G protein-mediated inhibitory effect of a nitric oxide donor on the L-type Ca^{2+} current in rat ventricular myocytes

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1. The role of the cGMP pathway in the modulation of the cardiac L-type Ca^{2+} current ($I_{Ca,L}$) by nitric oxide (NO) was examined in rat ventricular myocytes.

2. The NO donors DEANO, SIN-1, SNP, SNAP and GSNO had no significant effects on basal $I_{Ca,L}$. However, DEANO (100 μM) inhibited $I_{Ca,L}$ after the current had been previously stimulated by either isoprenaline (Iso, 1–10 nM), a β-adrenergic agonist, or isobutylmethylxanthine (IBMX, 10–80 μM), a wide spectrum phosphodiesterase (PDE) inhibitor.

3. The anti-adrenergic effect of DEANO on $I_{Ca,L}$ was not mimicked by other NO donors (SIN-1, SNAP and SPNO).

4. The NO-sensitive guanylyl cyclase inhibitor ODQ (10 μM), antagonized the inhibitory effect of DEANO on $I_{Ca,L}$. Likewise, inhibitors of the cGMP-dependent protein kinase (cG-PK), Rp-8-chloro-phenylthio-cGMP (10 μM) and KT5823 (0·1 and 0·3 μM), also abolished the inhibitory effect of DEANO on Iso (1–10 nM)-stimulated $I_{Ca,L}$.

5. Intracellular dialysis with exogenous cAMP (10–100 μM) blunted the inhibitory effect of DEANO (10 and 100 μM) on $I_{Ca,L}$. SNAP and SNP also had no effect on the cAMP-stimulated $I_{Ca,L}$.

6. Pre-treatment of the myocytes with pertussis toxin (0·5 μg ml^{-1}, 4–6 h at 37 °C) eliminated the inhibitory effect of DEANO (100 μM) on $I_{Ca,L}$, in the presence of either Iso (0·01 and 1 nM) or IBMX (10–80 μM).

7. These results demonstrate that DEANO produces anti-adrenergic effects in rat ventricular myocytes. This effect of DEANO occurs in a cGMP-dependent manner, and involves activation of cG-PK and regulation of a pertussis toxin-sensitive G protein.

The synthesis of nitric oxide (NO) plays an important role in the endothelium-dependent relaxation of various blood vessels. In the heart, the role of NO synthesis is rather controversial (reviewed in Méry *et al.* 1997; Feron *et al.* 1999; Kojda & Kottenberg, 1999). NO was soon recognized as an activator of cGMP synthesis in the whole heart, as well as in purified cardiac myocytes. In turn, cGMP coordinates the activity of various proteins, such as the cGMP-stimulated phosphodiesterase (PDE2), the cGMP-inhibited-PDE (PDE3) and the cGMP-dependent protein kinase (cG-PK). Accordingly, several NO donors can modulate myocyte shortening, ionic currents and contractile proteins, in a cGMP-dependent manner. More recently, nitrosothiols (SNAP, SNAC, GSNO) or the sydnonymine SIN-1 were found to elicit cGMP-independent effects in cardiac myocytes, including the modulation of calcium channels (Hu *et al.* 1997; Xu *et al.* 1998), the inhibition of the creatine kinase (Gross *et al.* 1996), and the modulation of mitochondrial respiration (Wolin *et al.* 1997).

While these results tend to support a physiological role of NO in heart muscle, a number of questions remain unanswered. First, in several studies performed on isolated cardiac myocytes, NO donors had no effect on cell shortening (Stein *et al.* 1993; McDonell *et al.* 1995, 1997), L-type Ca^{2+} current ($I_{Ca,L}$) (Thomas *et al.* 1997), or the cAMP-activated chloride current (Zakharov *et al.* 1996). Thus, subtle differences between cardiac preparations can blunt the effects of NO at the single cell level. Second, the exact mechanism by which NO or cGMP produces its effect in a given cardiac preparation is not clear. For instance, the inhibitory effect of NO or cGMP on $I_{Ca,L}$ in mammalian cardiac myocytes may take place either via activation of PDE2 (Feron *et al.* 1999) or activation of cG-PK (Méry *et al.* 1991; Sumii & Sperelakis, 1995; Whaler & Dollinger, 1995). Third, while the effect of cG-PK on $I_{Ca,L}$ appears to take place at the level of the L-type Ca^{2+} channel (or a closely associated protein Méry *et al.* 1991; Sumii & Sperelakis, 1995), the possibility exists that cG-PK might also act

upstream from Ca^{2+} channel phosphorylation. Indeed, in smooth muscle cells, cG-PK was shown to directly phosphorylate α_i subunits of GTP-binding proteins (G proteins) and/or receptors (Pfeiffer *et al.* 1995; G.-R. Wang *et al.* 1998). In addition, cG-PK can increase the spontaneous binding of GTP on α subunits and reduce the stimulatory effects of receptor agonists on their GTPase activity (Pfeiffer *et al.* 1995; Miyamoto *et al.* 1997; G.-R. Wang *et al.* 1998).

In the present study, we examined the effects of different NO donors on basal and stimulated $I_{Ca,L}$ in ventricular myocytes isolated from rat hearts. In this preparation, we had found previously that cG-PK mediates the inhibitory effect of exogenous cGMP on $I_{Ca,L}$ (Méry *et al.* 1991). While none of the NO donors tested produced any effect on basal $I_{Ca,L}$, the NONOate DEANO produced a profound inhibition of the current stimulated by isoprenaline. We show that cG-PK mediates this effect through the activation of a pertussis toxin-sensitive G protein.

Part of this work has been presented in an abstract form (Abi-Gerges *et al.* 1997*c*).

METHODS

The investigation conforms with our institution guidelines which are defined by the European Community guiding principles in the care and use of animals (86/609/CEE, *CE Off J* no. L358, 18 December, 1986) and the French decree no. 87/848 (*J Off République Française*, 20 October, 1987, pp. 12245–12248). Authorizations to perform animal experiments according to this decree were obtained from the French Ministère de l'Agriculture et de la Forêt (no. 04226, 12 April, 1991).

Cell isolation and storage

Male Wistar rats (160–250 g) were anaesthetized by intraperitoneal injection of urethane (2 g kg^{-1}) and heparin (2·5 mg kg^{-1}). After all reflex activity had ceased, the animal was killed by opening the chest and rapidly removing the heart. Myocytes were dispersed using collagenase A (0·255 mg ml^{-1}; Boehringer-Mannheim Biochemica, Mannheim, Germany) as previously described (Scamp *et al.* 1990; Abi-Gerges *et al.* 1997*a*).

Electrophysiology

The whole-cell configuration of the patch-clamp technique was used to record the L-type Ca^{2+} current ($I_{Ca,L}$) on Ca^{2+}-tolerant cells, as described (Scamps *et al.* 1990; Abi-Gerges *et al.* 1997*a*, 1999). The routine protocol consisted of a depolarizing pulse to 0 mV test potential (400 ms duration) elicited every 8 s from a holding potential of −50 mV. The test potential was set at 0 mV because, at this potential, the $I_{Ca,L}$ amplitude (Scamps *et al.* 1990; Méry *et al.* 1991; Abi-Gerges *et al.* 1999; see Fig. 2) and steady-state activation are at their maximal values in rat myocytes (Scamps *et al.* 1990). Current–voltage relationships and inactivation curves were performed as described (Abi-Gerges *et al.* 1999). The experiments were carried out at room temperature (22–32 °C, mean value 25·6 ± 0·1 °C, $n = 346$), and the temperature did not change by > 2 °C in any given experiment.

During patch-clamp experiments, the maximal amplitude of $I_{Ca,L}$ and the current at the end of the 400 ms test potential were measured as described (Abi-Gerges *et al.* 1997*a*). Currents were not compensated for capacitive and leak currents. The density of basal $I_{Ca,L}$ was 5·25 ± 0·14 pA pF^{-1} and the density of the current at the end of the 400 ms pulse was 0·37 ± 0·02 pA pF^{-1} ($n = 266$). The steady-state value of the end-pulse current was stable over the time course of the experiments (see individual current traces in figures). The effects of the agonists used in this study were not correlated with the amplitude of the end-pulse current (data not shown). The decay of the capacitive transient was fast (< 3 ms), and did not interfere significantly with the activation of the calcium current (mean time to peak 6·2 ± 0·1 ms, $n = 266$). On-line analysis of the recordings was made possible by programming a PC-compatible 486/50 microcomputer in Assembly language (Borland) to determine, for each membrane depolarization, peak and steady-state current values.

Solutions for patch-clamp recordings

The extracellular solution contained (mM): 107 NaCl, 10 Hepes, 20 CsCl, 4 $NaHCO_3$, 0·8 NaH_2PO_4, 1·8 $MgCl_2$, 1·8 $CaCl_2$, 5 D-glucose, 5 sodium pyruvate, and 6×10^{-4} tetrodotoxin, pH 7·4 adjusted with CsOH (294 mosmol kg^{-1}). Solutions were superfused onto floating myocytes as described (Abi-Gerges *et al.* 1997*a*). The patch pipettes (0·5–1·0 MΩ) were filled with an intracellular solution containing (mM): 119·8 CsCl, 5 EGTA (acid form), 4 $MgCl_2$, 5 sodium phosphocreatine, 3·1 Na_2ATP, 0·42 Na_2GTP, 0·062 $CaCl_2$ (pCa 8·5) and 10 Hepes, pH 7·3 adjusted with CsOH (292 mosmol kg^{-1}).

Drugs

3-Morpholino-sydnonimine (SIN-1) was a generous gift from Dr J. Winicki (Hoechst-Houdé Laboratories, Paris-La Défense). Spermine-NONOate (SPNO) and *S*-nitroso-L-glutathione (GSNO) were from Alexis Corp. (La Jolla, CA, USA); *S*-nitroso-*N*-acetyl-D,L-penicillamine (SNAP), 2-(*N*,*N*-diethylamino)-diazenolate-2-oxide (DEANO), 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxaline-1-one (ODQ) from Tocris-Cookson (Bristol, UK) or Alexis Corp.; 8-(4-chlorophenylthio)guanosine-3′-5′-cyclic monophosphorothioate, Rp-isomer (Rp8cG) were from Biolog L.S.I. (Bremen, Germany); 8-(4-chlorophenylthio)-guanosine-3′,5′-cyclic monophosphate (8-p-CPT-cGMP) and KT5823 were from Calbiochem-France Biochem (Meudon, France); pertussis toxin (from *Bordetella pertussis*) was from Sigma-Aldrich (Saint Quentin Fallavier, France); tetrodotoxin was from Latoxan (Rosans, France). All other drugs were from Sigma-Aldrich. Drugs were prepared and used according to manufacturer's instructions. Mock DEANO consisted of a DEANO (100 μM)-containing solution left at room temperature for > 20 h. Solutions were prepared by dilution to the desired concentration in the physiological solution at the beginning of each experiment.

Statistical analysis

Results are expressed as means ± S.E.M. Differences between mean values were tested for statistical significance by Student's paired *t* test, as indicated. In the text, the 'basal' condition for $I_{Ca,L}$ refers to the absence of extracellular isoprenaline or IBMX, or intracellular cAMP.

RESULTS

Effect of NO donors on basal $I_{Ca,L}$

NO donors can modulate basal $I_{Ca,L}$ in some, but not all cardiac myocytes (Méry *et al.* 1993; Kirstein *et al.* 1995; Campbell *et al.* 1996; Y. G. Wang *et al.* 1998). In the experiment of Fig. 1*A*, a rat myocyte was first exposed to control intracellular and extracellular solutions. Under this condition, the amplitude of the basal $I_{Ca,L}$ declined slowly

(~ −5·4 pA min^{-1}), a phenomenon known as 'run-down'. Superfusion of the myocyte with DEANO (100 μM) barely modified the amplitude and the kinetics of the basal $I_{Ca,L}$. As summarized in Fig. 1*B*, the basal $I_{Ca,L}$ amplitude was not significantly modified by different NO donors. These included the NONOate DEANO (100 μM), the nitrosothiols GSNO (1 mM) and SNAP (1 mM), the ferrocyanate SNP (500 μM), and the sydnonymine SIN-1 (1 mM). Lower concentrations of these compounds (down to 1 μM) also had no effect on basal $I_{Ca,L}$ (data not shown).

Since nitrosylation and/or oxidation might account for effects of NO donors on the basal $I_{Ca,L}$ in other preparations (Campbell *et al.* 1996; Hu *et al.* 1997) we further investigated the sensitivity of basal $I_{Ca,L}$ in rat myocytes to the effects of reducing or oxidizing agents. The basal $I_{Ca,L}$ was not changed when the myocyte was superfused with 1 mM reduced glutathione (5·9 ± 2·6 % over basal, $n = 5$), 1 mM DL-dithiothreitol (DL-DTT) (2·3 ± 1·6 % over basal, $n = 6$), 0·1 mM *N*-acetyl-penicillamine (−1·6 ± 0·9 % over basal, $n = 4$). In contrast, extracellular application of a superoxide anion generator LY83583 (10 μM) inhibited the basal $I_{Ca,L}$ (−36·9 ± 2·5 % over basal, $n = 17$, $P < 0·001$). Therefore, the basal activity of L-type Ca^{2+} channels in rat myocytes was sensitive to an oxidative treatment but not to NO donors.

Inhibitory effect of DEANO on the β-adrenergic stimulation of $I_{Ca,L}$

NO donors can reduce the β-adrenergic stimulation of $I_{Ca,L}$ in frog and guinea-pig ventricular myocytes (Méry *et al.*

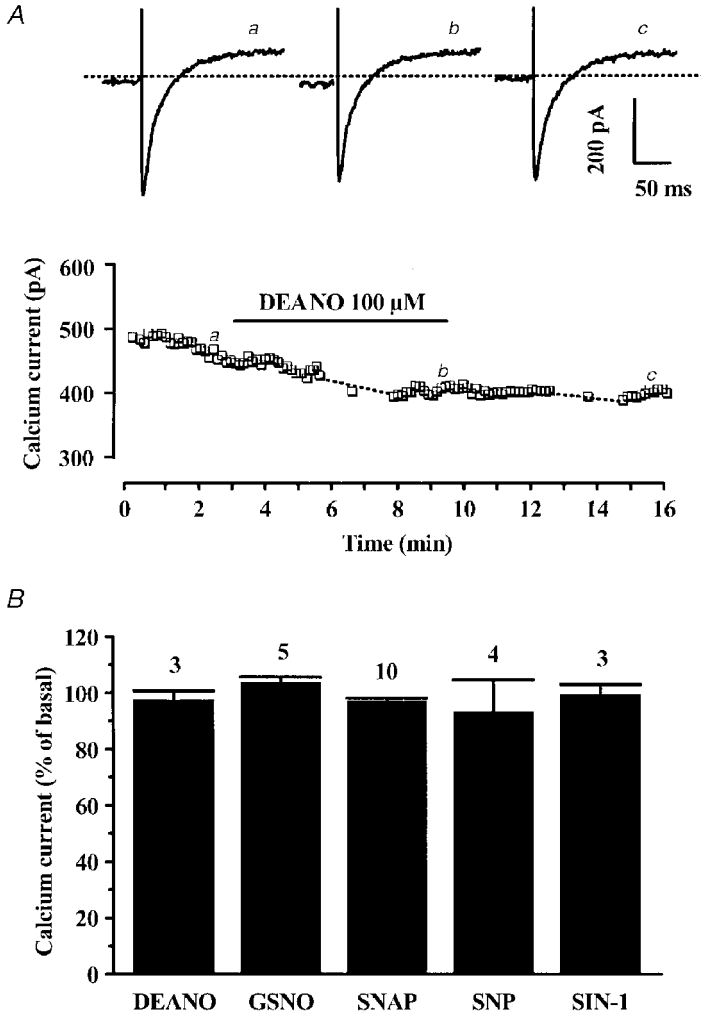

Figure 1. NO donors do not regulate basal $I_{Ca,L}$ in rat ventricular myocytes

A, a myocyte was first exposed to control intracellular and extracellular control solutions. $I_{Ca,L}$ (□) was elicited at 0 mV from a holding potential of −50 mV. Superfusion of the myocyte with 100 μM DEANO is indicated by the horizontal line. Current traces on top were recorded at the times indicated by the corresponding letters on the main graph. The dotted line indicates the zero-current level. *B*, summary of the effects of DEANO (100 μM), GSNO (1 mM), SNAP (1 mM), SNP (500 μM) and SIN-1 (1 mM) on basal $I_{Ca,L}$ amplitude. The amplitude of $I_{Ca,L}$ in the presence of NO donors was normalized to the amplitude of basal $I_{Ca,L}$ in control conditions (set to 100 %). Bars are the means and lines are the S.E.M. of the number of experiments indicated near the bars.

1993; Levi *et al.* 1994; Whaler & Dollinger, 1995; reviewed in Méry *et al.* 1997). We first investigated the effects of DEANO in the presence of isoprenaline (Iso), a non-selective β-adrenergic agonist. In the experiment of Fig. 2*A*, a myocyte was exposed to control solutions and the $I_{Ca,L}$ amplitude reached a steady state 11·8 min after the beginning of the recording of $I_{Ca,L}$. The myocyte was then superfused with 10 nM Iso, which induced a ~2-fold increase in $I_{Ca,L}$. In the continuing presence of Iso, superfusion with 100 μM DEANO elicited a pronounced reduction in the response of $I_{Ca,L}$ to Iso. The inhibitory effect of DEANO was reversible. Figure 2*B* shows the current–voltage relationships of $I_{Ca,L}$ (filled symbols) and of the steady-state current measured at the end (400 ms) of the test pulse (open symbols) in the same experiment as in Fig. 2*A*. The effects of Iso and DEANO on $I_{Ca,L}$ were homogeneous across the voltage range and neither drug produced a change in the steady-state current (similar findings were obtained in 10 other experiments). In addition, DEANO did not change the inactivation curve of $I_{Ca,L}$ (data not shown). Thus, DEANO produced an anti-adrenergic effect on $I_{Ca,L}$ which occurred in a voltage-independent manner.

The inhibitory effect of DEANO (100 μM) was studied at three different Iso concentrations (1, 3 and 10 nM). As summarized in Fig. 3, increasing the Iso concentration progressively reduced the inhibitory effect of DEANO on $I_{Ca,L}$ suggesting that the inhibitory effect of DEANO was somewhat competitive with the stimulatory effect of Iso.

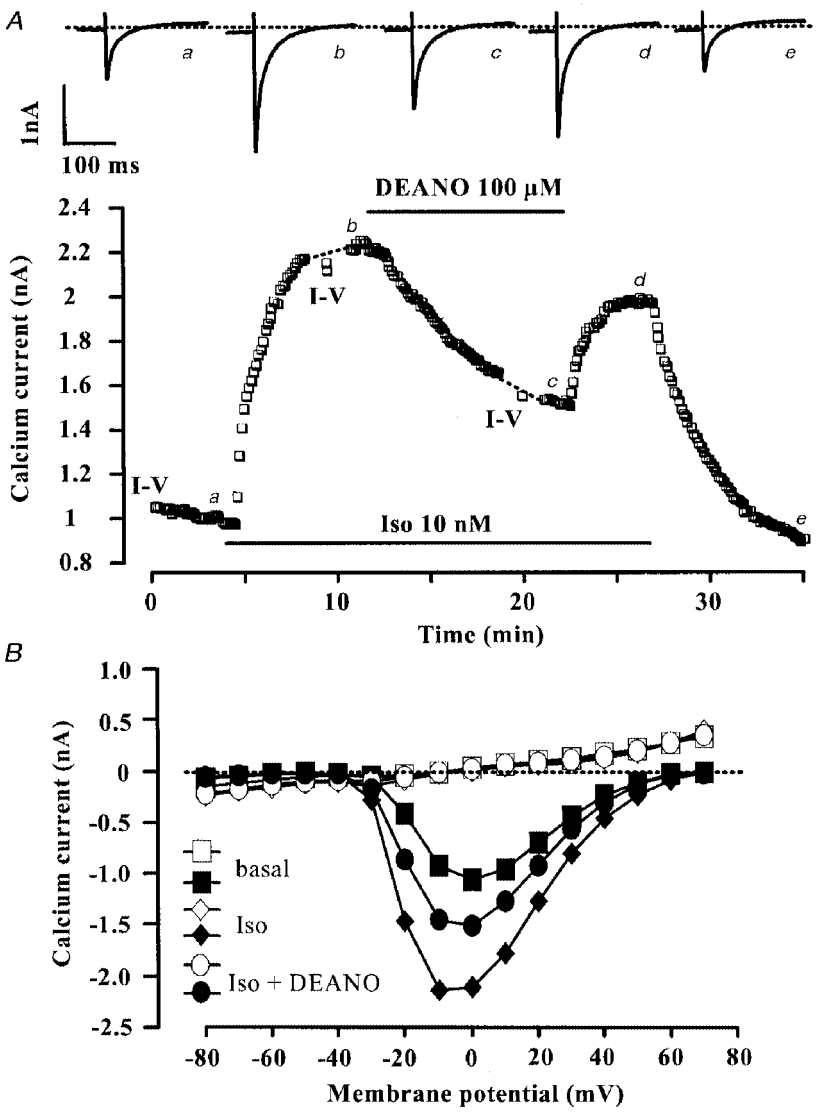

Figure 2. DEANO inhibits the β-adrenergic stimulation of $I_{Ca,L}$ in rat ventricular myocytes

A, a myocyte was first exposed to control intracellular and extracellular solutions. Applications of isoprenaline (Iso, 10 nM) and DEANO (100 μM) are indicated by the horizontal lines. Current traces on top were recorded at the times indicated by the corresponding letters on the main graph. The dotted line indicates the zero-current level. *B* (same experiment as in *A*), current–voltage relationships of $I_{Ca,L}$ (filled symbols) and of the steady-state current at the end of the pulse (open symbols) obtained under basal conditions, and in the presence of Iso (10 nM) with or without DEANO (100 μM).

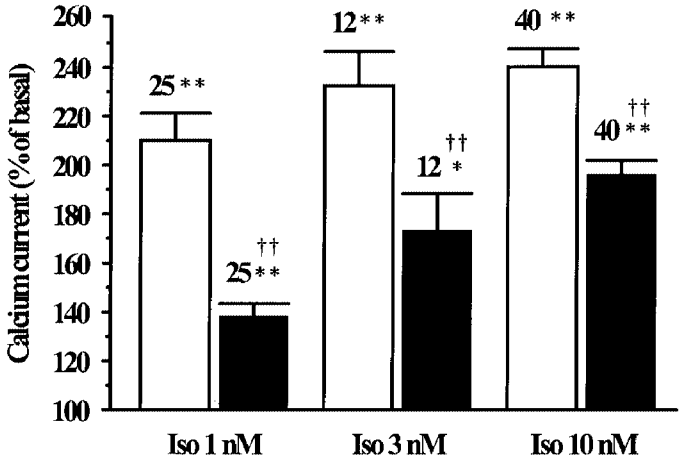

Figure 3. Summary of the effects of DEANO on the β-adrenergic stimulation of $I_{Ca,L}$ in rat ventricular myocytes

The amplitude of $I_{Ca,L}$ in the presence of Iso (1, 3 or 10 nM), without (□) or with DEANO (100 μM, ■), was normalized to the basal $I_{Ca,L}$ amplitude (set to 100%). Bars are the means and lines are the S.E.M. of the number of experiments indicated near the bars. Significant differences from basal (*) or Iso levels (†) are indicated as: * $P < 0{\cdot}001$; **, †† $P < 0{\cdot}0001$.

The involvement of NO in the anti-adrenergic effect of DEANO was investigated using mock DEANO (see Methods). The Iso (1 nM) stimulation of $I_{Ca,L}$ was identical in the absence and in the presence of 100 μM mock DEANO (176·8 ± 11·9% and 188·9 ± 10·1% of control, respectively, $n = 4$). In addition, the stimulatory effect of 1 nM Iso on $I_{Ca,L}$ was not changed in the presence of 100 μM diethylamine (179·1 ± 16·0% and 177·0 ± 20·0% of control, respectively, $n = 4$), the other metabolite of DEANO. Thus, the inhibitory effect of DEANO on Iso-stimulated $I_{Ca,L}$ was mediated by NO and not by one of its metabolites.

Effect of other NO donors on the β-adrenergic stimulation of $I_{Ca,L}$

For a comparison with the effect of DEANO, we examined the effects of other NO donors on the Iso-stimulated $I_{Ca,L}$, namely SPNO (a NONOate like DEANO), SIN-1 (a sydnonymine) and SNAP (a nitrosothiol). Figure 4*A* shows a typical experiment in which SIN-1 (100 μM) had no effect on $I_{Ca,L}$ stimulated by 10 nM Iso. As summarized in Fig. 4*B*, SIN-1 (100 μM) as well as the two other NO donors, SPNO (100 μM) and SNAP (100 μM), produced inconsistent and

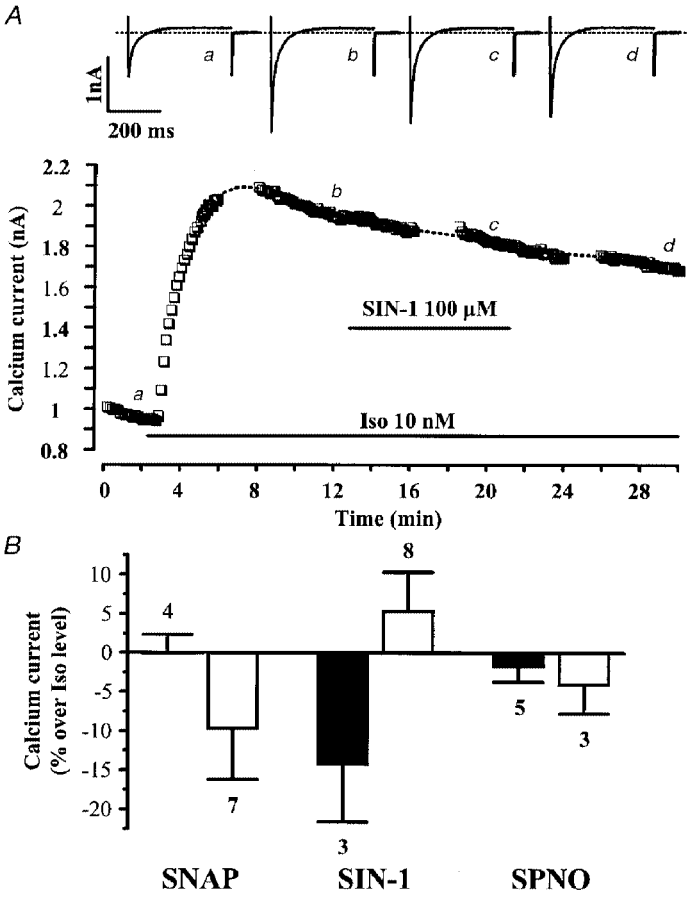

Figure 4. Other NO donors do not inhibit the β-adrenergic stimulation of $I_{Ca,L}$ in rat ventricular myocytes

A, a myocyte was first exposed to control intracellular and extracellular solutions. Applications of Iso (10 nM) and SIN-1 (100 μM) are indicated by the horizontal lines. Current traces on top were recorded at the times indicated by the corresponding letters on the main graph. The dotted line indicates the zero-current level. *B*, summary of the effects of three NO donors (SNAP, SIN-1, SPNO, at 100 μM) on $I_{Ca,L}$ in the presence of either 1 nM (■) or 10 nM Iso (□). The effects of NO donors on $I_{Ca,L}$ are presented as percentage variations from the amplitude of the Iso-stimulated $I_{Ca,L}$. Bars are the means and lines are the S.E.M. of the number of experiments indicated near the bars.

non-significant effects on $I_{Ca,L}$ stimulated with either 1 or 10 nM Iso. In addition, the shape of the current–voltage relationship of the Iso-stimulated $I_{Ca,L}$ was not modified in the presence of SIN-1, SNAP, or SPNO ($n = 8$, 5 and 2, respectively, data not shown). These negative results were not due to the wash-out of some cellular component in the whole-cell configuration of the patch-clamp technique since SNAP also failed to modify the Iso-stimulated $I_{Ca,L}$ when tested in the perforated-patch configuration (data not shown, see also Thomas *et al.* 1997). They were not due either to a difference in temperature, since the mean temperatures of the experiments were similar with DEANO (25·3 ± 0·3 °C, $n = 25$), SPNO (28·0 ± 0·5 °C, $n = 5$), SIN-1 (28·5 ± 0·5 °C, $n = 3$) and SNAP (28·3 ± 0·4 °C, $n = 4$) when tested on top of 1 nM Iso. Thus, among a total of four NO donors tested, DEANO was the only one to consistently produce an inhibitory effect on Iso-stimulated $I_{Ca,L}$ in rat ventricular myocytes (see Maragos *et al.* 1991; Feelisch *et al.* 1991).

Contribution of guanylyl cyclase to the anti-adrenergic effect of DEANO on $I_{Ca,L}$

To evaluate the contribution of cGMP to the modulation of $I_{Ca,L}$ by DEANO, we examined the effect of DEANO in the presence of ODQ, an inhibitor of the NO-sensitive guanylyl cyclase (Kojda *et al.* 1996; Abi-Gerges *et al.* 1997*b*). In the typical experiment of Fig. 5*A*, a myocyte was exposed to control solutions, and then superfused with 3 nM Iso. The

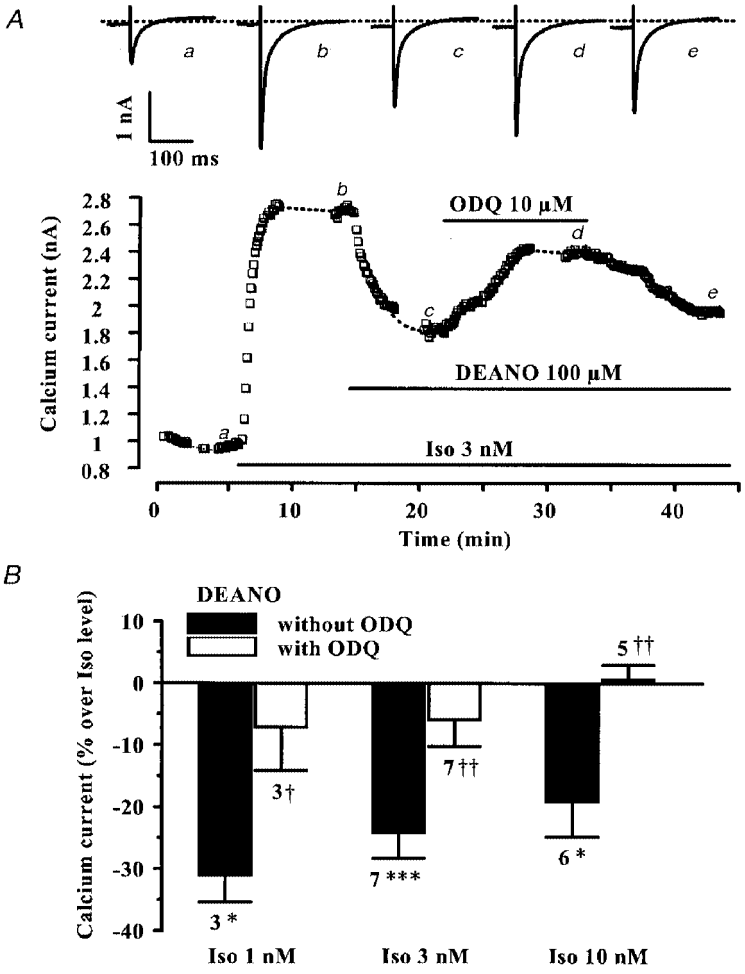

Figure 5. The guanylyl cyclase inhibitor ODQ antagonizes the effect of DEANO on the β-adrenergic stimulation of $I_{Ca,L}$ in rat ventricular myocytes

A, a myocyte was first exposed to control intracellular and extracellular solutions. Applications of Iso (3 nM), DEANO (100 μM) and ODQ (10 μM) are indicated by the horizontal lines. Current traces on top were recorded at the times indicated by the corresponding letters on the main graph. The dotted line indicates the zero-current level. *B*, summary of the effects of DEANO without (■) or with ODQ (10 μM, □), in the presence of 1, 3 or 10 nM Iso. DEANO was used at 10 μM (in the presence of Iso 10 nM) or 100 μM (in the presence of 1 and 3 nM Iso). The effects of DEANO on $I_{Ca,L}$ are presented as percentage variations from the amplitude of the Iso-stimulated $I_{Ca,L}$. Bars are the means and lines are the S.E.M. of the number of experiments indicated near the bars. Significant differences from Iso (*) or Iso + DEANO (†) levels are indicated as: *, †$P < 0{\cdot}05$; ††$P < 0{\cdot}01$; ***$P < 0{\cdot}005$.

stimulatory effect of the β-adrenergic agonist was reduced by about 50% upon addition of DEANO (100 μM). In the continuing presence of Iso plus DEANO, superfusion with ODQ (10 μM) increased $I_{Ca,L}$, eliminating most of the inhibitory effect of DEANO. The effect of ODQ was slowly reversible upon wash-out of the drug. On average (Fig. 5*B*), ODQ (10 μM) fully antagonized the inhibitory effects of DEANO on $I_{Ca,L}$, in the presence of 1, 3 or 10 nM Iso. However, ODQ (10 μM) did not modify Iso (10 nM)-stimulated $I_{Ca,L}$ in the absence of DEANO ($-2{\cdot}2 \pm 2{\cdot}1$% over Iso level, $n = 4$). Thus, these results indicate that the inhibitory effect of DEANO on Iso-stimulated $I_{Ca,L}$ in rat ventricular myocytes is mediated by activation of the NO-sensitive guanylyl cyclase.

Contribution of cG-PK to the anti-adrenergic effect of DEANO on $I_{Ca,L}$

We have shown previously that an intracellular application of cGMP in rat ventricular myocytes produces an inhibition of cAMP-stimulated $I_{Ca,L}$ that involves the activation of cG-PK (Méry *et al.* 1991). Similarly, the Iso (1 nM) stimulation of $I_{Ca,L}$ was strongly reduced in the presence of 8-p-CPT-cGMP (100 μM), a selective activator of cG-PK (from $235{\cdot}1 \pm 8{\cdot}9$% to $168{\cdot}2 \pm 6{\cdot}9$% stimulation, $n = 4$, $P < 0{\cdot}005$). Next, the role of the cG-PK in the anti-adrenergic effect of DEANO was examined using (Rp)-8-CPT-cGMP (Rp8cG), a membrane-permeant inactive analogue of cGMP which competes with cGMP for the binding on cG-PK (Butt *et al.* 1994). In the typical

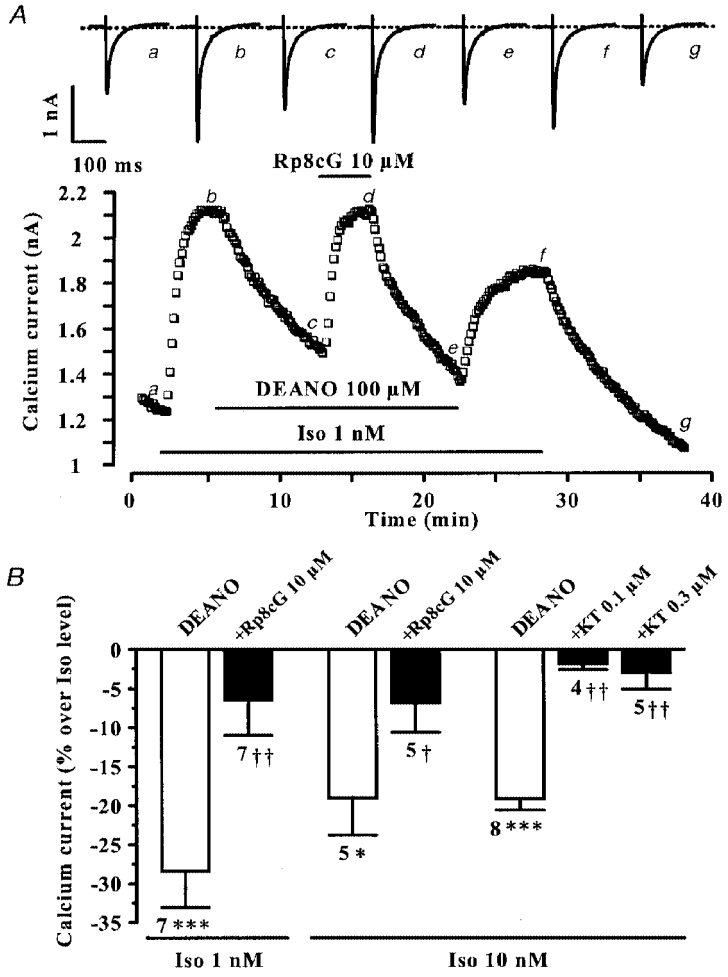

Figure 6. cG-PK inhibitors antagonize the effect of DEANO on the β-adrenergic stimulation of $I_{Ca,L}$ in rat ventricular myocytes

A, a myocyte was first exposed to control intracellular and extracellular solutions. Applications of Iso (1 nM), DEANO (100 μM) and Rp8cG (10 μM) are indicated by the horizontal lines. Current traces on top were recorded at the times indicated by the corresponding letters on the main graph. The dotted line indicates the zero-current level. *B*, summary of the effects of DEANO (100 μM) without (□) or with cG-PK inhibitors (■; Rp8cG, 10 μM; KT5823, 0·1 or 0·3 μM), in the presence of 1 or 10 nM Iso. The effects of DEANO on $I_{Ca,L}$ are presented as percentage variations from the amplitude of the Iso-stimulated $I_{Ca,L}$. Bars are the means and lines are the S.E.M. of the number of experiments indicated near the bars. Significant differences from Iso (*) or Iso + DEANO (†) levels are indicated as: *, †$P < 0{\cdot}05$; ††$P < 0{\cdot}01$; ***$P < 0{\cdot}001$.

experiment of Fig. 6*A*, the β-adrenergic stimulation of $I_{Ca,L}$ (with 1 nM Iso) was strongly inhibited by DEANO (100 μM). This anti-adrenergic effect of DEANO was fully antagonized by superfusion of the cell with 10 μM Rp8cG, in a reversible manner. Figure 6*B* summarizes the results of several similar experiments. At 1 and 10 nM Iso, Rp8cG (10 μM) fully reversed the inhibitory effect of DEANO (100 μM) on $I_{Ca,L}$. We also examined the effects of KT5823, another cG-PK inhibitor structurally unrelated to cGMP (Komalavila & Lincoln, 1996). As summarized in Fig. 6*B*, KT5823 (0·1 or 0·3 μM) also fully reversed the anti-adrenergic effect of DEANO (100 μM). In the absence of DEANO, neither Rp8cG (10 μM) nor KT5823 (0·1–0·3 μM) had any significant effect on Iso-stimulated $I_{Ca,L}$ (data not shown). Thus, these results demonstrate that cG-PK mediates the inhibitory effect of DEANO on $I_{Ca,L}$ in rat ventricular myocytes.

Effect of DEANO on cAMP-stimulated $I_{Ca,L}$ in rat ventricular myocytes

When elicited by intracellular dialysis of cGMP in rat ventricular myocytes, cG-PK-dependent inhibition of $I_{Ca,L}$ appeared to take place at the level of the Ca^{2+} channels (Méry *et al.* 1991; Sumii & Sperelakis, 1995). In other cell types, cG-PK can modulate the initial steps of signal transduction, at the level of receptors and/or G proteins (Pfeiffer *et al.* 1995; Miyamoto *et al.* 1997; G.-R. Wang *et al.* 1998). To discriminate between these possibilities, we first studied the effect of DEANO in myocytes dialysed with cAMP through the patch pipette, in order to bypass the steps involved in cAMP production. In the experiment of Fig. 7*A*, the patch pipette was filled with a control intracellular solution supplemented with 30 μM cAMP, and intracellular dialysis of the myocyte started when the membrane patch was ruptured. The diffusion of cAMP into the cytosol

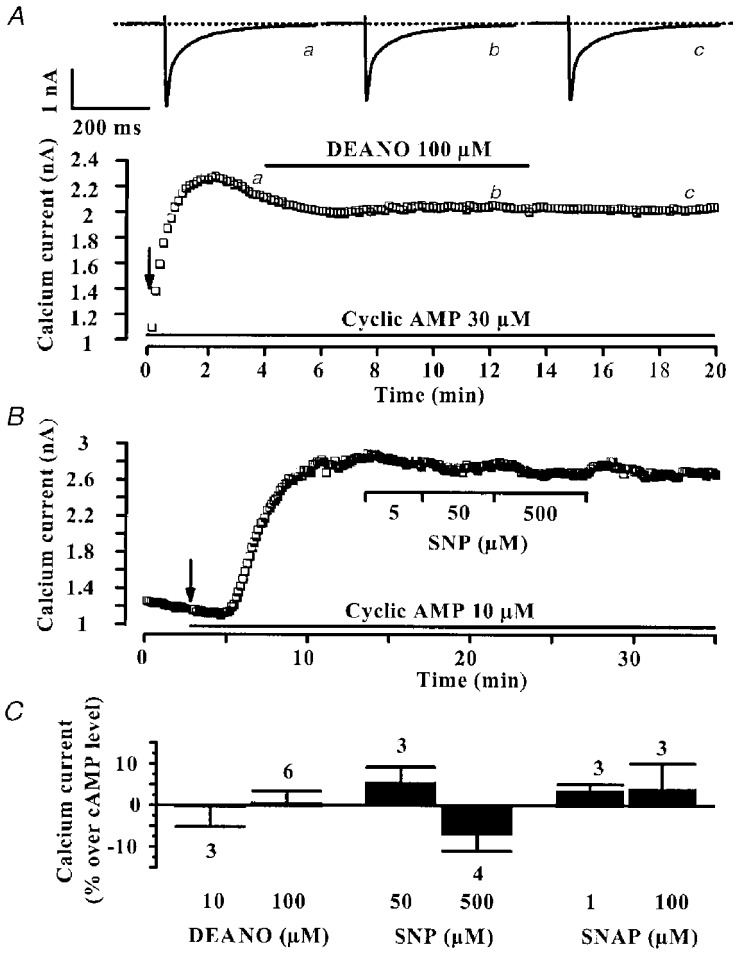

Figure 7. DEANO does not inhibit the stimulation of $I_{Ca,L}$ induced by exogenous cAMP

A, the pipette solution contained 30 μM cAMP, and cAMP dialysis started when the patch was ruptured (arrow). *B*, a myocyte was first dialysed with the control solution, and intracellular dialysis with 30 μM cAMP started at the time indicated by the arrow. Applications of DEANO (100 μM) in *A* or SNP (5, 50 and 500 μM) in *B* were performed as indicated by the horizontal lines. In *A*, current traces on top were recorded at the times indicated by the corresponding letters on the main graph. The dotted line indicates the zero-current level. *C*, summary of the effects of DEANO, SNP and SNAP on the cAMP (10–100 μM)-stimulated $I_{Ca,L}$. Variations are given as percentage change over the amplitude of the cAMP-stimulated $I_{Ca,L}$. Bars are the means and lines are the S.E.M. of the number of experiments indicated near the bars.

induced a large increase in $I_{Ca,L}$ (to 44·9 pA pF^{-1}). Surprisingly, superfusion of the myocyte with DEANO (100 μM) did not modify the cAMP-stimulated $I_{Ca,L}$. The effect of another NO donor, SNP, is shown in Fig. 7*B*. In this experiment, the myocyte was first exposed to control intracellular and extracellular solutions. Intracellular dialysis with cAMP (10 μM) started at the time indicated by the arrow, inducing a 2·5-fold increase in $I_{Ca,L}$ (to 26·7 pA pF^{-1}). Superfusion of the myocyte with increasing concentrations of SNP (5, 50 and 500 μM) had no effect on the cAMP-stimulated $I_{Ca,L}$.

The effects of DEANO, SNP and SNAP on $I_{Ca,L}$ in cAMP (10–100 μM)-dialysed myocytes are summarized in Fig. 7*C*. On average, the density of $I_{Ca,L}$ was elevated to 23·02 ± 3·57 pA pF^{-1} ($n = 12$). None of the NO donors significantly changed the cAMP-stimulated $I_{Ca,L}$. Thus, since DEANO inhibits the Iso-stimulated $I_{Ca,L}$ but not the cAMP-stimulated $I_{Ca,L}$, it must act at a step located upstream from cAMP production in the cAMP signalling cascade leading to $I_{Ca,L}$ stimulation. In the following, we tested the hypothesis that DEANO via activation of cG-PK interferes with the receptor-dependent modulation of adenylyl cyclase.

Effect of DEANO on Iso-stimulated $I_{Ca,L}$ in pertussis toxin-treated myocytes

In vitro experiments demonstrated that cG-PK could modulate the activity of G_i proteins, which are negatively coupled to adenylyl cyclase in rat cardiac myocytes (Pfeiffer *et al.* 1995; Miyamoto *et al.* 1997). Therefore, we studied the

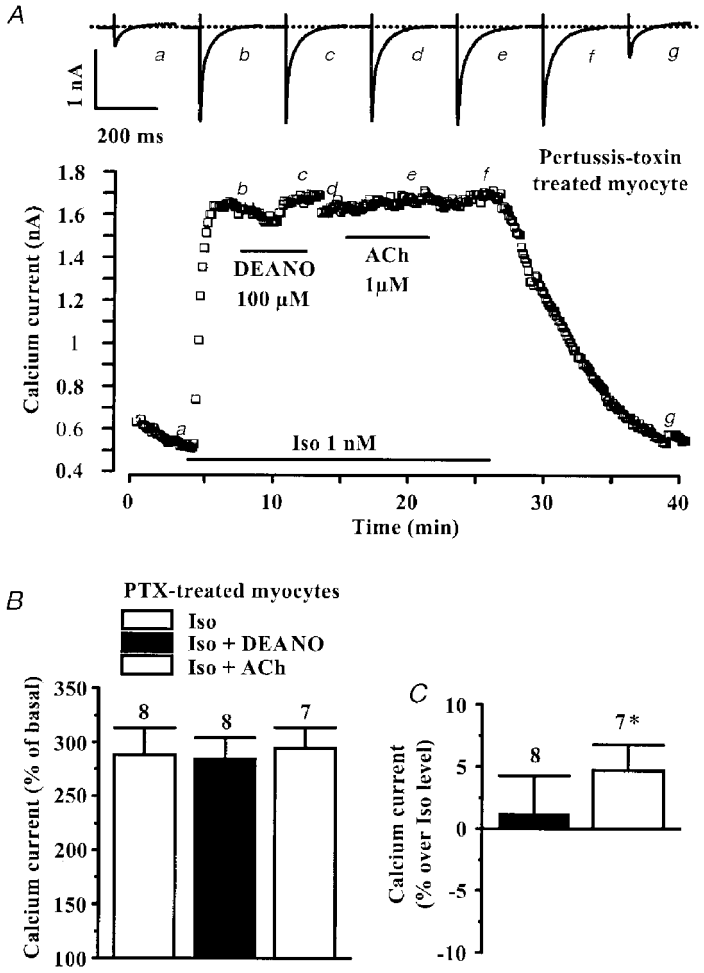

Figure 8. DEANO does not inhibit the β-adrenergic stimulation of $I_{Ca,L}$ in PTX-treated rat ventricular myocytes

A, a myocyte was incubated with pertussis toxin (PTX, 0·5 μg ml^{-1}, 4 h, 37 °C) prior to the experiment. It was first exposed to control extracellular and intracellular solutions, and applications of Iso (1 nM), DEANO (100 μM) and acetylcholine (ACh, 1 μM) were performed as indicated by the horizontal lines. Current traces on top were recorded at the times indicated by the corresponding letters on the main graph. The dotted line indicates the zero-current level. *B* and *C*, summary of the effects of DEANO (100 μM) and ACh (1 μM) on the Iso (0·01 and 1 nM)-stimulated $I_{Ca,L}$ in PTX-treated myocytes. The amplitude of $I_{Ca,L}$ is presented as percentage increase over basal amplitude (in *B*) or as percentage variations from the amplitude of the Iso-stimulated $I_{Ca,L}$ (in *C*). Bars are the means and lines are the S.E.M. of the number of experiments indicated near the bars. Significant differences from Iso level are indicated as: * $P < 0·05$.

effect of DEANO in pertussis toxin (PTX)-treated myocytes, where G_i/G_o proteins are irreversibly inactivated. Myocytes were incubated for 4–6 h (before patch-clamp experiments) in the presence of 0·5 μg ml^{-1} PTX, at 37 °C (Hilal-Dandan *et al.* 1992). In the typical experiment shown in Fig. 8*A*, Iso (1 nM) was applied to a PTX-treated myocyte, inducing a fast and large rise in $I_{Ca,L}$. However, under these conditions, superfusion of the myocyte with DEANO (100 μM) had no effect on $I_{Ca,L}$. Since the muscarinic receptor agonist acetylcholine (ACh) produces an anti-adrenergic effect on $I_{Ca,L}$ which clearly involves activation of PTX-sensitive G proteins (reviewed in Méry *et al.* 1997; Feron *et al.* 1999), we tested the efficacy of PTX to inactivate G_i/G_o proteins by investigating the effect of a subsequent application of ACh (1 μM) on Iso-stimulated $I_{Ca,L}$. As shown in Fig. 8*A*, and summarized in Fig. 8*B*, PTX treatment fully abrogated the anti-adrenergic effect of both ACh (1 μM) and DEANO (100 μM). Note that when normalized to the Iso-stimulated $I_{Ca,L}$ amplitude, ACh, but not DEANO, tended to potentiate the effect of the β-adrenergic agonist in PTX-treated myocytes (Fig. 8*C*).

We next examined whether the effect of PTX was taking place upstream or downstream from cG-PK activation. For this, we investigated the effect 8-p-CPT-cGMP (100 μM) in PTX-treated myocytes. In four such cells, the stimulation of $I_{Ca,L}$ by Iso 1 nM was only slightly attenuated by 8-p-CPT-cGMP (from 192·0 ± 11·0% to 171·7 ± 11·0% of control, $P = 0{\cdot}1$), demonstrating that PTX treatment blunted the inhibitory effect of a direct activation of the cG-PK on $I_{Ca,L}$.

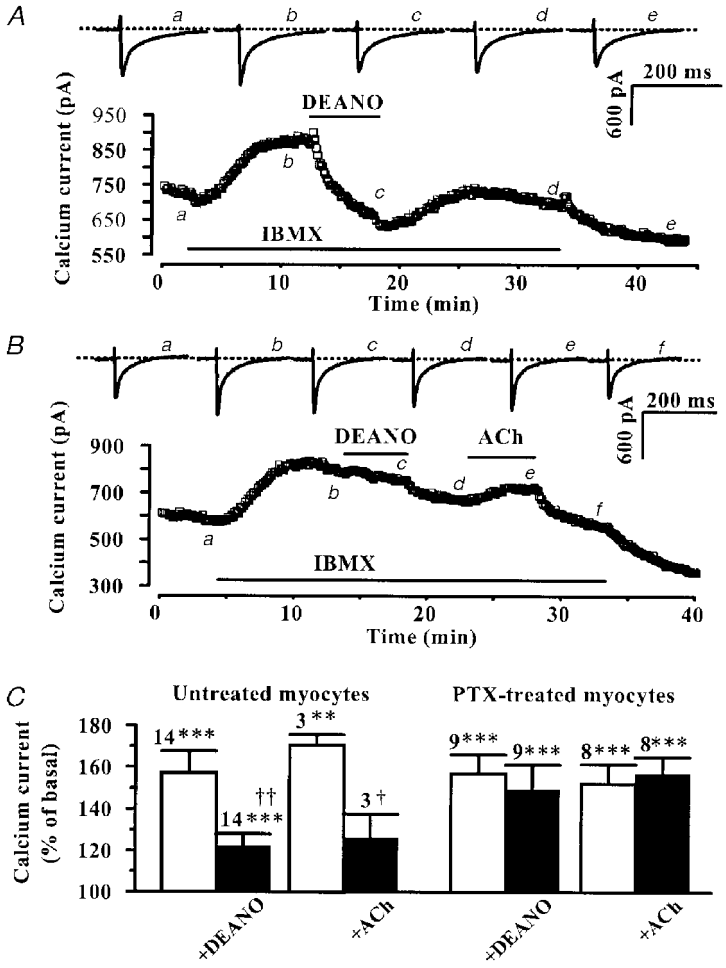

Figure 9. Effects of DEANO and ACh on IBMX-stimulated $I_{Ca,L}$ in rat ventricular myocytes

Untreated (*A*) and PTX-treated (*B*) myocytes were first exposed to control extracellular and intracellular solutions. Applications of IBMX (40 μM in *A*, 20 μM in *B*), DEANO (100 μM, in *A* and *B*) and ACh (1 μM, in *B*) are indicated by the horizontal lines. Current traces on top were recorded at the times indicated by the corresponding letters on the main graphs. The dotted lines indicate the zero-current level. *C*, summary of the effects of IBMX (10–80 μM) on $I_{Ca,L}$, used either alone (□) or in the presence of DEANO (100 μM) or ACh (1 μM) (■) in untreated (left) or PTX-treated myocytes (right). The effects of IBMX on $I_{Ca,L}$ are presented as percentage variations from the amplitude of basal $I_{Ca,L}$ (set to 100%). Bars are the means and lines are the S.E.M. of the number of experiments indicated near the bars. Significant differences from basal (*) and IBMX (†) levels are indicated as: †$P < 0{\cdot}05$; **, ††$P < 0{\cdot}01$; ***$P < 0{\cdot}005$.

In these cells, the Iso-stimulated $I_{Ca,L}$ was also not affected by ACh (1 μM, 198·9 ± 8·0 % of control). Altogether, these data demonstrate that DEANO inhibits $I_{Ca,L}$ via a cG-PK modulation of a PTX-sensitive G_i/G_o protein.

Effect of DEANO on IBMX-stimulated $I_{Ca,L}$ in pertussis toxin-treated myocytes

While PTX treatment of rat ventricular myocytes did not modify basal $I_{Ca,L}$ density (5·2 ± 0·4 pA pF^{-1}, $n = 23$, in PTX *vs.* 5·3 ± 0·1 pA pF^{-1}, $n = 266$, in control), it somewhat increased the Iso response (at 0·01 and 1 nM, compare Fig. 8*B* and Fig. 3). Thus, the anti-adrenergic effect of DEANO might have been blunted in PTX-treated myocytes as a result of a saturating production of cAMP. Therefore, we re-examined the involvement of G_i/G_o proteins in the modulation of $I_{Ca,L}$ by DEANO under conditions where intracellular cAMP was non-maximally increased. To do this, we used low concentrations of isobutyl-methyl-xanthine (IBMX), a broad spectrum phosphodiesterase inhibitor which, unlike Iso, does not activate cAMP synthesis but increases cAMP levels by reducing its degradation. Figure 9*A* shows a typical experiment performed in a control (untreated) myocyte. IBMX (40 μM) elicited a 25% increase in basal $I_{Ca,L}$ and this effect was totally abolished by further application of DEANO (100 μM). This effect of DEANO clearly involved NO generation, since the addition of the NO scavenger carboxy PTIO (Akaike *et al.* 1993) totally antagonized the effect of the NO donor. Indeed, in three myocytes where the stimulation of $I_{Ca,L}$ by 40 μM IBMX (154·2 ± 4·9 % of control) had been reduced by 100 μM DEANO (to 118·5 ± 2·9 % of control, $P < 0·005$), the addition of carboxy PTIO (100 μM) to the DEANO solution restored the stimulation of $I_{Ca,L}$ to its level in Iso alone (to 156·8 ± 1·7 % of control).

In contrast to control cells, DEANO failed to inhibit the response of $I_{Ca,L}$ to IBMX in PTX-treated myocytes. In the typical experiment of Fig. 9*B*, neither DEANO (100 μM) nor ACh (1 μM) produced any inhibition of $I_{Ca,L}$ stimulated by IBMX (20 μM). The results of several similar experiments are summarized in Fig. 9*C*. On average, IBMX (10–80 μM) exerted submaximal stimulatory effects on $I_{Ca,L}$. While ACh (1 μM) strongly reduced the IBMX response in control (untreated) myocytes, this inhibition was absent in PTX-treated myocytes indicating that G_i/G_o proteins were efficiently blocked by PTX. Similarly, DEANO (100 μM) strongly antagonized the IBMX-stimulated $I_{Ca,L}$ in untreated myocytes, and the effect was abolished in PTX-treated myocytes. Thus, the inhibitory effect of DEANO on $I_{Ca,L}$ shared some similarity with the effect of ACh, in that both effects required the integrity of PTX-sensitive G proteins. Note that in one out of the nine experiments in PTX-treated myocytes, ACh had no effect on IBMX-stimulated $I_{Ca,L}$ while DEANO still elicited an inhibitory effect.

DISCUSSION

In this study, we report that $I_{Ca,L}$ is regulated by NO in rat ventricular cardiac myocytes. The NO donor DEANO exerted a pronounced anti-adrenergic effect, which involved the activation of the NO-sensitive guanylyl cyclase and cG-PK. The main locus of action of cG-PK appeared to be a PTX-sensitive G protein.

In agreement with studies in frog and guinea-pig ventricular myocytes (Méry *et al.* 1993; Levi *et al.* 1994; Whaler & Dollinger, 1995), we found that a NO donor, DEANO, strongly inhibited the β-adrenergic stimulation of $I_{Ca,L}$ in rat ventricular myocytes. The inhibitory effect of DEANO occurred in the micromolar range of concentrations and was not mimicked by mock DEANO or diethylamine. This effect clearly involved NO generation, since the addition of the NO-scavenger carboxy PTIO (Akaike *et al.* 1993) totally antagonized the effect of the NO donor. In contrast, other NO donors (SIN-1, SNAP and SPNO, a NONOate like DEANO) did not significantly modify the Iso-stimulated $I_{Ca,L}$. However, the rate of NO release by these NO donors is slow compared with that of DEANO (Feelisch *et al.* 1991; Maragos *et al.* 1991; Ferrero *et al.* 1999). As suggested by Kojda *et al.* (1996), the time course of NO release appears to be critical when comparing the functional effects of different NO donors. Nevertheless, although DEANO is 1000-fold more potent than SIN-1, SNP or SNAP, all these NO donors inhibit the Iso-stimulated $I_{Ca,L}$ in frog ventricular myocytes (e.g. see Méry *et al.* 1993; Abi-Gerges *et al.* 1997*b*). A possible explanation for this discrepancy may come from the observation that rat and mouse ventricles exhibit higher myoglobin contents than other species (O'Brien *et al.* 1992). Since myoglobin is known as a potent NO scavenger, it may hinder the effects of slow NO sources, such as SIN-1, SNAP and SPNO, but not that of a fast NO source like DEANO (Ishibashi *et al.* 1992; Beckman & Koppenol, 1996).

Another interesting observation was that DEANO behaved as an apparent competitive antagonist of the response of $I_{Ca,L}$ to Iso in rat myocytes. We found that the inhibitory effect of DEANO on $I_{Ca,L}$ was reduced as the concentration of Iso was increased from 1 to 10 nM. This observation might help to explain the discrepancy between negative and positive results obtained in the literature when comparing the efficacy of a given NO donor in different cardiac preparations (Thomas *et al.* 1997).

DEANO was shown to enhance cGMP production in isolated rat ventricular myocytes (Kojda *et al.* 1996). In agreement with this finding, we found that activation of the cGMP pathway accounted for the anti-adrenergic effect of DEANO on $I_{Ca,L}$ in the same preparation. Indeed, the effect of DEANO on $I_{Ca,L}$ was antagonized by ODQ, a specific guanylyl cyclase inhibitor (Abi-Gerges *et al.* 1997*b*; Sandirasegarane & Diamond, 1999). Moreover, the effect of DEANO on $I_{Ca,L}$ was mimicked by 8-p-CPT-cGMP, a

cG-PK activator, and antagonized by Rp8cG and KT5823, two cG-PK inhibitors (Butt *et al.* 1994; Komalavila & Lincoln, 1996). Therefore, the endogenous activation of the cG-PK accounted for the anti-adrenergic effect of DEANO on $I_{Ca,L}$.

Earlier studies in rat ventricular myocytes have shown that cG-PK was involved in the inhibitory effects of exogenous cGMP on $I_{Ca,L}$ (Méry *et al.* 1991; Sumii & Sperelakis, 1995). Intracellular dialysis of isolated myocytes with cGMP or with constitutively active cG-PK elicited inhibitory effects on $I_{Ca,L}$ when the current was maximally stimulated with 100 μM IBMX or 100 μM intracellular cAMP. In addition, NO donors inhibited IBMX- or cAMP-stimulated $I_{Ca,L}$ in guinea-pig myocytes (Levi *et al.* 1994; Whaler & Dollinger, 1995). Accordingly, L-type Ca^{2+} channels were viewed as a major target of cG-PK in these cells (Méry *et al.* 1991; Levi *et al.* 1994; Whaler & Dollinger, 1995; Sumii & Sperelakis, 1995).

However, we found here that in rat ventricular myocytes the inhibitory effect of DEANO on $I_{Ca,L}$ was strongly reduced when the concentration of Iso was increased to 10 nM (Fig. 3) or in the presence of 200 μM IBMX (data not shown). Moreover, DEANO did not inhibit the stimulation of $I_{Ca,L}$ induced by intracellular cAMP. Therefore, the major target of cG-PK may differ when activated by endogenous cGMP production (this study) or by intracellular dialysis of exogenous cGMP (Méry *et al.* 1991). Our present results suggest that cG-PK acts at the level of G proteins when guanylyl cyclase is stimulated by NO since the inhibitory effect of DEANO on $I_{Ca,L}$ was antagonized by PTX, which inactivates G_i/G_o proteins. Both G_i and G_o proteins are involved in the regulation of $I_{Ca,L}$ (Méry *et al.* 1997; Valenzuala *et al.* 1997; Feron *et al.* 1999), but only G_i-dependent pathways were shown to be modulated by cG-PK (Pfeiffer *et al.* 1995). Activation of cG-PK was shown to phosphorylate α_i subunits of G proteins (Pfeiffer *et al.* 1995) and to increase the spontaneous binding of GTP (Miyamoto *et al.* 1997). Our results strongly suggest that this modulation of α_i subunits of G_i proteins accounts for the observed cG-PK-dependent, PTX-sensitive inhibitory effect of DEANO on Iso-stimulated $I_{Ca,L}$ in rat myocytes. Yet, we can only speculate on the contribution of this mechanism to the regulation of the Iso-stimulated $I_{Ca,L}$ by NO in guinea-pig myocytes (Levi *et al.* 1994; Whaler & Dollinger, 1995). Interestingly, rat ventricles express higher levels of myoglobin than guinea-pig ventricles (O'Brien *et al.* 1992), and high myoglobin content blunted cGMP production induced by NO (Ishibashi *et al.* 1992). Therefore, we propose that only high levels of cGMP can elicit the direct inhibition of L-type calcium channels by cG-PK (as seen during dialysis of cGMP, or in guinea-pig myocytes), while moderate levels of cGMP induce the specific regulation of G_i proteins by cG-PK (as seen in rat myocytes).

In addition to the regulation of cGMP production, NO can regulate directly the activity of several proteins in the heart, including the L-type Ca^{2+} channels. Nitrosothiols and SIN-1 enhanced $I_{Ca,L}$ in a cGMP-independent manner in ferret ventricular myocytes (Campbell *et al.* 1996), but nitrosothiols inhibited the activity of cloned L-type Ca^{2+} channels in HEK 293 cells (a human embryonic kidney cell line) (Hu *et al.* 1997). In rat ventricular myocytes, as in cardiac myocytes from other species and tissues (Méry *et al.* 1993; Levi *et al.* 1994; Kirstein *et al.* 1995; Wahler & Dollinger, 1995; Y. G. Wang *et al.* 1998; Feron *et al.* 1999), NO donors (including nitrosothiols and SIN-1) did not elicit a cGMP-independent effect on $I_{Ca,L}$. Interestingly, we found that the basal $I_{Ca,L}$ in rat ventricular myocytes was not modified by reducing agents but was inhibited by LY83583, a superoxide anion generator. Hu *et al.* (1997) also reported that the basal activity of the L-type Ca^{2+} channel was not changed by reducing agents but was inhibited by oxidant treatments. In contrast, $I_{Ca,L}$ was inhibited by reducing agents (glutathione, DL-DTT) and enhanced by an oxidant (DTNB) in ferret ventricular myocytes (Campbell *et al.* 1996). Altogether, these data suggest that the different effects of NO donors on the basal $I_{Ca,L}$ might require different redox environments. In addition, it is possible that the 'direct' effects of NO donors do not take place on the L-type Ca^{2+} channel itself, but on other auxiliary proteins and/or on lipids. While the reason for these discrepancies remains to be fully elucidated, our results clearly demonstrate that NO can recruit an endogenous cG-PK in rat ventricular myocytes, leading to a tight control of the β-adrenergic stimulation of L-type Ca^{2+} channel activity.

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