{2+} in mediating the positive chronotropic effect of NO donors.

Key Words: Nitric oxide signaling; Heart rate; Intracellular calcium; Sinoatrial node.

Introduction

Our previous work demonstrates that nitric oxide (NO) exerts a positive chronotropic effect[1–5] via a guanylate cyclase/cGMP dependent stimulation of the hyperpolarization-activated "pacemaker" current I_f.[1,2] In particular, we showed that the increase in heart rate (HR) seen with micromolar concentrations of NO donors in isolated guinea pig atria is maintained after inhibition of $I_{Ca\text{-}L}$ but it is abolished

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by specific I_f blockers (ZD7288 or 2 mmol/l Cs^+).[1,2] In agreement with these functional data, we found that (a) NO increases the amplitude of I_f (but not of $I_{Ca\text{-}L}$) and the beating rate of isolated sinoatrial node cells and (b) both effects are reversibly abolished by I_f blockade.[1,2] Inhibition of guanylyl cyclase (GC) by ODQ or LY835383 prevents the positive chronotropic effect of NO donors,[1,2] whereas a membrane permeable analog of cGMP mimicks this effect, indicating that the NO-I_f signaling pathway is GC-cGMP-dependent.[1,2]

It is known that cGMP can directly enhance I_f by binding to the intracellular domain of the channel,[6,7] but its action is several fold weaker than that of the "physiological" I_f gating nucleotide, cAMP,[6,7] suggesting that cellular signaling downstream of cGMP is likely to be involved. For instance, binding of cGMP to the cGMP-inhibited phosphodiesterase (PDE3) leads to an increase in intracellular cAMP,[8,9] which in turn could enhance I_f (a) directly, i.e. by binding to the channel and increasing its open probability,[6,7] and (b) indirectly, through the activation of cAMP-dependent protein kinase (PKA).[6,10,11] Furthermore, the NO-mediated stimulation of I_f could occur through an increase in the cytosolic calcium level (Ca_i^{2+}).[12] Indeed, recent findings indicate that NO donors can mobilize Ca^{2+} from intracellular stores in several tissues, including cardiocytes.[13–17]

The aim of the present study was to evaluate the role of cAMP/PKA signaling (via cGMP-mediated inhibition of PDE3) and of intracellular Ca^{2+} stores in the positive chronotropic effect of NO donors.

Materials and Methods

Guinea pig atrial preparation

The spontaneously beating guinea pig atrial preparation has been described in detail previously.[1] Briefly, before starting experiments, the atria were equilibrated for 120–200 min (with the Tyrode solution changed every 20 min) until their beating rate stabilized (within 5 bpm for 40 min). The Tyrode solution contained (mmol/l): NaCl 120, KCl 4, $MgCl_2$ 2, $NaHCO_3$ 25, $CaCl_2$ 1.8, NaH_2PO_4 0.1, glucose 11, and was aerated with 95% O_2 and 5% CO_2 (pH 7.4, temperature kept at 37 ± 0.1 °C).

Sinoatrial node cells

Cells were isolated from the sinoatrial node (SAN) of adult male guinea pigs.[18] Briefly, the heart was removed and perfused for *c.* 35 min via the Langendorff method with a re-circulating Tyrode solution (with 0 Ca^{2+} in the first 3 min) containing Type II collagenase [86 U/ml (Worthington Biochemicals)]. The SAN region was then excised, cut into small strips (*c.* 2 × 5 mm) perpendicular to the crista terminalis and dispersed in 2 ml high-potassium Krebs buffer (70 mmol/l K^+, 4 °C). After 1–4 h, cells were moved to a physiological Tyrode solution (see below). Only spindle-shaped cells showing faint striations, well defined membranes, and regular spontaneous activity were used.

Nitric oxide donors

We have previously shown that micromolar concentrations of sodium nitroprusside (SNP), 3-morpholinosydnonimine (SIN-1)[1] or diethylamine-NO (DEA-NO, unpublished observation) increase HR by enhancing basal I_f without affecting $I_{Ca\text{-}L}$ in SAN cells.[1] In the present study we used SIN-1 (with 100 U/ml of superoxide dismutase (SOD) to prevent formation of peroxynitrite[19]), DEA-NO and its carrier molecule (DEA) in a concentration range from 0.05 to 100 μmol/l. SNP was used at the concentration of 10 μmol/l in atrial preparations and 5 μmol/l in SAN cells.

Protocols

Role of PDE3, PKA and PKG in mediating the positive chronotropic effect of NO donors

The chronotropic effect of SIN-1 or DEA-NO was evaluated (a) alone and (b) in the presence of three inhibitors of PDE3: milrinone, Ro-13-6438 (0.2 μmol/l for 40 min) or trequinsin (50 nmol/l for 30 min). Moreover, we tested whether PKA inhibition with KT5720 (1 μmol/l for 30 min) or H-89 (0.3 μmol/l for 30 min) affects the chronotropic response to DEA-NO or SNP.

Similarly to PKA, the cGMP-dependent protein kinase (PKG) can stimulate Ca^{2+} release from SR microvesicles.[20] To evaluate whether PKG activation is coupled to the positive chronotropic effect of NO donors, we examined the HR response to DEA-NO in the presence of two structurally different PKG inhibitors: KT5823 (1 μmol/l for 40 min) or Rp-8-pCPT-cGMPs (40 μmol/l for 40 min). Similar concentrations of KT5823 and Rp-8-pCPT-cGMPs have been shown to inhibit PKG-dependent phosphorylation in cardiac or vascular tissues.[21,22]

Chronotropic response to NO donors after inhibition of sarcoplasmic Ca^{2+} release

The chronotropic response to SIN-1 or DEA-NO alone was compared with that seen after suppressing SR Ca^{2+} release by pretreatment of the atrial preparations with either ryanodine (Rya, 2 μmol/l for *c.* 40 min) or cyclopiazonic acid (CPA, 60 μmol/l for *c.* 100 min). Similar concentrations of Rya or CPA have been demonstrated to inhibit SR Ca^{2+} release channels or the SR Ca^{2+} ATPase without affecting plasmalemmal Ca^{2+} channels or the Na^+/Ca^{2+} exchanger.[23,24]

Calcium fluorescence in isolated spontaneously beating SAN cells

Qualitative evaluation of the effect of exogenous NO on cytosolic Ca^{2+} level in spontaneously beating SAN cells was performed by measuring the whole cell Ca_i^{2+} transient (Indo-1, 410/495 nm ratio)[25] before and 2–5 min after application of 5 μmol/l SNP. Briefly, cells were loaded with 3 μmol/l of Indo-1 AM (Sigma) for 15 min, and were then allowed to settle on the base of a flow chamber before being perfused with physiological Tyrode solution [NaCl 118.5, KCl 4.2, $MgSO_4$ 1.18, $NaHCO_3$ 14.5, $CaCl_2$ 2.5, KH_2PO_4 1.18, glucose 11.1 (mmol/l), pH 7.4, aerated with 95% O_2 and 5% CO_2] at 36 °C. Excitation light from a Xenon arc lamp (355 nm) was delivered to the cell via an oil immersion objective ($\times$100, numeric aperture 1.4, Leica) and a dichronic mirror reflecting light <375 nm. Two photomultipliers collected emitted fluorescence at 410 and 495 nm. Signals were filtered at 100 Hz, recorded onto video tape, and analysed off-line. Background fluorescence (recorded in the absence of a cell) was subtracted from the 410 and 495 nm signal, and then the Ca_i^{2+} transient was displayed as the 410/495 ratio.

All chemicals were purchased from Calbiochem with the exception of the NO donors, Rya, and CPA which were obtained from Sigma.

Statistical analysis

For protocols in atrial preparations, data are expressed as mean $\pm$ S.E.M. and analysed by one-way repeated measures ANOVA (within each treatment group) or by factorial ANOVA (for comparisons between different treatment groups). Changes in Ca_i^{2+} fluorescence in SAN cells (geometric mean $\pm$ S.D.) were evaluated with Wilcoxon signed-rank test. Statistical significance was accepted at $P<0.05$.

Results

Basal HR in atrial preparations

After 120–200 min of equilibration the spontaneous beating rate reached a stable basal (B/L) value which averaged at 173 ± 2 bpm ($n=91$).

Role of PDE3, PKA and PKG in the positive chronotropic effect of NO donors

At the concentrations employed in these studies, PDE3 inhibitors elicited a small but significant increase in basal HR ($+9 \pm 2$ bpm with 50 nmol/l trequinsin, $n=7$, $+11 \pm 2$ bpm with 0.2 μmol/l Ro-13-6438, $n=9$, and $+15 \pm 3$ bpm with 0.2 μmol/l milrinone, $n=8$, for all $P<0.05$). Pretreatment with PDE3 inhibitors markedly suppressed the positive chronotropic response to DEA-NO in a concentration range from 5 to 100 μmol/l [$+41 \pm 5$ bpm with 100 μmol/l DEA-NO alone *v* $+21 \pm 3$ bpm or $+22 \pm 4$ bpm with 100 μmol/l DEA-NO after PDE3 inhibition with trequinsin or Ro-13-6438, $P<0.05$, Fig. 1(a)] and to SIN-1 [1–100 μmol/l in the presence of 100 U/ml SOD; $+51 \pm 5$ bpm *v* $+22 \pm 3$ bpm after pretreatment with milrinone, $P<0.05$, data for 100 μmol/l SIN-1 shown in Fig. 1(b)].

PKA inhibition with KT5720 ($n=6$) or H-89 ($n=9$) did not change baseline HR (-2 ± 3 bpm and -1 ± 2 bpm, $P=$ N.S. for both) but significantly attenuated the chronotropic effect of NO donors. The peak increase in HR with DEA-NO was $+25 \pm 4$ bpm in the presence of KT5720 [*cf.* $+41 \pm 5$ bpm with 100 μmol/l DEA-NO alone, $P<0.05$, see Fig. 1(a)]. Similarly, H-89 markedly suppressed the increase in HR with 10 μmol/l SNP [$+54 \pm 9$ bpm with SNP alone *v* $+37 \pm 8$ bpm after pretreatment with H-89, $P<0.05$, Fig. 1(c)]. Conversely, PKG inhibition with KT5823 ($n=8$) or Rp-8-pCPT-cGMPs ($n=8$) had no effect on the magnitude of the chronotropic response to 5–100 μmol/l DEA-NO [data for 100 μmol/l shown in Fig. 1(a)].

Chronotropic response to SIN-1(SOD) or DEA-NO after inhibition of SR Ca^{2+} release

Both Rya ($n=7$) and CPA ($n=9$) reduced the spontaneous beating rate of guinea pig atrial preparations (by 43 ± 4 bpm and 30 ± 3 bpm respectively, $P<0.05$ for both). With CPA, the negative chronotropic effect was preceded by a transient

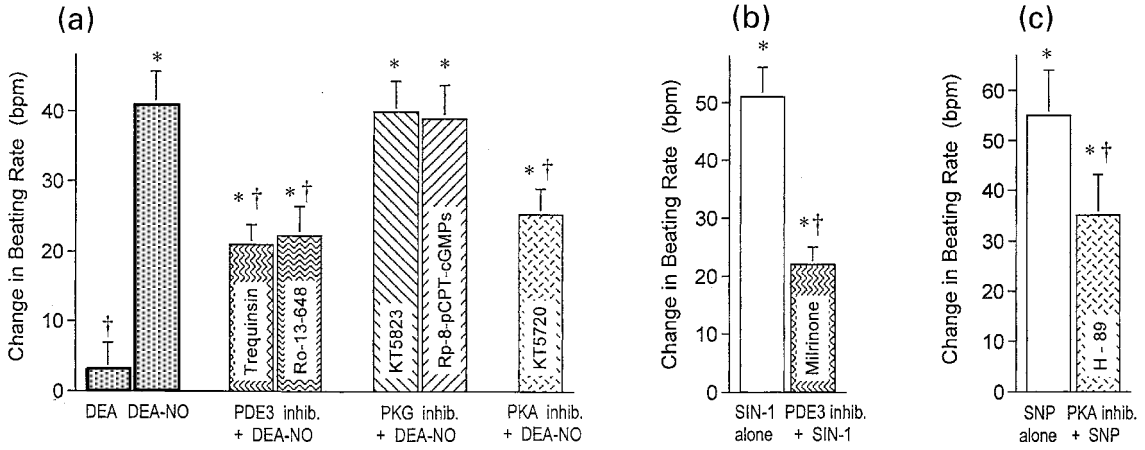

Figure 1 Effect of PDE3, PKG, or PKA inhibition on the chronotropic response to NO donors in isolated guinea pig atria. Bars show mean change in rate ± S.E.M. in response to a NO donor. (a) Change in beating rate with 100 μmol/l of DEA-NO ($n=8$, baseline HR 164 ± 8 bpm) or DEA ($n=10$, baseline HR 170 ± 6 bpm) compared with the effect of DEA-NO applied in the presence of inhibitors of PDE3 (50 nmol/l trequinsin, $n=7$, or 0.2 μmol/l Ro-13-648, $n=9$, note that basal HR increased with PDE3 inhibition from 177 ± 6 to 186 ± 7 bpm and from 177 ± 8 to 189 ± 7 bpm respectively, $P<0.05$ for both), PKG (1 μmol/l KT5823, $n=8$, or 40 μmol/l Rp-8-pCPT-cGMPs, $n=8$, PKG inhibitors had no effect on the basal HR of 173 ± 8 and 182 ± 9 bpm respectively, see text), or PKA (1 μmol/l KT5720, $n=6$, no significant effect of PKA inhibition on the basal HR of 182 ± 6 bpm, see text). * $P<0.05$ *v* baseline HR; † $P<0.05$ *v* the effect of DEA-NO on its own. (b) Effect of PDE3 inhibition with milrinone (0.2 μmol/l, $n=8$, causing an increase *per se* in basal HR from 182 ± 5 bpm to 198 ± 7 bpm, $P<0.05$) on the rate response to 100 μmol/l SIN-1 (with 100 U/ml SOD, $n=6$). * $P<0.05$ *v* baseline HR; † $P<0.05$ *v* the effect of SIN-1(SOD) in the absence of PDE3 inhibition. (c) Change in beating rate with 10 μmol/l SNP ($n=9$) before (basal HR 167 ± 9 bpm before SNP application and 165 ± 8 bpm following SNP washout) and after PKA inhibition with 0.3 μmol/l H-89 (basal HR of 164 ± 9 bpm following PKA inhibition). * $P<0.05$ *v* baseline HR; † $P<0.05$ *v* the response to SNP on its own.

increase in HR, consistent with a transient increase in Ca_i^{2+} following inhibition of the SR Ca^{2+} ATPase. Figure 2(a) shows an example of the HR response to increasing concentrations of SIN-1 in the presence of 100 U/ml SOD. A significant positive chronotropic effect was seen with 1–100 μmol/l of SIN-1 [$n=6$, see Fig. 2(c)], with the highest concentration increasing HR by 51 ± 3 bpm ($P<0.05$). Pretreatment with Rya ($n=7$) significantly attenuated the chronotropic response to SIN-1 [Fig. 2(b) and (c), + 19 ± 4 bpm with 100 μmol/l SIN-1, $P<0.05$]. Similarly, DEA-NO alone ($n=8$) elicited a dose-dependent positive chronotropic response [Fig. 3(a), peak effect of + 41 ± 5 bpm with 100 μmol/l, $P<0.05$, Fig. 3(d)] which was significantly suppressed by CPA ($n=9$, + 16 ± 3 bpm at 100 μmol/l, $P<0.05$ *v* the effect of DEA-NO alone, see Fig. 3). Similar concentrations of the carrier molecule (DEA, $n=10$) had no effect on HR (− 1 ± 1 bpm to + 3 ± 4 bpm, N.S., see Fig. 3).

Effect of SNP on the intracellular calcium transient in isolated SAN cells

Figure 4 shows a representative recording of Ca_i^{2+} transient in a spontaneously beating SAN cell before and after the application of 5 μmol/l SNP. SNP increased both diastolic and peak Ca^{2+} fluorescence signals (+ 13 ± 1% and + 28 ± 1% respectively, $P<0.05$, $n=6$ cells), consistent with an increase in cytosolic Ca^{2+}. In agreement with the data obtained in isolated atria, SNP caused an increase in the spontaneous beating rate of SAN cells (+ 26 ± 7 bpm, $P<0.05$).

Discussion

The present work further elucidates the signaling pathway responsible for the cGMP-I_f-mediated positive chronotropic effect of exogenous NO by showing that: (1) inhibition of PDE3 or PKA (but not PKG) and suppression of SR Ca^{2+} release markedly attenuate the positive chronotropic response to NO donors; (2) SNP, at a concentration which markedly enhances I_f without affecting basal $I_{Ca\text{-}L}$,[1] significantly increases the diastolic and peak Ca_i^{2+} fluorescence signal in SAN cells. Taken together with our previous work,[1] the present findings indicate that the NO-cGMP pathway leads to the I_f-mediated positive chronotropic effect by stimulating cAMP-dependent signaling (through the inhibition of PDE3) and SR Ca^{2+} release (Fig. 5).

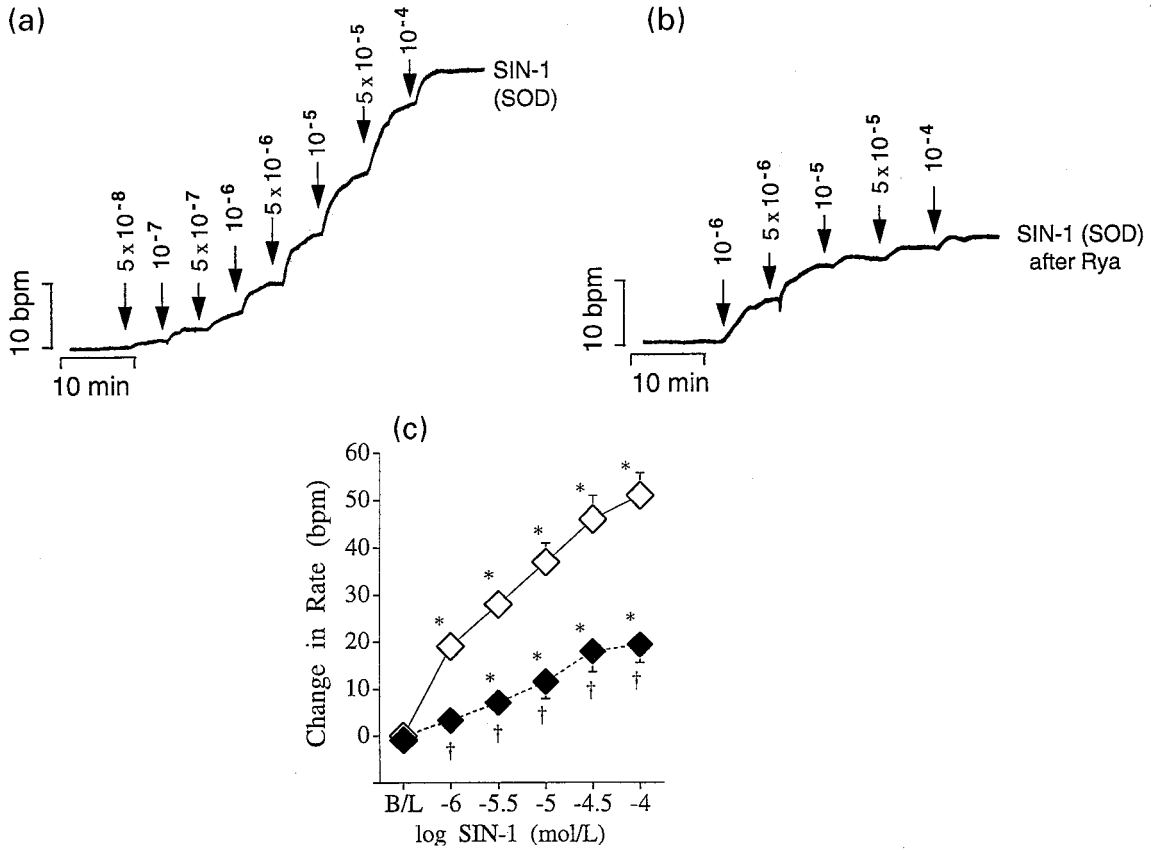

Figure 2 Chronotropic response of isolated atrial preparations to SIN-1(SOD) alone or after inhibition of SR Ca^{2+} release with Rya. (a) Raw data trace showing the effect of increasing concentrations of SIN-1 (mol/l) in the presence of 100 U/ml SOD on the atria spontaneous beating rate. Arrows indicate application of SIN-1. (b) Chronotropic response to SIN-1 (SOD) after pretreatment of spontaneously beating atria with 2 μmol/l Rya. (c) Mean change in HR ($\pm$S.E.M.) in response to the NO donor in six experiments with SIN-1(SOD) alone ($\diamond$, basal HR 169 ± 8 bpm), and in seven experiments with SIN-1(SOD) after pretreatment with Rya ($\blacklozenge$, basal HR 168 ± 8 bpm reduced by Rya to 125 ± 8 bpm, $P<0.05$, see text). * $P<0.05$ *v* baseline HR; † $P<0.05$ *v* the effect of SIN-1(SOD) alone.

Role of PDE3 and protein kinases in the positive chronotropic effect of NO donors

The increase in HR with NO donors is GC-dependent and can be mimicked by high concentrations of 8-Br-cGMP, consistent with an essential role of cGMP in the NO-mediated stimulation of I_f in SAN cells (Fig. 5).[1,2,26] In many tissues, cGMP action is mediated by activation of PKG or regulation of phosphodiesterase (PDE) activity.[9] Our findings indicate that cGMP-PKG signaling is not coupled to the positive chronotropic response to NO [Fig. 1(a)], consistent with the lack of effect of PKG inhibition on the increase in I_f with SNP or 8-Br-cGMP in SAN cells.[26] In contrast, we show that inhibition of PDE3 significantly attenuates the increase in HR with NO donors (Fig. 1). Degradation of intracellular cAMP in the myocardium is regulated by cGMP-binding PDEs, and both isoforms (i.e. cGMP-inhibited or PDE3 and cGMP-stimulated or PDE2) are present in the SAN.[27,28] Han *et al.*,[28] by using RT-PCR, suggested that PDE2 may be more abundant in rabbit SAN cells than PDE3;[28] however, cardiac PDE3 is known to be several-fold more sensitive to cGMP than PDE2, and PDE2 reduces cAMP levels only when this nucleotide is present in high concentrations.[9,29] Indeed, recent work by Yoo *et al.*[26] suggests that when I_f is prestimulated by a saturating concentration of isoprenaline, NO donors might inhibit the current via a cGMP stimulation of PDE2. Consistent with an important role of cGMP-PDE3 signaling in the NO stimulation of *basal* I_f,[1,2] we found that inhibition of PDE3 significantly reduces the positive chronotropic effect of exogenous NO [Fig. 1(a) and (b)].

Pharmacological PDE3 blockers increase the beating rate of guinea pig atria in a concentration-dependent fashion[30] and complete PDE3 blockade elevates the "basal" HR to *c.* 330 bpm, thereby exhausting the chronotropic reserve of this pre-

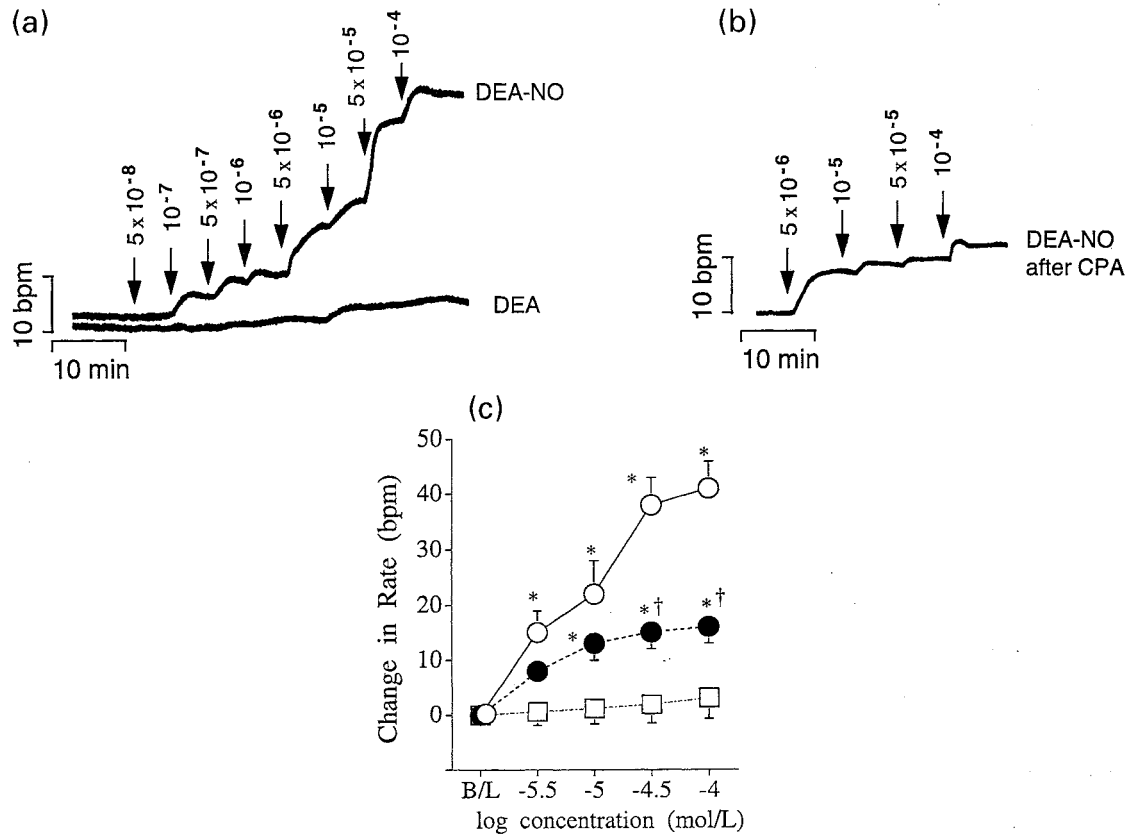

Figure 3 Chronotropic response to DEA-NO in isolated atrial preparations: Effect of supression of SR Ca^{2+} release by pretreatment with CPA. (a) Raw data traces showing the rate response to increasing concentrations of DEA-NO (mol/l, top) compared with the effect of similar concentrations of the carrier molecule DEA (bottom). Arrows indicate application of DEA-NO or DEA. (b) Rate response to DEA-NO after pre-incubation with 60 μmol/l CPA. (c) Mean change in beating rate ($\pm$S.E.M.) in response to DEA-NO for eight experiments with DEA-NO alone (○, basal HR 164 ± 8 bpm), with the carrier molecule DEA ($n=10$, □, basal HR 170 ± 6 bpm), and with DEA-NO after pretreatment with CPA ($n=9$, ●, basal HR 164 ± 6 bpm reduced by CPA to 133 ± 6 bpm, $P<0.05$, see text). * $P<0.05$ *v* baseline HR; † $P<0.05$ *v* the effect of DEA-NO on its own.

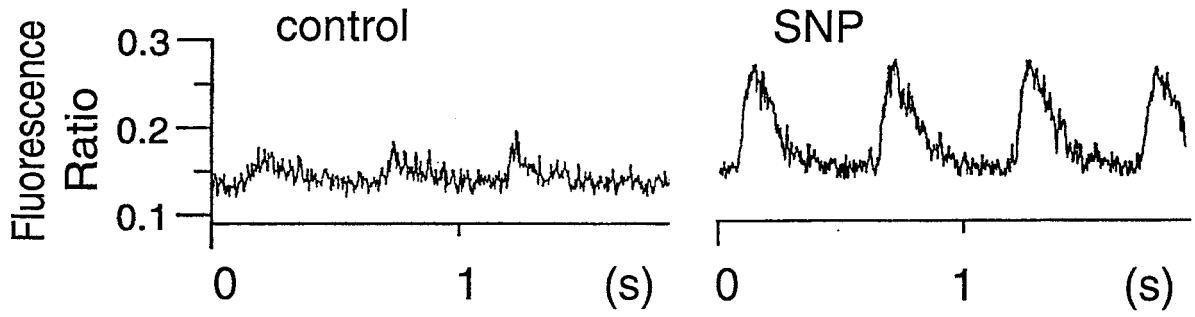

Figure 4 Effect of 5 μmol/l SNP on whole cell Ca^{2+} fluorescence signal (Indo-1, 410/495 nm ratio) in a spontaneously beating isolated guinea pig SAN cell. Note that SNP elicits an increase in diastolic and peak Indo-1 fluorescence, consistent with an increase in cytosolic Ca^{2+} and an increase in the cell beating rate.

paration.[30] To circumvent this problem we have used PDE3 blockers at concentrations <30% of their K_i.[31] Partial blockade of this phosphodiesterase[31] was consistently associated with a modest increase in basal HR (by approximately 10–15 bpm) and a significant reduction in the chronotropic response to NO donors (Fig. 1). Since our previous experiments show that the magnitude of the NO-mediated posi-

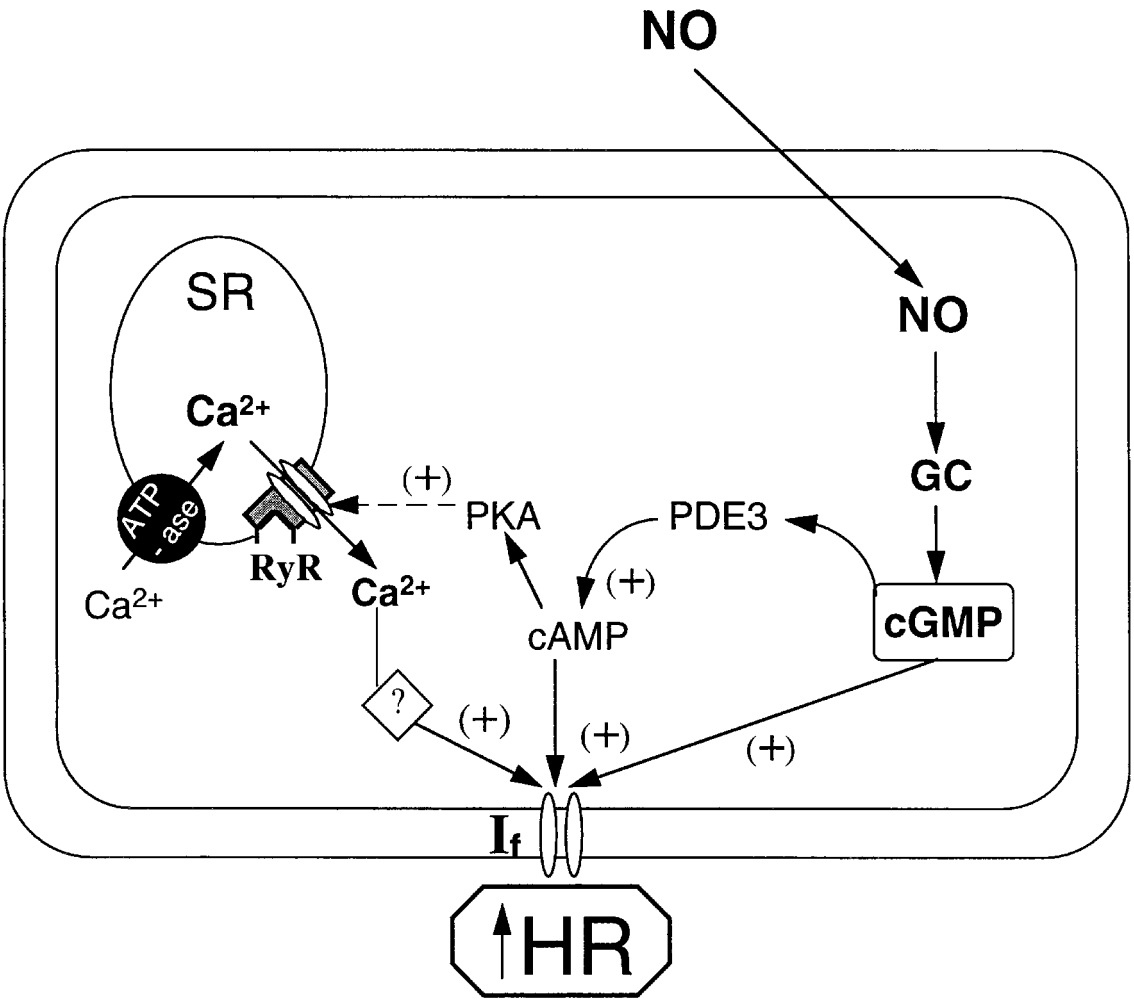

Figure 5 Schematic diagram to illustrate how several mechanisms downstream of cGMP may contribute to the increase in basal HR with exogenous NO (see text for abbreviations). Our previous work shows that a NO-GC-dependent stimulation of the "pacemaker" current I_f in SAN cells[1,2] provides the electrophysiological basis for a "direct" positive chronotropic action of micromolar concentrations of NO donors *in vitro* and *in vivo*.[1–5] The I_f current is known to be both voltage-gated and stimulated by cyclic nucleotides which bind directly to f-channels (cAMP being several-fold more potent than cGMP).[6,7] I_f is also modulated by protein kinase activity[10,11] but the mechanism does not involve direct phosphorylation of f-channels.[6–11] Furthermore, an increase in cytosolic Ca^{2+} is known to enhance I_f[12] via a yet-unidentified Ca^{2+}-dependent molecule.[12,35] Both the NO-dependent stimulation of I_f and the increase in HR with NO donors are mimicked by a cGMP analog, 8-Br-cGMP.[1,2,26] Our data indicate that cGMP binding to the cGMP-inhibited phoshodiesterase of cAMP (PDE3, expressed in both cytosolic and SR-associated isoform[40]) plays an important part in the positive chronotropic effect of NO donors (see Fig. 1). The increase in cAMP resulting from PDE3 inhibition[8,9] may stimulate I_f by directly increasing the channel open probability and by facilitating the release of SR Ca^{2+} via PKA-dependent modulation of RyRs.[20,39,41–43] Indeed, we show that PKA inhibition (Fig. 1) or suppression of SR Ca^{2+} release (Figs 2 and 3) markedly reduce the NO-mediated positive chronotropic effect whereas a low-micromolar concentration of SNP (known to be ineffective on basal $I_{Ca\text{-}L}$ in SAN cells[1,34]) increases Ca_i^{2+} fluorescence signal in SAN cells (Fig. 4) indicating an elevation in cytosolic Ca^{2+}.[25] Thus, the contribution of cGMP/cAMP, PKA and SR Ca^{2+} to mediating the I_f-dependent[1,2] increase in HR with NO is consistent with the established mechanisms of I_f regulation.[6,7,11,12]

tive chronotropic effect is unaffected by shifts in basal HR in the range of 120–200 bpm,[1,2] these findings are consistent with a significant contribution of PDE3 signaling to the increase in HR with NO.

SR Ca^{2+} release and the increase in HR with NO donors

Recent evidence shows that SR Ca^{2+} stores are present in SAN cells[18] and that modulation of SR Ca^{2+} release affects basal SAN activity (for example

see Refs 32, 33). In the present study, we demonstrate that suppression of SR Ca^{2+} release with Rya or CPA significantly reduces the positive chronotropic response to NO donors, providing functional evidence for a significant role of SR Ca^{2+} in mediating the NO-I_f-dependent[1] increase in HR. Our findings in isolated SAN cells show that SNP (5 μmol/l) increases both diastolic and peak Ca_i^{2+} fluorescence (Fig. 4), in keeping with an increase in cytosolic Ca^{2+}.[25] Since micromolar concentrations of SNP or SIN-1 have no effect on basal $I_{Ca\text{-}L}$ in SAN cells,[1,2,34] the present findings are consistent with a NO-mediated stimulation of SR Ca^{2+} release and suggest that this mechanism contributes to the increase in HR with NO (Fig. 5). A link between Ca_i^{2+} and the positive chronotropic effect of NO donors is not unexpected, since an elevation in Ca_i^{2+} is known to increase I_f[12] (through a mechanism which is most probably indirect[35]) whereas buffering Ca_i^{2+} with BAPTA reduces the current amplitude.[36]

In isolated cardiac SR vesicles NO donors can trigger Ca^{2+} release through a cGMP-independent nitrosylation of RyRs;[14,15] however, this mechanism is unlikely to play a major part in our findings since: (a) the positive chronotropic effect of NO donors is abolished by GC inhibition with ODQ or LY83583,[1,2] and (b) "direct" Ca^{2+} release from isolated cardiac microsomes requires *high* micromolar concentrations of NO donors (e.g. 100–300 μmol/l SIN-1, SNAP or CysNO[14,15]), whereas we observed a significant increase in Ca_i^{2+} transient with *low* micromolar concentrations of NO donors in SAN cells (Fig. 4). Likewise, it is unlikely that the increase in Ca^{2+} fluorescence with SNP would be a consequence of the increase in the beating rate of SAN cells (Fig. 4). A rate-dependent increase in Ca_i^{2+} transient is typically seen in ventricular myocytes but has not been demonstrated in atrial cardiocytes (where an increase in stimulation frequency produces a fall in Ca_i^{2+} transient[37]). Indeed, isolated SAN cells show an *inverse* relationship between the beating rate and Ca_i^{2+} (E.E. Verheijck, 23rd Meeting of the European Working Group on Cardiac Cellular Electrophysiology, Oxford, UK, 1999, unpublished data).

Evidence in cardiac tissue[16,17,38] and in other systems[13] indicates that several intracellular pathways may contribute to the NO-cGMP-dependent stimulation of SR Ca^{2+} release. For instance, NO could enhance SR Ca^{2+} release via a PKA-mediated stimulation of ryanodine receptors.[20,39] Indeed, we show that PDE3 (whose membrane-bound isoform is expressed on cardiac SR[40]) and PKA are functionally important in the NO signaling leading to an increase in HR (Fig. 1). Another (not exclusive[41]) mechanism might involve a NO-dependent sensitization of the cardiac RyR by endogenous cyclic adenosine diphosphate ribose (cADPR).[13,42,43]

Limitations

Although most of the cardiac biological effects of endogenously produced NO can be mimicked by NO donors, the opposite may not always be true as the metabolites or decomposition products of these agents may have biological effects in their own right.[19] To circumvent these problems[19] we have systematically used compounds from different classes of NO donors and found that, when applied in micromolar concentrations, they consistently produce an I_f-mediated positive chronotropic effect.[1,2] Although it would be difficult to ascertain whether endogenously produced NO stimulates I_f in physiological conditions (since isolated atria or SAN cells are not exposed[44] to the micromolar concentrations of NO which have been measured within the myocardium of the beating heart *in situ*[45]), there is some evidence that endogenous NO may exert a tonic positive chronotropic action. For instance, inhibiting NO synthase has been associated with a modest but significant reduction in HR in isolated cardiac preparations[46,47] and with a significant prolongation of the sinus node recovery time in humans.[48] Furthermore, conscious eNOS-deficient mice, which lack the major source of NO synthesis in the heart, are consistently found to have lower HR than the wild type controls.[47,49] As arterial baroreflex is known to re-set in chronic hypertension,[50] the bradycardia in eNOS knock out mice is in keeping with a tonic positive chronotropic effect of endogenous NO.

In conclusion, the present findings provide the first functional evidence for a role of PDE3-PKA signaling and SR Ca^{2+} in mediating the positive chronotropic effect of NO donors. Further studies will be required to evaluate the relative contribution of direct cGMP/cAMP f-channel gating[6,7] and PKA-Ca_i^{2+}[10–12] (Fig. 5) to the NO-mediated increase in basal I_f.[1,2,26]

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