Cyclic GMP regulation of the L-type Ca^{2+} channel current in human atrial myocytes

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1. The regulation of the L-type Ca^{2+} current (I_{Ca}) by intracellular cGMP was investigated in human atrial myocytes using the whole-cell patch-clamp technique.

2. Intracellular application of 0.5 μM cGMP produced a strong stimulation of basal I_{Ca} (+64 $\pm$ 5%, $n = 60$), whereas a 10-fold higher cGMP concentration induced a 2-fold smaller increase (+36 $\pm$ 8%, $n = 35$).

3. The biphasic response of I_{Ca} to cGMP was not mimicked by the cGMP-dependent protein kinase (PKG) activator 8-bromoguanosine 3′,5′ cyclic monophosphate (8-bromo-cGMP, 0.5 or 5 μM), and was not affected by the PKG inhibitor KT 5823 (100 nM).

4. In contrast, cGMP stimulation of I_{Ca} was abolished by intracellular perfusion with PKI (10 μM), a selective inhibitor of the cAMP-dependent protein kinase (PKA).

5. Selective inhibition of the cGMP-inhibited phosphodiesterase (PDE3) by extracellular cilostamide (100 nM) strongly enhanced basal I_{Ca} in control conditions (+78 $\pm$ 13%, $n = 7$) but had only a marginal effect in the presence of intracellular cGMP (+22 $\pm$ 7% in addition to 0.5 μM cGMP, $n = 11$; +20 $\pm$ 22% in addition to 5 μM cGMP, $n = 7$).

6. Application of erythro-9-[2-hydroxy-3-nonyl]adenine (EHNA, 30 μM), a selective inhibitor of the cGMP-stimulated phosphodiesterase (PDE2), fully reversed the secondary inhibitory effect of 5 μM cGMP on I_{Ca} (+99 $\pm$ 16% stimulation, $n = 7$).

7. Altogether, these data indicate that intracellular cGMP regulates basal I_{Ca} in human atrial myocytes in a similar manner to NO donors. The effect of cGMP involves modulation of the cAMP level and PKA activity via opposite actions of the nucleotide on PDE2 and PDE3.

The cardiac L-type Ca^{2+} channel current (I_{Ca}) is an important determinant of myocardial contractility. Its regulation by neurotransmitters, hormones, and paracrine factors contributes to the control of cardiac output to meet the demands of the body. A large number of these extracellular first messengers, acting on specific membrane receptors in cardiac myocytes, regulate the activity of adenylyl cyclase which in turn controls the intracellular concentration of cAMP, the activity of the cAMP-dependent protein kinase (PKA), and the degree of phosphorylation and stimulation of L-type Ca^{2+} channels (Hartzell, 1988; McDonald *et al.* 1994; Hove-Madsen *et al.* 1996; Striessnig, 1999). A typical example of such regulation is the control of heart function by the sympathetic and parasympathetic nervous systems, which act via adrenoceptors and muscarinic receptors (Brodde & Michel, 1999). In addition to the cAMP cascade, other factors regulate heart function by acting primarily on the cGMP cascade; these include atrial and brain natriuretic peptides (de Bold *et al.* 1996) and nitric oxide (NO) (Paulus & Shah, 1999; Shah & MacCarthy, 2000).

NO modulates cardiac contractility and rhythm in part via its ability to control the amplitude of I_{Ca} (for reviews see Fischmeister & Méry, 1996; Kelly *et al.* 1996; Kojda & Kottenberg, 1999; Paulus & Shah, 1999; Shah & MacCarthy, 2000). Classically, this regulation is mediated through the generation of cGMP by NO-stimulated soluble guanylyl cyclase activity. But, NO can also regulate cardiac contraction (Chesnais *et al.* 1999; Sandirasegarane & Diamond, 1999) or Ca^{2+} channel activity (Campbell *et al.* 1996; Hu *et al.* 1997) through cGMP-independent effects, so that the relative contribution of cGMP-dependent or -independent mechanisms to the overall effects of NO in the heart remains unresolved (Shah & MacCarthy, 2000).

Several studies in various animal species (reviewed in Lohmann *et al.* 1991; Fischmeister & Méry, 1996) have shown that exogenous cGMP can both stimulate or inhibit I_{Ca} (Hartzell & Fischmeister, 1986; Levi *et al.* 1989; Ono & Trautwein, 1991; Méry *et al.* 1991; Shirayama & Pappano, 1996; Han *et al.* 1998) and contractility (Nawrath, 1976; Trautwein & Trube, 1976; Endoh & Yamashita, 1981; Smith *et al.* 1991; Brady *et al.* 1993; Mohan *et al.* 1995; Kojda *et al.* 1996). These opposite effects can be explained by the presence of three different targets for cGMP with different affinities for the nucleotide (Lohmann *et al.* 1991; Butt *et al.* 1992): (1) the cGMP-inhibited phosphodiesterase (PDE3); (2) the cGMP-stimulated phosphodiesterase (PDE2); (3) the cGMP-activated protein kinase (PKG). The stimulatory effects on I_{Ca} or contractility observed during modest activation of the NO–cGMP pathway are best explained by cAMP elevation following PDE3 inhibition (Ono & Trautwein, 1993; Méry *et al.* 1993; Wahler & Dollinger, 1995; Kojda *et al.* 1996). But, the inhibitory effects of a strong activation of this pathway can be attributed either to PDE2 stimulation (in frog, Hartzell & Fischmeister, 1986; Méry *et al.* 1995) or to PKG activation (in embryonic chick heart, Wahler *et al.* 1990; Haddad *et al.* 1995; in adult mammalian heart, Levi *et al.* 1989; Méry *et al.* 1991; Wahler & Dollinger, 1995; Sumii *et al.* 1995; Kojda *et al.* 1996). Surprisingly, PKG was also reported to stimulate I_{Ca} in ventricular myocytes from newborn (Kumar *et al.* 1997) and young rabbit (Han *et al.* 1998). Altogether, these data indicate that the relative contribution of the different cGMP targets, as well as their final downstream modulation of I_{Ca} and heart function, may vary depending on the species, the developmental stage, and the region of the heart.

The variability in the results obtained in laboratory animals makes it difficult to extrapolate to humans and compelled us to directly assess the effects of NO and cGMP in human heart. Patch-clamp experiments performed in isolated human atrial myocytes demonstrated that NO donors and cGMP also regulate I_{Ca} in this preparation. At nanomolar concentrations, the NO donors SIN-1 and SNAP produced a stimulation of basal I_{Ca} (Kirstein *et al.* 1995; Vandecasteele *et al.* 1998*a*). This effect was blocked by intracellular methylene blue (Vandecasteele *et al.* 1998*a*), mimicked by PDE3 selective inhibitors (Kirstein *et al.* 1995; Kajimoto *et al.* 1997) or by an intracellular perfusion with cGMP (Rivet-Bastide *et al.* 1997). Although the molecular mechanisms involved have not been fully elucidated yet, these experiments suggested that in human atrial myocytes low concentrations of NO stimulated I_{Ca} via cGMP production and cGMP-inhibition of PDE3 (Kirstein *et al.* 1995). Surprisingly, when used at micromolar concentrations, the stimulatory effect of SIN-1 on I_{Ca} was strongly attenuated, suggesting the development of a secondary inhibitory effect at higher concentrations (Kirstein *et al.* 1995). Whether this secondary effect is also mediated by cGMP or results from a direct effect of NO or some of its by-products (e.g. resulting from the chemical reactions between NO, superoxide, and peroxynitrite) on L-type Ca^{2+} channels remains unknown.

In the present study, our aim was to dissect the mechanisms involved in the regulation of I_{Ca} by intracellular cGMP in human atrial myocytes. More specifically, we tried to address two questions: (1) to what extent can changes in intracellular cGMP mimic the bimodal regulation of I_{Ca} by NO?; (2) what are the respective contributions of the cGMP targets (PDE2, PDE3 and PKG) in the effect of exogenous cGMP on I_{Ca}?

A preliminary report of some of these results has appeared elsewhere (Vandecasteele *et al.* 1998*b*).

METHODS

Surgery

All protocols for obtaining human cardiac tissue were approved by the ethics committee of our institution (GREBB, Hôpital de Bicêtre, Université de Paris-Sud). Specimens of right atrial appendages were obtained from 33 patients (aged 10–83 years) undergoing heart surgery for congenital defects ($n = 2$), coronary artery diseases ($n = 25$) or valve replacement ($n = 6$). All patients but five received a pharmacological pre-treatment (Ca^{2+} channel blockers, digitalis, β-adrenergic antagonists, diuretics, ACE inhibitors, NO donors and/or anti-arrhythmic drugs). In addition, all patients received sedatives, anaesthesia and antibiotics prior to surgery. But, we found no obvious correlation between the Ca^{2+} current density or the effects on I_{Ca} of the drugs tested here and the long-term therapy received (if any) by the patient. Dissociation of the cells was realised immediately after surgery.

Human atrial cell dissociation

Myocytes were isolated as described previously (Kirstein *et al.* 1995) with some modifications. Briefly, quickly after excision, the tissue was washed and cut in small pieces in a Ca^{2+}-free Tyrode solution supplemented with 30 mM 2,3-butanedione monoxime (BDM). Small (~1 mm^3) pieces of atria were then incubated in a BDM- and Ca^{2+}-free Tyrode solution containing 40 i.u. ml^{-1} collagenase, 15 i.u. ml^{-1} protease and 5 mg ml^{-1} BSA. After 30 min, this solution was removed and replaced by fresh enzymatic solution containing only collagenase (200 i.u. ml^{-1}) for 10–20 min until a satisfactory cell yield was obtained. All steps were carried out at 37 °C, with continuous stirring at 200 r.p.m. and gassing with 95% O_2–5% CO_2. The cell suspension was filtered, centrifuged (for 1 min at 600–700 r.p.m.) and the pellet resuspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, non-essential amino acids, 1 nM insulin and antibiotics (penicillin, 100 i.u. ml^{-1} and streptomycin, 0.1 μg ml^{-1}). For patch-clamp experiments, 20–100 μl of this cell suspension was added to a control extracellular solution in a Petri dish.

Electrophysiological experiments

The whole-cell configuration of the patch-clamp technique (Hamill *et al.* 1981) was used to record the high-threshold L-type Ca^{2+} current (I_{Ca}) on Ca^{2+}-tolerant human atrial myocytes. In the routine protocols the cells were depolarised every 8 s from a holding potential of −50 mV to 0 mV for 400 ms. This holding potential was chosen to completely inactivate the fast Na^+ current. K^+ currents were blocked by replacing all K^+ ions with intracellular and extracellular Cs^+. Voltage-clamp protocols were generated by a challenger/09-VM programmable function generator (Kinetic Software, Atlanta, GA, USA). The cells were voltage clamped using a patch-clamp amplifier

(model RK-400; Biologic, Claix, France). Currents were sampled at a frequency of 10 kHz using a 12-bit analog–digital converter (DT2827; Data Translation, Marlboro, MA, USA) connected to a PC-compatible computer (386/33 System-pro; Compaq, Houston, TX, USA). All experiments were done at room temperature (19–25 °C) and the temperature varied by < 2 °C during the course of an experiment.

Solutions

Control extracellular solution contained (mM): NaCl 107.1, Hepes 10, CsCl 40, $NaHCO_3$ 4, NaH_2PO_4 0.8, $CaCl_2$ 1.8, $MgCl_2$ 1.8, D-glucose 5 and sodium pyruvate 5; pH 7.4 adjusted with NaOH. Patch electrodes (0.8–1.5 MΩ) were filled with control GTP-free intracellular solution that contained (mM): CsCl 119.8, EGTA (acid form) 5, $MgCl_2$ 4, creatine phosphate disodium salt 5, Na_2-ATP 3.1, Hepes 10, and $CaCl_2$ 62 μM (pCa 8.5); pH 7.3 adjusted with CsOH. In some experiments Na_2-GTP (420 μM) was added to the GTP-free intracellular solution, and the pH was readjusted. 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 tubing flowing at a rate of ~10 μl min^{-1}. Intracellular perfusion of the cell with cyclic nucleotides or the PKA inhibitor PKI during whole-cell recording was made possible by the use of a microcapillary inside the patch-clamp pipette, as already described (Hartzell & Fischmeister, 1986). This capillary was connected to little tanks containing intracellular solutions supplemented with cyclic nucleotides or PKI at different concentrations. Application of a modest negative pressure inside the patch electrode allowed flowing of the desired solution to the tip of the pipette and inside the cell by passive diffusion.

Materials

Collagenase type V and protease type XXIV, used for dissociation of human atrial cells, and Na_2-cGMP, Na_2-8-bromo-cGMP, erythro-9-[2-hydroxy-3-nonyl]adenine (EHNA), cAMP-dependent protein kinase inhibitor (PKI, rabbit sequence) used in patch-clamp experiments were from Sigma-Aldrich (L'Isle d'Abeau Chesnes, France). Cilostamide was from Tocris Cookson (Bristol, UK) and KT 5823 was from Calbiochem-France Biochem (Meudon, France). Cilostamide was dissolved at 10 mM in ethanol. KT 5823 was dissolved at 100 mM in DMSO. An equal amount of ethanol and/or DMSO corresponding to the concentration present in the final dilutions was added to all other solutions. All other drugs were dissolved in ionic aqueous solutions, made fresh daily and kept at 4 °C until use.

Data analysis

The maximal amplitude of I_{Ca} was measured as the difference between the peak inward current and the leak current (I_{400}), which was the current amplitude at the end of the 400 ms duration pulse (Kirstein *et al.* 1995). Currents were not compensated for capacitive and leak currents. Cell membrane capacitance and series resistances were measured by exponential analysis of current responses to 1 mV step changes in membrane potential. Membrane capacitance was 74.2 ± 21 pF (mean ± S.E.M.) and series resistance was 3.2 ± 0.2 MΩ ($n = 82$). On-line analysis was performed by programming a PC-compatible computer in PASCAL to determine peak and steady-state current values for each depolarisation.

The results are expressed as means ± S.E.M. In each experimental condition, the effects of the drugs tested on I_{Ca} are expressed as percentage change with respect to the values of the current under basal conditions, that is, in the absence of any hormonal stimulation. The variations in I_{Ca} induced by the different drugs were tested for statistical significance by Student's t test. Statistically significant differences between different conditions are indicated in the figures as: $*P < 0.05$; $**P < 0.01$; $***P < 0.005$.

RESULTS

Biphasic effect of cGMP on I_{Ca}

I_{Ca} was recorded in human atrial myocytes using the whole-cell configuration of the patch-clamp technique (Hamill *et al.* 1981). Basal I_{Ca} amplitude was measured 3–5 min after patch break to allow for equilibration between intracellular and pipette solutions. Basal I_{Ca} amplitude at a membrane potential of 0 mV was 241.0 ± 16.7 pA and I_{Ca} density, which represents the ratio of I_{Ca} amplitude to membrane capacitance, was 3.2 ± 0.2 pA pF^{-1} ($n = 82$). As in our previous studies (Kirstein *et al.* 1995; Rivet-Bastide *et al.* 1997; Vandecasteele *et al.* 1998*a*), I_{Ca} densities showed a large scatter between different patients and between individual cells from the same patient, with no obvious correlation with the diagnosis, sex, age or pretreatment of the patients. Figure 1*A* illustrates a typical experiment showing the effect of intracellular perfusion with cGMP on the time course of I_{Ca} amplitude measured at 0 mV from a holding potential of −50 mV. Two concentrations of cGMP (0.5 and 5 μM) were successively dialysed into the human atrial myocyte (see Methods). At the beginning of the experiment, the cell was dialysed with control intracellular (GTP-free) solution. After a stable baseline was achieved, the control solution was changed to a solution containing 0.5 μM cGMP (first arrow), which produced about a 2-fold increase in I_{Ca} amplitude. This effect was nearly abolished when the cGMP concentration was increased to 5 μM (second arrow). Washout of cGMP (third arrow) resulted in a rebound stimulation of I_{Ca} before it returned slowly to the control level. As summarised in Fig. 1*B*, on average 0.5 μM cGMP stimulated I_{Ca} by 64 ± 5% above control level ($n = 60$, $P < 0.001$ *vs.* control) and subsequent application of 5 μM cGMP resulted in an ~50% attenuation of this effect (36 ± 8% above basal level, $n = 35$, $P < 0.005$ *vs.* control and cGMP 0.5 μM). A lower concentration of cGMP (0.15 μM) was tested in six other cells, but I_{Ca} increased in only two of these cells, with an overall non-significant effect of the nucleotide (23 ± 20%, data not shown). Application of a single high concentration of cGMP (5 or 50 μM) stimulated basal I_{Ca} by 76 ± 16% ($n = 5$) and 53 ± 15% ($n = 5$), respectively. But these effects were only transient and the current amplitude returned to the basal level after a few minutes.

As illustrated by the individual current traces shown in Fig. 1*A*, the stimulatory effect of cGMP was not accompanied by any significant modification in the kinetics of I_{Ca}. This suggests that cGMP did not modify the voltage dependence of the Ca^{2+} channel gating but to examine this further, the effect of intracellular cGMP on the I_{Ca} current–voltage (Fig. 2*A*) and inactivation (Fig. 2*B*) relationships was investigated. The U-shape of both curves for basal I_{Ca} (■ in Fig. 2*A* and *B*), as well as their respective positions on the voltage axis, are characteristic of the high-threshold L-type Ca^{2+} current

in this preparation (Kirstein *et al.* 1995). As shown, 0.5 μM cGMP increased I_{Ca} by a similar amount at every membrane potential (Fig. 2*A*) and did not modify the inactivation curve of the current (Fig. 2*B*). Thus, cGMP modifies I_{Ca} in an essentially voltage-independent manner.

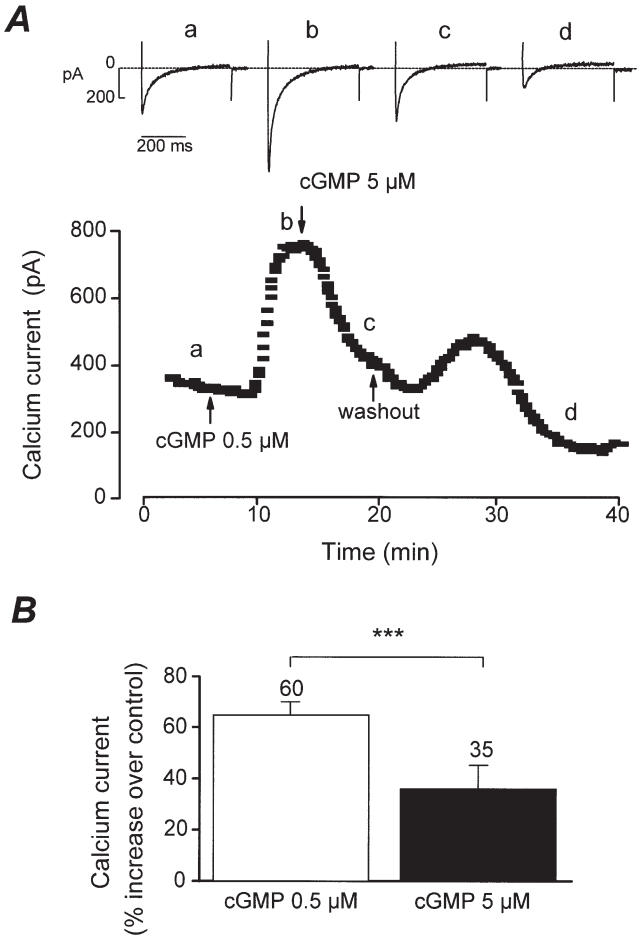

Figure 1. Effect of intracellular application of cGMP on basal I_{Ca} in human atrial myocytes

A, each symbol corresponds to a measure of I_{Ca} at 0 mV obtained every 8 s. The cell was first dialysed with control GTP-free intracellular solution and perfused with two concentrations of cGMP, first 0.5 μM and then 5 μM, at the time indicated by the arrows. After the applications of 0.5 and 5 μM cGMP the cell was dialysed with control intracellular solution (washout). The individual current traces shown in the upper part were obtained at the times indicated by the corresponding letters in the graph below. The horizontal line indicates the zero current level. *B*, mean stimulatory effect of cGMP (0.5 μM, □; 5 μM, ■), obtained using the same experimental protocol as in *A*. The size of the bars indicates the mean effects expressed as percentage increase over basal I_{Ca}, and the lines the S.E.M., with the number of experiments indicated above. *** $P < 0.005$, statistically significant difference between both conditions using Student's *t* test.

So far, our results indicate that cGMP activates two sequential mechanisms which affect I_{Ca} in an antagonistic manner; a stimulatory mechanism activated at concentrations below 0.5 μM and an inhibitory mechanism activated at concentrations above 5 μM. It should be noted that these cGMP concentrations do not necessarily reflect the actual concentrations of the cyclic nucleotide inside the cell. Indeed, access resistance to the cell and the presence of cyclic nucleotide phosphodiesterases are likely to lower drastically the intracellular cGMP concentration effectively used by the cell. The above experiments were performed in GTP-free intracellular solution, to limit the extent of endogenous cGMP synthesis by the myocytes (Rivet-Bastide *et al.* 1997) which might interfere with the effects of exogenous cGMP introduced through the patch pipette. Therefore, it was conceivable that the absence of intracellular GTP might lead to a progressive reduction of I_{Ca} due to a loss in a constitutive G protein activation of adenylyl cyclase activity (Skeberdis *et al.* 1997; Vandecasteele *et al.* 1998*a*), which could explain the biphasic effect of cGMP. To examine this, we tested the effect of cGMP in cells dialysed with GTP (420 μM). At

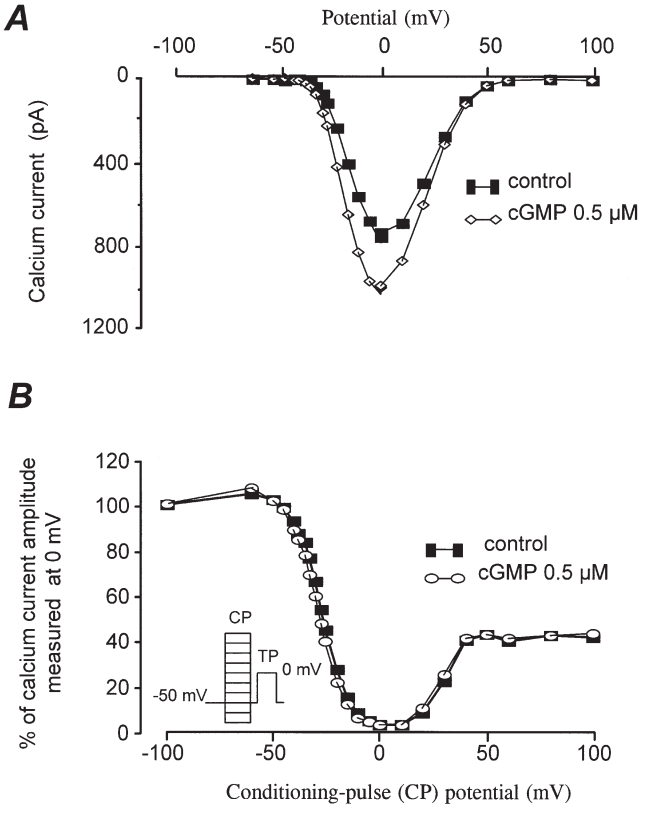

Figure 2. Voltage dependence of the stimulatory effect of cGMP on basal I_{Ca} in human atrial myocytes

Current–voltage relationships (*A*) and inactivation curves of I_{Ca} (*B*) in control condition (■) and in the presence of 0.5 μM intracellular cGMP (*A*, ◇; *B*, ○). Inactivation curves were obtained using the double-pulse protocol as indicated in the inset (see also Methods).

this high concentration of GTP, application of 0.5 μM cGMP ($n = 19$), followed by an application of either 5 μM ($n = 11$) or 50 μM cGMP ($n = 7$), modified I_{Ca} by $+36 \pm 4$% (at 0.5 μM, $P < 0.001$ *vs.* control), $+26 \pm 9$% (at 5 μM, $P < 0.05$ *vs.* control) and -2 ± 6% (at 50 μM, not significant). Thus, the biphasic effect of cGMP was still observed in the presence of GTP but addition of GTP reduced about 2-fold the stimulatory effect on I_{Ca} of the lowest cGMP concentration (0.5 μM; $P < 0.005$ *vs.* GTP free). Moreover, in the presence of intracellular GTP, it was necessary to increase the concentration of cGMP to higher levels (50 μM instead of 5 μM) to activate the secondary inhibitory mechanism. Thus, the presence of intracellular GTP, most probably via activation of endogenous cGMP synthesis (Rivet-Bastide *et al.* 1997), attenuated the stimulatory effect of cGMP on I_{Ca} and reduced the sensitivity of the inhibitory effect to the nucleotide. For a better dissection of the mechanisms involved in these two opposite effects, we returned to GTP-free conditions for all subsequent experiments.

Role of cGMP-dependent protein kinase (PKG)

Our first goal in this study was to determine the molecular mechanism by which cGMP stimulates I_{Ca} in human atrial myocytes. Since PKG was shown to be responsible for the stimulatory effect of cGMP on I_{Ca} in rabbit ventricle (Han *et al.* 1998), we examined whether 8-bromo-cGMP, a potent activator of this enzyme (Butt *et al.* 1992), could mimic the effect of cGMP. Figure 3*A* shows a typical experiment in which a human atrial myocyte was first dialysed with control (GTP-free) intracellular solution, and, after a few minutes (first arrow), was challenged with 5 μM 8-bromo-cGMP added to the patch pipette. As shown, 8-bromo-cGMP had no effect on basal I_{Ca} at this concentration. However, when used at 100 μM, 8-bromo-cGMP clearly enhanced I_{Ca}, an effect which amounted to ~50% of the maximal stimulation of the current obtained when the cell was dialysed with 50 μM cAMP (fourth arrow). Figure 3*B* summarises the results of several similar experiments in which three concentrations of 8-bromo-cGMP were tested (0.5, 5 and 100 μM). Whereas 0.5 and 5 μM of the cGMP derivative had no effect on basal I_{Ca} ($+2 \pm 5$%, $n = 3$, and -8 ± 11%, $n = 4$, respectively), 100 μM increased the current to 87 ± 18% above basal level ($n = 4$, $P < 0.05$).

The above results indicate that 8-bromo-cGMP is 200-fold less potent than cGMP in stimulating I_{Ca}. Since the cGMP-derivative is more potent than the native nucleotide in activating PKG (Butt *et al.* 1992), these results argue against an involvement of this enzyme in the stimulatory effect of cGMP on I_{Ca}. However, to examine this hypothesis further, we tested the effect of KT 5823, a highly selective (Komalavila & Lincoln, 1996) and commonly used PKG inhibitor (Wahler & Dollinger, 1995; Kumar *et al.* 1997). KT 5823 was used at 100 nM, a concentration which significantly reduced the inhibitory effect of NO donors on I_{Ca} in guinea-pig (Wahler & Dollinger, 1995) and rat ventricular myocytes (Abi-Gerges *et al.* 2001) but which is unlikely to inhibit PKA (Kase *et al.* 1987). As shown in Fig. 4, extracellular application of KT 5823 on human atrial myocytes did not modify the stimulatory effect of an intracellular application of either 0.5 μM cGMP (Fig. 4*A*) or 100 μM 8-bromo-cGMP (Fig. 4*B*) on I_{Ca}. As summarised in Fig. 4*C*,

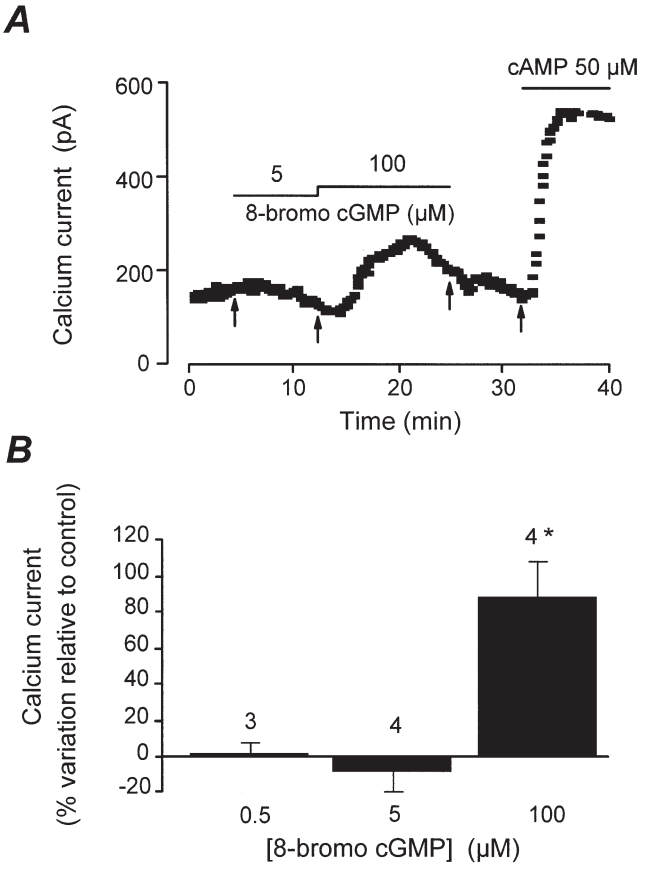

Figure 3. Effect of intracellular 8-bromo-cGMP and cAMP on basal I_{Ca} in human atrial myocytes

A, the cell was superfused with control extracellular solution throughout the experiment. Each symbol corresponds to a measure of I_{Ca} at 0 mV obtained every 8 s. At the beginning of the experiment, the cell was intracellularly dialysed with control GTP-free intracellular solution. The same solution containing 5 μM 8-bromo-cGMP was allowed to diffuse into the cytoplasm, without any notable effect on I_{Ca} (first arrow). In contrast, 100 μM of the cGMP derivative (second arrow) roughly doubled the current amplitude. After washout of 8-bromo-cGMP (third arrow), a maximal stimulation of I_{Ca} was obtained by perfusing the myocyte with 50 μM cAMP (last arrow). *B*, summary of the effects of 8-bromo-cGMP (0.5, 5 and 100 μM) on I_{Ca} in human atrial myocytes. The size of the bars indicates the mean effects expressed as percentage variation relative to basal I_{Ca}, and the lines the S.E.M., with the number of experiments indicated above. * $P < 0.05$, statistically significant difference from control I_{Ca} amplitude using Student's *t* test.

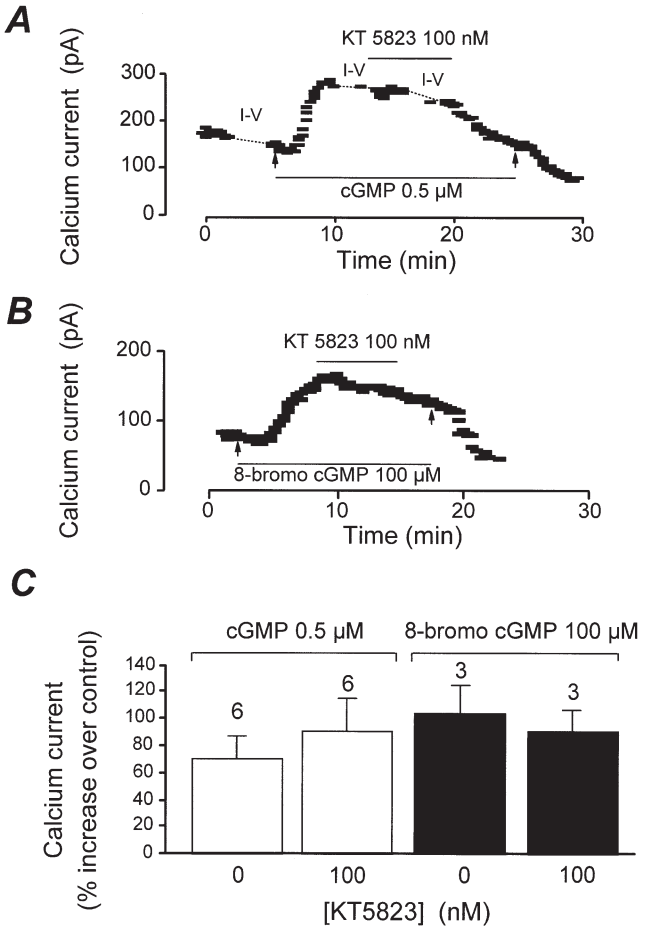

Figure 4. Role of cGMP-dependent protein kinase (PKG) in the stimulatory effect of cGMP on I_{Ca} in human atrial myocytes

A and *B*, two representative experiments showing the absence of effect of PKG inhibition by KT 5823 (100 nM) on I_{Ca} stimulated by cGMP (0.5 μM; *A*) and 8-bromo-cGMP (100 μM; *B*), in human atrial myocytes. Each symbol corresponds to a measure of I_{Ca} at 0 mV obtained every 8 s. In both experiments the cell was initially perfused with the usual GTP-free control intracellular solution, then dialysed with cGMP or 8-bromo-cGMP. As can be seen, both cyclic nucleotides clearly enhanced I_{Ca}. Superfusion of the cell with 100 nM KT 5823 in addition to cGMP or 8-bromo-cGMP failed to modify the amplitude of I_{Ca}. During the interruption of the curve in *A*, current–voltage (*I–V*) relationships were obtained, like those illustrated in Fig. 2*A*. Then the routine stimulation protocol was applied again. *C*, comparison of the mean effect of cGMP (0.5 μM, □) and 8-bromo-cGMP (100 μM, ■) in the absence and presence of KT 5823 (100 nM). The size of the bars indicates the mean effects expressed as percentage increase over basal I_{Ca}, and the lines the S.E.M., with the number of experiments indicated above.

0.5 μM cGMP produced a 72 ± 15% ($n = 6$) and 92 ± 24% ($n = 6$) stimulation of basal I_{Ca} in the absence or presence, respectively, of KT 5823. Similarly, in three cells, intracellular perfusion with 100 μM 8-bromo-cGMP induced a 102 ± 21% increase in basal I_{Ca}, and this effect remained unchanged after a subsequent application of 100 nM KT 5823 (92 ± 15%, Fig. 4*C*). Altogether, these results exclude the possibility that PKG plays a determinant role in the stimulatory effect of cGMP on I_{Ca}.

Role of cAMP-dependent protein kinase (PKA)

To examine the participation of PKA in the stimulatory effect of cGMP on basal I_{Ca} in human atrial myocytes, we performed experiments in which cGMP stimulation was followed by intracellular perfusion with PKI, a highly selective peptide inhibitor of PKA (Walsh *et al.* 1990). In four cells, intracellular perfusion with 0.5 μM cGMP increased I_{Ca} by 30 ± 4% above the control value. After stabilisation of I_{Ca} amplitude, intracellular perfusion was switched to a solution containing 10 μM PKI added to the cGMP (0.5 μM)-containing solution. This quickly resulted in abolition of the cGMP stimulation of I_{Ca} and in a decrease in the calcium current amplitude below the initial baseline (54 ± 6% below control value, see also Skeberdis *et al.* 1997). Thus, the stimulatory effect of cGMP on I_{Ca} is likely to be mediated by activation of PKA.

Role of cGMP-inhibited phosphodiesterase (PDE3)

One possible way by which cGMP could stimulate I_{Ca} in a PKA-dependent manner is through an increase in cAMP concentration due to inhibition of the cGMP-inhibited phosphodiesterase (PDE3). PDE3 was shown to regulate basal I_{Ca} in human atrial myocytes (Kirstein *et al.* 1995) and to be implicated in the stimulatory effect of cGMP on the isoprenaline-stimulated I_{Ca} in guinea-pig ventricular myocytes (Ono & Trautwein, 1991; Shirayama & Pappano, 1996). To examine a possible role of PDE3 in the effect of cGMP, we compared the effect of cGMP with that of cilostamide, a selective PDE3 inhibitor (Stoclet *et al.* 1995). As shown in Fig. 5, extracellular application of cilostamide (100 nM; Fig. 5*A*) and intracellular dialysis with cGMP (0.5 μM; Fig. 5*B*) produced comparable stimulatory effects on I_{Ca}. Moreover, when cilostamide was added as well as cGMP, it had only a marginal additional effect on I_{Ca} (Fig. 5*B*). The summary data of Fig. 5*C* allow for a comparison of the mean effects on basal I_{Ca} of cilostamide (100 nM), cGMP (0.5 μM) and of both compounds applied together. As shown, cilostamide and cGMP used alone produced very similar stimulatory effects on I_{Ca}, 78 ± 13% ($n = 7$) and 64 ± 8% ($n = 11$), respectively. When cilostamide was added to cGMP, the effect of the nucleotide was increased by 22 ± 7% ($n = 11$, $P < 0.05$, paired *t* test). These results indicate that the stimulatory effect of cGMP was mimicked by a selective PDE3 inhibitor, and the effect of PDE3 inhibition was greatly reduced in the presence of cGMP. Therefore, partial PDE3

inhibition is likely to be responsible for the cGMP-induced stimulation of I_{Ca} in human atrial myocytes.

Role of cGMP-stimulated phosphodiesterase (PDE2)

As shown above, the stimulatory effect of cGMP on I_{Ca} is reduced when the concentration of cGMP is increased. This suggests the development of a secondary inhibitory mechanism at higher cGMP concentrations. As stated in the Introduction, two possible mechanisms could account for a cGMP-dependent inhibition of I_{Ca}: activation of PKG or activation of the cGMP-stimulated phosphodiesterase (PDE2). In order to differentiate between these two possibilities, we tested the effects of KT 5823, the PKG inhibitor, and EHNA, a selective PDE2 inhibitor (Méry *et al.* 1995; Rivet-Bastide *et al.* 1997), for their ability to reverse the effect of 5 μM cGMP on I_{Ca}. In the experiment shown in Fig. 6*A*, a human atrial myocyte was first dialysed with 0.5 μM cGMP, resulting in approximately 60% stimulation of basal I_{Ca}. This effect was strongly reduced when the concentration of cGMP was increased to 5 μM. During the decrease in I_{Ca}, the cell was successively exposed to KT 5823 (100 nM), cilostamide (100 nM) and EHNA (30 μM). As shown, KT 5823 did not antagonise the inhibitory effect of cGMP, and cilostamide induced only a 15% increase in I_{Ca}. By contrast, application of EHNA induced a strong and reversible stimulation of I_{Ca} which recovered to the amplitude obtained in the presence of 0.5 μM cGMP. The results of several similar experiments are summarised in Fig. 6*B*. In 14 cells, 0.5 μM cGMP increased I_{Ca} by 70 ± 10% and a subsequent increase in cGMP concentration to 5 μM reduced this stimulation to 25 ± 11% above control level ($P < 0.01$ *vs.* 0.5 μM cGMP). In seven individual cells, extracellular application of either KT 5823 (100 nM) or cilostamide (100 nM) in the continuous presence of intracellular cGMP (5 μM) had no significant effect on the current amplitude ($+17 \pm 14$%, $P = 0.73$, and $+20 \pm 22$%, $P = 0.83$, respectively). This indicates that activation of PKG does not account for the inhibitory effect of cGMP on I_{Ca} and that PDE3 is already fully inhibited at 5 μM cGMP. Exposure of the cells to EHNA (30 μM) induced a strong stimulation of I_{Ca}, to 99 ± 16% above the control level ($n = 7$, $P < 0.005$ *vs.* 5 μM cGMP), an effect which was similar to that seen with 0.5 μM cGMP. These results demonstrate that the inhibitory effect of cGMP on I_{Ca} in human atrial myocytes involves an activation of PDE2.

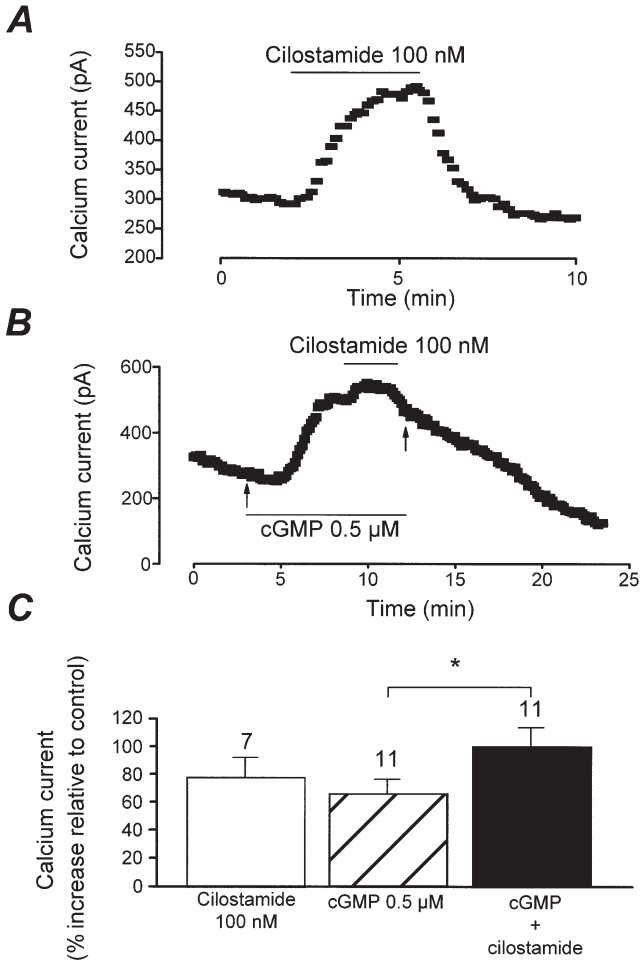

Figure 5. Effect of PDE3 inhibition by cilostamide on basal and cGMP-stimulated I_{Ca} in human atrial myocytes

A and *B*, each symbol corresponds to a measure of I_{Ca} at 0 mV obtained every 8 s. Both experiments were carried out using a GTP-free intracellular solution. *A*, after a few minutes during which the cell was superfused with control extracellular solution, extracellular application of 100 nM cilostamide during the period indicated by the horizontal line produced an ~60% increase of I_{Ca} above control level. *B*, the same extracellular application of 100 nM cilostamide had only a modest effect when I_{Ca} had been previously increased by intracellular perfusion with 0.5 μM cGMP (first arrow). The second arrow indicates the washout of intracellular cGMP. *C*, summary of the effect of cilostamide (100 nM, □), cGMP (0.5 μM, ▨) and the combination of both (■) on basal I_{Ca}. The size of the bars indicates the mean effects expressed as percentage increase over basal I_{Ca}, and the lines the S.E.M., with the number of experiments indicated above. * $P < 0.05$, statistically significant differences using Student's *t* test.

DISCUSSION

In the present study, we examined the effects of cGMP on the L-type Ca^{2+} current (I_{Ca}) in human atrial myocytes. Several main conclusions can be drawn from our experiments: (1) cGMP activates two sequential mechanisms which affect I_{Ca} in an antagonistic manner, a stimulatory mechanism activated at concentrations below 0.5 μM and an inhibitory mechanism activated at concentrations above

5 μM; (2) the regulation of I_{Ca} by cGMP is not accompanied by any modification in the voltage dependence of the Ca^{2+} current; (3) PKG does not seem to play a major role in either of these two opposite mechanisms; (4) the stimulatory effect of cGMP is due to activation of PKA resulting from a cGMP-dependent inhibition of PDE3; (5) the inhibitory effect is due to a reduction in PKA via cGMP-dependent stimulation of PDE2. We conclude that cGMP regulates I_{Ca} in human atrial myocytes by controlling the intracellular concentration of cAMP through opposing actions on PDE3 and PDE2.

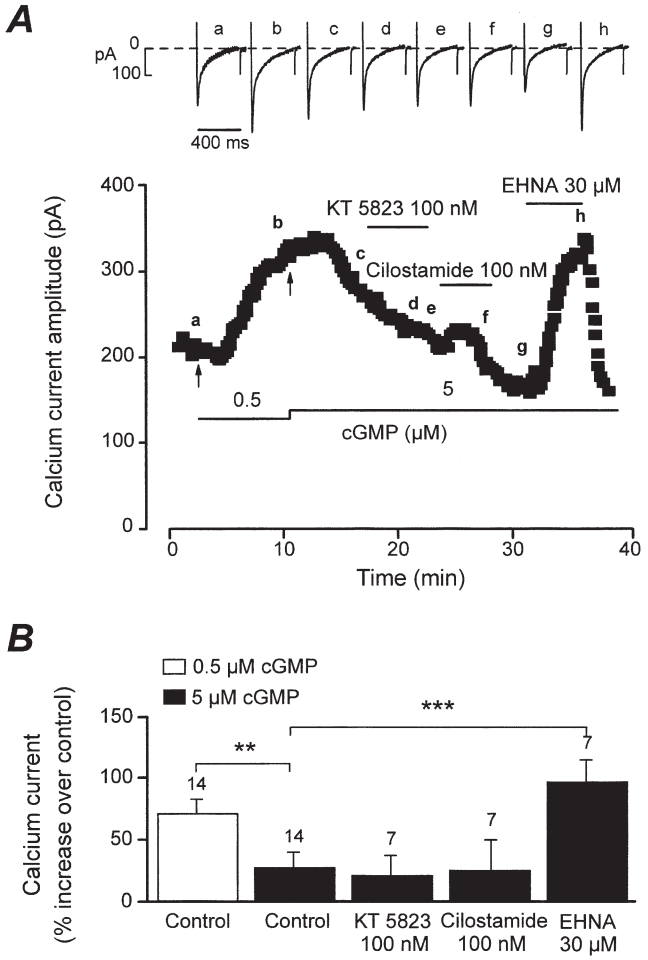

Figure 6. Contribution of PKG, PDE3 and PDE2 to the inhibitory effect of cGMP on I_{Ca} in human atrial myocytes

A, each symbol corresponds to a measure of I_{Ca} at 0 mV obtained every 8 s. The cell was first dialysed with intracellular GTP-free solution. Intracellular dialysis with 0.5 μM cGMP (first arrow) resulted in the usual stimulation of I_{Ca}, and switching to 5 μM cGMP (second arrow) partially reversed this stimulatory effect. Superfusion of the cell with KT 5823 (100 nM) or cilostamide (100 nM) had little or no effect on I_{Ca}, whereas EHNA (30 μM) restored the amplitude of the current observed in the presence of 0.5 μM cGMP. *B*, summary of several experiments similar to those shown in *A*. The effect of cGMP (0.5 μM, □; 5 μM, ■) on I_{Ca} is shown in the absence or presence of KT 5823 (100 nM), cilostamide (100 nM) or EHNA (30 μM). The size of the bars indicates the mean effects expressed as percentage increase over basal I_{Ca}, and the lines the S.E.M., with the number of experiments indicated above. ** $P < 0.01$, *** $P < 0.005$, statistically significant differences using Student's *t* test.

This study follows up and confirms earlier studies from our laboratory on the regulation of basal I_{Ca} by NO donors (Kirstein *et al.* 1995; Vandecasteele *et al.* 1998*a*) and by PDE2 in human atrial myocytes (Rivet-Bastide *et al.* 1997). In particular, we found that SIN-1 stimulates I_{Ca} in the nanomolar concentration range, an effect which is reduced when the concentration of the NO donor is increased in the micromolar range (Kirstein *et al.* 1995). The present experiments reveal that intracellular perfusion with cGMP, the second messenger of NO, produces very similar effects on I_{Ca} in human atrial myocytes.

Over the last 15 years, numerous studies have reported opposite and contradictory effects of intracellular cGMP on I_{Ca} in different cardiac preparations (for review, see Lohmann *et al.* 1991; Fischmeister & Méry, 1996). Although it is still difficult to draw a clear picture of the effects of the nucleotide on heart function, all these studies have contributed to our understanding that the cGMP signalling pathways are intimately linked to those of cAMP and involve three main enzymes, namely PDE2, PDE3 and PKG (Hove-Madsen *et al.* 1996). Thus, it is now accepted that cGMP will produce different effects depending on: (1) the presence and relative activities of these three enzymes; (2) their respective location inside the cell; (3) their respective affinities for cGMP; (4) whether adenylyl and/or guanylyl cyclases are constitutively active in the cells under study; (5) the concentration of cGMP used; (6) whether native cGMP or a cGMP analogue is used; (7) whether the effect of cGMP on I_{Ca} is examined under basal conditions or after the current has been enhanced by activation of the cAMP cascade. While the last three conditions are determined by the experimental conditions, the others are essentially determined by the animal species, the cardiac tissue, the developmental stage, and the pathophysiological condition of the preparation. For instance, cGMP stimulates basal I_{Ca} via PKG in ventricular myocytes isolated from neonatal (Kumar *et al.* 1997) and young rabbit hearts (Han *et al.* 1998), but has no effect on basal I_{Ca} in adult rabbit heart (Kumar *et al.* 1997) due to a lower expression of PKG in adult heart (Kumar *et al.* 1999). Isoprenaline-stimulated I_{Ca} is inhibited by cGMP in rat (Méry *et al.* 1991; Sumii & Sperelakis, 1995) and guinea-pig ventricular myocytes via activation of PKG (Levi *et al.* 1989), while cGMP inhibits the current via PDE2 activation in frog ventricular myocytes (Hartzell & Fischmeister, 1986).

Since PKG has been shown to mediate the stimulatory effect of cGMP on basal I_{Ca} in rabbit ventricular myocytes (Kumar *et al.* 1997; Han *et al.* 1998), we first examined whether PKG contributed to the stimulatory effect of

cGMP in human atrial myocytes. However, the negative results obtained with 8-bromo-cGMP or KT 5823 forced us to reject this hypothesis. In the case of 8-bromo-cGMP, a stimulation of I_{Ca} was observed only at 100 μM, which is a 200-fold higher concentration than necessary when using native cGMP. Nevertheless, since 8-bromo-cGMP is 10-fold more potent than cGMP in activating PKG (Butt *et al.* 1992), we suspect that the stimulation of I_{Ca} seen at such a high concentration was not due to PKG but rather to PKA activation. Indeed, 8-bromo-cGMP was shown to activate PKA with a K_d of 12 μM (Butt *et al.* 1992). In addition, 8-bromo-cGMP was shown to inhibit PDE3 with a K_i of 8 μM (Butt *et al.* 1992) which may lead to cAMP accumulation and activation of PKA (see below).

The stimulatory effect of cGMP on I_{Ca} in human atrial myocytes clearly involved activation of a cAMP-dependent phosphorylation process. Indeed, PKA inhibition with PKI completely abolished the stimulatory effect of cGMP. The PKA-mediated activation of I_{Ca} is most probably due to the phosphorylation of a subunit on the L-type Ca^{2+} channel (Gao *et al.* 1997; Bünemann *et al.* 1999; Striessnig, 1999). Interestingly, PKI not only antagonised the cGMP effect but also decreased basal I_{Ca} amplitude (see also Skeberdis *et al.* 1997). This indicates that, in human atrial myocytes, a constitutive PKA activity persists in the absence of any cAMP elevating stimulus and contributes to the basal amplitude of I_{Ca}. This constitutive PKA activity is most probably due to a substantial basal cAMP synthesis resulting from a constitutive activity of adenylyl cyclase. Indeed, acetylcholine decreases (Vandecasteele *et al.* 1998*a*) and phosphodiesterase inhibitors increase (Kirstein *et al.* 1995; Rivet-Bastide *et al.* 1997) basal I_{Ca} in this preparation.

To gain further insight into the mechanism by which cGMP enhances PKA activity, we tested the hypothesis that cGMP leads to cAMP elevation though an inhibition of PDE3. Our reasoning was that cGMP inhibits PDE3 in a submicromolar range of concentrations (Butt *et al.*1992), and also that PDE3 inhibition with milrinone mimicked the stimulatory effect of a low concentration of NO donors on I_{Ca} in human atrial myocytes (Kirstein *et al.* 1995; Vandecasteele *et al.* 1998*a*). We found indeed that cilostamide, a highly selective PDE3 inhibitor (Stoclet *et al.* 1995), mimicked the stimulatory effect of 0.5 μM cGMP on I_{Ca} and induced little additional effect in the presence of the cyclic nucleotide. Thus we conclude that cGMP stimulation of I_{Ca} in human atrium is due to inhibition of PDE3.

Surprisingly, increasing the concentration of cGMP from 0.5 to 5 μM reduced the stimulatory effect seen at the lowest concentration. This dual effect of cGMP was observed whether GTP was present or not in the pipette solution, refuting the hypothesis that a progressive reduction in the spontaneous activity of G_s proteins and adenylyl cyclase contributed to this phenomenon. But when GTP was present, the stimulatory effect of 0.5 μM cGMP on I_{Ca} was reduced approximately 2-fold, possibly because in this situation exogenous cGMP has to compete with endogenous cGMP production which is stimulated by the presence of GTP (Rivet-Bastide *et al.* 1997).

The attenuation of the stimulatory response of I_{Ca} to cGMP when the concentration of the nucleotide is increased suggests the existence of a secondary inhibitory mechanism with a lower sensitivity to cGMP than PDE3. Two possible candidates for such an inhibitory mechanism exist: (i) cGMP activation of PDE2 and (ii) cGMP activation of PKG (Lohmann *et al.* 1991; Fischmeister & Méry, 1996). Whereas PKG inhibition by KT 5823 was ineffective, PDE2 blockade by EHNA, a selective PDE2 inhibitor (Méry *et al.* 1995), fully reversed the inhibitory effect of 5 μM cGMP. Thus, PDE2 rather than PKG was responsible for the secondary inhibitory effect of cGMP on I_{Ca} in human atrial myocytes. In this respect, human atrial myocytes behave like frog ventricular myocytes where PDE2 activation is responsible for cGMP inhibition of pre-stimulated I_{Ca} (Hartzell & Fischmeister, 1986; Méry *et al.* 1995). This result was somewhat surprising because most previous electrophysiological studies suggested a predominant role of PKG in NO donor and/or cGMP inhibition of I_{Ca} in mammalian species (Thakkar *et al.* 1988; Levi *et al.* 1989; Wahler *et al.* 1990; Méry *et al.* 1991; Wahler & Dollinger, 1995; for review, see Lohmann *et al.* 1991). However, all these studies in mammalian heart were performed in ventricular tissues, so the possibility exists that atrial tissue differs from ventricular tissue in the amount of the respective cGMP target enzymes, their localisation within the cell and/or their coupling to L-type Ca^{2+} channels. The resemblance in the action of cGMP on I_{Ca} in human atrial and frog ventricular myocytes would support this hypothesis, since these two preparations share a number of similarities both on ultrastructural and functional grounds (see e.g. Morad & Cleeman, 1987).

Our demonstration that cGMP, like NO donors (Kirstein *et al.* 1995), exerts two opposite effects on I_{Ca} in human atrium may have pathophysiological relevance. Indeed, human cardiomyocytes possess a Ca^{2+}-dependent NO synthase (NOS) subtype which is constitutively expressed in endothelium (NOS3) (Wei *et al.* 1996), and are at reasonable diffusion distances from other NOS sources located in endothelial (NOS3) and neuronal cells (NOS1). Therefore, NO is likely to modulate myocardial contractility under physiological conditions (Shah & MacCarthy, 2000). Moreover, exogenous application of NO by NO donors modulates cardiac contractility (Kojda & Kottenberg, 1999; Paulus & Shah, 1999; Shah & MacCarthy, 2000), including in human atrium (Flesch *et al.* 1997). Finally, myocardial cells are exposed to high concentrations of NO upon induction of the Ca^{2+}-independent NOS (NOS2), which occurs in several pathological states, such as sepsis (Schulz *et al.* 1992; Thoenes *et al.* 1996), heart failure (De Belder *et al.* 1993; Haywood *et al.* 1996; Vejlstrup *et al.* 1998) and cardiac

allograft rejection (Yang *et al.* 1994; Paulus *et al.* 1997). Although it is likely that the cGMP-dependent alterations in I_{Ca} observed in this study contribute to the alterations in myocardial function in these pathological situations (but see Abi-Gerges *et al.* 1999), a number of other mechanisms may also be relevant, including a cGMP-dependent reduction of myofilament response to Ca^{2+} (Shah *et al.* 1994; Shah & MacCarthy, 2000), cGMP-independent effects on L-type Ca^{2+} channels (Campbell *et al.* 1996; Hu *et al.* 1997), ryanodine receptors (Xu *et al.* 1998), creatine kinase (Gross *et al.* 1996) or mitochondrial respiration (Wolin *et al.* 1997) and modulation by NO of cardiovascular reflexes (Zanzinger, 1999). Additional studies are needed to evaluate the respective contribution of each of these mechanisms to the overall cardiac response to NO.

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