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Role of nitric oxide/cyclic GMP in myocardial adenosine A_1 receptor-inotropic response

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1 In this study we have determined the different signalling pathways involved in adenosine A_1-receptor (A_1-receptor)-dependent inhibition of contractility in rat isolated atria.

2 N-cyclopentyladenosine (CPA) stimulation of A_1-receptor exerts: negative inotropic response, inositol phosphates accumulation, stimulation of nitric oxide synthase (NOS), increased production of nitric oxide (NO) and cyclic GMP.

3 Inhibitors of phospholipase C (PLC), protein kinase C (PKC), calcium/calmodulin, NOS and guanylate cyclase shifted the dose-response curve of CPA on contractility to the right. Those inhibitors also attenuated the A_1-receptor-dependent increase in cyclic GMP and activation of NOS.

4 These results suggest that CPA activation of A_1-receptors exerts a negative inotropic effect associated with increased production of nitric oxide and cyclic GMP. The mechanism appears to occur secondarily to stimulation of phosphoinositide turnover *via* PLC activation. This, in turn, triggers cascade reactions involving calcium/calmodulin and PKC, leading to activation of NOS and soluble guanylate cyclase.

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Abbreviations: A_1-receptor, adenosine A_1 receptor; A_2-receptor, adenosine A_2 receptor; NO, nitric oxide; NOS; nitric oxide synthase; PLC, phospholipase C; PKC, protein kinase C; 6-0HDA, 6-hydroxydopamine

Introduction

Adenosine is recognized as an important regulator of myocardial function providing myocardial protection during time of stress. Studies on paced rat left atria have demonstrated that adenosine compounds caused a dual inotropic effect: first a rapid decrease in contractility and second an increase in contractile tension. The negative inotropism was mediated by direct stimulation on A_1-adenosine receptors (A_1) and the positive inotropism by activation on A_2-adenosine receptors (A_2) (Froldi *et al*., 1994). Adenosine effects on myocardium are mediate predominantly by the type A_1 receptor and the effect were demonstrated in different mammalian preparations including human isolated cardiac preparations (de jong *et al*., 2000). An indirect anti ß-adrenergic effect of A_1 receptor activation on guinea-pig ventricular myocytes was also showed (Zhang *et al*., 2001).

Studies on signalling events coupled to adenosine receptor subtypes has revealed differences in the abilities to modulate many different signal transduction pathways. The signal transduction process subsequent to agonist binding to A_1 receptors include the pertussis-toxin sensitive G-protein (Endoh *et al*., 1993) that mediate the decrement of cyclic AMP production (Stein *et al*., 1994) while A_2 receptors increase intracellular levels of cyclic AMP *via* a pertussis-toxin insensitive Gs protein (Kitakaze *et al*., 1993; Stein *et al*., 1993; 1994). Neither the cyclic AMP elevating A_2 receptors (Stein *et al*., 1993; 1994) nor the cyclic AMP reducing A_1 receptors (Endoh *et al*., 1993) appears to be associated with changes in the direct inotropic effect of the specific receptor agonists. However, the anti ß-adrenergic effect of A_1 receptor stimulation is linked to a pertussis-toxin sensitive Gi protein (Zhang *et al*., 2001).

Thus, the signal transduction mechanism that mediate the action of A_1 receptor agonists on heart contractility have not been clearly defined. It is possible that A_1 receptor was coupled to more than one G protein regulating signal transduction pathways.

It is demonstrated that A_1 receptor activation decrease the basal influx of calcium acting on the L-type calcium channel current, but simultaneously elevating intracellular calcium concentration (Eckert *et al*., 1993). These responses were not sensitive to pertussis-toxin, but were reduced by guanosine-diphosphate- beta sulphate and by intracellular calcium release blockers; whereas intracellularly applied inositol-1,4,5-tris-phosphate (IP_3) mimicked the A_1 receptor activation (Eckert *et al*., 1993). These findings suggest a non-sensitive pertussis toxin-A_1 receptor dependent activation of cyclic GMP synthesis with increase in intracellular calcium.

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Among the mechanisms involved in the activation of receptor coupled to G proteins that activate cyclic GMP synthesis, an increase intracellular calcium are those mediated by phospholipase C (PLC) activation followed by generation of IP_3, which releases calcium from intracellular calcium stores, that in turn triggers cascade reactions involving calcium/calmodulin-dependent nitric oxide synthase (NOS) that leads to activation of soluble guanylate cyclase (Sterin-Borda *et al*., 1995).

It has been reported that myocytes produced endogenous nitric oxide (NO) and some of the physiological effect of NO appear to be mediated by activation of guanylate cyclase (Balligand *et al*., 1993; Sterin-Borda *et al*., 1995). Moreover, A_1 receptor activation and NO release from preconditioned heart have been implicated in protecting the myocardium from subsequent ischemic events (Liu *et al*., 1991; Vegh *et al*., 1992).

The aim of the present study was to determine the different signalling events involved in the A_1 receptor dependent inhibition of contractility in rat isolated atria. Therefore, we investigated whether (i) CPA-stimulation of A_1 receptor excerted negative inotropic response, stimulation of phosphoinositide (PI) turnover, activation of NOS and increase production of NO and cyclic GMP; (ii) if there is an association between PLC, PKC and calcium/calmodulin system with NOS activation, and (iii) if the negative inotropic effect of A_1 receptor activation is related with NOS activation and cyclic GMP accumulation at low concentration of CPA.

Methods

Animals

Adult male Wistar strain rats (250–300 g) were used. The animals housed in standard environmental conditions were fed with a commercial pelleted diet and water *ad libitum*. Experimental protocol were performed following the Guide to The Care and Use of Experimental Animals (DHEW Publication, NIH 80-23).

Radioligand binding assay

Membranes were prepared as described previously (Goin *et al*., 1994). In brief, atria were homogenized in an Ultraturrax at 4°C in 6 vol of potassium phosphate buffer, 1 mM $MgCl_2$, 0.25 M sucrose (buffer A) pH 7.5 supplemented with 0.1 mM phenylmethylsulphonylfluoride (PMSF), 1 mM EDTA, 5 μg ml^{-1} leupeptin, 1 μM bacitracin and 1 μM pepstatin A. The homogenate was centrifuged for 10 min at $3\,000 \times g$, then at $10{,}000 \times g$ and $40{,}000 \times g$ at 4°C for 15 and 90 min, respectively. The resulting pellets were resuspended in 50 mM phosphate buffer with the same protease inhibitors pH 7.5 (buffer B). Receptor ligand binding was performed as described previously (Bacman *et al*., 1990). Aliquots of the membrane suspension (30–50 μg protein) were incubated with different concentrations of [^{3}H]-cyclopentyl 1,3-dipropilxantine ([^{3}H]-DPCPX) (Tocris, Sp. Act: 98.2 Ci mmol) for 60 min at 25°C in a total volume of 150 μl of buffer B. Binding was stopped by adding 2 ml ice-cold buffer followed by rapid filtration (Whatman GF/c). Filters were rinsed with 12 ml of ice-cold buffer, transferred into vials containing 10 ml of scintillation cocktail and counted in a liquid scintillation spectrometer. Nonspecific binding was determined in the presence of 5×10^{-6} M DPCPX and never exceeded 10% of total binding. Radioactivity bound was lower than 10% of total counts.

Atrial preparation for contractility

Male Wistar rats were killed by decapitation. The left atria were carefully dissected from the ventricles, attached to a glass holder and immersed in a tissue bath containing Krebs–Ringer Bicarbonate (KRB) solution gassed with 5% CO_2 in oxygen and maintained at pH 7.4 and 30°C. KRB solution was composed as described previously (Sterin-Borda *et al*., 1986). A preload tension of 750 mg was applied to the atria and tissues were allowed to equilibrate for 1 h. The initial control values for contractile tension of the isolated atria were recorded by use of a force transducer coupled to an ink writing oscillograph (Borda *et al*., 1984). The preparations were paced with a bipolar electrode and an SK4 Grass Stimulator. The stimuli had a duration of 2 ms and the voltage was 10% above threshold. Inotropic effects (dF/dt) were assessed by recording the maximum rate of isometric force development during electrical stimulation at a fixed frequency of 150 beats min^{-1}. Control values (=100%) refer to the dF/dt before the addition of drugs. The absolute value for dF/dt at the end of the equilibration period (60 min) was 7.8 ± 0.5 g s^{-1}. Cumulative dose-response curves were obtained according to the method of Van Rossum (1963). A maximal effect was achieved within 5 min after each dose. Dose-response curves of CPA were done on untreated atria and those from chemically sympathectomized rats injected intravenously 24 h prior to sacrifice with 6-hydroxydopamine (6-OHDA) (16.5 mg Kg^{-1}). In order to assess an adequate denervation, the *in vitro* influence of tyramine (10^{-6} M) and norepinephrine (NE) (10^{-8} M) were assayed. As expected 6-OHDA-treated atria showed supersensitive to NE and refractory to tyramine (Sterin-Borda *et al*., 1996a).

Measurement of total labelled inositol phosphates (IPs)

Rat atria were incubated for 120 min in 0.5 ml of KRB gassed with 5% CO_2 in O_2 with 1 μCi [myo-^{3}H]-inositol ([^{3}H]-MI) (Sp. Act. 15 Ci mmol^{-1}) from Dupont/New England Nuclear. LiCl (10 mM) was added for inositol monophosphate accumulation, according to the technique of Berridge *et al*. (1982). CPA was added 30 min before the end of the incubation period and the blockers 30 min before the addition of CPA. Water-soluble IPs were extracted after 120 min incubation as previously described (Sterin-Borda *et al*., 1995). Determination of nitric oxide synthase activity

Nitric oxide synthase (NOS) activity was measured in atria by production of [U^{-14}C]-citrulline from [U^{-14}C]-arginine according to the procedure described by Bredt & Snyder (1989) for brain slices and by Sterin-Borda *et al*. (1995) for rat atria. Briefly, after 20 min preincubation in KRB solution, atria were transferred to 500 μl of prewarmed KRB equilibrated with 5% CO_2 in O_2 in the presence of [U-^{14}C]-arginine (0.5 μCi). Appropriate concentrations of drugs were added and the atria were incubated for 20 min under 5% CO_2 in O_2 at 37°C. Measurement of basal NOS activity in whole atria by the above mentioned procedure was inhibited 95% in the presence of 0.5 mM N^G-monomethyl-L-arginine (L-NMMA). The results (pmol g^{-1} tissue wet wt) obtained for whole atria were expressed

Table 1 Action of adenosine receptor antagonist on the inotropic negative effect of adenosine receptor agonists

Drugs	*Kd* (1×10^{-8} M)	*n*
CPA	0.8 ± 0.06	8
DPCPX + CPA	120.3 ± 4.1*	7
DMPX + CPA	1.3 ± 0.2	6
Atropine + CPA	1.0 ± 0.1	6
CPCA	80.3 ± 0.5*	6

Atria were preincubated for 30 min in the absence or in the presence of 5×10^{-8} M DPCPX (A_1 antagonist) or 5×10^{-7}M DMPX (A_2 antagonist) or atropine (5×10^{-7}M) before concentration-response curves to CPA (A_1 agonist) or CPCA (A_2 agonist) were constructed. Values are mean$\pm$ s.e.mean of n experiments of each group. *Statistically different from CPA value with $P<0.001$.

as the difference between values in the absence (252 ± 12; $n=9$) and in the presence (12 ± 2, $n=9$) of L-NMMA.

Cyclic GMP assay

Tissues were incubated in 1 ml KRB for 30 min and CPA was added in the last 5 min. When blockers were used, they were added 25 min before the addition of CPA. After incubation, atria were homogenized in 2 ml of absolute ethanol and centrifuged at $6000\times g$ for 15 min at 4°C. Pellets were then rehomogenized in ethanol-water (2 : 1) and supernatants collected and evaporated to dryness as indicated above. Cyclic GMP in the residue was dissolved in 400 μl of 0.05 M sodium acetate buffer pH 6.2. Aliquots of 100 μl were taken for the nucleotide determination using RIA procedure with a cyclic GMP ^{125}I-RIA KIT from Dupont/New England Nuclear.

Drugs

N-cyclopentyladenosine (CPA), 8-cyclopentyl-1,3-dipropylxantine (DPCPX), 5′-(N-cyclopropyl)-carboxamidoadenosine (CPCA), and 3,4-dimethyl-1-propargylxanthine (DMPX) were purchased from RBI; Atropine, L-arginine, N^G-monomethyl-L-arginine (L-NMMA), trifluoperazine (TFP) and staurosporine from Sigma Chemical Company; 1-6-17ß-3-methoxgestra-1,3,5 (10)-trien-17yl-aminohexyl-1-H-pirrole-2,5-dione (U-73122) from ICN Pharmaceuticals Inc; 1H-[1,2,4]-oxadiazola-[4,3-2]-quinoxaline-1-one (ODQ) from Tocris Cookson Inc. Stock solutions were freshly prepared in the corresponding buffers. The drugs were diluted in the bath to achieve the final concentration stated in the text.

Statistical analysis

Student's *t*-test for unpaired values was used to determine the levels of significance. When multiple comparisons were necessary, after analysis of variance, the Student-Newman-Keuls test was applied. Differences between means were considered significant if $P<0.05$.

Results

To assess the adenosine receptor subtypes involved in the inotropic negative effect of adenosine in rat atria, the concentration response curves of both A_1 (CPA) and A_2 (CPCA) agonists were performed. Table 1 shows that the potency (Kd) of CPA was greater than CPCA, while the efficacy (E_{max}) was similar (100% of inhibition). Moreover, a selective A_1 antagonist DPCPX could antagonize the negative inotropism of CPA, but the A_2 antagonist DMPX and atropine were without effects. It is note that the effect of CPA was not modified when experiments were carried out on atria

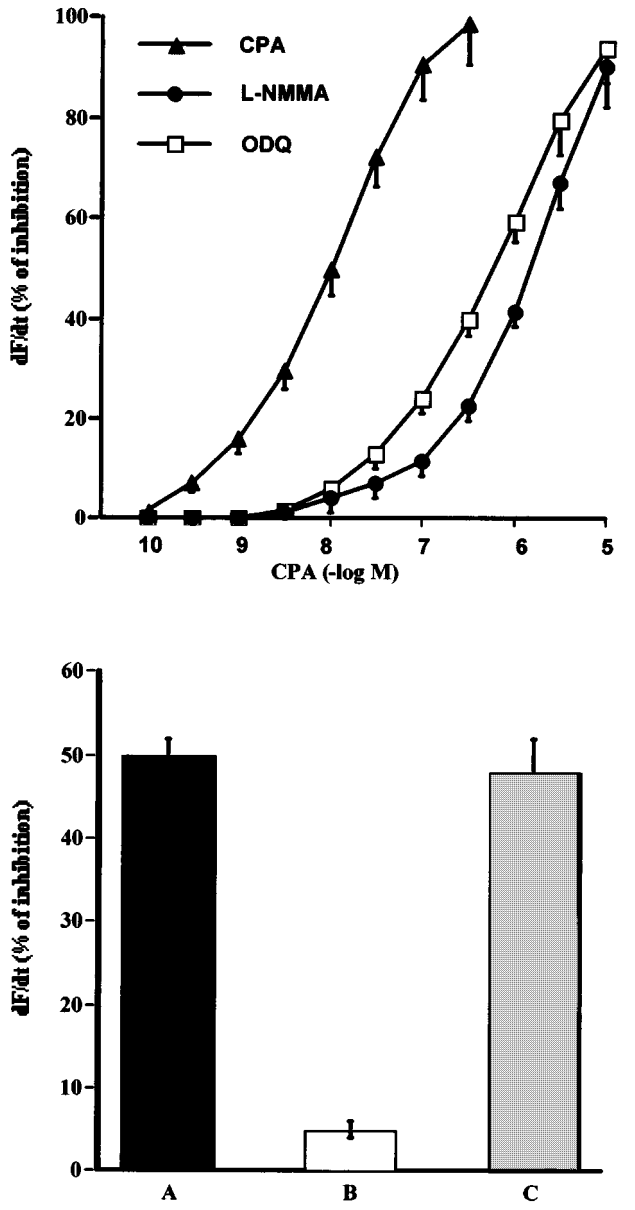

Figure 1 Decrease in contractility (dF/dt) of rat isolated atria by increasing concentrations of CPA (upper panel). The inhibitory action of L-NMMA (2×10^{-6} M) and ODQ (5×10^{-5} M) is also shown. Inhibition of negative inotropic effect of 1×10^{-8} M CPA (A) by treatment of atria with 2×10^{-6} M L-NMMA (B) and reversal of inhibition by L-arginine 1×10^{-5} M (C) (lower panel). Values represent the mean$\pm$s.e.mean of six different determinations in each group. Tissues were incubated for 30 min in presence or absence of different enzymatic inhibition and then CPA was added. Values are expressed as percentage changes calculated by comparison with the absolute values prior to the addition of CPA. No inotropic effects were observed with either inhibitors or L-arginine at the concentrations used.

from rats that had been chemically sympathectomized with 6-OHDA (normal: Kd 8.5×10^{-9} M; 6-OHDA 8.7×10^{-9} M; $n=5$). Results show that the negative inotropism is a direct effect triggered by adenosine receptor activation mediated preferentially by A_1 receptor subtype. The binding characteristics of [^{3}H] DPCPX in rat atria calculated by scatchard plots were Kd: 1.35 ± 0.08 nmol l^{-1} and Bmax: 24.2 ± 2.6 fmol mg^{-1} protein ($n=9$). [^{3}H] DPCPX binding to atria membranes was a saturable process to a single binding site and reversible manner with high affinity.

To determine if an endogenous NO signalling system participates in the contractile action of CPA, rat isolated atria were incubated with different inhibitors of the enzymatic pathways involved in the activation of NO and cyclic GMP synthesis. As can be seen in Figure 1 upper panel, the inhibition of NOS activity by L-NMMA (2×10^{-6} M) (Sterin-Borda *et al*., 1995) or the selective inhibition of NO-sensitive guanylyl cyclase by ODQ (5×10^{-5} M) (Garthwaite *et al*., 1995) shifted to the right the dose response curve of CPA. The inhibitory action of L-NMMA on the A_1 receptor agonist effect was reversed by L-arginine (Figure 1 lower panel).

To determine the participation of PLC in the negative inotropic effect of CPA, the action of U-73122 (5×10^{-6} M) (Smith *et al*., 1996) was explored. As can be seen in Figure 2, the inhibition of PLC shifted to the right the dose-response curve of the A_1 receptor agonist. To elucidate which pathways gated by PI turnover could be involved in this effect, atria were incubated in the presence of an inhibitor of protein-kinase C (PKC), staurosporine (1×10^{-9} M) (Sterin-Borda *et al*., 1996b) and an inhibitor of calcium-calmodulin (TFP, 5×10^{-6} M) (Sterin-Borda *et al*., 1995). Figure 2 shows that in the presence of either staurosporine or TFP the dose-response curve of CPA was also shifted to the right.

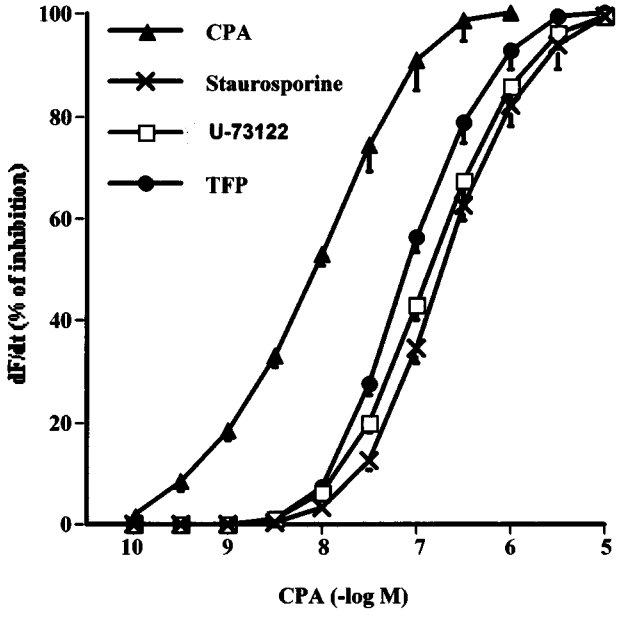

Figure 2 Effect of 5×10^{-6} M of U-73122, 5×10^{-6} M TFP and 1×10^{-9} M staurosporine on the dose-response curve of CPA upon atria *dF*/*dt*. Tissues were incubated for 30 min in presence or absence of different inhibitors and then the dose-response curves to CPA were obtained. Values represent the mean$\pm$s.e.mean seven experiments in each group. For more details see Figure 1.

To assess if A_1 receptor is coupled to PI turnover in rat atria, the effect of CPA in the presence and absence of A_1 receptor antagonist was investigated. As can be seen in Table 2, 1×10^{-8} M of CPA (at the EC_{50}, which induced the inotropic negative effect) increased PI turnover. The specific A_1 antagonist DPCPX but not DMPX (A_2 antagonist), significantly inhibited the stimulatory action of CPA. As a control, U-73122 abrogated this effect, indicating the PLC activation would be implicated in A_1 receptor activation of CPA upon PI hydrolysis.

The A_1 receptor agonist also increased in a concentration dependent manner the NOS activity and cyclic GMP production in rat isolated atria (Figure 3). But, while NOS activity showed a bell-shaped curve, cyclic GMP did not follow this scheme. Determination of nitrate/nitrite by Griess reaction in the incubation media followed similar profile (basal: 5.2 ± 0.3; 10^{-8} M CPA: 11.2 ± 0.4; 10^{-6} M CPA: 4.3 ± 0.2 μM/100 μl, $n=5$). So the increment on cyclic GMP accumulation triggered by high CPA concentrations did not reflect parallel change in NOS activity and in cardiac NO release. The inhibition of NOS activity by L-NMMA (2×10^{-6} M) or the selective inhibition of NO-sensitive guanylyl cyclase by ODQ (5×10^{-5} M); blocked the action of CPA upon the enzymes (Figure 3). To verify the nature of the mechanism by which the activation of A_1 receptor increased cyclic GMP synthesis and NOS activity, rat atria were incubated with several inhibitors. Table 3 shows that DPCPX, but not atropine or DMPX inhibited the stimulatory action of CPA on both NOS activity and cyclic GMP production. Also the inhibition of PLC (by U-73122), calcium-calmodulin (by TFP), and PKC (by staurosporine) attenuated the A_1 receptor-dependent activation of cyclic GMP production and NOS activity. As expected, L-NMMA (5×10^{-5} M) inhibited 95% basal NOS activity and was reversed by L-arginine (5×10^{-4} M) (see Methods). On the other hand, ODQ (5×10^{-5} M) blunted cyclic GMP-CPA stimulation. Atropine (1×10^{-7} M) was without any effect. All inhibitors at the concentration used, had not effect *per se* on basal values of PI hydrolysis (Table 2), NOS activity and cyclic GMP levels (Table 3) or cardiac contractility (data not shown). It is important to note that on contractile

Table 2 Effect of CPA upon phosphoinositide (PI) turnover

Drugs	*PI* (area units mg wet weight)
None (basal)	145 ± 6
CPA	210 ± 8*
DPCPX + CPA	139 ± 7
U-73122 + CPA	137 ± 6
DMPX + CPA	217 ± 7*
DPCPX	132 ± 5
U-73122	135 ± 7
DMPX	139 ± 5

Atria were incubated for 60 min with myo-[2-^{3}H]inositol and for additional 30 min in the presence of DPCPX (5×10^{-8} M), DMPX (5×10^{-7}M), U-73122 (5×10^{-6}M). Tissue were then left for a further 40 min in the absence (basal) or presence of 1×10^{-8} M CPA. Results correspond to the second peak. Values are mean$\pm$s.e.means of six experiments in each group performed by duplicates. *Statistically different from basal values ($P<0.001$).

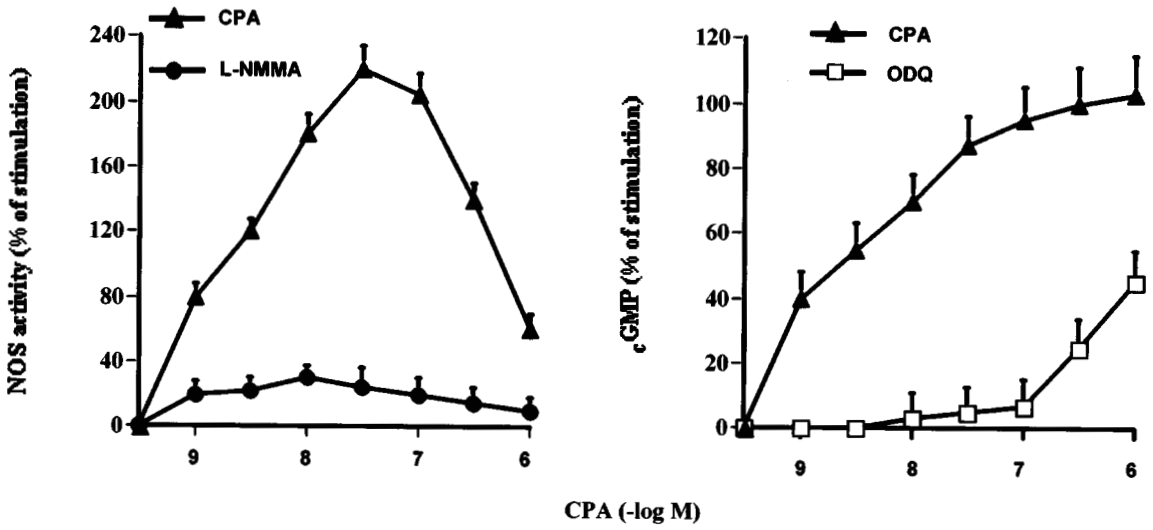

Figure 3 Increasing in NOS activity and cyclic GMP accumulation by increasing concentration of CPA. Inhibition of CPA effects by treatment atria with L-NMMA (2×10^{-6} M) or ODQ 5×10^{-5} M. Values are expressed as percentage of changes calculated by comparison with the absolute values prior to the addition of CPA. Values are mean ± s.e.mean of eight different determination in each group. For other details see legend of Table 3.

Table 3 Effect of CPA upon NOS activity and cyclic GMP production by rat isolated atria: influence of enzymatic inhibitors.

Drugs	*NOS activity* (pmol g^{-1} tissue wet wt)	*CGMP* (pmol g^{-1} tissue wet wt)
None (basal)	152 ± 12	49 ± 3
CPA	456 ± 18*	84 ± 4*
DPCPX + CPA	201 ± 13	52 ± 4
DPCPX	148 ± 9	52 ± 4
Atropine + CPA	450 ± 19*	82 ± 5*
Atropine	147 ± 8	47 ± 2
DMPX + CPA	461 ± 21*	86 ± 5*
DMPX	150 ± 10	51 ± 4
U-73122 + CPA	263 ± 13	52 ± 4
U-73122	145 ± 8	45 ± 2
TFP + CPA	391 ± 14	51 ± 3
TFP	143 ± 6	48 ± 3
Staurosporine + CPA	282 ± 12	53 ± 3
Staurosporine	161 ± 13	52 ± 3
L-NMMA + CPA	12 ± 2	50 ± 4
L-NMMA	8 ± 3	39 ± 5
L-NMMA + L-arginine + CPA	492 ± 20*	96 ± 6*
ODQ + CPA	435 ± 15*	47 ± 2
ODQ	153 ± 10	45 ± 2

Nitric oxide synthase (NOS) activity and cyclic GMP were measured incubating atria with or without enzymatic inhibitors for 20 min and then for additional 10 min with 1×10^{-8}M CPA. Values are mean ± s.e.means of six experiments performed in duplicate in each group. The final concentration of inhibitors were: DPCPX: 5×10^{-8} M; DMPX: 5×10^{-7} M; atropine: 1×10^{-7} M; L-NMMA: 5×10^{-5} M; U-73122 and TFP: 5×10^{-6} M; Staurospine: 1×10^{-9} M; L-arginine: 5×10^{-4} M and ODQ: 5×10^{-5} M. *Statistically different from basal values (none) with $P<0.001$.

experiments L-NMMA was used at 2×10^{-6} M, concentration that inhibited by 57% basal NOS activity (basal: 153 ± 11; basal + L-NMMA 2×10^{-6} M: 86.6 ± 0.5, $n=6$) but did not modify basal dF/dt values.

Discussion

The current studies give a new insight into the pathway by which NO and cyclic GMP are involved in the A_1 receptor-mediated negative inotropic effect of CPA in rat isolated atria. The results point to a role for PKC and calcium mobilization in the rapid activation of NOS and the accumulation of cyclic GMP. The presence of A_1 receptors on rat atria membranes which activation triggered negative inotropism on rat atria was demonstrated (de Jong *et al*., 2000); being CPA the most potent functional agonist (Gurden *et al*., 1993).

Several results support these conclusions: the concentration response curve of the CPA acting on A_1 receptor were shifted to the right when either NOS or soluble guanylate cyclase activity were inhibited. The mechanism seems to involve an increase of PI hydrolysis, whose intermediates would turn on a calcium/calmodulin constitutive NOS; since agents known to interfere with PLC and with calcium mobilization also cause a right-ward shift of the negative inotropic effect of CPA. The inhibition of PKC activity exerted the same effect.

In support of this, CPA activation of A_1 receptor led to increased inositol phosphate, cyclic GMP accumulation and NOS activation. The CPA-induced contractile response NOS activity and cyclic GMP production were blunted by the stereospecific NOS inhibitors and by the selective inhibition of NO-sensitive guanylate cyclase at low concentrations of the A_1 receptor agonist. The nature of this inhibition suggest that NO-cyclic GMP-mediate pathway predominate at low doses of CPA, while more than one signaling cascade accounts for the maximal negative inotropic effect of the A_1 receptor agonist in rat atria (Cinel & Gur, 2000; Zhang *et al*., 2001). Martynyuk *et al*. (1997) have shown that the inhibitory effect of adenosine could be related to a mechanism involving the interaction with superoxide anion in addition to the NO and cyclic GMP pathway. The lack of the correlation between myocardial A_1 receptor stimulation

promonted increase in NOS activation and NO release with cyclic GMP accumulation could indicate that cardiac cyclic GMP content does not necesarilly reflect changes in cardiac NO (Csont *et al*., 1998). The physiological effect of NO are believed to be mediated by cyclic GMP, however recent studies suggested that the glyceryl trinitrate derived NO induced direct cardioprotective effect involves a cyclic GMP-independent activation of K_{ATP} in the isolated rat heart (Csont *et al*., 1999).

It is known that myocardial contractility is regulated by changes in the free intracellular calcium concentrations which is determined by concerted interaction of calcium influx through voltage dependent calcium channel, release of calcium from intracellular pools and calcium extrusion systems (Xu & Narayanan, 2000). Adenosine through A_1 receptors decreases the L-type calcium channel but simultaneously elevate intracellular calcium concentration (Eckert *et al*., 1993). According to our results, the increment of intracellular calcium concentration by A_1-receptor activation could be related to activation of PLC followed by the generation of IPs, which releases calcium from intracellular calcium stores. This, in turn, activate constitutive atrial NOS with cyclic GMP accumulation. Both messengers mediate the CPA-induced negative inotropic effect.

Our results showed that A_1 receptor stimulation activating PKC, increased the NOS-cyclic GMP pathway. In support of this, adenosine receptor activation modulating rat myocardial PKC activity was demonstrated (Lasley *et al*., 1994). Also, PKC translocation had been previously proposed as a transduction mechanism of ischemic preconditioning mediated by A_1 receptor (Borst *et al*., 1999). Moreover, preconditioning through the activation of phosphatidylinositol-3-kinase, upstream PKC and NO has been reported (Tong *et al*., 2000).

Adenosine has been demonstrated that interacts with adrenergic neurons at both pre and post junctional sites through altering catecholamine release and through direct alteration of catecholamine effect at the receptor level (Pelleg, 1987). Adenosine modulates ß-adrenoceptor contractile response *via* activation of both A_1 and A_2 myocardial receptors (Sawmiller *et al*., 1996). A_1 receptor activation coupled to Gi protein attenuates ß-adrenoceptor mediated increase in myocardial contractility accounts to indirect adenosine-inhibited adenylate cyclase system (Kitakaze *et al*., 1993; Mudumbi *et al*., 1995). The fact that in 6-hydroxydopaminized rat atria persisted the negative inotropic effect of CPA observed in this paper, demonstrated a direct action on atria A_1 receptor independent of endogenous adrenergic mechanism.

On the other hand, adenosine has several muscarinic cholinergic interactions. Vagal stimulation will have enhanced negative chronotropic effect (Pelleg *et al*., 1985). Cardiac muscarinic receptor activation mediates inotropic negative response *via* NOS-cyclic GMP pathway in rat isolated atria (Sterin-Borda *et al*., 1995). However, in this work, atropine did not modify the CPA-induced contractile inhibition, indicating that muscarinic receptor are not involved in A_1 receptor induced contractile effect. A dissociation between adenosine induced bradichardia and M_2 receptor mediated muscarinic response, was described in M_2 receptor knockout mice (Stengel *et al*., 2000).

So far, adenosine A_1 receptor (Liu *et al*., 1991), NO (Vegh *et al*., 1992) and cGMP (Szilvassy *et al*., 1994) have been identified as potential mediators of preconditioning Here we give a new insight into the pathway by which A_1 receptor activation induced rapid stimulation of NOS with subsequent accumulation of cyclic GMP that may be the final mediators of protection by ischemia preconditioning.

These results suggest that CPA activation of A_1-receptors exerts a negative inotropic effect associated with increased production of nitric oxide and cyclic GMP. The mechanism appears to occur secondarily to stimulation of phosphoinositide turnover *via* PLC activation. This, in turn, triggers cascade reactions involving calcium / calmodulin and PKC, leading to activation of NOS and soluble guanylate cyclase.

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