ON THE ROLE OF CYCLIC AMP AND Ca^{2+}–CALMODULIN-DEPENDENT PHOSPHORYLATION IN THE CONTROL OF (Ca^{2+} + Mg^{2+})-ATPase OF CARDIAC SARCOLEMMA

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1. Introduction

Several reports [1–3] have provided in vitro evidence that cAMP, acting through protein kinase, may regulate the cardiac SL Ca^{2+}-pump. Thus it is a viable hypothesis that one site of action, whereby catecholamines modulate contraction, is via stimulation of phosphorylation of the plasma membrane. It was indicated in [4] that cAMP-dependent protein kinase catalyzed the phosphorylation of SL proteins of app. M_r 24 000 and 9000. Subsequently, it was shown that these proteins were interconvertible. In agreement with [5–7] the phosphoproteins were demonstrated to be present in both SL and SR. The phosphoprotein in SR has also been reported to dissociate into smaller protein fragments [4,8].

Calmodulin has been recognized as a general regulator of Ca^{2+}-controlled functions too [9,10] and some of its effects may be mediated through phosphorylation of specific proteins [11]. For the stimulation of the Ca^{2+}-pump in SR by cAMP-dependent phosphorylation to become effective, a prior phosphorylation by Ca^{2+}–calmodulin-dependent protein kinase was necessary [12,13]. These findings differed from [14–16], which showed an effect of cAMP-dependent protein kinase on (Ca^{2+} + Mg^{2+})-ATPase without the need of a Ca^{2+}-dependent phosphorylation. Using crude cardiac microsomes, stimulatory effects on the Ca^{2+}-pump by either cAMP or calmodulin have been found to be independent of each other [17].

We now report the cardiac SL protein of M_r 9000 to be a substrate for both a Ca^{2+}–calmodulin and a cAMP-dependent protein kinase. Also some other substrate proteins were detected specific for either type of protein kinase. cAMP-dependent phosphorylation caused a 1.6-fold increase in affinity of the Ca^{2+}-pump enzyme for Ca^{2+}, without changing its maximal rate. No effects were observed if membranes were phosphorylated by Ca^{2+}–calmodulin-dependent kinase. Half-maximal activation of the latter enzyme was reached at free [Ca^{2+}] as high as 1 μM, suggesting that it becomes only maximally operative in vivo at free [Ca^{2+}] present during systole.

2. Materials and methods

The calmodulin used was purified to homogeneity from bovine brain according to [18]. Half-maximal stimulation of a cAMP phosphodiesterase, purified from rat brain according to [19], was reached at ~10 nM calmodulin. Trifluoroperazine was a gift from Janssen Pharmaceutica, Beerse. cAMP-dependent protein kinase was purchased from Sigma (cat. no. P5511). The origin and quality of the other chemicals and biochemicals used were as in [4,20].

Cardiac SL membranes were isolated from dog heart as in [4]. The putative plasma membrane markers 5′-nucleotidase, ouabain-sensitive K^+-pNPPase and adenylate cyclase (48 ± 8, 83 ± 7 and 2.1 ± 0.5 nmol . min^{-1} . mg^{-1}, n = 5, respectively) were enriched 25-, 10- and 12-fold in respect to the homogenate, respectively. Conversely the SR marker rotenone-

Abbreviations: cAMP, cyclic adenosine 3′,5′-monophosphate; DTT, dithiothreitol; SDS, sodium dodecylsulphate; PAGE, polyacrylamide gel electrophoresis; EGTA, ethylene glycol bis(β-aminoethyl ether)N,N'-tetraacetic acid; SL, sarcolemma; SR, sarcoplasmic reticulum

insensitive NADPH cyt. *c* reductase, the mitochondrial marker succinic dehydrogenase and myofibrillar K^+-ATPase (11 ± 5, 10 ± 2 and 0 nmol . min^{-1} . mg^{-1}, $n = 5$, respectively) had low activities relative to the homogenate (6 ± 2, 28 ± 3 and 163 ± 21 nmol . min^{-1} . mg^{-1}, respectively). Determination of all marker enzymes was performed as in [4,20]. The membrane preparation contained an ATP-dependent Ca^{2+}-transport system as shown for rat heart [20]. The Ca^{2+}-uptake could not be stimulated by oxalate, but was inhibited by extravesicular Na^+ due to the Na^+/Ca^{2+}-antiporter present. These properties have been shown to be valuable criteria for differentiating cardiac SL from SR membranes, as shown for dog heart [21].

(Ca^{2+} + Mg^{2+})-ATPase activity was quantitated by monitoring the $^{32}P_i$ release from [γ-^{32}P]ATP by the method in [22]. Reactions were done in 200 μl medium containing 20–36 μg membrane protein/ml, 0.1 mM [γ-^{32}P]ATP, 50 mM Tris/maleate (pH 6.8), 100 mM KCl and 5 mM $MgCl_2$ at 37°C. Free [Ca^{2+}] was controlled in the sub-μM range by buffering with 100 μM EGTA. The association constants used for Ca^{2+}–EGTA, Mg^{2+} EGTA, Ca^{2+}–ATP and Mg^{2+}–ATP were 6.8×10^7, 1.1×10^2, 8.5×10^3 and 4.1×10^4 M^{-1}, respectively, at pH 7.5 obtained from [23,24]. Ion strength, pH corrections and free [Ca^{2+}] were calculated by the computer programs as in [24]. (Ca^{2+} + Mg^{2+})-ATPase was usually determined after a 4 min incubation period by subtracting values with EGTA alone from those obtained with Ca^{2+} + EGTA.

Phosphorylation of SL membranes was performed by incubation either at 25°C in 50 μl medium containing 20 mM Tris/maleate (pH 7.5), 15 mM K-phosphate, 10 mM theophylline, 5 mM $MgCl_2$, 100 mM KCl and 0.25 mM [γ-^{32}P]ATP or at 37°C in 50 μl medium with a similar composition as used in the (Ca^{2+} + Mg^{2+})-ATPase assay. After termination of the reaction samples were submitted to SDS–PAGE, after which the radioactive bands were identified and counted by autoradiography, as in [4].

3. Results and discussion

3.1. *Phosphorylation of vesicles by cAMP and Ca^{2+}–calmodulin-dependent protein kinases*

When SL membranes were incubated with [γ-^{32}P]-ATP, cAMP and cAMP-dependent protein kinase and the ^{32}P-incorporation into proteins was examined by

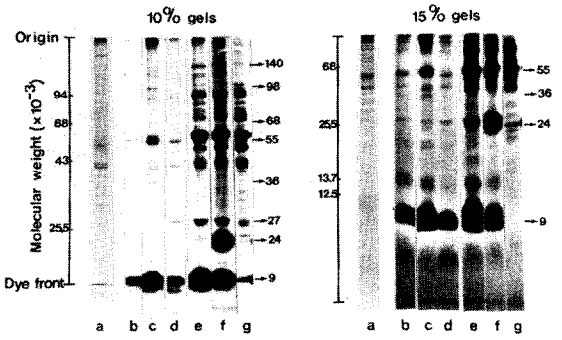

Fig.1. Autoradiographs illustrating Ca^{2+}–calmodulin- and cAMP-dependent phosphorylation of dog heart SL proteins. Membranes (0.5 mg protein/ml) were phosphorylated at 25°C under various conditions: (b) no additions; (c) 0.5 μM calmodulin + 0.2 mM $CaCl_2$; (d) 0.5 μM calmodulin, 0.2 mM $CaCl_2$ and 1 mM EGTA; (e) 10 μM cAMP and 15 mU/ml cAMP-dependent protein kinase; (f) autoradiograph of the same sample in (e) which was not treated at 95°C prior to SDS–PAGE; (g) autograph of cAMP-dependent protein kinase with no membranes added. Gels stained with Coomassie blue are shown in (a). The left and right portion of the figure shows, respectively, 10% and 15% PAGE runs of corresponding samples.

SDS–PAGE, 4 distinct protein bands were phosphorylated (fig.1). The major band had M_r 9000, the other membrane-associated proteins showed M_r 24 000, 27 000 and 140 000. The rate of ^{32}P-incorporation into these proteins was cAMP-dependent and could be blocked by the protein kinase inhibitor (not shown). The 24 000 M_r band shifted to the 9000 M_r region if samples were heated at 95°C prior to SDS–PAGE, suggesting that these proteins were analogous to those for rat heart SL [4]. The low level of phosphorylation observed if exogenous cAMP-dependent protein kinase was omitted, was due to an intrinsic cAMP-dependent protein kinase (not shown, cf. [6]). Alternatively, addition of Ca^{2+} + calmodulin to the vesicles instead of exogenous cAMP-dependent protein kinase, resulted in a completely different phosphorylation pattern (fig.1). Although the major ^{32}P-incorporation again occurred into the M_r 9000 band, distinct bands of M_r 98 000, 68 000, 55 000 and 36 000 also appeared to be Ca^{2+}–calmodulin-dependent. Ca^{2+}–calmodulin-dependent phosphorylation of SL proteins of M_r 9000 and 55 000 was also observed in rat heart SL. However, the intrinsic kinase involved in this process seemed to be more labile during the membrane isolation [25]. The substrate protein of M_r 55 000, first identified in cardiac microsomes [11], may be

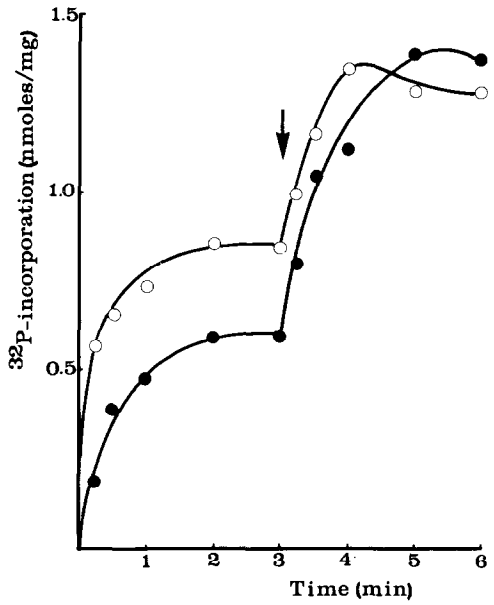

Fig.2. Time-course of the phosphorylation of the M_r 9000 protein in cardiac SL. Vesicles (0.20 mg protein/ml) were incubated under the conditions in fig.1 in the presence of 5 mU/ml cAMP-dependent protein kinase + 10 μM cAMP (○). After 3 min (→) 0.5 μM calmodulin + 0.2 mM $CaCl_2$ was added. In another series of experiments (●) Ca^{2+} and calmodulin were added first followed by addition of cAMP-dependent protein kinase at the time indicated by the arrow.

unique for SL, because we and other groups were unable to detect a similar phosphorylated protein in cardiac SR [4,12,25].

The time-course of cAMP- and Ca^{2+}–calmodulin-dependent ^{32}P-incorporation into M_r 9000 protein is shown in fig.2. It is likely that two different sites are phosphorylated, since the maximal ^{32}P-incorporation reached by each type of kinase, was almost additive. Assuming a 1 : 1 stochiometric ^{32}P-incorporation into the M_r 9000 protein, the results suggest that the Ca^{2+}-dependent substrate site is present in a partially phosphorylated state before the start of the experiments. Half-maximal inhibition of the rates of cAMP- and Ca^{2+}–calmodulin-dependent phosphorylation of M_r 9000 protein were reached at 10 μg/ml protein kinase inhibitor (specific for cAMP-dependent protein kinase) and 30 μM trifluoroperazine, respectively, indicating the specificity of each kinase. Apart from the similar M_r of the phosphorylated product obtained with both types of protein kinases, also its identical shift in mobility on SDS gels upon heating at 95°C (M_r change from 24 000 to 9000) suggests that the same substrate proteins are involved.

3.2. *Characterization of the intrinsic Ca^{2+}–calmodulin-dependent kinase*

Prior to studying the possible effect of phosphorylation on (Ca^{2+} + Mg^{2+})-ATPase activities in SL membranes, we started to investigate some properties of the Ca^{2+}–calmodulin-dependent kinase under conditions of the (Ca^{2+} + Mg^{2+})-ATPase assay: phosphate and theophylline were omitted, the pH was chosen at 6.8 and a Ca^{2+} buffering system was included. As can be seen from table 1, with 0.5 μM calmodulin present the Ca^{2+}-dependent kinase reached a half-maximal rate at ~1 μM free Ca^{2+}. This is consistent with findings in other calmodulin-dependent systems [10,11]. Half-maximal rate of phosphorylation was reached at 60 nM calmodulin, using a saturating free [Ca^{2+}] of 12 μM (table 1). Thus the intrinsic Ca^{2+}–calmodulin-dependent protein kinase had a much lower affinity for calmodulin than cAMP phosphodiesterase [10]. Under conditions of the (Ca^{2+} + Mg^{2+})-ATPase assay 5 mU/ml cAMP-dependent protein kinase incorporated phosphate at 1.7 nmol . min^{-1} . mg^{-1}, which was independent of the free [Ca^{2+}] used.

3.3. *(Ca^{2+} + Mg^{2+})-ATPase activity in relation to the degree of phosphorylation*

The use of a Ca^{2+}–EGTA buffer in studies of Ca^{2+} uptake at different Ca^{2+} levels caused rather low transport values probably due to disruption of vesicles by EGTA. Moreover, the possibility remained that the

Table 1
Calcium and calmodulin dependence of the rate of ^{32}P-incorporation into the 9000 M_r protein of cardiac SL

Ca^{2+}total (μM)	Calc. Ca^{2+}free (μM)	Calmodulin (μM)	Specific activity (nmol ^{32}P . mg^{-1} . min^{-1})
0	0	0.5	0.03
36	0.30	0.5	0.06
51	0.52	0.5	0.11
67	1.0	0.5	0.51
200	100	0.5	0.85
107	12	0	0.03
107	12	0.1	0.62
107	12	0.2	0.85
107	12	0.5	0.91
107	12	1.0	0.84

Membranes (0.2 mg membrane protein/ml) were phosphorylated at 37°C under the conditions of the (Ca^{2+} + Mg^{2+})-ATPase assay as in section 2. The initial rates of ^{32}P-incorporation were taken from the first 15 s of the incubation

Ca^{2+}–calmodulin complex bound to the membranes would contribute to the apparent Ca^{2+}-uptake. Therefore we preferred to investigate the possible relationship between (Ca^{2+} + Mg^{2+})-ATPase reaction, which promotes ATP-dependent Ca^{2+}-uptake, and the membrane phosphorylation. Fig.3 shows the activity of the enzyme as a function of free Ca^{2+}. The app. $K_{0.5}$ (0.53 μM) is 5-fold lower than that obtained in canine heart SL [26]. This discrepancy most probably results from the 10-fold higher Ca^{2+}–EGTA binding constant of 2.5×10^5 used in [26]. From the results in section 3.2, it could be inferred that in the (Ca^{2+} + Mg^{2+})-ATPase assay maximal phosphorylation of M_r 9000 protein at optimal amounts of Ca^{2+}, calmodulin, cAMP and protein kinases must be reached within 30 s. Fig.3 shows that the app. $K_{0.5}$ for Ca^{2+} is decreased 1.6-fold by the addition of cAMP and cAMP-dependent protein kinase. No effects were observed on the maximal rate of (Ca^{2+} + Mg^{2+})-ATPase, which clearly differs from findings in cardiac SR [13–16]. The most pronounced effect of cAMP-dependent protein kinase was observed at 0.3 μM Ca^{2+} (fig.3), a Ca^{2+} level at which calmodulin-dependent phosphorylation will have a minimal activity (cf. table 1). In the presence of 0.5 μM calmodulin, (Ca^{2+} + Mg^{2+})-ATPase activity was unaffected, whether stimulated at low or high [Ca^{2+}]. This result suggests that the effect of cAMP-dependent phosphorylation on the Ca^{2+}-pump of SL is not critically dependent on prior phosphorylation through Ca^{2+}–calmodulin-dependent protein kinase, which contrasts with observations reported for cardiac SR [12]. Our results were similar to a report on Ca^{2+}-uptake in cardiac microsomes, although a small effect of calmodulin on Ca^{2+} accumulation was observed [13]. However, in this paper measurements on Ca^{2+} uptake were not combined with phosphorylation studies. Therefore it remains possible that effects of calmodulin only become apparent if Ca^{2+}-uptake is studied. Indeed, evidence for this has already been provided in case of cardiac SR [12] and pancreatic islet plasma membranes [23]. To solve this problem, procedures to estimate Ca^{2+}-uptake and calmodulin binding in parallel are now under investigation.

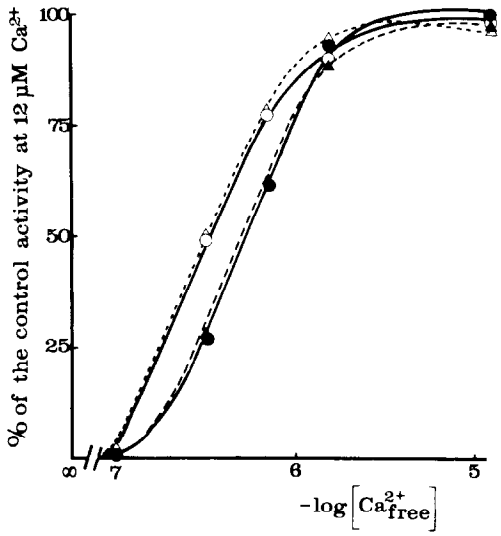

Fig.3. The effect of cAMP-dependent protein kinase and calmodulin on the Ca^{2+} dependence profile of SL (Ca^{2+} + Mg^{2+})-ATPase. Enzymic activity was estimated at different [Ca^{2+}] in the absence (•) or presence of 10 μM cAMP and 5 mU/ml cAMP-dependent protein kinase (○). The effect of adding 0.5 μM calmodulin under both conditions (respectively, ▲ and △) was also tested. The mean absolute activity at 10 μM Ca^{2+} amounted to 320 ± 36 nmol . min^{-1} . mg^{-1} (n = 6). Free [Ca^{2+}] are expressed as mol/liter.

3.4. *The effect of phosphorylation inhibitors on the (Ca^{2+} + Mg^{2+})-ATPase*

As can be seen from fig.4, the stimulation of (Ca^{2+} + Mg^{2+})-ATPase activity caused by cAMP-dependent protein kinase, could be prevented with increas-

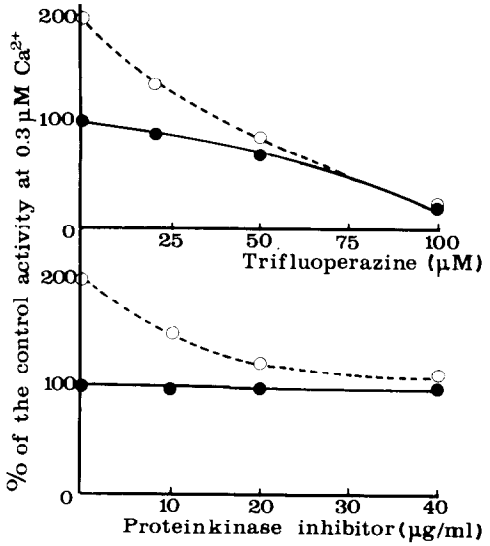

Fig.4. Effect of different concentrations of protein kinase inhibitor and trifluoperazine on (Ca^{2+} + Mg^{2+})-ATPase. Enzymic activity was estimated at a fixed [Ca^{2+}] (0.3 μM) in the absence (•) or presence of cAMP-dependent protein kinase (○). Activities were calculated as percentage of the control activity at 0.3 μM Ca^{2+} without any additions.

ing amounts of protein kinase inhibitor. Addition of 20–100 μM trifluoperazine caused a pronounced inhibition of both cAMP-stimulated and control (Ca^{2+} + Mg^{2+})-ATPase activities, measured at 0.3 μM Ca^{2+} in the absence of exogenously added calmodulin. Even the basal Mg^{2+}-ATPase was inhibited (not shown). Although at this drug concentration range inhibition of calmodulin-independent membrane protein activities may occur (e.g., α-adrenergic receptor blockade [27]), the inhibitory action of trifluoperazine fits into a model analogous to the erythrocyte [28], in which endogenous calmodulin tightly bound to the (Ca^{2+} + Mg^{2+})-ATPase is essential for most of its enzyme activity. Endogenous calmodulin was found in our preparation and varied between 26–50 pmol/mg. A relatively high resistance to low concentrations of the drug (<20 μM) has also been found for other calmodulin-associated enzymes, e.g., phosphorylase kinase [29].

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