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European Journal of Pharmacology 512 (2005) 207–213

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The phosphodiesterase 3 inhibitor cilostamide enhances inotropic responses to glucagon but not to dobutamine in rat ventricular myocardium

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Received 14 October 2004; received in revised form 25 January 2005; accepted 31 January 2005
Available online 31 March 2005

Abstract

The effects of phosphodiesterase (PDE) inhibitors (1–3) on tissue cAMP concentrations and the inotropic responses to dobutamine and glucagon were investigated in electrically driven right ventricular strips of the rat heart.

Dobutamine (0.3–100 μM) produced a concentration-dependent positive inotropic effect which was not affected by 50 nM (±)-1-(2,3-(dihydro-7-methyl-1*H*-inden-4-yl)oxy)-3-((1-methylethyl)amino)-2-butanol hydrochloride (ICI 118551), a β_2-receptor antagonist, but was virtually abolished by 0.3 μM (±)-2-hydroxy-5-(2-((2-hydroxy-3-(4-(1-methyl-4-(trifluoromethyl)-1*H*-imidazol-2-l)phenoxy)propyl)amino)ethoxy)-benzamide methanesulfonate (CGP 20712A), a β_1-receptor antagonist. Glucagon (0.01–1 μM) also enhanced the contractility of the preparation in a concentration-dependent way. Selective inhibitors of PDE 1 8-methoxymethyl-3-isobutyl-1-methylxantine (MIMX, 1 μM), PDE 2 erythro-9-[2-hydroxy-3-nonyl]adenine (EHNA, 1 μM) and PDE 3 cilostamide (0.1 μM) did not affect basal contractility. Cilostamide increased the positive inotropic effects of glucagon but not those of dobutamine. MIMX and EHNA did not alter the effects of either dobutamine or glucagon. Dobutamine (3 μM), but not glucagon (0.1 μM), increased tissue levels of cAMP. 1 μM of MIMX or EHNA were devoid of effects and failed to alter the effects of dobutamine and glucagon on cAMP. Cilostamide (0.1 μM) did not increase the effects of dobutamine but caused glucagon to enhance cAMP.

The pharmacological and biochemical data presented in this study can be explained quantitatively by a cell compartment model in which PDE 3 appears to be colocalized with the contractile machinery responsible for the effects of glucagon but not those of dobutamine. Neither PDE 1 nor PDE 2 appears to regulate the inotropic effects of dobutamine and glucagon in rat ventricular myocardium.

Keywords: Glucagon; Dobutamide; Phosphodiesterase inhibitor; Cilostamide; Rat ventricle; Cardiac contractility; cAMP

1. Introduction

Dobutamine and glucagon increase cardiac contractility by stimulating 3′,5′-cyclic adenosine monophosphate (cAMP) production in the myocardium and are useful therapeutic agents (Ewy and Plachetka, 1992; White, 1999). Stimulation of Gs protein, which causes adenylyl cyclase activation through β-adrenoceptors or glucagon receptors, is an important mechanism responsible for the contractile effects of dobutamine and glucagon, respectively (White, 1999).

The effects of cAMP-dependent positive inotropic agents are regulated by the activity of the cyclic nucleotide phosphodiesterase (PDE) enzymes which break down cAMP into its chemically inactive product 5′AMP (Beavo 1995). On the basis of structure, kinetic properties, substrate specificity and ability to regulate, PDE can be grouped into different families (Conti and Jin, 1999) and at least four of these families (PDE 1–4) are present in the heart of a variety of animal species, including man (Nicholson et al., 1991). Each PDE type could differentially regulate the effects of cAMP-dependent positive inotropic agents and this can be

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doi:10.1016/j.ejphar.2005.01.056

ascertained by using selective PDE inhibitors. For example, in isolated rat papillary muscle, the contractile effect of isoprenaline is potentiated by the PDE 4 inhibitor rolipram but not by the PDE 3 inhibitor milrinone (Katano and Endoh, 1992). It has recently been reported that PDE 4 activity limits the contractile responses of both glucagon and dobutamine in rat myocardium (Juan-Fita et al., 2004); however, the involvement of PDE 1–3 in regulating the contractile effects of these two inotropic agents is not known. The aim of the present study, therefore, was to gain an insight into the respective roles of the phosphodiesterase subtypes 1–3 in the regulation of the contractile effects of dobutamine and glucagon. To this end, we have studied the effects of dobutamine and glucagon, in the absence and in presence of selective inhibitors of the different phosphodiesterase subtypes, on the increase of contractile force and cAMP tissue levels in the rat ventricle. Selective phosphodiesterase inhibition was achieved using 8-methoxymethyl-3-isobutyl-1-methylxantine (MIMX) for PDE 1, erythro-9-[2-hydroxy-3-nonyl]adenine (EHNA) for PDE 2, and cilostamide for PDE 3 (Beavo, 1995).

2. Material and methods

The study was reviewed and approved by the Ethical Committee of the University of Murcia.

2.1. Paced right ventricular strips of the rat

Sprague–Dawley rats of either sex (250–350 g) were stunned and exsanguinated. The chest was opened, the heart rapidly removed and placed into Tyrode's solution saturated with 95% O_2–5% CO_2 and the free wall of the right ventricle was excised. All procedures were performed in the presence of Tyrode's solution of the following composition (mM): NaCl 136.9, KCl 5.0, $CaCl_2$ 1.8, $MgCl_2$ 1.5, NaH_2PO_4 0.4, $NaHCO_3$ 11.9 and dextrose 5.0. Right ventricular strips were mounted longitudinally between two platinum electrodes under 1 g tension in Tyrode solution maintained at 37 °C, pH 7.4 and gassed with 95% O_2–5% CO_2. The preparations were electrically stimulated (Grass SD-9 stimulator) at a frequency of 1 Hz and 1 ms of duration and supramaximal (threshold + 25%) voltage. Contractions were measured using a force-displacement transducer (Grass FT-03) and recorded on a Dynograph Beckman polygraph. Tissues were allowed to equilibrate for 45–60 min before drug challenge.

2.2. Experimental protocols

Phentolamine 1 μM, corticosterone 30 μM and desmethylimipramine 2 μM were present in all the experiments with dobutamine to block α-adrenoceptors and to inhibit extraneuronal and neuronal uptake, respectively. Cumulative concentration–response curves to dobutamine and glucagon were performed in the absence and presence of either MIMX, EHNA, or cilostamide. We used a concentration of 1 μM MIMX, 1 μM EHNA and 0.1 μM of cilostamide. These concentrations are around their corresponding IC_{50} values for selective inhibition of PDE 1–3 (Beavo, 1995). EHNA is also an inhibitor of the enzyme adenosine deaminase which catalyzes the metabolism of adenosine (Schaeffer and Schwender, 1974). To investigate a possible involvement of adenosine in the results obtained with EHNA, we used the selective antagonist of A_1 adenosine receptors 1,3-dipropyl-8-cyclopentylxantine (DPCPX) as well as 2′-deoxycoformycin (DCF) which inhibits adenosine deaminase but does not affect PDE 2 (Rivet-Bastide et al., 1997). Drugs were added to a 30-ml organ bath in a volume smaller than or equal to 0.1 ml. In each experiment only a single preparation was taken from each rat heart. Experiments were concluded by raising the Ca^{2+} concentration to 9 mM which produce a maximal inotropic response and results are expressed as percentages of this effect.

2.3. Measurement of cAMP

Levels of cAMP were measured by radioimmunoassay [^{125}I]-tyrosine methyl ester-cAMP (Immunotech, France) according to the manufacturer's instructions. The experiments were carried out with groups of rat right ventricular strips taken from different animals. cAMP was measured either under control conditions or after a 13-min exposure to a 1 μM concentration of either MIMX or EHNA, or 0.1 μM cilostamide. The incubation time was similar to that used by other authors for the study of the interaction between sympathomimetic agents and phosphodiesterase inhibitors on intracellular cAMP levels (Katano and Endoh 1992; Verde et al. 1999). To investigate the effects of dobutamine and glucagon, the agonists were incubated for 3 min in the absence of PDE inhibitors or for 3 min after a 10-min incubation period with the PDE inhibitors. After incubation with drugs, the tissue was immediately frozen. The preparation was then weighed and homogenized in cold perchloric acid 1.5 ml (0.3 mol/l) using a Polytron homogenizer (setting 4 for 30 s) and centrifuged (10,000 × g, 4 °C, 15 min). The supernatants were treated with potassium phosphate until pH 6.2 was reached. The sensitivity of the assay was 2 pmol/ml. The intra- and inter-assay coefficients of variation were 7.7% and 8.2%, respectively. The antibody cross-reacted 100% with 3′,5′-cAMP and less than 0.3% with other nucleotides. cAMP concentrations were expressed in pmol/g of tissue.

2.4. Drugs

The following drugs were used: glucagon, generously supplied by Novo Nordisk Pharma S.A. (Madrid, Spain). 2′-Deoxycoformycin was a gift from Pfizer S.A. (Madrid,

Spain). Dobutamine, phentolamine, desmethylimipramine, corticosterone, 8-methoxymethyl-3-isobutyl-1-methylxantine (MIMX), erythro-9-[2-hydroxy-3-nonyl]adenine (EHNA), cilostamide, 1,3-dipropyl-8-cyclopentylxantine (DPCPX), (±)-2-hydroxy-5-(2-((2-hydroxy-3-(4-(1-methyl-4-(trifluoromethyl)-1*H*-imidazol-2-l)phenoxy)propyl)amino)ethoxy)-benzamide methanesulfonate (CGP20712A), and (±)-1-(2,3-(dihydro-7-methyl-1*H*-inden-4-yl)oxy)-3-((1-methylethyl)amino)-2-butanol hydrochloride (ICI118551) were obtained from Sigma Chemicals Co. (Madrid, Spain) and dimethyl sulphoxide (DMSO) from Probus (Barcelona, Spain).

Dobutamine, phentolamine, CGP20712A, ICI185,551 and desmethylimipramine were dissolved in freshly distilled H_2O. Glucagon, corticosterone, MIMX, EHNA, cilostamide, DPCPX and 2′-deoxycoformycin were dissolved in DMSO and Tyrode solution (20% DMSO in Tyrode); this stock solution was diluted into pre-warmed and pre-aerated bathing solution to achieve the final concentration desired. The drug was added to the organ bath at an appropriate concentration so that the concentration of DMSO in the test solution was less than 0.3%, which produced no effect on the ventricular preparation.

2.5. Statistics

The results are expressed as mean values ± S.E.M. Student's *t*-test or one-way analysis of variance followed by Tukey's method for multiple comparisons was used. The criterion for significance was that *P* values should be less than 0.05.

3. Results

3.1. Effects of dobutamine and glucagon

Dobutamine (0.1–100 μM) produced a concentration-dependent positive inotropic effects (Fig. 1). CGP20712A (300 nM) virtually abolished the inotropic effects of dobutamine. In contrast, the selective β_2-receptor antagonist ICI118551 (50 nM) neither modified the dobutamine concentration–response curve nor significantly ($P>0.05$) changed its $-\log EC_{50}M$ (5.78 ± 0.10, $n=7$, alone and 5.67 ± 0.08, $n=5$, in the presence of ICI118551). Consequently, the effects of dobutamine are mediated through β_1-adrenoceptors.

Contractile force also increased gradually with glucagon in a concentration-dependent pattern (Fig. 1). The glucagon solvent DMSO, at the same concentrations as those present in the glucagon solutions, was devoid of effect in this preparation (data not shown). The $-\log EC_{50}$ of glucagon (7.30 ± 0.12, $n=5$) is significantly higher than that of dobutamine 5.78 ± 0.10 ($n=7$) ($P<0.05$); however, the E_{max} of glucagon (32.2 ± 6.5, $n=5$) was significantly lower than that of dobutamine (56.2 ± 3.9, $n=7$) ($P<0.05$).

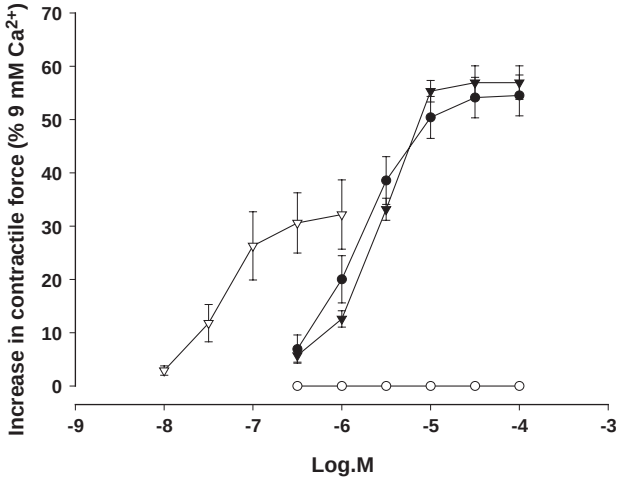

Fig. 1. Cumulative concentration–response curves for the inotropic activity of glucagon (▽), dobutamine alone (●) and in the presence of either 0.3 μM CGP20712A (○) or 50 nM ICI 118551 (▼) (β_1- and β_2-adrenoceptor selective receptor antagonists, respectively). Inotropic responses are expressed as a percentage of the effect caused by 9 mM Ca^{2+}. Each point represents the mean value±S.E.M. of 5–9.

3.2. Effects of dobutamine in combination with phosphodiesterase inhibitors

The PDE inhibitors 1–3 MIMX, EHNA at 1 μM and cilostamide at 0.1 μM concentration, which were devoid of effect alone, failed to alter the contractile effect of dobutamine (Fig. 2A). Neither of these PDE inhibitors significantly changes the $-\log EC_{50}$ or the E_{max} of dobutamine (Table 1). The selective antagonist of A_1 adenosine receptors DPCPX (50 nM), which was devoid of effects alone, did not alter the effect of EHNA alone or in combination with dobutamine. The $-\log EC_{50}$ of dobutamine+EHNA was 5.56 ± 0.06 ($n=6$) and 5.51 ± 0.03 ($n=5$) ($P>0.05$) in the absence and in the presence of DPCPX, respectively. Also, the E_{max} values of dobutamine in combination with EHNA in the absence (53.0 ± 4.6, $n=6$) or the presence (61.3 ± 5.2, $n=5$) of DPCPX were not statistically significant ($P>0.05$). The concentration–response curve of dobutamine was not affected by the inhibitor of adenosine deaminase, but not of PDE 2, DCF. In fact, neither the $-\log EC_{50}M$ (5.78 ± 0.10 $n=7$ in the absence and 5.64 ± 0.10, $n=5$ in the presence of DCF, $P>0.05$) nor the E_{max} (56.2 ± 3.9, $n=7$ in the absence and 55.2 ± 2.5, $n=5$ in the presence of DCF, $P>0.05$) of dobutamine was modified by DCF.

3.3. Effects of glucagon in combination with phosphodiesterase inhibitors

Cilostamide (0.1 μM) significantly enhanced the contractile effects and E_{max} of glucagon (Fig. 2B and Table 1).

The PDE 1–2 inhibitors MIMX (1 μM) and EHNA (1 μM) failed to alter the contractile effects of glucagon (Fig. 2B). These two PDE inhibitors did not change significantly

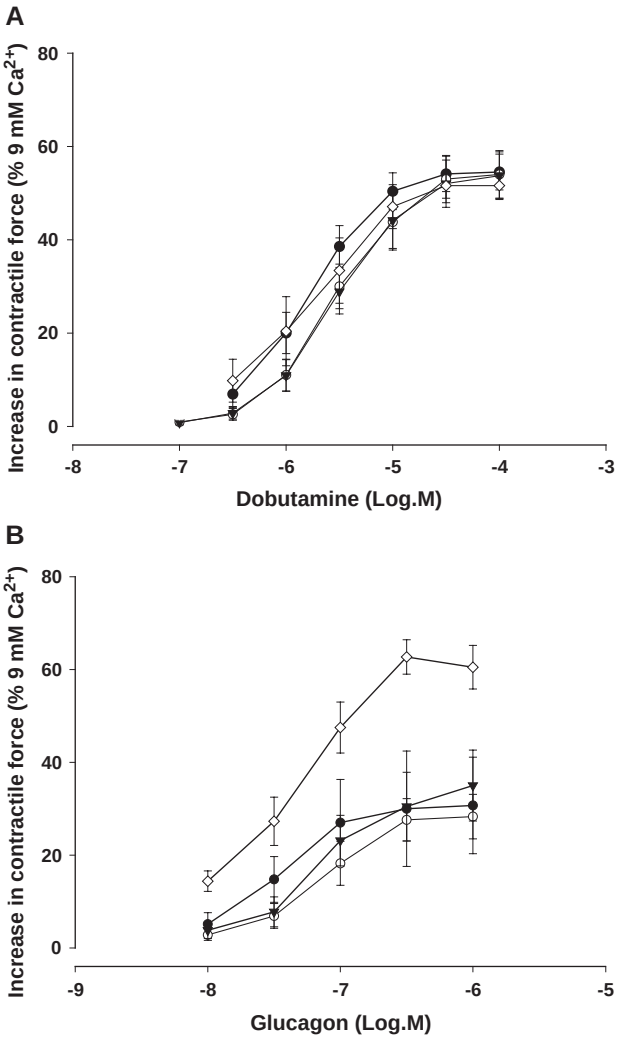

Fig. 2. Cumulative concentration–response curves for the inotropic effects of dobutamine (A) and glucagon (B) in the absence (●) and in the presence of the selective inhibitors of PDE 1 (○, MIMX), PDE 2 (▼, EHNA), and PDE 3 (◇, cilostamide). Each point represents the mean value ± S.E.M. of 4–7. Further details as in legend to Fig. 1.

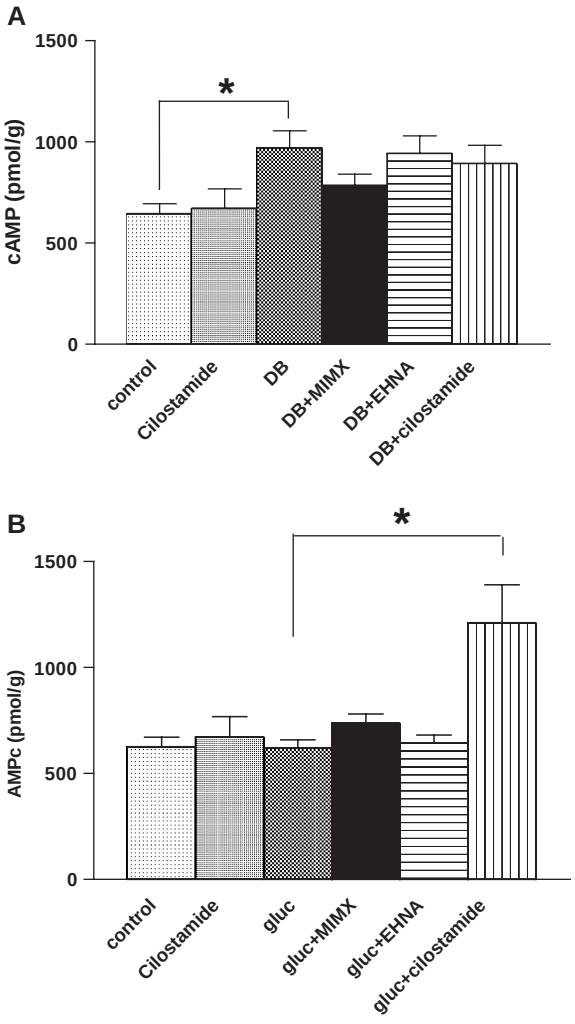

Fig. 3. Changes in cAMP levels in right ventricle of the rat heart. The effect of dobutamine (3 μM) and glucagon (0.1 μM) and the combination of 1 μM of either of the selective inhibitors of PDE 1 MIMX and PDE 2 EHNA or PDE 3 cilostamide 0.1 μM with dobutamine (A) or glucagon (B) are shown. Values are the mean ± S.E.M. of 5–9 experiments. *$P<0.05$ between two compared values.

the $-\log EC_{50}$ and E_{max} of glucagon (Table 1). The selective antagonist of A_1 adenosine receptors DPCPX (50 nM) did not alter the effect of EHNA alone or in combination with glucagon. The $-\log EC_{50}M$ of glucagon+EHNA were 7.00+0.20 ($n=5$) and 7.19 ± 0.07 ($n=5$) ($P>0.05$), respectively, in the absence and in the presence of DPCPX. Also, the E_{max} of glucagon in combination with EHNA in the absence (35.0 ± 7.7, $n=5$) and presence (36.3 ± 2.9, $n=5$) of DPCPX were not statistically significant ($P>0.05$). The concentration–response curve of glucagon was not affected by the inhibitor of adenosine deaminase DCF. Neither the $-\log EC_{50}$ (7.30 ± 0.12, $n=5$ in the absence and 7.40 ± 0.05, $n=5$ in the presence of DCF, $P>0.05$), nor the E_{max} (32.2 ± 6.5, $n=5$ in the absence and 32.9 ± 3.2,

Table 1
Inotropic potency of glucagon and dobutamine and effects of selective inhibitors of PDE (1–3)

	Glucagon				Dobutamine			
	Control	MIMX	EHNA	Cilostamide	Control	MIMX	EHNA	Cilostamide
n	5	5	5	4	7	6	6	4
$-\log EC_{50}$	7.3 ± 0.1	7.1 ± 0.1	7.0 ± 0.2	7.5 ± 0.1	5.7 ± 0.1	5.5 ± 0.1	5.5 ± 0.0	5.8 ± 0.1
E_{max}	32.2 ± 6.5	28.3 ± 4.8	35.0 ± 7.7	62.7 ± 3.7*	56.2 ± 3.9	53.7 ± 5.1	53.0 ± 4.6	51.6 ± 5.2

* $P<0.05$ when compared to its control.

$n=5$ in the presence of DCF, $P>0.05$) of glucagon showed statistical significance.

3.4. Effects of dobutamine and glucagon in combination with phosphodiesterase inhibitors on the tissue levels of cAMP

The experiments were designed to investigate whether the inotropic effects of dobutamine and glucagon and their modification by phosphodiesterase inhibitors correlated with tissue levels of cAMP. For this purpose, we studied the effect of dobutamine (3 μM) and glucagon (0.1 μM), which are concentrations similar to their corresponding inotropic EC_{50}, in the absence and the presence of the different phosphodiesterase inhibitors.

The cAMP content in right ventricle of rat heart was increased by dobutamine but it was not affected by either glucagon, MIMX, EHNA or cilostamide. Cilostamide, but not MIMX, or EHNA, caused glucagon to enhance cAMP tissue levels. However, the effect of dobutamine on cAMP levels was not significantly modified by any of the PDE inhibitors used in this study (Fig. 3).

4. Discussion

The major finding of this study is that although both agonists induce a positive inotropic effect through Gs protein-mediated cAMP production, cilostamide augments the contractile effect of glucagon but not of dobutamine in rat ventricular myocardium.

Dobutamine produces a cAMP-dependent positive inotropic effect mainly due to β_1-adrenoceptor activation (Ewy and Plachetka, 1992). This is consistent with our findings which show that the effect of dobutamine is completely blocked by the highly selective β_1-receptor antagonist CGP-20712A (Kitagawa et al. 1995) but is not modified by the selective β_2-receptor antagonist ICI118551.

Glucagon also generates cAMP, which causes positive inotropic effects (White, 1999). The maximal inotropic response to glucagon is considerably smaller than the maximal response to β-adrenoceptor stimulation (MacLeod et al., 1981), which also agrees with the results of the present study where the maximum concentration of glucagon used (1 μM) produced a maximum inotropic response about one-half that of the E_{max} of dobutamine.

Intracellular distribution of PDEs modulates the molecular machinery resulting from activation of different Gs-linked receptors (Houslay and Milligan, 1997). Indeed, inhibition of PDE 4, which is the predominant PDE in rat myocardium (Nicholson et al., 1991; Verde et al., 1999; Mongillo et al., 2004), enhances contractile responses to dobutamine and glucagon in this tissue (Juan-Fita et al., 2004). In addition to PDE 4, PDE 1–3 are also present in rat heart and appears to be responsible for modulating the response to cAMP-dependent positive inotropic agents (Verde et al., 1999; Mongillo et al., 2004). However, the contribution of PDE 1-3 in shaping the inotropic response to dobutamine and glucagon remains unknown but it was investigated in the present work by using selective inhibitors of these PDEs. The inhibitors of PDE 1 and PDE 2, MIMX and EHNA were shown to be devoid of effect alone and they also failed to potentiate the positive inotropic effect of either dobutamine or glucagon in rat right ventricular myocardium. This agrees with previous results showing that neither MIMX nor EHNA caused inotropic effects on their own and did not potentiate the effects of noradrenaline in the rat heart (Juan-Fita et al., 2003). The results with MIMX are to be expected since although PDE 1 is present in rat heart, it is predominantly confined to the nonmyocyte tissue (Bode et al., 1991; Verde et al., 1999).

PDE 2 inhibition by EHNA significantly enhances the effects of the β-adrenoceptor agonist isoprenaline on L-type Ca^{2+} current in rat ventricular myocytes (Verde et al., 1999) and this may play an important role in triggering myocardial contraction. However, our present results from rat right ventricle show that EHNA did not increase the contractile responses to dobutamine or glucagon. One possibility for the discrepancy between our results and those of Verde et al. (1999) is that the inhibitory effect of EHNA on the enzyme adenosine deaminase which metabolizes adenosine (Schaeffer and Schwender, 1974) may enhance the cardiodepressant effect of this nucleoside mediated by adenosine A_1 receptors (Romano et al., 1989) and thereby counteracts the possible potentiation of EHNA on the positive inotropic effects of both dobutamine and glucagon; however, this is not the case because the selective adenosine A_1 receptor antagonist DPCPX failed to modify the inotropic responses of either dobutamine or glucagon in the presence of EHNA and the inhibitor of adenosine deaminase 2′-deoxycoformycin (DCF) which is devoid of PDE 2 inhibitory activity (Rivet-Bastide et al., 1997) did not alter the contractile effect of dobutamine or glucagon. The reason why EHNA should potentiate the effect of isoprenaline on L-type Ca^{2+} current but not of dopamine and glucagon on contractility remains unknown but it could be related to the ability of EHNA to inhibit actin assembly (Schliwa et al., 1984).

Cilostamide at a concentration 0.1 μM which is similar to its K_i value for inhibition of PDE 3 (Beavo, 1995) was found to be devoid of a direct effect on its own in the present study. However, cilostamide potentiated the positive inotropic effect of glucagon but not that of dobutamine. This indicates that the hydrolysis of cAMP produced through Gs-linked glucagon receptors is more sensitive to PDE 3 inhibition, critically affecting the contractile machinery, than for Gs-linked β_1-adrenoceptors stimulated by dobutamine. Our findings with dobutamine agree with previous results showing that inhibition of PDE 3 fails to enhance the positive inotropic effect mediated by the nonselective β-adrenoceptor agonist isoprenaline (Nicholson et al., 1991; Katano and Endoh, 1990) or the

β_1-adrenoceptor-selective agonist noradrenaline in rat myocardium (Juan-Fita et al., 2003).

From the results of the present study it can be concluded that PDE 3 is involved in regulating the positive inotropic effect of glucagon but not that of dobutamine. The E_{max} of glucagon in our results is about one-half that of dobutamine. This, however, is not the consequence of less effective stimulation of the Gs-adenylate cyclase system by glucagon but of the role of PDE 3 in terminating its effect. In fact, when PDE 3 is inhibited, the maximal response is similar for both inotropic agents and the potency is higher for glucagon (see Results). The reported PDE 3 inhibition by glucagon does not play a role in our results because although it occurs in different species, it does not occur in the rat heart (Méry et al., 1990).

The selective inhibitors of PDE 1–3 used in the present study failed to modify cAMP concentrations on their own, which agrees with results obtained with these agents in rat myocardium (Verde et al., 1999; Juan-Fita et al., 2003). Dobutamine, however, increased cAMP in accord with previous findings (McNeill, 1978), but the effect was not enhanced by cilostamide, MIMX or EHNA. This is consistent with our inotropic dobutamine results and supports the notion that PDE 1–3 are not readily involved in the hydrolysis of cAMP generated by dobutamine, thereby not limiting its contractile response. Glucagon, 0.1 μM, which did not alter tissue levels of cAMP alone, enhanced cAMP levels in the presence of cilostamide but not in the presence of the inhibitors of PDE 1–2. Consequently, there is a good correlation between the functional and biochemical results obtained from interaction between these two inotropic agents and the different PDE inhibitors used in this study. Although PDE 3 does not appears to be responsible for modulating cAMP responses associated with β-adrenoceptors stimulation in rat ventricular myocardium (Mongillo et al., 2004), it seems to be particularly associated with the cellular compartment where the inotropic responses to glucagon are generated, thereby limiting its contractile responses.

The finding that glucagon, 0.1 μM, did not alter tissue levels of cAMP but enhanced cardiac contractility agrees with previous results (Juan-Fita et al., 2004). Only when PDE 3 (present results) or PDE 4 (Juan-Fita et al., 2004) are inhibited, glucagon 0.1 μM enhances cAMP levels. Therefore, it seems that cAMP generated by glucagon, although enough to trigger an inotropic response, is rapidly hydrolyzed by PDE 3 and PDE 4 and therefore is not detected biochemically, at least one of these enzymes is inhibited.

We conclude that inhibition of PDE 3 selectively enhances the inotropic response and cAMP levels mediated by glucagon receptors but not the β_1-adrenoceptor responses of dobutamine. This could be of interest, particularly when cardiac contractility is depressed and cAMP-dependent inotropic agents and PDE 3 inhibitors are co-administered (White, 1999; Emerman, 2004). However, the clinical relevance of the interaction described in the present work remains to be determined.

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