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# Cyclic AMP induced stimulation and inhibition of $Ca^{2+}$-uptake in rat cardiac sarcolemma vesicles

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Modulation of cardiac contractility by catecholamines is assumed to be mediated by an increase in intracellular levels of cyclic AMP which in turn stimulate cyclic AMP dependent protein kinase (PK). Phophorylation of isolated cardiac sarcolemma (SL) or sarcoplasmic reticulum (SR) by cyclic AMP dependent PK has been found to result in an enlarged and accelerated $Ca^{2+}$-accumulation by those preparations [1–3]. It has been suggested that the mechanical effects of catecholamines on the heart may be attributed to stimulation of $Ca^{2+}$-transport in SR [4, 5] as well as in SL [1, 6] as a result of cyclic AMP induced phosphorylation. Unfortunately, in a number of studies on $Ca^{2+}$-transport by cardiac membranes, contamination of the SL preparations with SR or vice versa was not considered very carefully [5–7], which made the interpretation of the results not unambiguous. For this reason and because of the contrasting views on the role of the SL and the SR in the modulation of intracellular $Ca^{2+}$-levels [4, 9], we developed an isolation procedure for cardiac SL [10] and studied the modulation of sarcolemmal $Ca^{2+}$-uptake by cyclic AMP induced phosphorylation. A stimulation as well as an inhibition of this $Ca^{2+}$-uptake was found, which was dependent on the SL protein concn and on the PK/SL protein concn ratio.

*Methods and materials*. SL vesicles were prepared from Wistar albino rat cardiac tissue at 4° as described previously [10]. Briefly: 20 cardiac ventricles were homogenized in 80 ml 20 mM Tris–HCl, 1 mM EDTA (pH 7.0) using a Virtis 45 blender for four periods of 7 sec at full speed with rest intervals of 15 sec. The homogenate was centrifuged by three successive runs of 20 min at 8800, 12,500 and 12,500 *g*, respectively, and 1 run of 60 min at 44,000 *g*. The resulting pellet was resuspended in 15 ml 20 mM Tris–oxalate, 0.6 M KCl, 1 mM EDTA (pH 6.8) and recentrifuged at 44,000 *g* for 60 min. The membrane preparations used for enzyme determinations and alprenolol binding were stored at −70° in 20 mM Tris–HCl, 10 mM $MgCl_2$ (pH 7.2). The membrane preparations used for $Ca^{2+}$-uptake experiments were stored at −70° in a medium consisting of 40 mM histidine, 5 mM $MgCl_2$, 75 mM KCl (pH 6.8). The membrane preparations used for marker enzyme determinations were stored at −70° in a medium consisting of 20 mM Tris–HCl (pH 7.2), 10 mM $MgCl_2$. The SL preparation was characterized by the following marker enzymes: ouabain sensitive ($Na^+$–$K^+$)ATPase (SL marker [11]), adenylate cyclase (SL marker [12]), NADH–ferricyanide oxidase (SR marker [13]), succinate cytochrome *c* reductase (mitochondrial marker [14]). Enzyme activities were measured by the method reported in the papers indicated by the reference numbers. Protein was determined by the method of Lowry *et al.* [15] using bovine serum albumin as standard. (−)-[$^3$H]Dihydroalprenolol (DHA) binding sites in the SL fraction were determined in freshly prepared preparations according to the method of Lefkowitz [16]. Specific binding was measured as the radioactivity displaceable with 0.1 mM (−)-alprenolol and was greater than 80% of total binding in the SL fraction and greater than 60% of total binding in the homogenate. Protein kinase type I was isolated from rat heart tissue by the method of Miyamoto *et al.* [17] through the DEAE-cellulose chromatography step. Only the two highest peak fractions of PK type I were used. Cyclic AMP dependent membrane protein phosphorylation was measured by the method of Erlichman *et al.* [28] in 20 mM Tris–HCl (pH 7.2), 10 mM $MgCl_2$ and was carried out at 30° for 5 min using [$\gamma$-$^{32}$P]ATP (2–40 nCi/nmole ATP). The reaction was stopped by the successive addition of 1 ml cold 10% (w/v) trichloroacetic acid (TCA), 0.1 ml 0.63% (w/v) bovine serum albumin and 1 ml TCA. Following centrifugation at 3000 rpm for 5 min, the pellet was washed twice, solubilized in 0.3 ml 0.1 N NaOH, dissolved in 4 ml Lumagel and counted in a Searle Isocap 300 liquid scintillation counter. $Ca^{2+}$-uptake was carried out at 30° in the histidine storage buffer solution, supplemented with 2.5 mM Tris–oxalate, 125 $\mu$M [$^{45}$Ca]$CaCl_2$ (5000–10,000 cpm/nmole), 5 mM ATP with or without 1 $\mu$M cyclic AMP and PK according to the method of Hui *et al.* [6]. In some experiments an ATP-regenerating system of 4 mM phosphoenolpyruvate and 20 I.U. pyruvate kinase was used. The free $Ca^{2+}$-ion concentration in the reaction mixture was set at 10 $\mu$M by the addition of 87 $\mu$M EGTA as calculated by the method of Katz *et al.* [18]. Five minutes after the addition of the appropriate amount of SL protein to the mixture the $^{45}$Ca-uptake reaction was started by the addition of ATP. The reaction was terminated by rapid filtration of duplicate samples of 0.1 ml of the reaction mixture through Millipore HAWP filters (0.45 $\mu$m pore size). After washing with 2 ml ice-cold histidine buffer and drying, the filters were dissolved in 4 ml Lumagel and counted. Corrections were made for the $^{45}Ca^{2+}$-radioactivity bound to the filters in the absence of SL protein. Sarcolemmal ($Ca^{2+}$–$Mg^{2+}$)ATPase activity was determined using the same incubation conditions as for the $Ca^{2+}$-uptake experiments, by measuring the decline of the ATP concn by the glucose-6-phosphate dehydrogenase, hexokinase method and measuring the production of ADP and AMP using the pyruvate kinase, lactate dehydrogenase, adenylate kinase method, both described by Gruber *et al.* [26].

*Results*. Table 1 shows that a 22-fold enrichment of specific ($Na^+$–$K^+$)ATPase activity was found in the SL fraction as compared with the original homogenate, whereas the sp. act. of the SR marker, NADH–ferricyanide oxidase, increased only 3-fold. In addition, the recovery of the ($Na^+$–$K^+$)ATPase in the SL fraction amounted to 10.8% whereas only 1.6% of the NADH–ferricyanide oxidase activity of the original homogenate was present in the SL fraction. A comparison of the ratio of the sp. acts and of the recoveries of these enzymes in the homogenate and the SL fraction reveals that the latter is enriched seven-fold or eight-fold in SL fragments compared to SR fragments. The number of [$^3$H]DHA binding sites and the adenylate cyclase activity was also increased markedly in the SL fraction. In relation to this it should be noticed that in our SL preparation 3.5 times more [$^3$H]DHA binding sites are present than in the rat ventricular membrane fraction described recently by Lefkowitz and coworkers, which was also prepared by differential centrifugation [25]. No attempt was made to unmask latent [$^3$H]DHA binding, adenylate cyclase and ($Na^+$–$K^+$)ATPase activity, which is to be expected due to the presence of inside-out (IO) and right side-out (RO) vesicles in the SL preparation as was determined by freeze-fracturing electron microscopy [10]. The high ($Na^+$–$K^+$)ATPase activity presumably represents already all the (patent + latent) activity, since the large difference in osmolarity between the storage buffer and the

Table 1. Marker enzyme activities in rat heart membrane fractions

| | Homogenate | Sarcolemma |
|---|---|---|
| Ouabain-sensitive ($Na^+$–$K^+$)ATPase* | 8.2 ± 1.1 (100) | 180.0 ± 17.7 (10.8) |
| NADH–ferricyanide oxidase* | 63.6 ± 6.8 (100) | 177.8 ± 30.3 (1.6) |
| Succinate cytochrome *c* reductase* | 2.4 ± 0.4 (100) | 1.9 ± 0.4 (0.4) |
| (−)-[$^3$H]Dihydroalprenolol binding†‡ | 27.1 ± 9.3 (100) | 124.3 ± 12.9 (2.3) |
| Adenylate cyclase†§ | | |
| Basal | 4.0 ± 1.2 (100) | 13.1 ± 2.6 (1.6) |
| + 20 mM NaF | 19.9 ± 3.7 | 51.5 ± 10.6 |
| + 10 μM (−)-isoprenaline | 6.7 ± 1.3 | 18.0 ± 2.6 |

Activities were measured as described in text.

Sp. act. values represent means ± S.E.M. from duplicate experiments on four to eight individual membrane preparations.

Recoveries of all activities are indicated between parentheses.

* Expressed as μmoles substrate/mg protein/hr.

† Values are underestimated due to IO, RO vesicle distribution.

‡ Expressed as fmoles [$^3$H]DHA bound/mg protein.

§ Expressed as pmoles cyclic AMP/mg protein/10 min.

ATPase assay medium induces an osmotic shock just before the assay. Evidence for this is based on the observation, described previously [10], that SDS incubation according to Besch *et al.* [19] did not increase sp. act. However, the IO, RO vesicle distribution will interfere with the determination of the number of [$^3$H]DHA binding sites and the adenylate cyclase activity in the SL preparation and of the recoveries, resulting in underestimated values. Evidence for this was obtained after a substantial separation and purification of RO vesicles from IO vesicles achieved recently by using the wheat germ lectin Sepharose affinity chromatography procedure of Mas-Oliva *et al.* [27]. In the RO vesicle preparation 609 fmoles/mg of specific [$^3$H]DHA binding sites were present, which implicates an approximately 22-fold enrichment of β-adrenoceptor sites in the SL fraction compared to the original homogenate. This agrees extremely well with the enrichment of the ($Na^+$–$K^+$)ATPase activity in the SL preparation.

The ($Ca^{2+}$–$Mg^{2+}$)ATPase activity of the SL preparation amounted to 32.4 ± 1.8 μmoles/mg protein/10 min. In the presence of 10 μg/ml oligomycin or 5 mM $NaN_3$, the enzyme activity amounted to 34.1 and 33.0 μmoles/mg protein/10 min, respectively. This indicates that mitochondrial ATPase activity is not present.

Fig. 1 (panel A) shows that at a protein concn of 0.1 mg/ml, the $Ca^{2+}$-uptake is linear with time up to 6 min. Cyclic Amp (1 μM) and PK at a low concn (0.04 mg protein/ml) induces a 20% increase of uptake velocity. On the other hand, $Ca^{2+}$-uptake is no longer linear with time and about 2.5 times lower when a higher SL protein concn of 0.7 mg/ml was applied (panel B). This lower $Ca^{2+}$-uptake was probably due to exhaustion of ATP by $Mg^{2+}$-ATPase, since by using a somewhat lower SL protein concn (0.47 mg/ml) plus an ATP-regenerating system, the $Ca^{2+}$-uptake is enhanced approximately 3 times (panel B). In Fig. 1 it is also shown that after phosphorylation of the

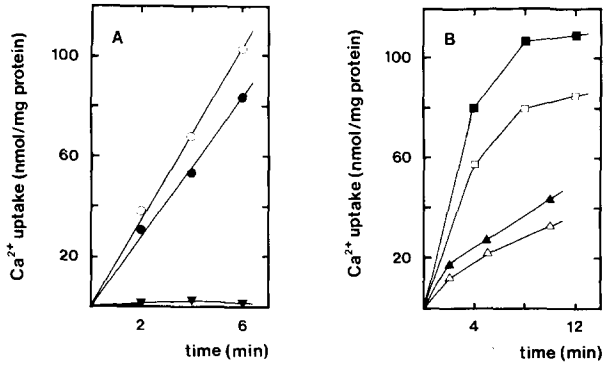


Fig. 1. Time course of sarcolemmal $Ca^{2+}$-uptake in the absence and presence of cyclic AMP and protein kinase type I. $Ca^{2+}$-uptake was carried out as described in the text. The incubation volume was 0.7 ml. Membrane protein concns used were 0.1 mg/ml (●,○,▼), 0.47 mg/ml (■,□) and 0.7 mg/ml (▲,△). At the time points indicated, duplicate samples of 0.1 ml were taken and processed as described. The ATP dependency of the sarcolemmal $Ca^{2+}$-uptake is demonstrated by incubating 0.1 mg/ml membrane protein in the presence (●) and absence (▼) of 5 mM ATP. The protein kinase concns used were 0.04 mg/ml (○), 0.32 mg/ml (□) and 0.8 mg/ml (△). One micromolar cyclic AMP was added to all incubations with protein kinase. The ATP-regenerating system was used in the experiments with a membrane protein concn of 0.47 mg/ml. Values are means of duplicate experiments on three individual membrane preparations.

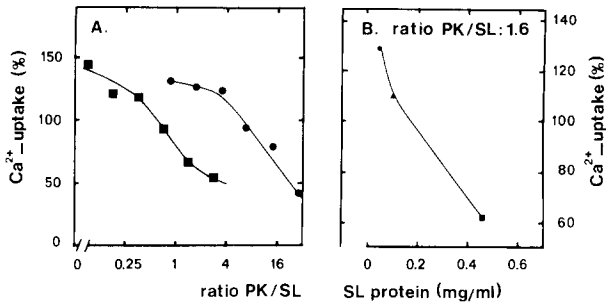


Fig. 2. Dualistic effect of cyclic AMP and protein kinase type I on sarcolemmal $Ca^{2+}$-uptake. $Ca^{2+}$-uptake was carried out for 6 min as described in the text in the presence of the ATP-regenerating system and various protein kinase concns. The incubation volume was 0.3 ml and duplicate samples of 0.1 ml were filtered through Millipore HAWP filters (pore size 0.45 $\mu$m). Membrane protein concns used were 0.046 mg/ml (●), 0.1 mg/ml (▲) and 0.46 mg/ml (■). One micromolar cyclic AMP was added to all incubations with protein kinase. (A) The ratio of protein kinase protein/sarcolemma protein (PK/SL ratio) is plotted vs the sarcolemmal $Ca^{2+}$-uptake expressed as a percentage of the control values without cyclic AMP and protein kinase. Values are means of duplicate experiments on one membrane batch. (B) The sarcolemmal $Ca^{2+}$-uptake at a PK/SL ratio of 1.6, expressed as a percentage of the control value, is plotted vs the sarcolemma protein concn in the assay. Values are means of duplicate experiments on two individual membrane preparations. In the absence of cyclic AMP and protein kinase, the $Ca^{2+}$-uptake amounted to 103, 84 and 61 nmoles $Ca^{2+}$/mg protein/6 min at sarcolemma protein concns of 0.046, 0.1 and 0.46 mg/ml, respectively.

higher SL concns with cyclic AMP and PK, the $Ca^{2+}$-uptake is not stimulated but inhibited by approximately 20%.

The dual effect of cyclic AMP and PK is further substantiated in Fig. 2, where it is shown that the stimulation and the inhibition of SL $Ca^{2+}$-uptake depends on the SL protein concn and the ratio of PK/SL protein. These experiments were performed in the presence of the ATP-regenerating system. At 0.046 mg SL protein/ml and a PK/SL ratio of 1, the $Ca^{2+}$-uptake is stimulated by 30%. This stimulation gradually changes into inhibition with increasing PK/SL ratios. At a SL protein concn of 0.46 mg/ml, the $Ca^{2+}$-uptake shows essentially the same picture but at lower PK/SL ratios (panel A), implicating that at intermediate PK/SL ratios both stimulation and inhibition should be observed independent of the SL protein concentration. Fig. 2B depicts the dual effect of cyclic AMP and PK at three different SL protein concns all with a constant PK/SL ratio of 1.6. A stimulation of 30% is found at the lowest protein concn, whereas a 40% inhibition is found at the highest protein concn.

Evidence that the amount of cyclic AMP induced membrane protein phosphorylation increases with increasing PK/SL protein ratios is shown in Fig. 3.

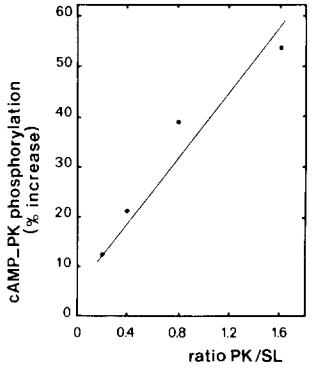


Fig. 3. Phosphorylation of SL protein independent of the PK/SL ratio. Phosphorylation was carried out as described in the text. The SL protein concn amounted to 0.33 mg/ml and the incubation volume was 0.3 ml. One micromolar cyclic AMP was added to all incubations with protein kinase. The PK/SL ratio was reached by adding the appropriate amount of PK protein. Cyclic AMP and PK induced phosphorylation of SL protein is expressed as a percentage of the basal phosphorylation in the absence of cyclic AMP and PK, which amounted to 40 pmoles $P_i$/mg SL protein/5 min. Values are means of duplicate experiments on four individual membrane preparations.

*Discussion.* ATP-dependent $Ca^{2+}$-uptake in SL vesicles is believed to represent the active outward directed $Ca^{2+}$-transport *in vivo* [9], which is essential for $Ca^{2+}$-homeostasis. This outward $Ca^{2+}$-transport may also participate in lowering the cytoplasmic $Ca^{2+}$-concn to achieve myocardial relaxation [1, 21]. Stimulation of $Ca^{2+}$-uptake in SL vesicles by cyclic AMP and PK was thus interpreted to contribute to the relaxation acceleration of catecholamines [1, 3, 6, 21]. The stimulation of SL $Ca^{2+}$-uptake by cyclic AMP and PK at low PK/SL ratios, as described here, agrees with the work of several investigators [1, 3, 6, 21]. In addition however, using higher PK/SL ratios, we found an apparent inhibition of the $Ca^{2+}$-uptake, which is not reported before. This inhibition of $Ca^{2+}$-uptake suggests that higher levels of SL protein phosphorylation caused by increasing the PK concn (Fig. 3) induce an inhibition of the $Ca^{2+}$-pump. However, in line with the suggestion of Ziegelhoffer *et al.* [8] that opening of the slow $Ca^{2+}$-channel in the SL is modulated by cyclic AMP dependent phosphorylation, another possibility could be that such phosphorylation apparently inhibits $Ca^{2+}$-uptake by opening $Ca^{2+}$-channels in the membrane, as a consequence of which the efflux of $Ca^{2+}$ from the vesicles increases. One could argue that the inhibition of the $Ca^{2+}$-uptake could be due to the presence of some unknown factor in the protein kinase preparation able to inhibit the $Ca^{2+}$-pump. However, this is in contradiction with the occurrence of the dual effect on the $Ca^{2+}$-uptake in the experiments, in which increasing SL membrane protein concns at a constant PK/SL ratio were used, as is shown in Fig. 2B.

Both the inhibition of the $Ca^{2+}$-pump and the opening of $Ca^{2+}$-channels may be interpreted as contributing to the positive inotropic action of catecholamines, the former by raising the $Ca^{2+}$-concn in the cytoplasm available for con-

traction, the latter by increasing the $Ca^{2+}$-flux into the cell [3, 8, 22, 23]. Moreover a combination of these two mechanisms could be considered as well.

In this context, it is noteworthy that several groups have reported the cyclic AMP induced phosphorylation of several different SL proteins *in vitro* [3, 21, 22, 24]. Although at present any physiological role of one or more of these SL membrane proteins remains to be established, we may now have an indication that more than one phosphorylateable SL protein is involved in the cyclic AMP dependent modulation of $Ca^{2+}$-fluxes across the SL.

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*Department of Medicinal Chemistry*
*Molecular Pharmacology Section*
*Free University*
*De Boelelaan 1083*
*1081 HV Amsterdam*
*The Netherlands*

JOHAN VELEMA
GERT R. BOLT
JOHAN ZAAGSMA

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## The accumulation of polyamines and paraquat by human peripheral lung

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The herbicide paraquat (1,1′-dimethyl-4,4′-bipyridylium) can accumulate in the lung of various species including humans [1, 2]. This accumulation has been shown to obey saturation kinetics and to be energy-dependent [2, 3]. The system responsible for the accumulation in rat lung is different from that reported for the uptake of the monoamine 5-hydroxytryptamine [4], although it may be the same as that responsible for the accumulation of the diamine putrescine [5]. Recently Smith *et al.* [6] also decribed the energy-dependent accumulation of the endogenous polyamines, spermine and spermidine, by rat lung slices, which appears to be similar to that previously described for the uptake of paraquat and putrescine [5].

The existence of systems for the accumulation of spermidine and spermine are not unique to the lung. Similar systems have been described in mouse and rabbit brain [7, 8] and human leukocytes [9]. In the mouse brain [7], the accumulation of spermidine and spermine is sodium-independent and appears to be similar to the accumulation of putrescine in rat lung and brain [5]. More recently two very high-affinity systems have also been described for the accumulation of spermine in rat brain slices [10] but these systems were sodium-dependent and therefore may be different from the sodium-independent system previously