Role of the NO-cGMP pathway in the muscarinic regulation of the L-type Ca 2+ current in human atrial myocytes

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1. The whole-cell patch-clamp technique was used to examine the participation of nitric oxide synthase (NOS) and soluble guanylyl cyclase in the muscarinic regulation of the L-type Ca^{2+} current (I_{Ca}) in freshly isolated human atrial myocytes.
2. Acetylcholine (ACh, 1 μM) decreased basal I_{Ca} by $39{\cdot}1 \pm 5{\cdot}5\,\%$ ($n = 8$) under control conditions, and by $38{\cdot}0 \pm 6{\cdot}1\,\%$ ($n = 6$) in the presence of 1*H*-[1,2,4]oxadiazolo[4,3-*a*]-quinoxaline-1-one (ODQ, 10 μM), a potent guanylyl cyclase inhibitor, and N^G-monomethyl-L-arginine (L-NMMA, 1 mM), a competitive NOS inhibitor. L-NMMA alone had no effect on I_{Ca}, whilst ODQ increased I_{Ca} in 50 % of the cells.
3. The accentuated antagonism of ACh on I_{Ca}, i.e. its ability to antagonize the stimulatory effect of β-adrenergic agonists and, by extension, of other cAMP-elevating agents, was examined after the current was stimulated by either the β-adrenergic agonist isoprenaline (Iso) or serotonin (5-HT). ACh (100 nM or 1 μM) completely blocked the stimulatory effects of 10 nM Iso or 10 nM 5-HT on I_{Ca}.
4. Extracellular application of Methylene Blue (MBlue, 10 μM), a guanylyl cyclase inhibitor, antagonized the inhibitory effect of 1 μM ACh on Iso- or 5-HT-stimulated I_{Ca}. However, this effect was overcome by a 100-fold higher ACh concentration and was not mimicked by an intracellular application of MBlue.
5. Inhibition of NOS and soluble guanylyl cyclase activities by addition of ODQ (10 μM) and L-NMMA (1 mM) to both extracellular and intracellular solutions, or by a 2 h pre-incubation of the cells with these inhibitors, modified neither the Iso (10 nM) response nor the inhibitory effect of ACh (100 nM or 1 μM) on Iso-stimulated I_{Ca}.
6. Extracellular application of the NO donor SNAP (*S*-nitroso-*N*-acetyl-D,L-penicillamine) at 100 nM produced a stimulatory effect on I_{Ca} in control conditions. This stimulatory effect was abolished by intracellular MBlue (20 μM) or by intracellular and extracellular application of ODQ (10 μM) in combination with L-NMMA (1 mM).
7. We conclude that the NO–cGMP pathway does not contribute significantly to the muscarinic regulation of I_{Ca} in human atrial myocytes.

The cardiac L-type calcium current (I_{Ca}) plays a determinant role in the dual regulation of the inotropic state of the heart by the autonomic nervous system (McDonald, Pelzer, Trautwein & Pelzer, 1994). Whereas considerable evidence supports the view that the cAMP cascade is the unique route for the β-adrenoceptor-mediated sympathetic stimulation of I_{Ca} (reviewed in Hove-Madsen, Méry, Jurevicius, Skeberdis & Fischmeister, 1996), the intracellular signalling pathways that underlie the acetylcholine (ACh)-mediated parasympathetic antagonism of the β-adrenergic response, i.e. the indirect negative inotropic effect of vagal stimulation, are less clear (reviewed in Méry, Abi-Gerges, Vandecasteele, Jurevicius, Eschenhagen & Fischmeister, 1997).

The cardiac negative inotropic effect of ACh is generally accompanied by a reduction in the concentration of cAMP (Fleming, Strawbridge & Watanabe, 1987). This effect mainly results from an inhibition of adenylyl cyclase via a pertussis toxin-sensitive G protein (Breitwieser & Szabo, 1985; Hescheler, Kameyama & Trautwein, 1986; Fleming *et al.* 1987; Jurevicius & Fischmeister, 1996). However, other mechanisms such as a muscarinic activation of a cyclic nucleotide phosphodiesterase or activation of a phosphatase have also been documented in heart muscle (for a brief review, see Méry *et al.* 1997). Moreover, ACh and other muscarinic receptor agonists have been shown to elevate cGMP levels in the heart muscle (George, Polson, O'Toole

& Goldberg, 1970) as well as in isolated cardiac myocytes (Stein, Drögemüller, Mülsch, Schmitz & Scholz, 1993; MacDonnel, Tibbits & Diamond, 1995). The recent discovery of the existence of a nitric oxide synthase (NOS) in a variety of cardiac cells including cardiomyocytes (Balligand *et al.* 1995; Kelly, Balligand & Smith, 1996) led to the hypothesis that ACh-mediated increases in cGMP level might result from a stimulation of NOS activity (Kelly *et al.* 1996).

Since cGMP and nitric oxide (NO) donors were shown to produce negative inotropic effects and an inhibition of I_{Ca} in several cardiac preparations (for review see Fischmeister & Méry, 1996), it was tempting to speculate that the NO–cGMP pathway might participate in the cardiac effects of ACh. This hypothesis was recently confirmed in cardiac preparations from different animal species using inhibitors of the NO–cGMP pathway which were found to attenuate or even abolish the inhibitory response to muscarinic agonists (Balligand, Kelly, Mardsen, Smith & Michel, 1993; Han, Shimoni & Giles, 1994, 1995; Levi, Alloatti, Penna & Gallo, 1994; Balligand *et al.* 1995; Wang & Lipsius, 1995; Han, Kobzik, Balligand, Kelly & Smith, 1996). However, this hypothesis was contradicted in several other cardiac preparations in which the muscarinic response was found to be insensitive to these inhibitors (Stein *et al.* 1993; Kennedy, Hicks, Brian & Seiffen, 1994; Nawrath, Baumner, Rupp & Oelert, 1995; Habuchi, Nishio, Tanaka, Yamamoto, Lu & Yoshimura, 1996; Méry, Hove-Madsen, Chesnais, Hartzell & Fischmeister, 1996; Abi-Gerges, Méry & Fischmeister, 1997*c*). For example, in human papillary muscle strips, the anti-adrenergic effect of carbachol remained unaffected by a pre-treatment of the preparation with the competitive NOS inhibitor N^G-monomethyl-L-arginine (L-NMMA), or the guanylyl cyclase inhibitor Methylene Blue (MBlue: Kilter *et al.* 1995). Moreover, in human atria, where transcripts of the constitutive endothelial NOS (NOS 3) were detected by *in situ* hybridization (see Kelly *et al.* 1996), the participation of this enzyme in the muscarinic response was challenged by the observation that ACh inhibits contraction in this preparation (Böhm, Gierschik, Schwinger, Uhlmann & Erdmann, 1994) but NO donors have no effect (Nawrath *et al.* 1995) or even stimulate I_{Ca} in isolated human atrial myocytes (Kirstein, Rivet-Bastide, Hatem, Bénardeau, Mercadier & Fischmeister, 1995).

To get further insights into the mechanism of action of ACh in the human heart, we have re-examined in the present study the effect of ACh on I_{Ca} recorded in freshly isolated human atrial myocytes using the whole-cell patch-clamp technique. The effects of ACh on basal and stimulated I_{Ca} were investigated in the absence or presence of extracellular and/or intracellular L-NMMA (a NOS inhibitor) and of two guanylyl cyclase inhibitors, MBlue and 1*H*-[1,2,4]oxadiazolo-[4,3-*a*]quinoxaline-1-one (ODQ). This allowed us to examine whether NO and/or cGMP participate in the muscarinic regulation of basal and phosphorylated I_{Ca}.

Preliminary results have appeared in abstract form (Vandecasteele, Eschenhagen & Fischmeister, 1997).

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 twenty-two patients (aged 8–78 years) undergoing heart surgery for congenital defects, coronary artery diseases or valve replacement at the Hôpital Marie-Lannelongue, Le Plessis-Robinson, France. Most patients received a pharmacological pre-treatment (Ca^{2+} channel blockers, digitalis/glycosides, β-adrenergic antagonists, diuretics, angiotensin converting enzyme (ACE) inhibitors, NO donors and/or anti-arrhythmic drugs). In addition, all patients received sedatives, anaesthesia, and antibiotics. Four patients did not receive any cardiovascular pharmacotherapy. We found no obvious correlation between the calcium current density or the effects on I_{Ca} of the drugs tested here and the therapy received (if any) by the patient. Dissociation of the cells was realized immediately after surgery.

Human atrial cell dissociation

Myocytes were isolated as described previously (Kirstein *et al.* 1995) with some modifications. Briefly, after excision of the atrial tissue, the tissue was cut up and washed in a calcium-free Tyrode solution supplemented with 30 mM 2,3-butanedionemonoxime for 10 min and then incubated in the same solution containing 40 i.u. ml^{-1} collagenase, 15 i.u. ml^{-1} protease and 5 mg ml^{-1} BSA for 30 min. The solution was then replaced by fresh enzymatic solution containing only collagenase for 15–60 min until a satisfactory cell yield was obtained. All steps were carried out at 37 °C, with continuous gassing with 95% O_2 and 5% CO_2. The digestion mix was then poured on a nylon filter (pore diameter, 250 μm) in order to separate the dissociated myocytes from the non-digested part of the tissue. The resulting cell suspension was then centrifuged, and the pellet resuspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, non-essential amino acids, insulin (1 nM) and antibiotics (penicillin, 100 i.u. ml^{-1} and streptomycin, 0·1 μg ml^{-1}). For patch-clamp experiments, 100–200 μl of this cell suspension were put in a Petri dish containing control external solution.

Electrophysiological experiments

The whole-cell configuration of the patch-clamp technique (Hamill, Marty, Neher, Sakmann & Sigworth, 1981) was used to record the high-threshold calcium current (I_{Ca}) on Ca^{2+}-tolerant human atrial myocytes. In the routine protocols the cells were depolarized 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^+. Occasionally (as in Fig. 2), the cell was held at −80 mV and depolarized to −50 mV for 50 ms, prior to the test pulse to 0 mV. 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 filtered at 3 kHz and sampled at a frequency of 10 kHz using a 12-bit analog-to-digital converter (DT2827, Data Translation, Marlboro, MA, USA) connected to a PC-compatible computer (386/33 System-pro, Compaq, Houston, TX, USA). While all points were used for data analysis (see below),

only one out of four points was used for the drawings of current traces (Figs 1 and 2). All experiments were done at room temperature (19–25 °C).

Solutions

Control external solution contained (mM): 107·1 NaCl, 10 Hepes, 40 CsCl, 4 $NaHCO_3$, 0·8 NaH_2PO_4, 1·8 $CaCl_2$, 1·8 $MgCl_2$, 5 D-glucose, 5 sodium pyruvate, pH adjusted to 7·4 with NaOH. Control or drug-containing solutions were applied to the exterior of the cell, at a flow rate of ~10 μl min^{-1}, by placing the cell at the opening of a 250 μm (inner diameter) capillary tube. Patch electrodes (0·8–1·5 MΩ) were filled with control internal solution that contained (mM): 119·8 CsCl, 5 EGTA (acid form), 4 $MgCl_2$, 5 creatine phosphate disodium salt, 3·1 Na_2-ATP, 0·42 Na_2-GTP, 10 Hepes, 62 μM $CaCl_2$ (pCa, 8·5), pH adjusted to 7·3 with CsOH. In some experiments, MBlue (10 μM) or L-NMMA (1 mM) + ODQ (10 μM) were added to the intracellular solution.

Materials

DMEM was obtained from Gibco-BRL. L-NMMA, ODQ and SNAP were from Tocris Cookson (Bristol, UK). All other drugs, including collagenase Type V and protease Type XXIV were purchased from Sigma. Stock solution of ODQ (100 mM) was made in DMSO. All other drugs were dissolved in ionic aqueous solutions. All stock solutions were made fresh daily and kept at 4 °C until use.

Data analysis

The maximum amplitude of I_{Ca} was measured as the difference between the peak inward current and the end-pulse current (I_{400}), i.e. 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 resistance were measured by exponential analysis of current responses to 1 mV step changes in membrane potential. Membrane capacitance was 64·6 ± 3·1 pF (mean ± S.E.M.) and series resistance was 4·3 ± 0·3 MΩ ($n = 47$). The on-line analysis was made possible by programming a PC-compatible computer in PASCAL language to determine, for each depolarization, peak and steady-state current values.

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, i.e. 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.

RESULTS

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 3–15 min after patch break to allow for equilibration between intracellular and pipette solutions. When the cells were superfused with control external solution and the patch pipette was filled with control internal solution, basal I_{Ca} amplitude at 0 mV membrane potential was on average −257 ± 32 pA, and I_{Ca} density, which represents the ratio of I_{Ca} amplitude to membrane capacitance, was −4·1 ± 0·5 pA pF^{-1} ($n = 28$). As in our previous studies (Kirstein *et al.* 1995; Rivet-Bastide, Vandecasteele, Hatem, Bénardeau, Mercadier & Fischmeister, 1997), 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.

Effect of L-NMMA and ODQ on basal I_{Ca}

To examine whether NOS and/or guanylyl cyclase activities modulate basal I_{Ca}, the cells were exposed to L-NMMA and ODQ. Superfusion of human atrial myocytes with 1 mM L-NMMA did not significantly modify the basal amplitude of I_{Ca} (−4·6 ± 3·5% variation, $n = 4$; data not shown). The effect of 10 μM ODQ was examined in eight atrial myocytes and showed a high variability. In four cells, ODQ produced an increase of basal I_{Ca} ranging from 12 to 104% above the control amplitude, whereas ODQ had no effect in the four other cells. Consequently, the mean effect of ODQ on the eight cells (+22·8 ± 13·7% increase over basal amplitude) was not statistically significant ($P = 0·66$). Thus, in our experimental conditions, NOS does not participate in the regulation of basal I_{Ca}, whereas soluble guanylyl cyclase may play a role, at least in some cells.

ACh inhibition of basal I_{Ca} in the presence of L-NMMA and ODQ

Figure 1 shows the effect of ACh (1 μM) on basal I_{Ca} in a human atrial myocyte. The effect of ACh was first examined under control conditions. Superfusion of the cell with a 1 μM concentration of the muscarinic agonist decreased the amplitude of I_{Ca} from −100 to −80 pA within 2–3 min. Upon washout of ACh, I_{Ca} increased rapidly to a value which was ~20% above control, and this rebound was followed by a progressive reduction towards a new steady-state amplitude, which was reached after ~5 min. The cell was subsequently exposed to 1 mM L-NMMA for 3 min and then to a combination of L-NMMA plus 10 μM ODQ for the rest of the experiment. As shown in Fig. 1, exposure of the cell to L-NMMA or to L-NMMA + ODQ had no apparent effect on I_{Ca}. In addition, it did not prevent either the inhibitory effect of ACh, or the rebound stimulation upon ACh washout elicited by a second application and washout of the agonist in the continuing presence of L-NMMA and ODQ. In a total of eight experiments, ACh (1 μM) alone decreased basal I_{Ca} by 39 ± 7%. In six of them, the effect of 1 μM ACh was tested in the presence of extracellular L-NMMA (1 mM) + ODQ (10 μM) and similarly decreased I_{Ca} (38 ± 6%). In these eight cells, a rebound stimulation of I_{Ca} upon ACh washout was observed in only five cells, and this phenomenon was also not modified by the presence of the NOS and guanylyl cyclase inhibitors. Thus, inhibition of NOS and soluble guanylyl cyclase activities does not modify the muscarinic regulation of basal I_{Ca} in human atrial myocytes.

Accentuated antagonism of ACh on stimulated I_{Ca}

In the ventricular muscle, activation of muscarinic receptors has relatively little inhibitory effect on the force of contraction and Ca^{2+} current amplitude in the absence of β-adrenergic stimulation. However, activation of muscarinic receptors after a stimulation of the β-adrenergic receptors

leads to a profound inhibition of the β-response. As shown in Fig. 2, this phenomenon, referred to as accentuated antagonism of ACh, is also present in human atrial myocytes. In the experiment shown in Fig. 2*A*, 10 nM of the non-selective β-adrenergic agonist isoprenaline (Iso) produced an ~3-fold increase in I_{Ca}. This stimulation was completely abolished by the addition of 100 nM ACh to the Iso-containing extracellular solution (inhibition of the β-response was 106%). The inhibitory effect of ACh was due to activation of muscarinic receptors, since application of 1 μM atropine, a muscarinic receptor antagonist, antagonized most of the response to ACh ($88 \pm 6\%$, $n = 3$). Activation of β-adrenergic receptors was not a prerequisite for the accentuated antagonism of ACh to take place. Indeed, as shown in Fig. 2*B*, application of ACh (1 μM) also fully antagonized (102% inhibition) the 5-HT_4 receptor-mediated stimulatory effect induced by serotonin (5-HT, 10 nM) on I_{Ca} (Ouadid, Seguin, Dumuis, Bockaert & Nargeot, 1991). On average, 5-HT (10 nM) and Iso (10 nM) increased I_{Ca} by $+376 \pm 77\%$ ($n = 3$) and $+170 \pm 22\%$ ($n = 14$) over basal I_{Ca} amplitude, respectively, and both responses were fully antagonized by 1 μM ACh (to, respectively, $-19 \pm 27\%$ ($n = 3$) and $+4 \pm 6\%$ ($n = 3$) of basal amplitude).

Accentuated antagonism of ACh in the presence of Methylene Blue

To determine whether a soluble guanylyl cyclase activity was implicated in the accentuated antagonism of ACh on I_{Ca}, we examined the effect of ACh in the presence of Methylene Blue (MBlue), a classical inhibitor of this enzyme (for review, see Tremblay, Gerzer & Hamet, 1988). In the experiment shown in Fig. 3*A*, I_{Ca} was stimulated by 10 nM Iso. After I_{Ca} reached its maximal amplitude, 10 μM MBlue was added to the Iso-containing solution, which had little effect on the current. However, the presence of MBlue prevented the inhibitory effect of 1 μM ACh added subsequently to the solution. MBlue inhibited the effect of ACh in a competitive manner, since addition of ACh at a concentration of 100 μM overcame the inhibitory effect of 10 μM MBlue, and I_{Ca} was decreased to basal level. Figure 3*B* summarizes the results of three similar experiments. On average, Iso (10 nM) enhanced the current by 116 ± 26 and $123 \pm 16\%$ in the absence and presence of 10 μM MBlue, respectively, demonstrating that the guanylyl cyclase inhibitor did not interfere with the β-adrenergic response. The subsequent addition of ACh at 1 μM in the

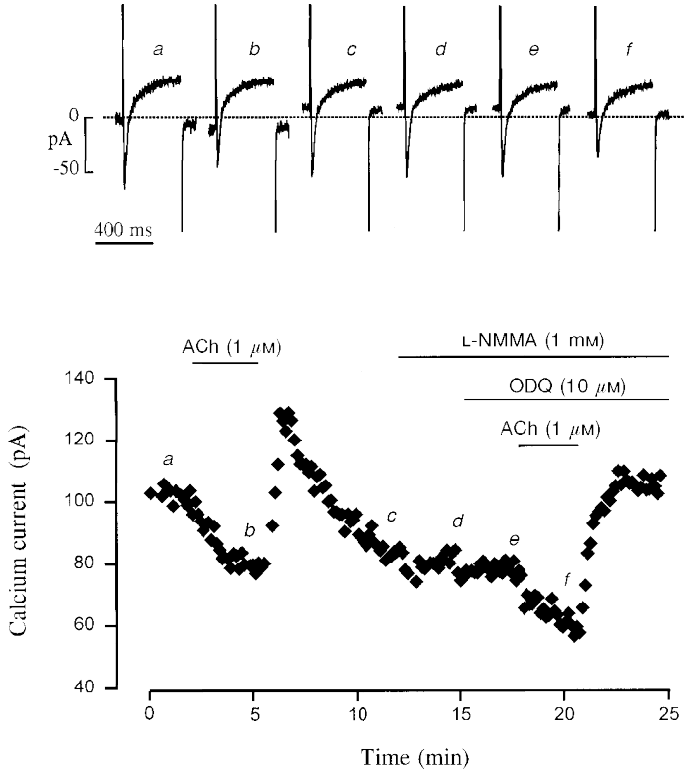

Figure 1. Effect of ACh on basal I_{Ca} in a human atrial myocyte

Each symbol on the graph corresponds to a measure of I_{Ca} at 0 mV, obtained every 8 s. The cell was first superfused with control solution and then exposed to the drugs during the periods indicated by the bars. 1 μM ACh alone, or together with ODQ (10 μM) + L-NMMA (1 mM) produced the same inhibition of I_{Ca}, which was followed in both cases by a rebound stimulation of the current. The individual current traces shown in the upper part of the figure were obtained at the times indicated by the corresponding letters in the bottom graph. The dotted line indicates the zero current level.

presence of MBlue produced a slight decrease in I_{Ca} (to $100 \pm 34\%$ above control level) which was not statistically significant. However, a 100-fold higher concentration of the muscarinic agonist clearly inhibited I_{Ca} to $-15 \pm 6\%$ below basal amplitude.

MBlue was shown recently to behave as a muscarinic receptor antagonist in frog and rat cardiac myocytes (Abi-Gerges, Eschenhagen, Hove-Madsen, Fischmeister & Méry, 1997*a*; Abi-Gerges, Hove-Madsen, Fischmeister & Méry, 1997*b*). Thus, the possibility exists that the antagonistic effect of MBlue on the muscarinic inhibition of I_{Ca} in human atrial myocytes seen here was not due to guanylyl cyclase inhibition but rather to the binding of MBlue to the muscarinic receptor. In order to test this hypothesis, we performed experiments in which MBlue was applied intracellularly to exclude any direct interaction of MBlue with the agonist-binding site on the muscarinic receptors. To do this, the patch-pipette was filled with an intracellular

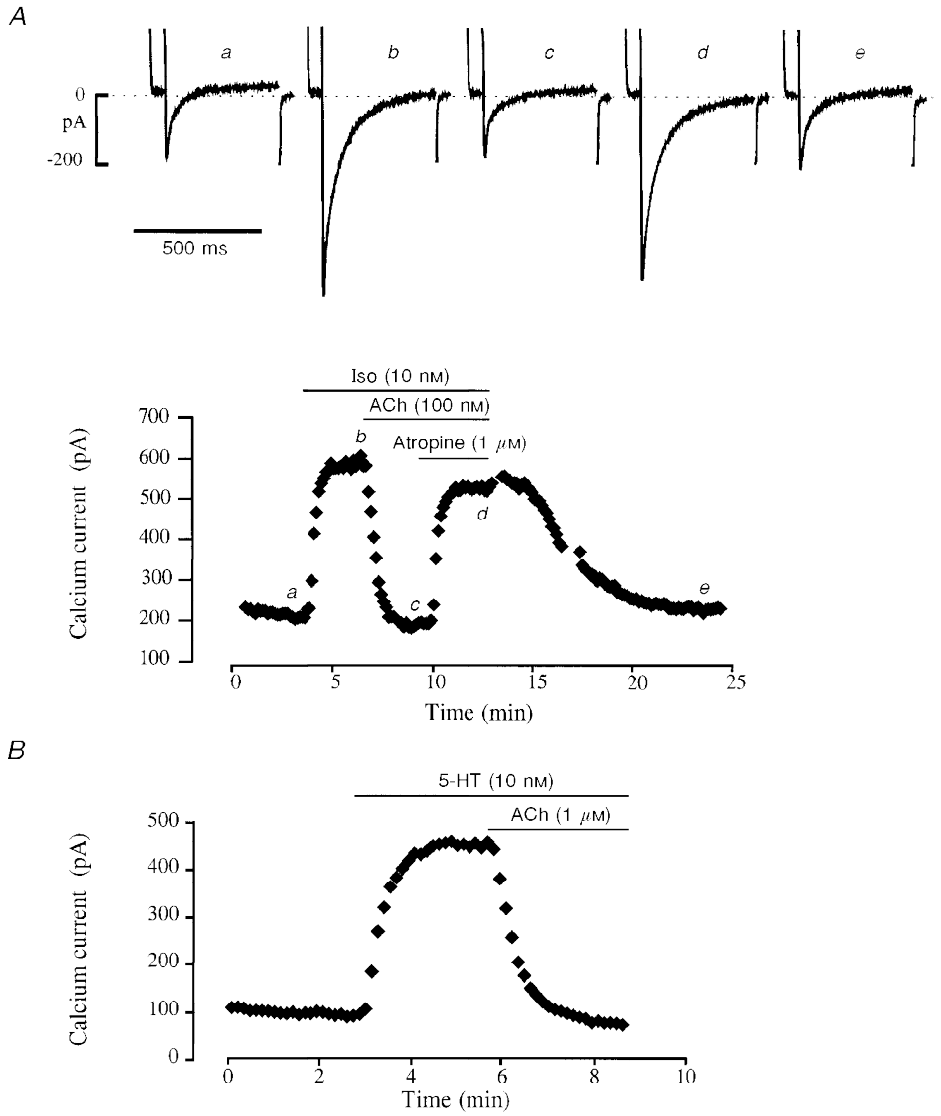

Figure 2. Accentuated antagonism of ACh on I_{Ca} in human atrial myocytes

The cells were first superfused with control solution and then exposed to the drugs during the periods indicated in the graphs by the bars. *A*, superfusion of the cell with 10 nM Iso produced a nearly maximal stimulation of I_{Ca}, which was fully abolished by 100 nM ACh. Addition of the muscarinic antagonist atropine (1 μM) restored I_{Ca} to an amplitude which was close to the Iso-stimulated level. Upon washout of the drugs and return to control conditions, I_{Ca} slowly recovered its control amplitude. The individual current traces shown in the upper part of the figure were obtained at the times indicated by the corresponding letters in the graph. The dotted line indicates the zero current level. *B*, application of 10 nM 5-HT to another myocyte also produced a strong stimulation of I_{Ca}. Application of 1 μM ACh in the continuous presence of 5-HT induced a complete inhibition of the serotoninergic response.

solution which contained 20 μM MBlue. As shown in Fig. 4*A*, the presence of MBlue in the intracellular solution did not interfere with the 5-HT stimulatory effect on I_{Ca}. On average (Fig. 4*B*), 10 nM 5-HT increased I_{Ca} by $251 \pm 9\%$ ($n = 5$) in the presence of intracellular MBlue. In contrast to the previous experiments, the presence of intracellular MBlue did not prevent the inhibitory effect of 1 μM ACh. In the experiment shown in Fig. 4*A*, 1 μM ACh completely and reversibly inhibited the 5-HT response of I_{Ca}. This result was confirmed in a total of five identical experiments (Fig. 4*B*) where the 5-HT stimulation of I_{Ca} was fully antagonized by 1 μM ACh in the presence of intracellular MBlue. In these experiments, 5-HT (10 nM)-stimulated I_{Ca} was decreased to a value which was $-18 \pm 18\%$ of basal amplitude after addition of 1 μM ACh, an effect which was not different from the effect of ACh in the absence of MBlue (Fig. 4*B*). The inhibitory effect of ACh (1 μM) was also complete in three cells perfused with intracellular MBlue where Iso (10 nM) was used instead of 5-HT to stimulate I_{Ca} (data not shown).

Accentuated antagonism of ACh in the presence of ODQ and L-NMMA

The lack of effect of intracellular MBlue on the inhibition of I_{Ca} by ACh suggests that this effect is independent of its action on soluble guanylyl cyclase. However, MBlue may not be the most efficient guanylyl cyclase inhibitor. Unlike MBlue, ODQ is not a superoxide anion generator, and inhibits, selectively, the NO-sensitive isoform of guanylyl cyclase (Garthwaite, Southam, Boulton, Nielsen, Schmidt & Mayer, 1995; Abi-Gerges *et al.* 1997*a*). Thus, in the following experiments, we re-examined the effect of ACh in human atrial cells in the presence of ODQ. Moreover, to eliminate a possible NO-dependent, guanylyl cyclase-independent effect of ACh, the cells were also exposed to L-NMMA, a competitive NOS inhibitor. Figure 5*A* shows a typical experiment in which ODQ (10 μM) and L-NMMA (1 mM) were introduced both in the intracellular and extracellular solutions. As shown, application of 10 nM Iso produced a large increase in I_{Ca} in the presence of the two

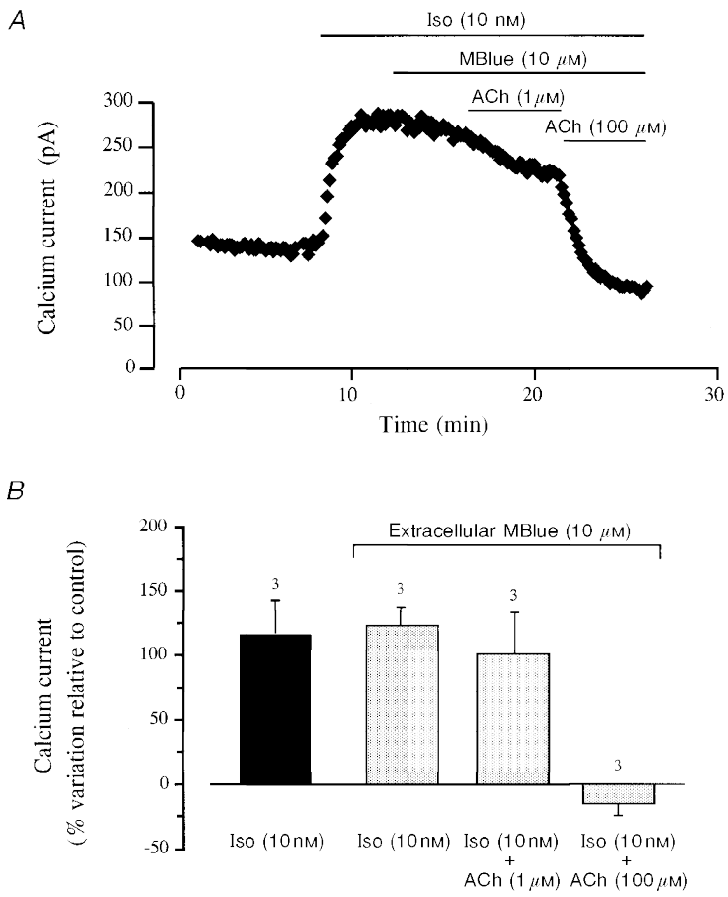

Figure 3. Accentuated antagonism of ACh on I_{Ca} in the presence of extracellular Methylene Blue
A, the cell was superfused for several minutes with a control solution and then challenged with different drugs during the periods indicated by the bars. Application of 10 nM Iso induced an ~2-fold increase in I_{Ca}, which was unchanged when the cell was superfused with the same solution containing 10 μM MBlue. In the presence of MBlue, 1 μM ACh had only a weak inhibitory effect on I_{Ca}, whilst 100 μM ACh completely blocked the Iso response. *B*, summary of the results of three experiments similar to that shown in *A* (means ± S.E.M.; number of experiments indicated above the vertical columns).

inhibitors. The mean stimulation of I_{Ca} by 10 nM Iso was not different in control experiments (+170 ± 22% above basal I_{Ca} amplitude, $n = 14$) and in the presence of ODQ and L-NMMA (+205 ± 38%, $n = 11$; Fig. 5*B*). Figure 5*A* also shows that the presence of ODQ and L-NMMA did not interfere with the muscarinic regulation of I_{Ca}. Indeed, addition of 1 μM ACh fully antagonized the Iso-response in the presence of the two inhibitors. The summary data shown in Fig. 5*B* indicate that for two different concentrations of ACh (100 nM and 1 μM), the presence of intracellular and extracellular ODQ (10 μM) and L-NMMA (1 mM) did not significantly modify the inhibitory effect of the muscarinic agonist.

Although L-NMMA is supposed to act rapidly (Han *et al.* 1995), the possibility remained that, in the relative short time of a patch-clamp experiment (usually around 20 min), ODQ and/or L-NMMA were unable to completely block guanylyl cyclase and/or NOS activity. Thus, a residual activity of these enzymes might remain in the presence of the two inhibitors, which could be sufficient to support the action of ACh on I_{Ca}. To test this hypothesis, we pre-incubated the cells with ODQ (10 μM) and L-NMMA (1 mM) for at least 2 h before the beginning of the patch-clamp experiment. Furthermore, the two inhibitors remained present in both intracellular and extracellular solutions throughout the experiments. However, this protocol did not prevent the accentuated antagonism of ACh (1 μM) on Iso (10 nM)-enhanced I_{Ca}. Indeed, as illustrated in Fig. 5*B*, in three cells taken from three different patients, the mean stimulatory effect of Iso (10 nM) was a 231 ± 111% increase above basal amplitude, and ACh (1 μM) reduced the current to a value which was −2 ± 18% below basal amplitude.

Positive control for the efficiency of MBlue and ODQ

Although unlikely, the above results do not rule out the possibility that MBlue and/or ODQ are ineffective in blocking NO-mediated pathways in human atrial myocytes under our experimental conditions. To address this question specifically, we have examined the effect of MBlue and ODQ

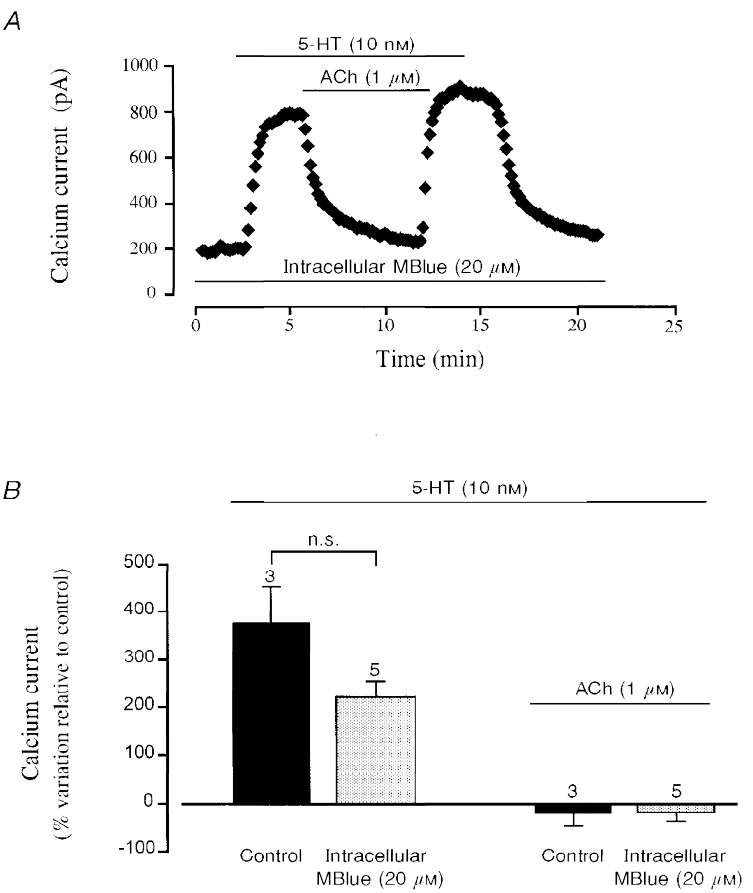

Figure 4. Accentuated antagonism of ACh on I_{Ca} in the presence of intracellular Methylene Blue
A, the cell was internally perfused with a pipette solution containing 20 μM MBlue throughout the entire experiment, as indicated by the bar. After superfusion of the cell with control solution, the cell was exposed to 5-HT (10 nM) and subsequently to the same solution containing 1 μM ACh during the periods indicated by the upper bars. *B*, comparison of the effects of 5-HT and ACh in the absence (Control) and presence of 20 μM intracellular MBlue (means ± S.E.M.; number of experiments indicated above the vertical columns). n.s., not significant.

on the regulation of I_{Ca} by NO. We have shown previously that the NO donor SIN-1 (3-morpholino-sydnonimine) stimulates basal I_{Ca} in human atrial myocytes under control conditions (Kirstein *et al.* 1995). This effect is probably due to an increase in intracellular cGMP concentration, since it is mimicked by intracellular dialysis of cGMP (Rivet-Bastide *et al.* 1997). Here, we tested the effect of another NO donor, SNAP (*S*-nitroso-*N*-acetyl-D,L-penicillamine) on I_{Ca} in human atrial myocytes. Extracellular application of 100 nM SNAP increased I_{Ca} by $52 \pm 13\%$ above basal level ($n = 16$, $P < 0{\cdot}01$; data not shown) under control conditions. However, when 20 μM MBlue was dialysed inside the cells, the same concentration of SNAP had no significant effect on I_{Ca} ($12{\cdot}9 \pm 9{\cdot}3\%$ increase above basal I_{Ca}, $P = 0{\cdot}14$, $n = 7$). Similarly, the stimulatory effect of SNAP was abolished in the presence of intracellular and extracellular ODQ (10 μM) and L-NMMA (1 mM) ($1{\cdot}4 \pm 16{\cdot}8\%$ increase above basal I_{Ca}, $n = 4$).

DISCUSSION

In the present study, we used several inhibitors of the NO–cGMP pathway to examine its role in the effect of ACh on the L-type calcium current in human atrial myocytes. The main conclusion of our study is that neither NOS nor guanylyl cyclase activity appeared to be involved in the muscarinic regulation of I_{Ca} in this preparation.

The constitutive form of endothelial NOS (NOS 3 or eNOS) was found recently to be expressed in intact myocytes from rat ventricular and human atrial tissues (Balligand *et al.* 1995; Kelly *et al.* 1996). Since NO donors modulate cardiac I_{Ca} in human heart (Kirstein *et al.* 1995), as in a number of other animal species (Fischmeister & Méry, 1996), it was legitimate to question the potential role of NOS activity in the regulation of I_{Ca}. For this reason, we investigated the effect of L-NMMA on I_{Ca} in human atrial myocytes. L-NMMA is an L-arginine analogue that was shown to fully

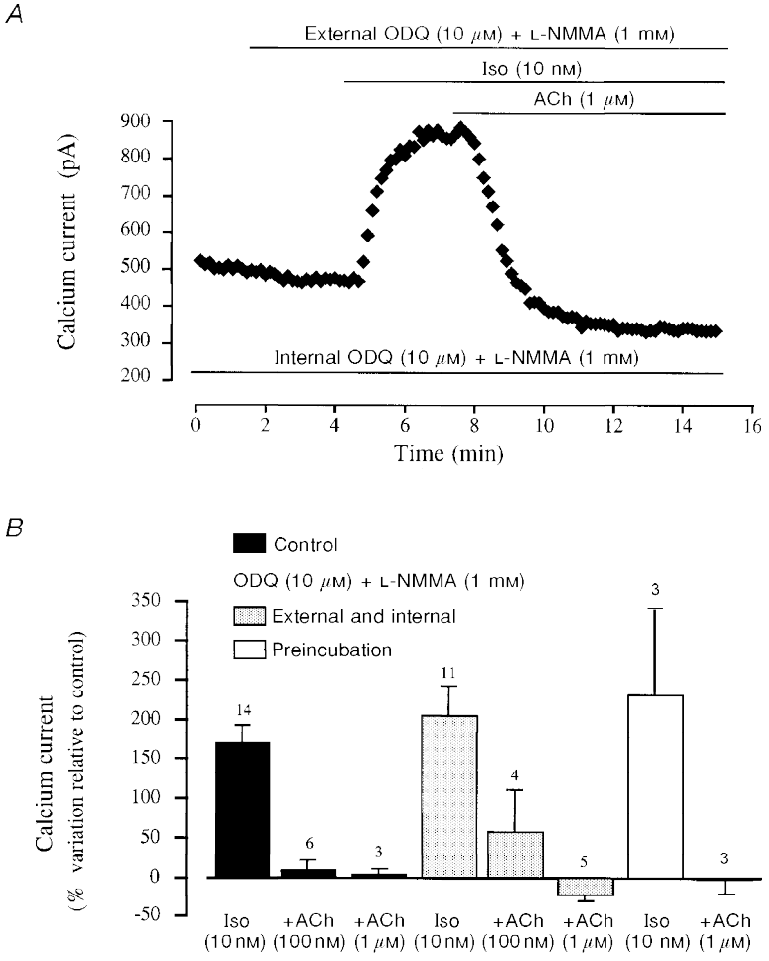

Figure 5. Accentuated antagonism of ACh on I_{Ca} in the presence of ODQ and L-NMMA

A, ODQ (10 μM) and L-NMMA (1 mM) were added to the pipette solution (lowermost bar) throughout the entire experiment, and applied extracellularly during the period indicated by the uppermost bar. The cell was then superfused with Iso (10 nM) and Iso + ACh (1 μM). *B*, comparison of the effect of Iso (10 nM) and ACh (100 nM and 1 μM) in the absence (Control, ■) or presence of intracellular and extracellular ODQ (10 μM) and L-NMMA (1 mM) (▨), and after > 2 h preincubation of the cells with these compounds and introduction of the compounds in both extracellular and intracellular solutions (□). (Means ± S.E.M.; number of experiments indicated above the vertical columns.)

inhibit both the constitutive and inducible forms of NOS at submillimolar concentrations (Marletta, 1994). We used L-NMMA at a high concentration (1 mM) to ensure a complete inhibition of NOS activity (Marletta, 1994). However, we did not observe any effect on basal I_{Ca} in human atrial myocytes. This suggests that, either NOS activity is absent in our experimental conditions, or it is not functionally coupled to L-type Ca^{2+} channels in the absence of any exogenous modulator.

One main effector of NO in cardiac myocytes is a soluble form of guanylyl cyclase. Since cGMP was shown to regulate cardiac I_{Ca} in a number of animal species including human (Rivet-Bastide *et al.* 1997), it was of interest to examine whether an endogenous production of cGMP might regulate basal I_{Ca}. Recently, we found that inhibition of cGMP-stimulated phosphodiesterase (PDE2), one of the main targets of intracellular cGMP in heart (Fischmeister & Méry, 1996), with erythro-9-[2-hydroxy-3-nonyl]-adenine (EHNA) stimulates basal I_{Ca} in human atrial myocytes (Rivet-Bastide *et al.* 1997). Since the stimulatory effect of EHNA on I_{Ca} required the presence of intracellular GTP (the substrate of guanylyl cyclase), it was concluded that human atrial myocytes possess a basal guanylyl cyclase activity that provides a sufficient amount of cGMP to stimulate PDE2 (Rivet-Bastide *et al.* 1997). Inhibition of guanylyl cyclase should lead to an effect similar to PDE2 inhibition, i.e. to a stimulation of I_{Ca}. However, cardiac myocytes possess other cGMP-effectors besides PDE2, including a cGMP-inhibited phosphodiesterase (PDE3) and a cGMP-dependent protein kinase (PKG), which regulate I_{Ca} in different directions (Fischmeister & Méry, 1996; Méry *et al.* 1997). In the present study, we found that inhibition of soluble guanylyl cyclase with ODQ induced a variable stimulatory effect on basal I_{Ca} in 50 % of human atrial cells and had no effect in the other cells. We used a 10 μM concentration, which was ~500 times higher than the IC_{50} reported by Garthwaite *et al.* (1995) for the inhibition of soluble guanylyl cyclase in slices of cerebellum. At the same concentration, ODQ had no effect on basal, Iso-, forskolin- or cAMP-stimulated I_{Ca} in frog ventricular myocytes but only reversed the inhibitory effect of NO donors on Iso-stimulated I_{Ca} (Abi-Gerges *et al.* 1997*b*). These results in frog and the stimulatory effect of ODQ observed in half of the cells in human suggests that ODQ was also acting as a guanylyl cyclase inhibitor in human atrial myocytes, but that a cell-to-cell variability may exist with respect to the relative efficacy of the different cGMP effectors; namely PDE2, PDE3 and PKG, and/or their coupling to Ca^{2+} channels (Rivet-Bastide *et al.* 1997).

Our finding that ACh inhibits basal I_{Ca} in human atrial myocytes may indicate that the intracellular concentration of cAMP, in the absence of any agonist, is sufficient to ensure a substantial phosphorylation of the L-type Ca^{2+} channels through cAMP-dependent protein kinase. Abrupt washout of ACh elicited a rebound stimulation of I_{Ca}, a phenomenon already observed by Wang & Lipsius (1995) in cat atrial myocytes, and by Tareen, Ono, Noma & Ehara (1991) on the Iso-stimulated Cl^- current in guinea-pig ventricular myocytes. However, in our study, the rebound was not systematically observed in human atrial myocytes. Moreover, in contrast with cat atrial myocytes (Wang & Lipsius, 1995), superfusion of human atrial cells with ODQ and L-NMMA did not modify the inhibitory effect of ACh or the rebound stimulation (when present). Thus, NOS and guanylyl cyclase activities do not seem to play an important role in the regulation of basal I_{Ca} by muscarinic receptors in human atrial myocytes.

The finding that, in ventricular myocytes from frog (Hartzell & Fischmeister, 1986) and rat (Méry *et al.* 1991), cGMP antagonizes the effect of a β-adrenergic stimulation on I_{Ca} suggested a potential role for cGMP in the anti-adrenergic effect of ACh. However, studies specifically addressing this question led to conflicting results (see Méry *et al.* 1997). Since some of the discrepancies may be due to species differences, it was important to test this hypothesis directly in human tissue. In human atrial myocytes, it was reported that both 5-HT and Iso produce a similar maximal increase in I_{Ca}, and that 5-HT_4 receptors, like β-adrenergic receptors, mediate their effect via cAMP and cAMP-dependent protein kinase (Ouadid *et al.* 1991). Our results showing that ACh antagonized in a similar manner both 5-HT- and Iso-stimulation of I_{Ca} confirm these findings. Extracellular application of MBlue, a soluble guanylyl cyclase inhibitor, had no effect on the β-adrenergic stimulation of I_{Ca}. However, at the same concentration (10 μM), MBlue prevented the accentuated antagonism of ACh on I_{Ca} in human atrial myocytes. This is similar to results obtained in guinea-pig (Levi *et al.* 1994) and rat (Balligand *et al.* 1993) ventricular myocytes as well as in rabbit nodal myocytes (Han *et al.* 1995, 1996). However, this antagonistic effect of MBlue is unlikely to be due to guanylyl cyclase inhibition since it was not mimicked by an intracellular perfusion with MBlue. Moreover, MBlue was shown recently to bind to muscarinic M_2 receptors in rat cardiac myocytes (Abi-Gerges *et al.* 1997*a*). Thus, the anti- muscarinic effect of extracellular MBlue seen here is probably due to this detrimental side effect of the drug. In a second series of experiments, we used ODQ, a more selective guanylyl cyclase inhibitor (Garthwaite *et al.* 1995; Abi-Gerges *et al.* 1997*b*), in combination with L-NMMA to inhibit the two key enzymes of the NO–cGMP pathway. However, no difference in the β-adrenergic stimulation or in the accentuated antagonism of ACh was found in comparison with control experiments. In several studies in which the muscarinic regulation of I_{Ca} was found to depend upon NO (Han *et al.* 1994, 1995, 1996), pre-incubation of the myocytes with NOS antagonists was used. However, incubation of human atrial myocytes with ODQ and L-NMMA for as long as 2 h before the experiment did not attenuate the efficacy of ACh. Finally, the lack of effect of MBlue and ODQ on the muscarinic response of I_{Ca} was not due to an inefficiency of these compounds in blocking NO-mediated pathways, since both drugs antagonized the stimulatory effect of the NO donor SNAP on I_{Ca}.

In conclusion, our present results demonstrate that NOS and guanylyl cyclase activities do not play a determinant role in the muscarinic regulation of basal and stimulated I_{Ca} in human atrial myocytes. This conclusion may not only apply to atrial tissue since Kilter *et al.* (1995) reported recently that pre-treatment of human papillary muscle strips with L-NMMA or MBlue modified neither the basal force of contraction, nor the positive inotropic effect of Iso, nor the anti-adrenergic effect of carbachol. Moreover, a similar conclusion was reached recently in a study performed in frog ventricular myocytes (Méry *et al.* 1996). The discrepancy with other results may partly be due to species differences in the respective contributions of the NO–cGMP pathway and the inhibition of adenylyl cyclase (Hescheler *et al.* 1986; Jurevicius & Fischmeister, 1996) to the signalling cascades mediated by activation of cardiac muscarinic receptors. Nevertheless, species- and tissue-independent discrepancies remain (Balligand *et al.* 1993; Habuchi *et al.* 1996; Han *et al.* 1996; Abi-Gerges *et al.* 1997*c*) that argue against a determinant role of the NO–cGMP pathway for the muscarinic control of the heart.

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