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In situ detection of cyclic AMP-phosphodiesterase activity in the heart of Lewis and Sprague-Dawley rats: the effect of restraint stress or amphetamine application

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Summary. Cyclic AMP plays an important role in heart functions under normal as well as pathological conditions. Since phosphodiesterase (PDE), responsible for the hydrolysis of cAMP, is equally important as synthesizing adenylyl cyclase, we decided to determine its activity by cytochemical procedure after exposure of rats to restraint stress or an acute dose of amphetamine. Sprague-Dawley (S-D) and Lewis (LE) rats, the latter known to have a deficient hypothalamo-pituitary-adrenal axis activity, were used in order to disclose the possible significance of rat strain on PDE activity. Animals were divided into 3 groups: controls, rats treated with an acute dose of amphetamine (8 mg/kg, i.p., for 60 min) and rats under restraint stress for 60 min. Control hearts of both strains revealed PDE activity on sarcolemma of cardiomyocytes and plasmalemma of endothelial cells of microvessels. In LE rats we observed an additional enzyme reaction in junctional sarcoplasmic reticulum. In addition, cardiomyocytes of LE rats revealed a higher PDE activity when compared to S-D rats. Restraint stress decreased PDE activity in cardiomyocytes of LE rats while amphetamine markedly inhibited enzyme activity in cardiomyocytes of S-D rats. Endothelial PDE was more resistant to stress. Our results indicate differences in PDE localization and variations in sensitivity of myocardial cAMP-PDE of LE and S-D rat strains to restraint stress and amphetamine application.

Key words: Phosphodiesterase, Cytochemistry, Heart, Restraint stress, Amphetamine

Introduction

The second messenger cyclic 3',5'-adenosin monophosphate (cAMP) is arguably the most important modulator of cardiac contractility, not only regulating the function of proteins directly involved in excitation-contraction coupling but also regulating proteins that shape the resulting calcium transient (Katz, 1982).

The myocardial cAMP level at each moment is determined by the opposing activities of adenylyl cyclase (AC) and phosphodiesterase (PDE), the enzymes forming and hydrolysing cAMP, respectively. Unlike AC, whose activity is readily regulated by G-protein-coupled receptors, e.g. activated by adrenomimetics, the activity of PDE does not have receptor-mediated activity regulation.

PDE is known to exist in multiple soluble and membrane-bound molecular forms. The myocardium contains four major families of PDE hydrolysing cAMP (Beavo and Reifsnyder, 1990). Many studies have demonstrated interspecies variations in PDE activity (Shahid et al., 1990; Shahid and Nicholson, 1990; Lugnier et al., 1993; Okruhlicova et al., 1997). Observed variations indicate that this enzyme is also a subject for activity modifications, especially under pathological conditions. Many pathological stimuli, like ischemia, stress and various drugs are known to affect cAMP concentrations and, consequently, heart function (Krause et al., 1978; Miroshnichenko et al., 1983; Okruhlicova et al., 1988, 2000; Petkova and Milkov, 1991; Roth et al., 1998; Tribulova et al., 2000). Our previous studies showed differences in the response of AC to various stimulants in the human heart (Hynie et al., 1996) and divergences of myocardial AC activity in several inbred rat strains (Šída et al., 2003). In the inbred rat strains Klenerova et al. (1998, 1999) demonstrated different brain AC activity during physiological and stress conditions that were associated with behavioural changes in the animals (Kaminský et al., 2001; Klenerova et al., 2002).

The aim of this study was to determine by cytochemical methods whether two pathological conditions which were shown to share some common biochemical and behavioural responses (Klenerova et al.,

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1997-1999), namely restraint stress and amphetamine treatment, would change PDE activity; furthermore, we wanted to determine whether possible observed responses are dependent on the rat strain used.

Material and methods

Animals

The experiments were carried out on male Sprague-Dawley (S-D) and Lewis (LE) rats (Charles River Laboratories, Schulzfel, Germany). At the start of the experiments the average body weight of the rats was 240 g. Animals had free access to standard pellet food and water. Rats were housed five per cage (42x26 cm) and maintained on a 12 h light/12 h dark cycle (change performed at 6:00 and 18:00 hours), at a constant temperature (21±1 ºC) and relative humidity (50-70%). Treatment of animals was in accordance with the Declaration of Helsinki Guiding Principles on Care and Use of Animals [DHEW Publication, NHI 80-23].

Restraint stress and amphetamine treatment

During the exposure to restraint (immobilization) stress (IMO), rats (n=6 per group) were immobilized by fixing the front and hind legs with adhesive plaster; then the animal was restrained in a snug-fitting vertical plastic-mesh. This mesh was bent to conform to the size of the individual animal (Klenerova et al., 2003). After stressor exposure for 1 h, rats were returned to the home cage and left undisturbed for another 1 h when the passive avoidance procedure started or animals were sacrificed. Another group of rats (n=6) was treated with an acute dose of amphetamine (AMPH) (8 $mg.kg^{-1}$ b.w.). This i.p. treatment was performed 60 min before decapitation of the rats; the heart was rapidly excised and the left ventricle was frozen in liquid nitrogen and kept at -70 ºC.

Cytochemistry of membrane-bound cAMP-dependent phosphodiesterase

Detection of cAMP-PDE was done according to our previous method (Okruhlicova et al., 1996). Briefly, 40-μm-thick cryostat slices of the left ventricle were fixed for 5 min in 1% glutaraldehyde, pH 7.4, then pre-incubated for 45 min at 4 ºC in a medium consisting of 50 mmol/l Tris-maleate, pH 7.4, 2 mmol/l $MgCl_2$, 5 mg/ml 5'-nucleotidase (5'-NC, originating from snake venom *Crotalux atrox*) and 0.25 mol/l sucrose and then incubated for 30 min at 37 ºC in the medium containing 180 mmol/l Tris-maleate, pH 7.4, 3 mg/ml 5'-NC, 3 mmol/l cAMP, 0.25 mol/l sucrose and 2 mmol/l $Pb(NO_3)_2$. For control enzyme reactions either the substrate or 5'-NC were omitted. The specimens were post-fixed for 30 min at 4 ºC in 40 mmol/l OsO_4, dehydrated in a graded series of alcohol and propylene oxide and embedded in Epon 812. Ultrathin sections were cut with a LKB ultramicrotome and evaluated with a Tesla BS 500 electron microscope. The enzyme reaction was evaluated in unstained sections.

All chemicals were purchased from Sigma Chemicals Co.

Results

In our studies using the in situ cytochemical method we examined the effects of restraint stress or acute amphetamine on cAMP-PDE activity in the left ventricle of two rat strains, namely Lewis and Sprague-Dawley rats.

Strain-dependent differences in basal cAMP-PDE

Control hearts of both LE and S-D rats revealed enzyme activity on the sarcolemma of cardiomyocytes and plasmalemma of endothelial cells of capillaries and small arterioles. In LE rats we observed an additional PDE reaction on subsarcolemmal cisternae and junctional sarcoplasmic reticulum (Fig.1). We also observed a higher intensity of enzyme reaction in cardiomyocytes of LE rats when compared with S-D rats.

Effect of IMO on PDE activity

Restraint stress induced a decrease in PDE activity in cardiomyocytes of both strains. However, in the myocardium of LE rats the effect of stress was stronger than in the S-D rats (Fig.2).

Table 1. Intensity of cAMP-PDE reaction product in control hearts of Sprague-Dawley and Lewis rats.

	S-D	LE
SI	++	++, +++
JSR	-	++
EC	++	++

Classification: +++, strong reaction; ++, normal; -, absent. SI: sarcolemma; JSR: junctional sarcoplasmic reticulum; EC: endothelial cell; S-D: Sprague-Dawley rat; LE: Lewis rat.

Table 2. Effect of restraint stress and amphetamine treatment on cAMP-PDE activity in the heart of Lewis and Sprague-Dawley rats.

	IMO	AMPH
SI	± / +	+ / ±
JSR	± / -	± / -
EC	++ / ++	++ / ++

Classification: ++, normal reaction; +, moderate; ±, weak; -, absent. SI; sarcolemma; JSR: junctional sarcoplasmic reticulum; EC: endothelial cell; IMO: immobilisation stress; AMPH: amphetamine.

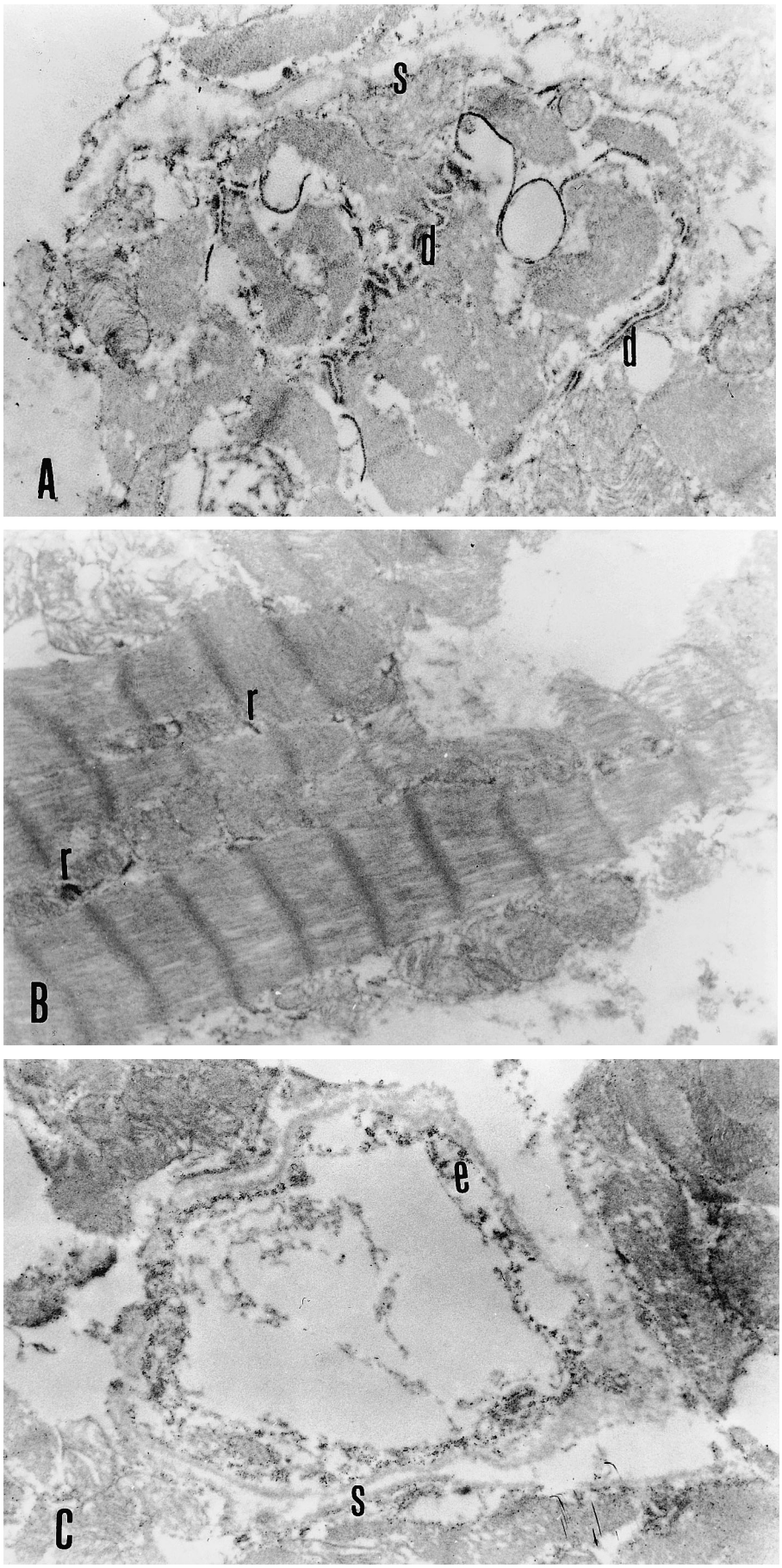

Fig. 1. Ultrastructural localization of basal cAMP-PDE activity in the sarcolemma (s) and the junctional sarcoplasmic reticulum (r) in a control heart of a Lewis **(A, B)** and Sprague-Dawley rat **(C)**. e: capillary, d: intercalated discs. A, x 12,000; B, x 10,000, C, x 13,000

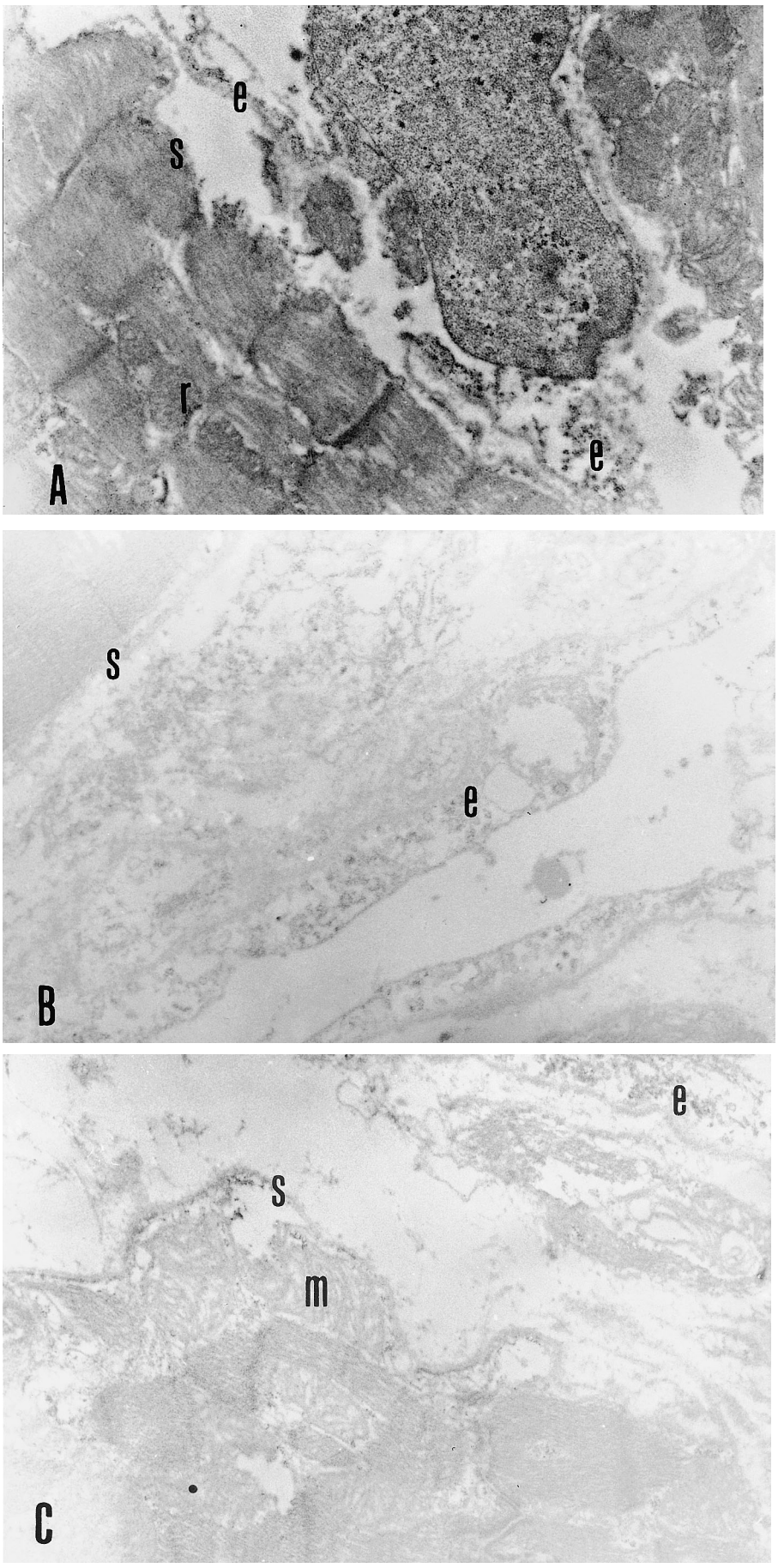

Fig. 2. Effect of restraint stress on cAMP-PDE activity in the myocardium of LE **(A, B)** and S-D **(C)** rats. s: sarcolemma; r: junctional sarcoplasmic reticulum; e: capillary, m: mitochondria. A, x12,000; B, x 16,000; C, x 22,000

Effect of AMPH on PDE activity

AMPH treatment produced a stronger inhibition of PDE activity in cardiomyocytes of S-D rats in comparison with LE rats (Fig. 3).

Effect of stress on PDE activity in endothelial cells

The cAMP-PDE activity in endothelial cells of capillaries and small arteries in the myocardium of both strains was more resistant to both restraint stress and amphetamine treatment in comparison with the sarcolemma of cardiomyocytes (Figs. 2, 3). Semi-quantitative evaluation of enzyme reactions is shown in Tables 1 and 2.

Restraint and AMPH treatment resulted in a heterogeneous structural damage of cardiomyocytes and endothelial cells of capillaries. Injured cardiomyocytes displayed intracellular oedema and weak to moderate ischemia-like injury of mitochondria and contraction bands of myofilaments. In some capillaries, the endothelial layer integrity was damaged (Figs. 2, 3).

Discussion

Cyclic nucleotides and calcium ions are well-known

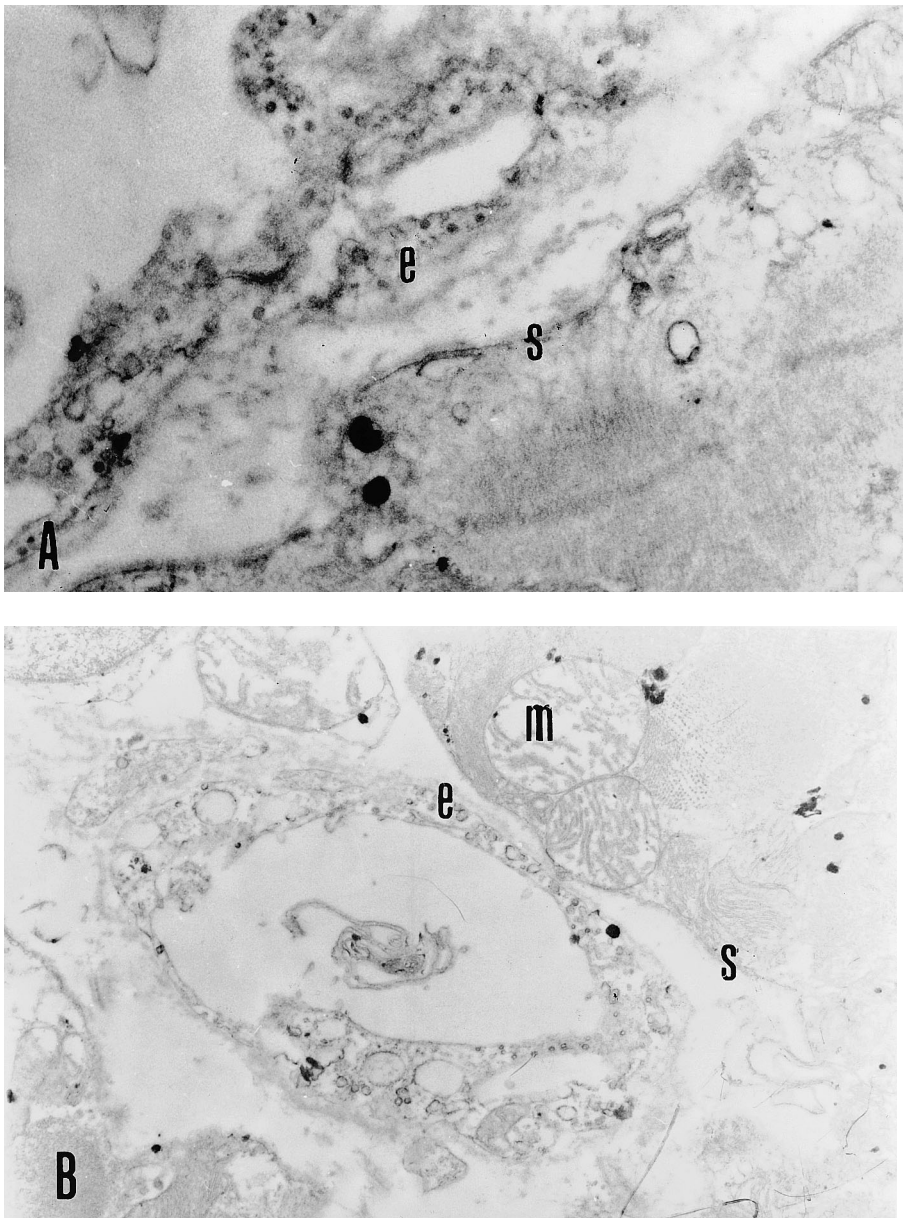

Fig. 3. Effect of amphetamine treatment on cAMP-PDE activity in the myocardium of LE **(A)** and S-D **(B)** rats. e: capillary, m: mitochondria, s: sarcolemma. Magnification: A: x 24,000; B x 14, 000

second messengers regulating the rate and contraction of the heart. Cyclic AMP was studied more intensively than cGMP. It is formed by adenylyl cyclase that is coupled by G-proteins to beta-adrenergic receptors. During the fast changing of heart contraction and relaxation (systole and diastole) cardiomyocytes have to have a device for the degradation of cAMP which is done by the action of PDE.

Cyclic nucleotide phosphodiesterases, on the basis of their structural and kinetic properties, were classified into at least seven families; four of them hydrolyse cAMP in the heart (Beavo et al., 1994) and they are differentially regulated by a variety of signals (Conti et al., 1991; Beltman et al., 1993). Species- and tissue-dependent differences in activities and subcellular distribution of PDEs reported in many studies (Shahid et al., 1990; Lugnier et al., 1993; Okruhlicova et al., 1996, 1997) were suggested to be responsible for a wide range of potential disturbances of cardiovascular functions, i.e. cardiac and vascular inotropy, cardiac rhythm and excitability and inflammatory responses to injury.

Both enzymes responsible for the actual level of cAMP–adenylyl cyclase and cAMP-PDE have already been cytochemically detected on the sarcolemma and the junctional sarcoplasmic reticulum of cardiomyocytes (Katz et al., 1974; Slezak and Geller, 1979; Schulze, 1984; Okruhlicova et al., 1988, 1997, 2000). The co-localization of both enzymes producing and hydrolysing cAMP may represent a functional entity regulating cAMP concentration in sub-membrane compartments (Jurevicius and Fischmeister, 1996), where some targets for cAMP-induced phosphorylation (calcium channels, intercalated discs, sarcoplasmic reticulum) are also present (De Mello, 1988; Darow et al., 1996). Dysfunction of PDE-related activity may play an important role in pathological conditions, and the balance between both enzyme systems seems to be important for normal function..

When we determined cAMP-PDE under basal conditions, in S-D rat myocardium the cAMP-PDE was detected on the sarcolemma of cardiomyocytes while in LE rat myocardium this enzyme activity was also seen on the junctional sarcoplasmic reticulum and subsarcolemmal cisternae. The results indicate differences in the presence of membrane-bound isoforms of cAMP-PDE in the myocardium of the two rat strains tested.

Our results correspond with our previous studies demonstrating species and tissue differences between cAMP-PDE in situ localisation in rat and dog cardiomyocytes (Okruhlicova et al., 1996-1998) that may be associated with species differences concerning inotropic response to PDE inhibitors.

The increase of cAMP-PDE activity in cardiomyocytes of LE rats seems to accompany the changes in other components of cAMP system and the myocardial function. For example, Barbato et al. (1998, 2002) observed differences in cardiac functions of various rat strains in Langendorf heart preparation; cardiac performance of LE rats was significantly lower than in S-D rats. Our results demonstrated higher cAMP-PDE activity in cardiomyocytes of LE rats, which probably reduces levels of cAMP in these cells and thus also their function. It seems that cAMP-PDE may be one of the main mechanisms, together with adenylyl cyclase activity, which are involved in the variations in heart functions in genetically diverse rat strains. The difference in localization of myocardial cAMP-PDE in the two studied rat strains may play a role not only in the intensity of physiological responses but also in the differential vulnerability to pathological conditions, and in the heart contractile responses of the particular rat strains to the cAMP-PDE inhibitors.

Differences in the activity and localization of myocardial PDE in the two rat strains studies are in accordance with the finding of adenylyl cyclase activity. Šída et al. (2003) measured the highest AC activity in the left ventricle of S-D rats and the lowest one in LE rats. Inbred rat strains were also characterized with variations in brain adenylyl cyclase activity during physiological conditions (Klenerova et al., 1998, 1999). Some of these changes may be attributed to differences in hypothalamo-pituitary-adrenal axis activity in various rat strains.

From our experiments it is evident that there were differences in the activity of basal cAMP-PDE after restraint stress or amphetamine treatment in the myocardium of both rat strains; these differences must result in changes of the intracellular cAMP levels. PDE in cardiomyocytes of LE rats was more sensitive to restraint stress compared to the ones of S-D rats, while amphetamine induced a higher inhibition of PDE in cardiomyocytes of S-D rats than in LE rats. It is of interest in both rat strains that the PDE activity persisted on the endothelium of capillaries and microvessels both after restraint stress as well as after amphetamine treatment. This may be due to genetic diversity resulting in different tissue sensitivity to experimental conditions and different tolerance to amphetamine cumulation (Spaber and Fosson, 1984). It may also be associated with the presence of various membrane-bound cAMP-PDE isoforms in endothelial cells and cardiomyocytes, and their different modulation by NO/cGMP (Lugnier and Komas, 1993; Eckly and Lugnier, 1994; Okruhlicova et al., 1996). In our experiments we observed changes in cAMP-PDE activities in the myocardium of both strains that were similar after ischemia and after conditions which are associated with disturbances in metabolism, formation of energy and ion concentrations.

Klenerova et al. (1998, 1999, 2002) demonstrated that amphetamine treatment and restrain stress stimulated adenylyl cyclase activity in the brain of Wistar rats; the expected increase in cAMP levels may be the cause for behavioural changes. Amphetamine as a sympathomimetic drug can increase cAMP level in the heart via stimulation of adrenoceptor/AC system and affect the heart rate (Simpson, 1975; Gallardo-Carpentier

et al., 1997). However, there are some controversial results in the literature concerning the effect of stress on cAMP level in the heart (Okada et al., 1983; Khananashvili and Karsanov, 1987; Petkova and Milkov; 1991; Szekeres, 1996); changes in cAMP levels are responsible for modulation of heart rate (Nagaraja and Jeganathan, 1999). Differences in cAMP-PDE activity in the myocardium of LE and S-D rats during restraint stress and amphetamine treatment can reflect changes in cAMP concentration due to dysfunction of PDE and suggest the important role of a balance between both enzyme systems regulating cAMP level.

Taken together, our results indicate differences in cAMP-PDE activity and localization in the cardiomyocytes of two rat strains as well as different sensitivity of myocardial cAMP-PDE activity to restraint stress and amphetamine treatment in both Lewis and Sprague-Dawley rat strains.

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