THE EPINEPHRINE-INDUCED ACTIVATION OF THE CARDIAC SLOW Ca^{2+} CHANNEL IS MEDIATED BY THE cAMP-DEPENDENT PHOSPHORYLATION OF CALCIDUCTIN, A 23 000 M_r SARCOLEMMAL PROTEIN

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1. Introduction

Epinephrine and other β-adrenergic agonists are known to increase the cardiac contractile force and to abbreviate systole. The latter effect is readily explainable by the cAMP-dependent phosphorylation of phospholamban, the activator of the cardiac sacroplasmic reticulum calcium pump, resulting in the acceleration of the rate of Ca^{2+}-uptake [1–3]. In contrast, the positive inotropic effect is as yet unexplained. β-Adrenergic agonists act through cAMP-dependent phosphorylation of target proteins and such phosphate acceptors are likely to be found either in the contractile apparatus or at the membrane level. The former possibility was ruled out because cardiac troponin I phosphorylation results in a decrease, rather than an increase, of the Ca^{2+}-sensitivity of myofibrils (review [4]) and because cardiac myosin light-chain kinase was not found to be modulated by cAMP-dependent phosphorylation [5,6]. On the other hand, epinephrine may act through a cAMP-mediated increase in Ca^{2+}-entry through the sarcolemma. Extracellular Ca^{2+} is indeed required for cardiac contraction which is triggered by a voltage-dependent slow Ca^{2+}-channel, shown by electrophysiological studies to be activated by epinephrine (review [7]). Cardiac sarcolemma was found to contain a number of phosphate-acceptor proteins [8–11], among which a substrate of cAMP-dependent protein kinase was identified with M_r = 22 000–27 000 [9,11].

The recent availability of highly purified preparations of cardiac sarcolemmal vesicles [12] and of the pure protein components of the cAMP pathway [13, 14] prompted a re-examination of the effect of cAMP-dependent phosphorylation on Ca^{2+} fluxes in sarcolemmal vesicles. This report shows that the cAMP-dependent phosphorylation of a 23 000 M_r membrane protein, for which we propose the name calciductin, is associated with a ~2-fold increase in the ATP-independent, depolarization-induced Ca^{2+}-uptake, the in vitro equivalent of the slow Ca^{2+}-channel [15], thereby explaining the positive inotropic effect of epinephrine.

2. Materials and methods

Bovine serum albumin, PMSF[1] and cAMP were from Sigma. Carrier-free [^{32}P]orthophosphoric acid and $^{45}CaCl_2$ (>20 mCi/g) were purchased from New England Nuclear. [γ-^{32}P]ATP (500 Ci/mol) was prepared according to [16]. Polyacrylamide gel electrophoresis reagents were from Bio-Rad and Serva. All other chemicals were either from Merck or analytical grade. The catalytic subunit (C subunit) of cAMP-dependent protein kinase (type II from bovine heart) was prepared and stored as in [14]. Its heat-stable protein kinase inhibitor (PKI) was purified to homogeneity from rabbit skeletal muscle as in [13], and used without prior separation of the iso-inhibitors [17]. Glycogen phosphorylase *b* kinase was prepared

Abbreviations: PMSF, phenyl methyl sulfonyl fluoride; cAMP, cyclic adenosine 3′:5′-monophosphate; EGTA, ethylene glycol bis(β-aminoethyl ether) *N,N*′-tetraacetic acid; C subunit, the catalytic subunit of cAMP-dependent protein-kinases; PKI, the protein kinase inhibitor of cAMP-dependent protein-kinases; cGMP, cyclic guanosine 3′:5′-monophosphate

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from rabbit skeletal muscle as in [18] and calmodulin from ram testis as in [19].

2.1. *Purification of sarcolemmal vesicles*

Sarcolemmal vesicles were isolated from dog heart ventricles as in [12] and suspended in 10 mM Tris–HCl buffer (pH 7.4) at a final protein concentration of 4 mg/ml. Protein concentration was determined as in [20]. The preparation was either used immediately or stored at 4°C for <48 h. The membrane preparation was characterized by measuring the (Na^+,K^+)-ATPase [12] and 5′-nucleotidase (21) activities, the initial rate of the ATP-dependent Ca^{2+}-uptake and the maximal Ca^{2+} accumulation into the vesicles in the presence of 5 mM oxalate, as in [2]. Sonication was performed at 0°C for 1 s by using a 100 W MSE sonifier.

2.2. *Phosphorylation of sarcolemmal vesicles*

Sarcolemmal vesicles (100 μg protein) were incubated at 37°C for 10 min in 75 μl of a 10 mM $MgCl_2$, 0.3 mM [γ-^{32}P]ATP (~500 cpm/pmol), 30 mM potassium phosphate buffer (pH 7.5), in the presence of one of the following reagents: 4 mM EGTA; 4 mM EGTA plus boiled or native C subunit (1 μg, E/S = 0.01); 4 mM EGTA plus 1 μM cAMP; 4 mM EGTA plus 1 μM cGMP; 0.1 mM Ca^{2+}; 0.1 mM Ca^{2+} plus 1 μM calmodulin; 0.1 mM Ca^{2+} plus 1 μg glycogen phosphorylase *b* kinase (E/S = 0.01). Phosphorylation reactions were quenched by addition of a final concentration of 1% (w/v) dodecyl sulfate and analyzed by 0.1% dodecyl sulfate/5–20% gradient polyacrylamide slab gel electrophoresis as in [22]. The electrophoretogram was stained with Coomassie blue, destained, dried and processed for autoradiography with Kodak X-Omat R films. Autoradiograms were scanned by using a Joyce-Loebl microdensitometer MK III CS.

2.3. *Depolarization-induced Ca^{2+} uptake*

The ATP-independent, depolarization-induced Ca^{2+} uptake was measured essentially according to [15] except that addition of valinomycin was not found mandatory. Also the incubation time in the polarizing buffer (2.5 mM KCl, 147.5 mM NaCl) was 5 min, and 0.22 μm Millipore filters were used. Vesicles (100 μg) were first sonicated in the loading buffer containing 150 mM KCl, 10 mM $MgCl_2$, 0.3 mM ATP and either boiled or native C subunit (1 μg) and then incubated overnight at 4°C in the presence or absence of 5 μg pure PKI, i.e., a 20-fold molar excess over C subunit, capable of totally inhibiting C subunit in solution as shown in [13]. In each case, unlabeled ATP was used in the overnight incubation for the bulk of the suspension that served for Ca^{2+}-uptake studies while [γ-^{32}P]ATP was used in an aliquot for subsequent dodecyl sulfate–polyacrylamide gel electrophoresis and analysis of [^{32}P]phosphate-labeled proteins.

This allowed the comparison of the phosphorylation and Ca^{2+}-uptake of 3 different preparations:

(i) Control vesicles incubated with boiled C subunit and Mg-ATP inside and outside the vesicles;
(ii) Vesicles incubated with native C subunit and Mg-ATP inside and outside the vesicles, i.e., both rightside- and inside-out vesicles were phosphorylated;
(iii) Vesicles incubated with native C subunit and Mg-ATP inside the vesicles while the C subunit remaining outside was totally inhibited by excess PKI.

Under the latter conditions, sarcolemmal proteins of only right side out vesicles were phosphorylated.

3. Results and discussion

3.1. *Preparation of cardiac sarcolemmal vesicles*

Sarcolemmal vesicles were obtained with a yield of 1 mg protein/10 g canine heart ventricle and were highly enriched in the characteristic plasma membrane markers, i.e., ouabain-sensitive (Na^+,K^+)-ATPase and 5′-nucleotidase, which exhibited spec. act. 80–100 μmol/(mg . h) and 5–10 μmol/(mg . h), respectively.

These values are comparable to those reported either for this [12] or different [23–25] preparations. The rate of ATP-dependent Ca^{2+}-uptake was only 12–16 nmol/(mg . min) pointing to a maximal 4% contamination by sarcoplasmic reticulum vesicles, which exhibit an initial rate of 300–450 nmol/(min . mg) [2]. Also, cardiac sarcolemmal vesicles were shown to exhibit an ATP-dependent Ca^{2+}-pumping system [25]. A maximal amount of 300–400 nmol Ca^{2+}/mg protein was accumulated in the presence of oxalate.

3.2. *The major phosphate acceptor of sarcolemmal vesicles is the 23 000 M_r calciductin*

As shown in fig.1, a minor phosphate acceptor of M_r 56 000 was found to be labeled under all condi-

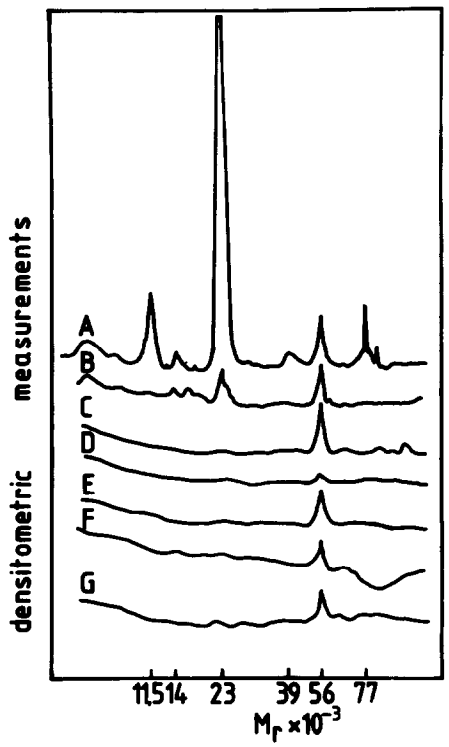

Fig.1. The phosphate-acceptor proteins of cardiac sarcolemmal vesicles. Vesicles were incubated 10 min at 37°C as in section 2 in the presence of: (A) native C subunit and EGTA; (B) cAMP and EGTA; (C) cGMP and EGTA; (D) EGTA; (E) Ca^{2+}; (F) Ca^{2+} and calmodulin; (G) Ca^{2+} and glycogen phosphorylase *b* kinase. ^{32}P-Labeled proteins were separated by polyacrylamide gel electrophoresis and detected by scanning the autoradiogram. Absorbance units are arbitrary.

tions. Its phosphorylation appeared to increase slightly in the presence of either C subunit or of the cyclic nucleotides (fig.1 A–C), suggesting that this band may correspond to the regulatory subunit of type II cAMP-dependent protein kinase, as suggested in [26]. Also a very faintly labeled peptide of M_r 14 000–16 000 was found to be present under all conditions tested, and its phosphorylation was increased slightly upon addition of either cAMP or C subunit. This band may be analogous to the 15 000 M_r protein in [10].

Addition of C subunit or, to a lower extent, of cAMP, resulted in a marked increase in the phosphorylation of 2 bands of M_r 22 000–24 000 and 11 500, which are probably identical to substrates 1 and 2 in [26] and to the 24 000 and 9000 M_r interconvertible bands in [27]. Phosphorylation of these components appears to be exclusively cAMP-dependent. Two other faint bands were visible after C subunit addition, which correspond to the autophosphorylation of the 39 000 M_r C subunit and to a phosphate-acceptor of M_r 77 000.

3.3. *Effect of cAMP-dependent phosphorylation of right side out sarcolemmal vesicles on the ATP-independent, depolarization-induced Ca^{2+} uptake*

When vesicles were sonicated in the presence of native C subunit and Mg ATP, and then incubated overnight at 4°C, only the M_r 23 000 calciductin was found to be [^{32}P]phosphate-labeled (fig.2A). Addition of excess protein kinase inhibitor to the suspension after sonication resulted in only a 17% decrease of calciductin phosphorylation, suggesting that up to 83% of the vesicles were oriented rightside-out after sonication (fig.2B). The non-phosphorylated vesicles (boiled C subunit), which are mostly rightside-out as shown above, exhibit a significant ATP-independent, depolarization-induced Ca^{2+} uptake up to 140 nmol Ca^{2+}/mg protein (fig.3). This value is comparable to those in [15]. The uptake was amplified 2.2-fold upon cAMP-dependent phosphorylation of calciductin. As expected, addition of excess PKI to the outer

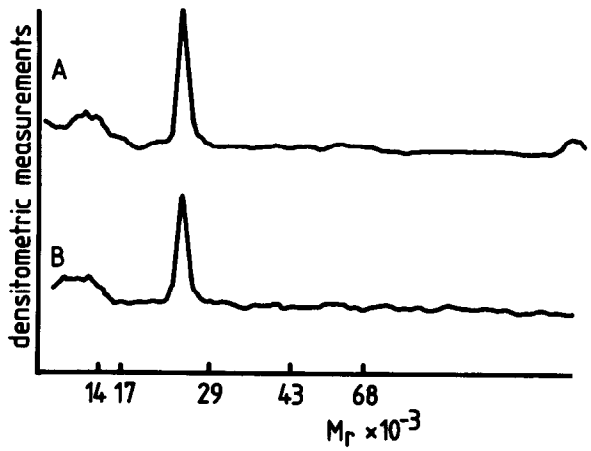

Fig.2. Cardiac sarcolemmal proteins phosphorylated after overnight incubation at 4°C in the loading 150 mM KCl buffer (see section 2): (A) Vesicles sonicated in the presence of C subunit and Mg-[γ-^{32}P]ATP; (B) Vesicles sonicated in the presence of C subunit and Mg-[γ-^{32}P]ATP, excess protein kinase inhibitor was added to the suspension after sonication. ^{32}P-Labeled proteins were analyzed as in fig.1. Molecular mass markers are bovine serum albumin, ovalbumin, carbonic anhydrase, β-lactoglobulin and lysozyme, with M_r = 68 000, 43 000, 29 000, 17 000 and 14 000, respectively.

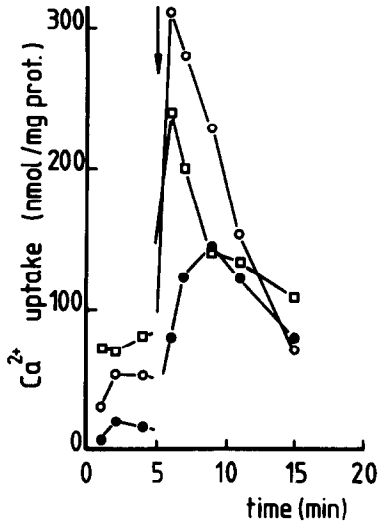

Fig.3. The ATP-independent, depolarization-induced Ca^{2+} uptake by cardiac sarcolemmal vesicles. Vesicles were sonicated in the loading buffer containing 150 mM KCl, 10 mM $MgCl_2$, 0.3 mM ATP and either boiled (•) or native (open symbols) C subunit. Then excess PKI was either omitted (○) or added (□) to the suspension after sonication. At time 0, vesicles were polarized by dilution in 2.5 mM KCl, 147.5 mM NaCl in the presence of $^{45}CaCl_2$. Partial depolarization was achieved at 5 min (→) by diluting to 105.75 mM KCl, 44.25 mM NaCl. Ca^{2+} uptake was determined by millipore filtration [15].

medium after sonication, which only abolishes phosphorylation of inside-out vesicles, did not abolish the C subunit-dependent stimulation of the Ca^{2+} channel, which remained 1.7-fold higher than the unphosphorylated control.

4. Conclusion

Under conditions where calciductin is in rightside-out vesicles the only protein phosphorylated in a pure cAMP-dependent manner (fig.2), the slow Ca^{2+} inward current, that is mimicked by the depolarization-induced Ca^{2+}-uptake of sarcolemmal vesicles [15], is stimulated ~2-fold (fig.3). This lends additional support to the hypothesis that the epinephrine-induced increase in Ca^{2+}-entry and thereby in contractile force is mediated by the activation of adenylate cyclase and the resulting dissociation of cAMP-dependent protein kinase which is in part bound to sarcolemma ([26], this paper). The cAMP-dependent phosphorylation of sarcolemmal proteins is apparently restricted to calciductin, a 23 000 M_r membrane protein that is either the Ca^{2+}-channel or, more likely, a channel-bound activator protein. This is, to the best of our knowledge, the first example of a gating system modulated by phosphorylation–dephosphorylation.

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