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CALCIUM AND PAPAVERINE INTERACTION WITH SOLUBLE CARDIAC PHOSPHODIESTERASE

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Abstract—The *c*AMP phosphodiesterase (PDEase), found in the 105,000 *g* supernatant fraction of calf heart homogenate, was regulated by Ca^{2+}, being inhibited by low and stimulated by high calcium concentrations. Such a sensitivity to Ca^{2+} was maintained during the purification of the enzyme through a DEAE–cellulose column. EGTA demonstrated through indirect evidence that the PDEase-inhibiting effect of papaverine was not Ca^{2+}-dependent. Moreover, the inhibitory effect of papaverine was not removed by imidazole. An increase in cAMP content and synthesis induced by noradrenaline was observed in the presence of papaverine. From these observations the effect of papaverine on cAMP accumulation could be ascribed to its inhibitory effect on PDEase, through both a direct interaction with the enzyme and an altered availability of Ca^{2+}.

Recently, attention has been focused on the fact that the positive inotropic effect of many drugs stimulating adenyl-cyclase [1–3] or inhibiting phosphodiesterase (PDEase) [4] was due to an increase of intracellular cAMP. A rise and a fall of cAMP during each myocardial contraction was also observed [5], thereby confirming that changes in intracellular cAMP content are correlated to, or responsible for, the cardiac inotropism.

The intimate mechanism of this effect is not quite clear. The hypothesis that cAMP facilitates the entrance of Ca^{2+} across the plasma membrane with a consequent increased availability of this ion at intracellular level was put forth (for references see Kukovetz and Pöch [1]). On the other hand, evidence that Ca^{2+} shows a modulating effect on the systems responsible for the content of cAMP in the cell is widely documented. In the brain, low calcium concentrations stimulate the adenyl-cyclase activity, high calcium concentrations inhibit it [6–9]. Similarly, in fat cells and in adrenal medulla Ca^{2+} regulates cAMP formation in the absence and in the presence of hormones [10–14]. In the liver [15], adrenal tumour [16], and heart [17], high calcium concentrations inhibit adenyl-cyclase activity.

Besides, different sensitivity to calcium ions is also exhibited by PDEases of several tissues: soluble PDEase from brain is stimulated by low Ca^{2+} and inhibited [18] or stimulated [19] by high Ca^{2+} concentrations, soluble smooth muscle PDEase in inhibited by all Ca^{2+} concentrations but more strongly by the lower ones [20], soluble rat heart PDEase is slightly stimulated by all calcium concentrations [21], soluble liver PDEase is not sensitive to calcium ions [21].

Moreover, papaverine, the well-known inhibitor of PDEase, influences calcium movements in skeletal [22] and in smooth [20, 23] muscle.

In this paper, a biphasic effect of calcium ions on soluble bovine heart PDEase is presented. On this basis a double inhibitory influence of papaverine on soluble cardiac PDEase is hypothesized.

METHODS

Materials. All reagents were commercial grade. TRIS, cAMP, DEAE-cellulose, 5′-nucleotidase (Grade II—from *Crotalus adamanteus* venom), histone and bovine serum albumin were from Sigma; papaverine and theophylline from Erba S.p.A., imidazole from Schuchardt, (ethylene bis[oxyethylenenitrilo])-tetraacetic acid (EGTA) from Eastman Organic Chemicals, noradrenaline from Recordati, AG 50W-X8 resin from Biorad Laboratories, γ^{32}P-ATP was from Radiochemical Centre, Amersham.

PDEase preparation. The enzyme preparation and purification procedure followed partially the methods of Butcher and Sutherland [24] with some modifications. Fresh or −45° frozen bovine heart was cut into strips and homogenized for 1 min with 2 vol 10% sucrose–3·5 mM Tris HCl pH 7·3 in a Braun homogenizer at high speed. The homogenate was then filtered through cheesecloth and centrifuged at 105,000 *g* for 60 min. The precipitate was discarded and the supernatant adjusted to 20% saturation with solid ammonium sulphate. The sediment was discarded and the supernatant was adjusted to 50% saturation with solid ammonium sulphate. The precipitate was taken up in a little volume of 10% sucrose–Tris 3·5 mM pH 7·3 and dialyzed overnight against 20 vol of the same solution. This soluble PDEase preparation, called 20–50% $(NH_4)_2SO_4$ dialyzed fraction, was then applied to a DEAE–cellulose column and eluted with a step KCl gradient from 0·1 to 0·2 then to 0·5 M according to the method of Wang *et al.* [25].

Assay of PDEase activity. PDEase activity was measured according to the procedure of Butcher and Sutherland [24]. Each tube contained 40 mM Tris HCl pH 7·3, 2 mM $MgCl_2$, 0·4 mM cAMP, 50 μg 5′-nucleotidase in the last 10 min of incubation, 20–200 μg of protein and, where indicated, 1 mM EGTA in a final vol of 1 ml. Low stabilized calcium concentrations were calculated by Ca–EGTA complex according to Portzehl *et al.* [26]. Minor variations are indicated in the legends of tables and figures. Reactions, carried out at 37° for 30 min, were stopped

Table 1. Ventricle bovine heart PDEase activity at different steps of purification

Fraction	Sp. act. (± S.E.) (μmoles cAMP hydrolyzed. mg^{-1}. hr^{-1})	% of total protein recovered	% of total PDEase recovered
105,000 *g* supernatant	1·37 ± 0·04		
20–50% $(NH_4)_2SO_4$ precipitate dialyzed	5·40 ± 0·50		
DEAE–cellulose eluates:			
0·1 M KCl	0	62·0	0
0·2 M KCl	11·76 ± 0·98	34·5	88·3
0·5 M KCl	15·40 ± 2·70	3·5	11·7

Experimental conditions are described in Methods.

The total protein recovery after fractionation from DEAE–cellulose is 85%, the total PDEase recovery is 70%.

The data are the mean ± S.E. of values obtained from four preparations.

with 0·1 ml TCA 50%. Proteins were determined according to the method of Lowry [27]. P_i was analyzed by the method of Fiske and Subbarow [28].

Incubation for cAMP assay. Each vessel contained 1·9 ml Krebs–Ringer bicarbonate pH 7·2, 2·5% bovine albumin and, where indicated, papaverine, and/or noradrenaline, 195–205 mg ventricular rat heart cut into small squares. Reactions, carried out at 37° for 10 min, were stopped with 1 ml of 3% TCA. Isolation of, and assay for cAMP partially followed the method of Kuo and Greengard [29, 30] with some modifications according to Dorigo *et al.* [31]. The assay of the nucleotide is based upon the ability of a protein kinase from bovine heart to catalyze the transfer of ^{32}P to histone from $\gamma^{32}P$-ATP in a cyclic AMP-dependent reaction. The enzyme preparation was that obtained after the DEAE–cellulose step of purification. Histone-bound ^{32}P was measured in a Beckman liquid scintillation counter.

RESULTS

Effect of Ca^{2+} on PDEase activity. Table 1 shows the recovery and the progressive purification of PDEase activity during its fractionation. In particular the elution of absorbed PDEase from DEAE–cellulose column with a step KCl gradient, results in a first fraction very rich in proteins but without enzymatic activity, a second with a good content of proteins and a good PDEase activity, and a third with very low protein concentration and the highest enzymatic activity. It appears that the second fraction is quantitatively the most relevant.

Table 2 reports the effect of EGTA on PDEase at different steps of purification. The Ca^{2+}-chelating agent lowers the enzymatic activity in the supernatant, in the 20–50% $(NH_4)_2SO_4$ precipitate dialyzed, and in the 0·2 M KCl eluate, but not in the 0·5 M KCl fraction. This indicates that a part of PDEase activity is supported by a stimulating effect of Ca^{2+} ions and that the greater part of PDEase keeps the sensitivity to Ca^{2+} ions after the passage through the DEAE–cellulose column. Experiments not reported showed that a further chromatographic purification of the 0·2 M KCl fraction through a DEAE-cellulose column and elution with an exponential gradient of 20 mM Tris–HCl pH 7·3 and 0·5 M $(NH_4)_2SO_4$ in 20 mM Tris HCl according to Cheung [32], increases its specific activity and maintains the same sensitivity to EGTA.

In view of the similar specific activities and similar K_m of the second and the third fractions, the enzyme seems to be the same.

The effect of increasing concentrations of Ca^{2+} on PDEase at the step of 20–50% $(NH_4)_2SO_4$ dialyzed precipitate, is reported in Fig. 1. There is evidence that calcium concentrations under 1–1·5 nmoles/mg

Table 2. Effect of EGTA on PDEase from different fractions

Fraction	PDEase activity (μmoles cAMP hydrolyzed.mg^{-1}.hr^{-1}) no EGTA	1 mM EGTA	% of inhibition	Apparent K_m
105,000 *g* supernatant	0·94	0·43	45·7	
20–50% $(NH_4)_2SO_4$ precipitate dialyzed	4·56	2·39	47·6	
DEAE-cellulose eluates:				
0·2 M KCl	11·01	6·58	40·2	$3·3 \times 10^{-4}$
0·5 M KCl	21·20	19·75	6·8	4×10^{-4}

Experimental conditions are described in Methods.

The data are from a single preparation.

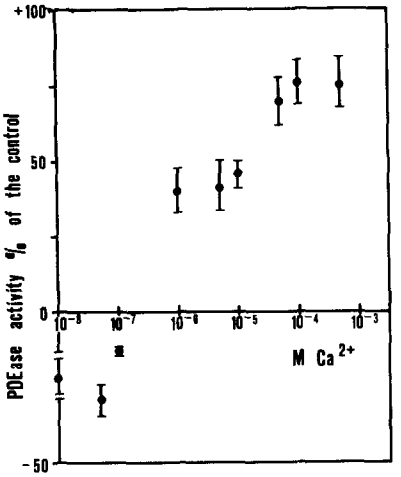

Fig. 1. Effect of increasing concentrations of Ca^{2+} on PDEase of 20–50% $(NH_4)_2SO_4$ precipitate dialyzed fraction. Experimental conditions are described in Methods. The 'free' Ca^{2+} concentrations from 10^{-8} to 10^{-5} are calculated by the Ca^{2+}-EGTA complex according to Portzehl *et al.* [26], the higher concentrations are achieved with $CaCl_2$. Enzyme proteins: 150 μg/ml. The % refers to the activity in the presence of 1 mM EGTA. Data are the mean ± S.E. of values obtained from four preparations.

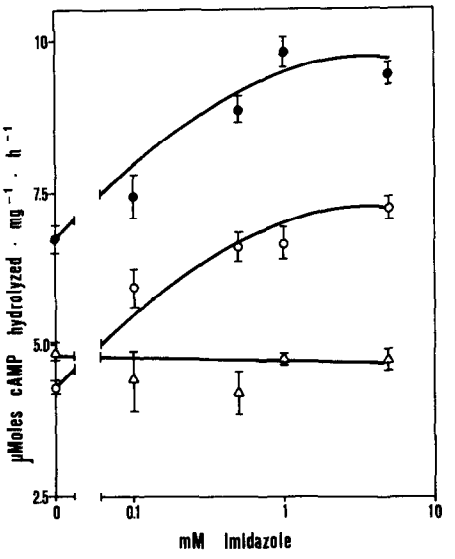

Fig. 3. Effect of EGTA and papaverine on imidazole-stimulated PDEase activity of 0·2 M KCl fraction. Experimental conditions are described in Methods. ●——● control, ○——○ 1 mM EGTA, △——△ 0·1 mM papaverine. Enzyme proteins: 60 μg/ml. Data are the mean ± S.E. of five assays.

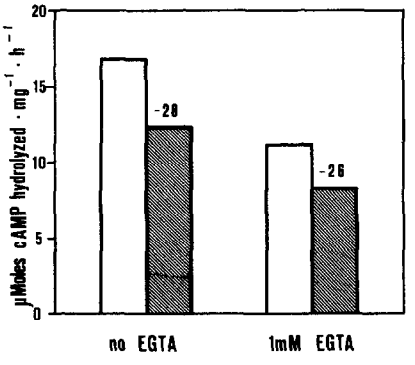

Fig. 2. Effect of papaverine on PDEase of 0·2 M KCl fraction in the absence and in the presence of EGTA. Experimental conditions are described in Methods. Enzyme proteins: 60 μg/ml. White columns = controls, black columns = 0·1 mM papaverine. The values up the black columns are the per cent of inhibition. Data are from a single experiment.

protein inhibit PDEase activity, that, in contrast, is stimulated by higher calcium concentrations.

Effect of papaverine on PDEase and on cAMP content and synthesis. PDEase is inhibited by papaverine at all different stages of its purification. 0·26 mM is the drug concentration which gives 50% inhibition of PDEase of EGTA-sensitive 0·2 M KCl fraction. Moreover, Fig. 2 shows that the effect of papaverine is the same, both in the absence and in presence of EGTA, thus indicating its 'direct' effect on the enzyme.

In Table 3 the effect of papaverine on cAMP content and synthesis induced by noradrenaline in rat heart is reported. It is evident that papaverine increases, with respect to the control, the cAMP content about 2 times and the cAMP accumulation induced by noradrenaline 9 times.

Effect of imidazole on PDEase activity. Imidazole exhibits on 0·2 M KCl-fraction PDEase a biphasic

Table 3. Effect of papaverine on cAMP content and synthesis induced by noradrenaline in rat heart

	cAMP pmoles/g fresh tissue	
—	187·77 ± 16·74	
0·2 mM Papaverine	298·41 ± 18·43	P < 0·005*
0·01 mM Noradrenaline	212·96 ± 13·73	P = NS*
0·01 mM Noradrenaline + 0·2 mM Papaverine	1828·18 ± 148·72	P < 0·001†

Experimental conditions are described in Methods.
* P vs control values. NS = not significant.
† P vs control and 0·01 mM noradrenaline values.
The data are the mean ± S.E. of four assays.
Preincubation time with papaverine: 30 min to allow the drug to penetrate into the cells.

effect by increasing enzymatic activity at concentrations below 5 mM and inhibiting it at higher concentrations. Figure 3 shows that the stimulating effect of imidazole is still evident in the presence of EGTA, and therefore is not Ca^{2+} mediated. The same stimulating concentrations of imidazole do not antagonize the inhibitory effect of papaverine.

DISCUSSION

Present results show that the PDEase soluble activity isolated from bovine heart is dependent on Ca^{2+}. The sensitivity of the enzyme to this ion is maintained during the purification by the DEAE–cellulose column. Calcium ions affect PDEase biphasically, low concentrations inhibiting it, high concentrations stimulating it. This effect, together with the previously reported influence of Ca^{2+} on PDEase from different tissues [18–21], suggests the possibility that calcium ions could vary the intracellular content of cAMP through a modification of the activity of these hydrolyzing enzymes. In particular, recent findings that, in the heart, cAMP varies during the myocardial contraction cycle [5], rising just before the contraction (when Ca^{2+} is still low) and lowering after, when the relaxation begins (when Ca^{2+} is still high), agree with the possibility that soluble PDEase could be blocked or could work very slowly when Ca^{2+} is low in the cell, thus permitting the cAMP increase. PDEase would work to its highest degree when Ca^{2+} is high in the cell, thus destroying cAMP.

In this context, papaverine is of particular interest: it is furnished with inotropic activity [33–35] and inhibits PDEase activities isolated from many tissues and also from the heart [4]. Papaverine interferes also with the movements of Ca^{2+}, by increasing its uptake in to sarcotubular membranes in skeletal muscle [22] and its binding in smooth muscle [20], or by impairing its utilization , through a decrease of permeability to it across the plasma membrane in smooth muscle (for references to papaverine in smooth muscle see Ferrari [23]).

In the present results, papaverine inhibits soluble cardiac purified PDEase by directly interacting with the enzyme. In this respect imidazole and calcium, which both possess a stimulating effect on soluble PDEase, cannot remove the block induced by the drug. This could mean that papaverine has a strong affinity to the enzyme and/or that the interaction sites of imidazole, calcium and papaverine with the enzyme are not the same. Further, these results agree with the non-antagonism observed on smooth muscle between contracting concentrations of imidazole and relaxing doses of papaverine [36]. On the other hand, if papaverine—as in smooth and in skeletal muscle—modifies the intracellular calcium distribution in the heart too, then an indirect effect of the drug on PDEase activity, through the altered availability of Ca^{2+}, cannot be excluded.

Together with the inhibition of PDEase, papaverine increases the cAMP content and the cAMP synthesis induced by noradrenaline in isolated heart tissue, incubated *in vitro*. An increase of cAMP content during the relaxation with papaverine was found in intestinal and vascular smooth muscle [37] but not in uterine muscle [38]. In adipose tissue too the inhibiting effect on PDEase by papaverine was not accompanied by an increase of cAMP [39]. These contrasting results suggest that the pharmacological actions of the drug in different tissues are probably the result of a different balancing of its effects on PDEase, on the movements of calcium, and on energy production [40, 41].

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