Species-dependent isoenzyme subtypes of membrane-bound cyclic AMP-dependent protein kinase in highly purified cardiac sarcolemma

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Highly purified sarcolemma from dog and pig cardiac muscle has been shown to contain significant activities of a membrane-bound cyclic AMP-dependent protein kinase. In addition, these membranes undergo endogenous phosphorylation when incubated with Mg^{2+} and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. By comparing ^{32}P -labelled patterns obtained with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and the photoaffinity label 8-azidoadenosine 3':5'- $[\text{P}^{32}]\text{monophosphate}$ (8-azido-cyclic $[\text{P}^{32}]\text{AMP}$), we have demonstrated that, whereas the major kinase isoenzyme in dog sarcolemma was Type II, that in the pig membrane was the Type I isoenzyme.

INTRODUCTION

Mammalian tissues have been shown to contain two major types of soluble cyclic AMP-dependent protein kinases, namely protein kinase Type I and Type II (Nimmo & Cohen, 1977). The two isoenzymes are quite similar in size, tetrameric subunit composition (R_2C_2) and mode of activation by cyclic AMP (Rubin *et al.*, 1972; Erlichman *et al.*, 1973; Beavo *et al.*, 1975), and they appear to share a common catalytic subunit (Corbin *et al.*, 1975), but the two types are readily distinguishable by their unique regulatory subunits. Whereas the M_r of the Type I regulatory subunit (R_I) is approx. 48 000, that of the Type II (R_{II}) is approx. 55 000 (Beavo *et al.*, 1975; Rosen *et al.*, 1975). As well as differences in regulatory-subunit M_r values, it has been demonstrated that the Type II kinase can catalyse the intramolecular phosphorylation of R_{II} (Erlichman *et al.*, 1974; Maeno *et al.*, 1974), whereas the Type I kinase cannot. The purified protein kinase II from bovine cardiac muscle incorporates 1 molecule of phosphate from ATP into each of two serine residues in its cyclic AMP-binding protein dimer (Erlichman *et al.*, 1974).

Since the late 1970's, it has been widely held (Rubin *et al.*, 1979) that the Type I cyclic AMP-dependent protein kinase existed only as a soluble enzyme, whereas the Type II isoenzyme was both soluble and particulate-associated. In cardiac muscle from a number of mammalian species, it has been claimed that this particulate-associated kinase was mainly or entirely the Type II isoenzyme (Corbin *et al.*, 1977).

In the present paper, we report significant differences in types of cyclic AMP-dependent protein kinase in plasma membrane prepared from dog and pig ventricular muscle.

EXPERIMENTAL

Materials

Marker proteins used for M_r determination of endogenous proteins in SDS/polyacrylamide-gel electrophoresis (phosphorylase *b*, 94 000; bovine serum albumin, 67 000; albumin, 43 000; carbonic anhydrase, 30 000; soya-bean trypsin inhibitor, 20 100; lactalbumin, 14 400) were from Pharmacia. $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (200–400 Ci/mmol) and 8-azido-cyclic $[\text{P}^{32}]\text{AMP}$ (20–50 Ci/mmol) were purchased from ICN Chemicals and Radioisotope Division. $[\text{H}^3]\text{Ouabain}$ (33 Ci/mmol) was obtained from Amersham. Cyclic AMP and 3-isobutyl-1-methylxanthine were purchased from Sigma. All electrophoresis materials, including silver-staining reagents, were from Bio-Rad, X-ray film (XAR-5) was obtained from Eastman Kodak. All other materials used were of reagent grade.

Preparation of sarcolemma

Sarcolemmal vesicles were prepared from approx. 100 g of dog and pig ventricular muscle, removed from the animals under sodium thiopental anaesthesia, as described previously (Jones *et al.*, 1980). Yield was 5–10 mg of sarcolemma protein/100 g of ventricular muscle. The vesicles, from both animals, at a concentration of 0.5–1.0 mg of protein/ml, were resuspended in 20 mM Tris/HCl (pH 7.4)/0.25 M-sucrose, frozen in liquid N_2 and stored at -70°C in 1 ml batches until use. The vesicles were characterized as to orientation and purity, and this has been reported elsewhere (Church & Sen, 1983). Briefly, the sarcolemmal vesicles isolated by this technique were shown to be approx. 96% inside-out by measuring $(\text{Na}^+, \text{K}^+)\text{-dependent ATPase}$ activity and membrane sialic acid content under hypo- and iso-osmotic conditions. The membranes were demonstrated to be free

Abbreviations used: R_2C_2 , cyclic AMP-dependent protein kinase holoenzyme; R_I and R_{II} , regulatory subunits of Type I and Type II cyclic AMP-dependent protein kinases respectively; C, catalytic subunit of cyclic AMP-dependent protein kinase; 8-azido-cyclic $[\text{P}^{32}]\text{AMP}$, 8-azido-adenosine 3':5'- $[\text{P}^{32}]\text{monophosphate}$.

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of significant contamination by both mitochondria and sarcoplasmic reticulum by showing that ATP-dependent ^{45}Ca uptake was not altered by addition of oligomycin or Tris/oxalate respectively (Church & Sen, 1983). Recognizable cell debris was minimal, as judged by electron microscopy (Church & Sen, 1983).

Photoaffinity-labelling of cyclic AMP-dependent protein kinase regulatory subunits

The covalent labelling of cyclic AMP-binding subunits of protein kinase by 8-azido-cyclic [^{32}P]AMP was carried out by a modification of the method of Walter *et al.* (1977). The reaction mixture (100 μl) containing cardiac sarcolemma protein (50–100 μg) in 20 mM-Tris/HCl, pH 7.4, 0.25 M-sucrose, 10 mM-MgCl₂, 1 mM-isobutyl-methylxanthine and 0.2 μM -8-azido-cyclic [^{32}P]AMP was incubated at 4 °C in the dark for 15 min and then irradiated for 15 min at 4 °C with a Mineralight u.v. lamp ($\lambda_{\text{max.}}$ = 254 nm) at a distance of 9 cm. The reaction was halted by addition of 45 μl of a stop solution [4 parts of 10% (w/v) SDS:1 part of mercaptoethanol/20% (w/v) sucrose]. Non-specific binding was determined by carrying out photoaffinity labelling in the presence of 20 μM -cyclic AMP.

Phosphorylation of endogenous proteins

Phosphorylation of endogenous cardiac membrane proteins was carried out in a reaction mixture (100 μl) containing cardiac sarcolemma protein (50–100 μg), 10 mM-MgCl₂ and 1.5 μM -[γ - ^{32}P]ATP, in the absence or presence of cyclic AMP as indicated in individual Figures and legends. Incubation was carried out at 37 °C, with a 5 min preincubation and a phosphorylation period of 30 s after addition of [γ - ^{32}P]ATP. The reaction was stopped by the addition of 45 μl of a stop solution.

SDS/polyacrylamide-gel electrophoresis and autoradiography

SDS/polyacrylamide-gel electrophoresis of the phosphorylated cardiac membranes was carried out by the method of Laemmli (1970), with 9% or 15% -acrylamide separating gels and 4.5% -acrylamide stacking gels, both containing 0.1% SDS. After electrophoresis, gels were silver-stained, dried under vacuum and exposed to Kodak XAR-5 film for 24–72 h. In some experiments, the autoradiographic profiles were quantified by scanning the autoradiographs with a Hoefer GS 300 transmittance/reflectance scanning densitometer and then measuring the areas under the various peaks with a planimeter.

Other methods

Plasma-membrane markers were assayed as follows. Ouabain-inhibitable (Na^+ , K^+)-dependent ATPase activity was measured as described by Post & Sen (1967). Ca^{2+} -stimulated Mg^{2+} -dependent ATPase activity was assayed in a medium as outlined by Caroni & Carafoli (1980), with Tris-ATP containing 10^6 d.p.m. of [γ - ^{32}P]ATP/ml as substrate. The hydrolysed ^{32}P released was extracted as described by Post & Sen (1967), and 1 ml of the butyl acetate extraction medium was then assessed for ^{32}P by liquid-scintillation counting. Specific [^3H]ouabain binding was carried out as outlined by Lichtstein & Samuelov (1980). Protein content was measured by the method of Lowry *et al.* (1951).

RESULTS

Identification of autophosphorylated R_{II} subunit of cyclic AMP-dependent protein kinase in dog and pig sarcolemma

A particulate fraction isolated from dog ventricular muscle, as outlined in the Experimental section, has been previously shown to be highly enriched in plasma-membrane markers, relatively devoid of other subcellular-organellar contamination, and contain significant activities of a membrane-bound cyclic AMP-dependent protein kinase (Church & Sen, 1983). As might be expected when the same isolation procedure is used, and as illustrated in Table 1, sarcolemma from pig ventricular muscle displayed plasma-membrane marker-enzyme activities comparable with those obtained from dog heart. Furthermore, we have shown that this enriched sarcolemmal fraction from either pig or dog heart can undergo endogenous phosphorylation when incubated with Mg^{2+} and [γ - ^{32}P]ATP. In the absence of added cyclic nucleotide, only one protein band, of M_r 55000, is phosphorylated (Figs. 1 and 2). It has been demonstrated previously by the use of photoaffinity labelling with 8-azido-cyclic [^{32}P]AMP that this phosphorylation takes place on the cyclic AMP-binding regulatory component of membrane-bound type II cyclic AMP-dependent protein kinase (Jones *et al.*, 1980; Haley, 1975). It has been proposed that this is a manifestation of a cyclic nucleotide-independent autophosphorylation of the regulatory subunit by the bound catalytic subunit (Rangel-Aldao & Rosen, 1976a). To confirm these studies and the relationship between [γ - ^{32}P]ATP labelling and cyclic AMP binding in our own preparations, we undertook experiments with 8-azido-cyclic [^{32}P]AMP too. As shown in Fig. 1, results in dog sarcolemma were as might be

Table 1. Comparison of specific plasma-membrane markers between sarcolemma from dog and pig ventricular muscle, both isolated by the procedure of Jones *et al.* (1980)

Markers were assayed as outlined in the Experimental section. Both ATPase activities are expressed as μmol of P_i released/h per mg of protein, and [^3H]ouabain binding is reported as pmol of [^3H]ouabain bound/mg of protein. Data are means $\pm$ S.E.M. for the numbers of preparations in parentheses.

Sarcolemmal marker	Dog	Pig
(Na^+ , K^+)-dependent ATPase	28.0 $\pm$ 1.5 (15)	19.0 $\pm$ 1.9 (8)
Specific [^3H]ouabain binding	66.5 $\pm$ 1.8 (6)	58.3 $\pm$ 1.0 (6)
Ca^{2+} -stimulated Mg^{2+} -dependent ATPase	3.1 $\pm$ 0.6 (6)	2.0 $\pm$ 0.03 (5)

Fig. 1. Autoradiograph depicting phosphorylation of dog sarcolemmal substrates by intrinsic cyclic AMP-dependent protein kinase and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (left) and photoaffinity incorporation of 8-azido-cyclic $^{32}\text{P}]\text{AMP}$ (right)

Intrinsic phosphorylation of sarcolemma (left) was measured in the absence (–) and the presence (+) of $1\ \mu\text{M}$ -cyclic AMP added 5 min before the $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. Phosphorylation was allowed to continue for 20 s. Specific labelling of membrane proteins with 8-azido-cyclic $^{32}\text{P}]\text{AMP}$ (right) was achieved by measuring binding in the absence (–) and presence (+) of $20\ \mu\text{M}$ exogenously added unlabelled cyclic AMP (approx. 34.0% specific cyclic AMP binding to R_{II}). Membranes were labelled and autoradiographed essentially as described in the Experimental section. Pictured are autoradiographs prepared from 15%-polyacrylamide gels exposed for 48 h. ' R_{II} ' designates the autophosphorylated and photoaffinity-labelled cyclic AMP-dependent protein kinase regulatory subunit, and 'I' and 'II' designate the two major cyclic AMP-dependent substrate proteins in mammalian cardiac sarcolemma. In the centre, ' $M_r\ 55\text{K}$ ' (55000), ' $M_r\ 27\text{K}$ ' (27000) and ' $M_r\ 12\text{K}$ ' (12000) refer to the M_r values of the labelled proteins. To each well $20\ \mu\text{g}$ of sarcolemmal proteins was applied.

predicted from work published elsewhere, i.e. significant specific binding of 8-azido-cyclic $^{32}\text{P}]\text{AMP}$ took place only on a M_r -55000 protein which exactly corresponded to the protein that was phosphorylated by $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. Results of identical experiments in pig sarcolemma were significantly different (Fig. 2). In contrast with the dog, most of the specific binding of 8-azido-cyclic $^{32}\text{P}]\text{AMP}$ in the pig membranes were located on a protein of M_r

Fig. 2. Autoradiograph showing autophosphorylation of pig sarcolemmal substrates by intrinsic cyclic AMP-dependent protein kinase and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (left) and photoaffinity incorporation of 8-azido-cyclic $^{32}\text{P}]\text{AMP}$ (right)

Conditions and abbreviations were the same as described for Fig. 1, except that pig sarcolemmal membranes were used in these experiments. Specific binding of cyclic AMP was as follows: 24.8% to R_{I} , 2.3% to R_{II} .

48000. This protein was not phosphorylated when the sarcolemma was incubated with Mg^{2+} and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (Fig. 2).

DISCUSSION

Two prominent forms of cyclic AMP-dependent protein kinase, designated as Types I and II, have been identified in mammalian tissues on the basis of their order of elution from columns of DEAE-cellulose (Corbin *et al.*, 1975; Hofmann *et al.*, 1975). These two forms appear to differ primarily in the properties of their distinctive regulatory subunits. Along with differences in size (Beavo *et al.*, 1975; Hofmann *et al.*, 1975), immunoreactivity (Fleischer *et al.*, 1976), amino acid composition (Zoller *et al.*, 1979) and cyclic AMP-binding properties (LaPorte *et al.*, 1980), the two types of regulatory subunits (R_{I} and R_{II}) differ also in their susceptibility to phosphorylation by the catalytic subunit (C). R_{I} is not phosphorylated by C at a significant rate. R_{II} , on the other hand, is readily phosphorylated in the absence of cyclic AMP to yield a modified subunit that exhibits altered kinetic parameters towards C and cyclic AMP (Erlichman *et al.*, 1974; Rangel-Aldao & Rosen, 1976a,b). Significant amounts of cyclic AMP-dependent protein kinase activities have been shown both by our

laboratory (Church & Sen, 1983) and by others (Jones *et al.*, 1980; Manalan & Jones, 1982) to reside in purified sarcolemma from mammalian heart. As we have shown here (Figs. 1 and 2), incubation of both highly enriched dog and pig sarcolemma with Mg^{2+} and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ alone results in the strong phosphorylation of a single protein band of M_r 55000. It has been rigorously demonstrated that this autophosphorylation is characteristic of Type II cyclic AMP-dependent protein kinase (Erlichman *et al.*, 1974; Schwartz *et al.*, 1979) and can occur by means of an intramolecular reaction (Rangel-Aldao & Rosen, 1976b). Indeed, in highly purified sarcolemmal vesicles isolated from dog heart, Jones *et al.* (1980) first demonstrated that the only major substrate protein phosphorylated in the absence of cyclic AMP had M_r 55000. The same group (Jones *et al.*, 1980; Manalan & Jones, 1982) clearly demonstrated, by use of the photoaffinity probe 8-azido-cyclic $[\text{P}^{32}]\text{AMP}$, that the M_r -55000 band in purified dog sarcolemma was indeed the regulatory subunit of the membrane-bound cyclic AMP-dependent protein kinase. It was not surprising that, by utilizing substantially the same methodology for both membrane isolation and R_{II} labelling, we obtained evidence that concurs totally with that of Jones and co-workers (Figs. 1 and 2) (Jones *et al.*, 1980; Manalan & Jones, 1982).

The situation in the purified plasma-membrane fraction from pig ventricles was dramatically different (Fig. 2). Although autophosphorylation experiments revealed the presence of significant activity of Type II cyclic AMP-dependent protein kinase in the pig sarcolemma, cyclic AMP labelling with 8-azido-cyclic $[\text{P}^{32}]\text{AMP}$ revealed that the vast majority of protein kinase associated with the plasma membrane was of the Type I variety. This conclusion is based on the facts that, firstly, the M_r of the cyclic AMP-binding protein was 48000, compared with 55000 for R_{II} , and secondly, this M_r -48000 moiety did not undergo autophosphorylation. It was unexpected that the largest proportion of cyclic AMP-dependent protein kinase activity of pig sarcolemma was of the Type I variety, in light of much work performed in the mid-1970's. Particulate fractions isolated from rat, rabbit and ox hearts (Corbin *et al.*, 1977) or bovine cerebral cortex (Rubin *et al.*, 1979) have been shown to contain mainly or entirely the Type II isoenzyme, suggestive of enzyme compartmentalization (Corbin *et al.*, 1977). These findings have subsequently been extended to dog myocardial membrane fractions too (Jones *et al.*, 1980; Manalan & Jones, 1982). This is the first report that we are aware of that shows large amounts of a membrane-bound Type I isoenzyme in any organelle yet isolated from a nucleated cell system. The only report similar to this was that by Rubin *et al.* (1979), who reported that the membrane-derived protein kinase isolated from human erythrocytes was characterized as a Type I enzyme on the basis of a variety of biochemical parameters. This, combined with the fact that the pig is known to be a close anatomical and physiological model to the human, may result in important implications to human cardiac biochemistry. In addition, in view of this present finding, we may be forced to be more cautious in

forming generalizations concerning the distribution and specificity of cyclic AMP-dependent protein kinase isoenzymes.

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