Cyclic AMP-dependent protein kinase phosphorylates residues in the C-terminal domain of the cardiac L-type calcium channel α_1 subunit

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Abstract

The molecular basis of the regulation of cardiac L-type calcium channel activity by cAMP-dependent protein kinase (cA-PK) remains unclear. Direct cA-PK-dependent phosphorylation of the bovine ventricular α_1 subunit in vitro has been demonstrated in microsomal membranes, detergent extracts and partially purified $(+)$-$[^3H]$PN 200–110 receptor preparations. Two ^{32}P-labelled phosphopeptides, derived from cyanogen bromide cleavage, of 4.7 and 9.5 kDa were immunoprecipitated specifically by site-directed antibodies against the rabbit cardiac α_1 subunit amino acid sequences 1602–1616 and 1681–1694, respectively, consistent with phosphorylation at the cA-PK consensus sites at Ser^{1627} and Ser^{1700}. No phosphopeptide products consistent with phosphorylation at three other C-terminal cA-PK consensus phosphorylation sites (Ser^{1575}, Ser^{1848} and Ser^{1928}) were identified using similar procedures suggesting that these sites are poor substrates for this kinase. Ser^{1627} and Ser^{1700} may represent sites of cA-PK phosphorylation involved in the physiological regulation of cardiac L-type calcium channel function.

Keywords: α_1 subunit; Cardiac; Cyclic AMP-dependent protein kinase; Dihydropyridine; L-type calcium channel; Phosphorylation site

1. Introduction

Autonomic nervous system control of the force of cardiac contraction and beat frequency is likely to occur through regulation of L-type voltage-sensitive calcium channel activity [1,2]. β-Adrenergic stimulation results in increased L-type Ca^{2+} current due to modification of the millisecond kinetics of channel opening and closing and an increased availability of channels for opening [3]. These changes in kinetic properties are thought to be the result of

direct channel phosphorylation by cyclic AMP-dependent protein kinase (cA-PK) [1–3]. While there is evidence to implicate phosphorylation of both the α_1 [4,5] and β [6,7] subunits, the molecular basis of the regulation of cardiac L-type channel activity remains to be established.

None of the phosphorylation sites for cA-PK identified in the skeletal muscle α_1 subunit [8–10] are present at equivalent positions in the cardiac α_1 isoform [11]. However, a number of consensus cA-PK sites present in the C-terminal sequence following the final putative transmembrane segment (IVS6) [11] have been proposed to mediate cA-PK activation of this isoform [1]. In this study we have investigated the possibility that the cardiac α_1 subunit is a substrate for cA-PK and have utilised antibody mapping methods in phosphopeptide analysis [9] to identify two predominant cA-PK phosphorylation sites in the C-terminal region of this subunit. Parts of this work have been presented to the Biochemical Society [12].

2. Materials and methods

$[^3H]$Azidopine (52 Ci/mmol) and $[\gamma-^{32}P]$ATP, 3000 Ci/mmol) were obtained from Amersham International.

Abbreviations: CNBr, cyanogen bromide; EDT, ethanedithiol; ELISA, enzyme-linked immunosorbent assay; cA-PK, cyclic AMP-dependent protein kinase; MOPS, 3-[*N*-morpholino]propanesulfonic acid; PBS, phosphate-buffered saline; $(+)$-$[^3H]$PN 200–110, $(+)$-isopropyl-4-(1,2,3,-benzoxadiazol-4-yl)-1,4-dihydro-2,6-dimethyl-5-([^3H]methoxycarbonyl)-pyridine-3-carboxylate; PVDF, polyvinylidene difluoride; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Sulfo-MBS, maleimidobenzoyl-*N*-hydroxysulfosuccinimide ester; TFA, trifluoroacetic acid; WGA, wheat germ agglutinin.

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(+)-Isopropyl-4-(1,2,3,-benzoxadiazol-4-yl)-1,4-dihydro-2,6-dimethyl-5-([³H]methoxycarbonyl)pyridine-3-carboxylate ((+)-[³H]PN 200–110, 85 Ci/mmol) was obtained from New England Nuclear. Unlabelled nitrendipine was kindly provided by Bayer. All chemicals were of the highest grade available.

2.1. Preparation of cardiac ventricular muscle microsomes

Ventricular muscle microsomes were prepared from fresh bovine hearts by the method of Schneider and Hofmann [13], using 40 mM 3-[N-morpholino]propanesulfonic acid (MOPS) (pH 7.5) and 0.3 M sucrose instead of Tris and mannitol, respectively. All solutions contained the protease inhibitors: 10 mM EGTA, 1 mM benzamidine, 1 mM iodoacetamide, 1 mM 1,10 phenanthroline, 0.1 mM phenylmethylsulfonyl fluoride and 10 μg/ml soybean trypsin inhibitor. Microsomal membranes were used fresh or stored at a protein concentration of 20–40 mg/ml in 0.3 M sucrose, 40 mM MOPS (pH 7.5) at −70°C.

2.2. Measurement of (+)-[³H]PN 200–110 binding to membrane fractions

Membranes were incubated at a protein concentration of 100 μg/ml with 0.2–2.0 nM (+)-[³H]PN 200–110 in the absence and presence of 1 μM unlabelled nitrendipine in assay buffer (20 mM Tris-HCl (pH 7.5), 1.5 mM CaCl₂) for 90 min at 4°C. Duplicate 400 μl aliquots were vacuum filtered on Whatman GF/C filters, washed twice with 5 ml 20 mM Tris-HCl (pH 7.5) and counted in Optiscint 'Safe' (Pharmacia) in a scintillation counter.

2.3. Solubilisation and assay of (+)-[³H]PN 200–110 receptor

Cardiac microsomal membranes (750 mg of protein) were diluted to a protein concentration of 2.5 mg/ml in a final concentration of 10 mM Hepes-Tris (pH 7.4), 185 mM KCl, 1.5 mM CaCl₂. The following protease inhibitors were included in all buffers: 1 μg/ml antipain, 1 mM benzamidine, 1 mM iodoacetamide, 1 μg/ml leupeptin, 1.5 μM pepstatin A, 0.1 mM phenylmethylsulfonyl fluoride, 10 μg/ml soybean trypsin inhibitor. All steps were carried out at 4°C. Membranes were incubated with 0.25 nM (+)-[³H]PN 200–110 in the presence of 2 μM diltiazem. An aliquot (1 ml) was taken and unlabelled nitrendipine added to give a final concentration of 1 μM to determine non-specific binding. After incubation on ice for 45 min the two mixtures were subjected to ultracentrifugation at 116000 × g for 30 min. The pelleted membranes were resuspended in solubilisation buffer containing 10 mM Hepes-Tris (pH 7.4), 185 mM KCl, 1.5 mM CaCl₂, 1% (w/v) digitonin) at a detergent to protein ratio of 3:1 and incubated on ice for 45 min with occasional gentle agitation. Insoluble material was removed by cen-

trifugation at 100000 × g for 30 min. Aliquots (100 μl) were taken from the main solubilisation mixture and the non-specific binding incubation for assay of total radioactivity, protein-bound radioactivity and soluble protein. Protein-bound radioactivity was assayed by the polyethylene glycol precipitation method of Curtis and Catterall [14].

2.4. Purification of (+)-[³H]PN 200–110 receptor

The main solubilisation mixture was diluted immediately with 150 ml ice-cold dilution buffer (10 mM Hepes-Tris (pH 7.4), 1.5 mM CaCl₂) and applied onto a 150 ml column of DEAE-Sephadex 'Fast Flow' (Pharmacia) pre-equilibrated in 10 mM Hepes-Tris (pH 7.4), 1.5 mM CaCl₂, 92.5 mM KCl, 0.1% (w/v) digitonin at a flow-rate of 5 ml/min. The column was washed with 250 ml wash buffer (10 mM Hepes-Tris (pH 7.4), 1.5 mM CaCl₂, 0.1% (w/v) digitonin) at the same flow-rate. Elution of the column was performed at a flow-rate of 3 ml/min with 250 ml wash buffer containing 0.15 M KCl. The eluate was loaded directly onto 3 ml wheat germ agglutinin (WGA)-Sepharose (Pharmacia). The column was washed with 40 ml wash buffer at a flow-rate of 2 ml/min, then eluted with 10 ml wash buffer containing 0.3 M N-acetyl-D-glucosamine at a flow-rate of 0.5 ml/min. Fractions (1.5 ml) were collected and 50 μl aliquots were counted for radioactivity. Where necessary, enriched (+)-[³H]PN 200–110 receptor preparations were concentrated using Millipore Ultrafree Cl concentrators (> 10 kDa molecular mass exclusion) by centrifugation at 1500 × g at 4°C for 60 min.

2.5. Site-directed antibodies

Four synthetic peptides corresponding to amino acid sequences 1602–1616 (IKTEGNLEQANEELR), 1681–1694 (LQAGLRTLHDIGPE), 1774–1789 (SHEKLVDSTFTPSSYS) and 1889–1906 (LSYQD-DENRQLAPPEEEK) in the rabbit cardiac α₁ subunit [11] were prepared using the solid phase Nᵅ-fluorenylmethoxycarbonyl-polyamide method [15]. Each peptide was synthesised with a C-terminal cysteine residue to facilitate coupling to carrier protein and resin supports. Peptides were cleaved from the support using 60% (v/v) trifluoroacetic acid (TFA) in dichloromethane containing 2% (w/v) phenol and 2% (v/v) ethanedithiol (EDT) after which amino acid side-chain protecting groups were removed in 95% TFA in H₂O containing 2% phenol and 2% EDT. Peptides were washed in ether and characterised by high pressure liquid chromatography according to Davis et al. [16]. Peptides were conjugated to ovalbumin (molar ratio 8:1) with maleimidobenzoyl-N-hydroxysulfosuccinimide ester (Sulfo-MBS, Pierce) [17]. Antisera against ovalbumin conjugates were raised in New Zealand White rabbits by intravenous injection of 200 μg conjugate in 10 mM sodium phosphate (pH 7.2), 150 mM NaCl. Intra-

venous booster injections (200 μg) were made after 1 week and thereafter twice a week for 2 weeks. One week after the last injection rabbits, showing an immune response, were bled. Pre-immune serum was prepared from a bleed taken before immunisation. Serum was stored at $-70°C$. Antisera to the rabbit skeletal muscle α_1 amino acid sequence 1399–1418 (LDEFKAIWAEYD-PEAKGRIKC) was prepared using ovalbumin as carrier protein as described previously [18] as part of a separate study in collaboration with Dr. S.A. Baldwin and Professor D. Wray.

Antibody titre was estimated in ELISA using microtitre plates coated with either peptide (2 μg/ml) or DEAE Sephadex/WGA-Sepharose enriched calcium channel preparation (3 μg protein/ml). Plates were blocked with 0.5% casein, 10% calf serum, 0.05% Tween-20 in phosphate-buffered saline (PBS; 40 mM sodium phosphate (pH 7.4), 0.9% NaCl) for 1 h and then anti-peptide or control antisera were applied at the required dilution for 2 h. Antibody reaction was detected using sequential incubations (1 h) of swine anti-rabbit IgG (1:1000 dilution, Dakopatts) and rabbit peroxidase anti-peroxidase conjugate (1:1000 dilution, Amersham) in 1% calf serum, 0.05% Tween-20, PBS at room temperature. Positive wells were identified by the development of blue coloration following the application of 10 mg/ml 3,3',5,5'-tetramethylbenzidine (Miles) in 0.1 M sodium acetate/citrate (pH 6.0), 0.0045% H_2O_2. Between steps wells were washed three times with 0.05% Tween-20 in PBS.

For immunoprecipitation experiments, immunoaffinity matrices were prepared according to Schneider et al. [19] by the covalent coupling of bound antibodies to protein A-Sepharose (6 ml serum/ml of gel) using dimethylpimelimidate (Pierce). Cardiac ventricular microsomes were incubated with 2.5 nM [^{3}H]azidopine for 90 min at 4°C in the absence or presence of 1 μM nitrendipine. Photoaffinity labelling of the channel protein was achieved by illumination of the mixture on ice with a 440W MBFR/U U.V. lamp at a distance of 12 cm for 3 min. Labelled membranes were washed in incubation buffer (10 mM Hepes-Tris (pH 7.4), 185 mM KCl, 1.5 mM $CaCl_2$) and solubilised as above. Photoaffinity labelled detergent extracts (5000 dpm specifically labelled protein, 0.9 ml) were incubated with 0.1 ml immunoaffinity matrix for 16 h at 4°C. After centrifugation at 11 000 × g for 1 min, supernatant was removed and the pellets washed twice in 1.5 ml radioimmune assay buffer (10 mM Hepes-Tris (pH 7.4), 92.5 mM KCl, 0.5% digitonin (w/v)). Bound antigen was eluted with 2 × 0.5 ml 20 mM ethanolamine (pH 10), 0.5% sodium deoxycholate. Aliquots (100 μl) were counted for radioactivity in Optiscint 'Safe' (Pharmacia).

Immunoblots were performed as described previously [20] using DEAE Sephadex/WGA-Sepharose enriched calcium channel preparation (0.5 μg/lane) as antigen. Antibody overlay was with antiserum (1:100 dilution, 3 h) and anti-rabbit IgG (whole molecule) alkaline phosphatase

conjugate (1:1000 dilution, 1 h) and immunoblots were developed using 5-bromo-4-chloro-3-indoyl phosphate/nitroblue tetrazolium (Sigma Fast™ tablets).

2.6. Phosphorylation of membrane-bound and enriched (+)-[^{3}H]PN 200–110 receptor

Phosphorylation protocols were based on that described by De Jongh et al. [21]. Cardiac ventricular microsomes (50–100 μg of protein) or enriched (+)-[^{3}H]PN 200–110 receptor preparation (50–100 μg of protein) were incubated in 0.1 μM [γ-^{32}P]ATP (3000 Ci/mmol) in 50 mM Tris-HCl (pH 7.5), protease inhibitors as described above, 10 mM $MgCl_2$, 1 mM EGTA in a total volume of 150 μl. Reactions were initiated by the addition of cyclic AMP (125 pmol) or cA-PK catalytic subunit (0.5 μg) and samples incubated at 25°C for 30 min. For immunoprecipitation studies of intact (+)-[^{3}H]PN 200–110 receptor the phosphorylation reaction was terminated by the addition of 15 μl 200 mM EDTA, 50 mM Tris-HCl (pH 7.5). To remove unreacted [γ-^{32}P]ATP prior to immunoprecipitation, 45 μl bovine serum albumin in reaction buffer was added to a final concentration of 1 mg/ml. The sample was applied to a 2 ml Sephadex G-25 column equilibrated with 25 mM Tris-HCl (pH 7.5), 200 mM NaCl, 5 mM EDTA, 50 mM KF, 20 mM Na pyrophosphate, 0.5% (w/v) digitonin and subjected to centrifugation at 1000 × g for 1 min. For immunoprecipitation studies of phosphopeptides derived from (+)-[^{3}H]PN 200–110 receptor, the phosphorylation reaction was terminated by the addition of SDS-PAGE loading buffer and the ^{32}P-labelled α_1 subunit separated by SDS-PAGE [22].

2.7. Immunoprecipitation of ^{32}P-labelled (+)-[^{3}H]PN 200–110 receptor and peptides

Aliquots (0.4 ml) of ^{32}P-labelled (+)-[^{3}H]PN 200–110 receptor (2000–5000 dpm) or peptides (500–1000 dpm) were immunoprecipitated by incubation with 50 μl immunoaffinity matrix prepared as described above at 4°C for 16 h. The immunoaffinity matrix was washed with 25 mM Tris-HCl (pH 7.5), 0.1% Triton X-100 and eluted with SDS-PAGE loading buffer for analysis by SDS-PAGE.

2.8. Mapping of cA-PK phosphorylation sites within the primary sequence of the α_1 subunit

Enriched (+)-[^{3}H]PN 200–110 receptor was phosphorylated as described above. ^{32}P-labelled phosphoproteins were resolved by SDS-PAGE in 4–12% polyacrylamide gradients under reducing conditions [22] and transferred electrophoretically at 80 V for 2.5 h at 4°C to polyvinylidene difluoride membrane [23]. The phosphorylated α_1 polypeptide was located on the filter by autoradiography. The filter immobilised, ^{32}P labelled α_1 subunit was excised and incubated with 0.15 M cyanogen bromide (CNBr)

in 70% formic acid for 1 h at room temperature and 15 h at 4°C. CNBr cleavage products were eluted from the membrane in 250 mM Tris-HCl (pH 9.5), 1% Triton X-100 and the pH re-adjusted to pH 7.5 immediately. Immunoaffinity matrices of anti-peptide and control antibodies, prepared as described above, were incubated with aliquots of ^{32}P-labelled CNBr peptides in the absence or presence of 1 μg/ml corresponding competing peptide for 16h at 4°C. Immunoprecipitation was assessed by the level of ^{32}P label appearing in the matrix pellet after centrifugation at 1000 $\times$ g for 1 min. ^{32}P-labelled phosphopeptides were eluted from the immunoaffinity matrices using SDS-PAGE loading buffer and subjected to SDS-PAGE on 15% polyacrylamide gels. Gels were sliced (2 mm) and radioactivity in gel slices assessed by scintillation counting in Optiphase 'Safe'. Peptide masses were assigned by comparison with molecular mass standards (Sigma Molecular Weight Markers for Peptides) (molecular mass range 2.5–17 kDa) and Dalton Mark VII-L (molecular mass range 14.2–66 kDa).

2.9. Protein determination

Protein concentration was determined as described by Lowry et al. [24] or Bradford [25].

3. Results and discussion

3.1. Characterisation of anti-peptide antibodies

Antisera were prepared against four synthetic peptides corresponding to sequences in the C-terminal region of the rabbit cardiac α_1 subunit following the IVS6 putative transmembrane domain (1602–1616, 1681–1694, 1774–1789 and 1889–1906) (Fig. 1). Each sequence was selected to lie within a complete cyanogen bromide digestion product containing a consensus cA-PK phosphorylation site. All five anti-peptide antisera were shown to recognise the corresponding immunising peptide and enriched (+)-[^{3}H]PN 200–110 receptor preparation in ELISA at dilutions of 1:1000 and 1:100 or less, respectively (not shown). In each case, the specificity of immune recognition was demonstrated by the inhibition of immune reaction when antibody was preincubated with its corresponding peptide and the lack of inhibition when antibodies were tested against non-target peptides.

The specificity of all antibodies was further demonstrated in immunoprecipitation experiments. Immunoaffinity matrices of all five antibodies immunoprecipitated specifically calcium channel affinity labelled with [^{3}H]azidopine and ^{32}P-labelled α_1 subunit, phosphorylated in the presence of cA-PK (Table 1). No specific immunoprecipitation was observed when an anti-Escherichia coli or protein A protein matrix was used (Table 1). Detection of a 200 kDa polypeptide in Western
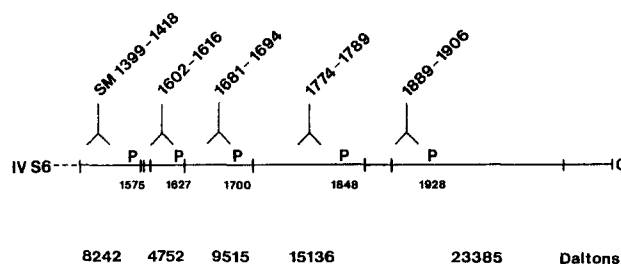

Fig. 1. Location of site-directed antibody binding sites (inverted Y) and consensus cA-PK phosphorylation sites (P) in the C-terminal domain of the cardiac L-type Ca^{2+} channel α_1 subunit. The bold line represents the polypeptide chain of the α_1 subunit. Tick marks indicate the position of methionine residues in the primary sequence of rabbit cardiac α_1 subunit [11] with distances between them being approximately proportional to the corresponding separations in the primary structure. Numbers below the line indicate the position of serines in consensus cA-PK phosphorylation sites in the rabbit primary sequence [11]. The predicted molecular mass of complete CNBr cleavage products containing cA-PK consensus sites are given at the foot of the figure.

blots of enriched (+)-[^{3}H]PN 200–110 receptor preparation was demonstrated for two of the antibodies, anti-α_1 1681–1694 and 1774–1789 in the absence but not the presence of the corresponding immunising peptide (Fig. 2).

3.2. cA-PK-dependent phosphorylation of the α_1 subunit in cardiac microsomal membranes

Phosphorylation of cardiac microsomal membranes in the presence of added exogenous cA-PK catalytic subunit

Table 1
Immunoprecipitation of affinity labelled and phosphorylated calcium channel protein

Antiserum	Radioactivity immunoprecipitated (dpm)	
	specific [^{3}H]azidopine labelled protein	^{32}P-labelled α_1 subunit
Anti-skeletal muscle α_1 1399–1418	204	457
Anti-α_1 1602–1616	257	406
Anti-α_1 1681–1694	347	409
Anti-α_1 1774–1789	156	368
Anti-α_1 1889–1906	260	301
Anti-E. coli	22	119
Protein A-Sepharose	7	26

Immunoprecipitations were carried out on antibody crosslinked covalently with dimethylpimelimidate to protein A-Sepharose. Cardiac microsomal membrane proteins were labelled with [^{3}H]azidopine in the absence or presence of 1 μM unlabelled nitrendipine to determine nonspecific labelling. Affinity labelled proteins were immunoprecipitated from digitonin extracts. The nonspecific signal was subtracted from the total signal to yield the specific [^{3}H]azidopine label immunoprecipitated. Data are means of two experiments. In phosphorylation experiments, cardiac microsomal proteins, phosphorylated in the presence of [γ-^{32}P]ATP and cA-PK catalytic subunit, were solubilised in digitonin as described in Section 2. ^{32}P-Labelled proteins (5000 dpm) were immunoprecipitated and proteins eluted from the immunomatrices were separated by SDS-PAGE on 4–12% linear acrylamide gradients. The region of gel corresponding to 200 $\pm$ 10 kDa was excised and counted for radioactivity. Data are the means of two experiments.
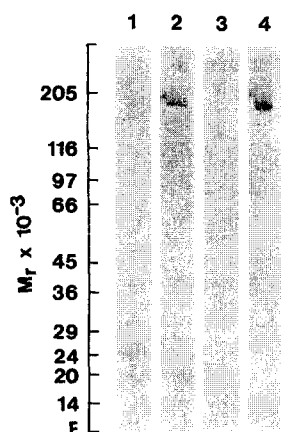
Fig. 2. Immunoblot analysis of (+)-[^{3}H]PN 200–110 receptor using anti-peptide antibodies. Enriched (+)-[^{3}H]PN 200–110 receptor preparation (2.5 μg, 67 pmol/mg protein) was subjected to SDS-PAGE under reducing conditions on 4–12% linear acrylamide gradient gels followed by immunoblotting with antibodies to cardiac α_1 subunit peptides 1681–1694 (lanes 1 and 2) and 1774–1789 (lanes 3 and 4). Incubation with diluted antiserum (1:100) was performed in the presence (1, 3) or absence (2, 4) of 1 μg/ml of the corresponding immunising peptide. Positions of molecular mass standards (kDa) are shown on the axis. F, ion front.

and [γ-^{32}P]ATP resulted in the labelling of multiple polypeptides (Fig. 3A). A phosphorylated polypeptide of approximately 200 kDa was immunoprecipitated by all five anti-α_1 subunit antibodies (Table 1) and was identified as the major substrate for cA-PK on dilution of the phosphorylated membrane protein sample before electrophoresis (Fig. 3B). No phosphorylation of the α_1 subunit was observed when cardiac ventricular microsomes were treated with cAMP only (Fig. 3B), indicating that endogenous cA-PK is not associated with the channel in the plasma membrane. cA-PK catalysed phosphorylation of the α_1 subunit was observed also in enriched (+)-[^{3}H]PN 200–110 receptor preparation in the presence of exogenous cA-PK and [γ-^{32}P]ATP (Fig. 3C). In the absence of cA-PK catalytic subunit labelling of the α_1 subunit was not observed indicating that phosphorylation was not a result of endogenous kinase activity in the receptor preparation.

The cardiac α_1 subunit has been reported to be a poor substrate for cA-PK [26–28]. It has been suggested [4,28] that this may be due to post-translational proteolytic processing of the C-terminal similar to that seen in skeletal muscle [21]. As a precaution in the present study a cocktail of protease inhibitors was included in most buffers to reduce the effects of proteolysis. The presence of the C-terminal region, at least up to residues in the region 1889–1906, was confirmed in this study by the recognition of the α_1 subunit by the anti-α_1 1889–1906 antibody in ELISA (not shown) and in immunoprecipitation experiments (Table 1). These results suggest that cA-PK phosphorylation sites are accessible in membrane bound and detergent solubilised α_1 subunit in which the C-terminal region remains unproteolysed.

3.3. Mapping of cA-PK phosphorylation sites

Further mapping of cA-PK phosphorylation sites within the primary structure of the α_1 subunit by amino acid microsequencing and/or autoradiography of isolated ^{32}P-labelled peptide fragments was precluded by the low yields of purified α_1 polypeptide in purification experiments. As an alternative approach, we have sought to identify sites susceptible to cA-PK phosphorylation by immunoprecipitation of ^{32}P-labelled phosphopeptides, derived from CNBr digestion of the α_1 subunit, using site-directed antibodies and peptide identification by scintillation counting of gel slices following peptide resolution by SDS-PAGE.

Complete CNBr cleavage of the α_1 subunit could not be achieved even with extended and repeated treatment of polyvinylidine difluoride membrane-bound protein with CNBr, nor in cleavage reactions of the soluble ^{32}P-labelled α_1 subunit (Fig. 4A), suggesting that there was oxidative modification of some methionine residues during the work up before the cleavage reaction. As a consequence, the immunological analysis of ^{32}P-labelled phosphopeptides was complicated by the possibility of immunoprecipitation of consensus phosphorylation sites in amino acid sequences adjacent to the CNBr fragment targeted by the
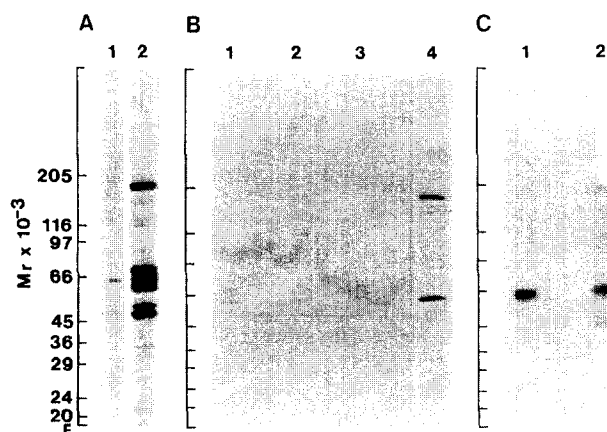

Fig. 3. Phosphorylation of membrane bound and enriched (+)-[^{3}H]PN 200–110 receptor preparations. (+)-[^{3}H]PN 200–110 receptor preparations were phosphorylated in the presence of 0.1 μM [γ-^{32}P]ATP and analysed by SDS-PAGE under reducing conditions in 4–12% linear acrylamide gradients. Fixed and dried gels were exposed to Kodak X-Omat film with an intensifying screen. Positions of molecular mass standards (kDa) are shown on the axes. (A) Cardiac microsomal membranes (500 μg protein/lane, 52 fmol (+)-[^{3}H]PN 200–110 binding sites/mg protein) phosphorylated in the absence (lane 1) and presence (lane 2) of 0.5 μg cA-PK catalytic subunit. (B) Cardiac microsomal membranes (5 μg protein/lane, 65 fmol (+)-[^{3}H]PN 200–110 binding sites/mg protein) phosphorylated as above in the absence (lanes 1 and 3) or presence of 125 pmol cyclic AMP (lane 2) or 0.5 μg cA-PK catalytic subunit (lane 4). (C) Phosphorylation of enriched (+)-[^{3}H]PN 200–110 receptor preparation (20 μg protein/lane, 81 pmol (+)-[^{3}H]PN 200–110 binding sites/mg protein) in the absence (lane 1) or presence (lane 2) of 0.5 μg cA-PK catalytic subunit. Direct comparison of the density of labelling of the α_1 subunit between panels could not be made as there were differences in the radioactive specific activity of [γ-^{32}P]ATP used and in exposure times between experiments.

particular antibody employed. The criterion for the identi-fication of a phosphorylation site for cA-PK was that, for a given antibody, a ^{32}P-labelled phosphopeptide of the pre-dicted molecular mass for the consensus site containing peptide from complete CNBr cleavage should be identified in the sample immunoprecipitated in the absence but not the presence of the corresponding immunising peptide.

In four separate experiments, phosphorylation at the consensus site Ser^{1627} (^{1619}IKKIWKRTSMKLLQ^{1632} [11]) was indicated by immunoprecipitation of a 4.7 kDa CNBr ^{32}P-phosphopeptide with anti-α_1 1602–1616 antibody, in the absence but not the presence of competing peptide (Fig. 4B, predicted mass 4.752 kDa). In addition to Ser^{1627}, three threonine residues were also present in the target peptide but none was contained in a consensus sequence and, therefore, were unlikely to be the substrate site of the cA-PK. A ^{32}P-labelled peptide of 9.5 kDa was immuno-precipitated specifically by anti-α_1 1681–1694 antibody in three separate experiments (Fig. 4C, predicted mass 9.515 kDa) suggesting possible cA-PK phosphorylation at the consensus site at Ser^{1700} (^{1691}IGPEIRRAISGDLTA^{1705} [11]) also. Within this targeted peptide a further two serine and three threonine residues were present. None was pre-

sent in a consensus sequence and they were considered to be unlikely sites for cA-PK phosphorylation.

Neither anti-skeletal muscle α_1 1399–1418 antibodies nor anti-cardiac α_1 1774–1789 or anti-α_1 1889–1906 antibodies immunoprecipitated ^{32}P-labelled CNBr peptides of the predicted molecular mass (8.242, 15.136 and 23.385 kDa, respectively, not shown). Since immunoprecipitation of intact ^{32}P-labelled α_1 subunit was demonstrated for each antibody, we conclude that the absence of detectable phosphopeptides for consensus serines at positions 1575, 1848 and 1928 indicates that these residues are likely to be poor substrates for cA-PK. This assumes equal efficiency of CNBr cleavage at each methionine residue and that the consensus serine residues are present in the bovine cardiac α_1 subunit. In all peptide immunoprecipitation experi-ments, no attempt was made to assign identities to immunoprecipitated products with molecular weights higher than the target peptide. There was no immuno-precipitation of ^{32}P-labelled peptides when a non-specific antiserum was used (Fig. 4D).

The physiological significance of cA-PK catalysed phosphorylation at sites on the cardiac α_1 subunit identi-fied in this in vitro study remains to be established.
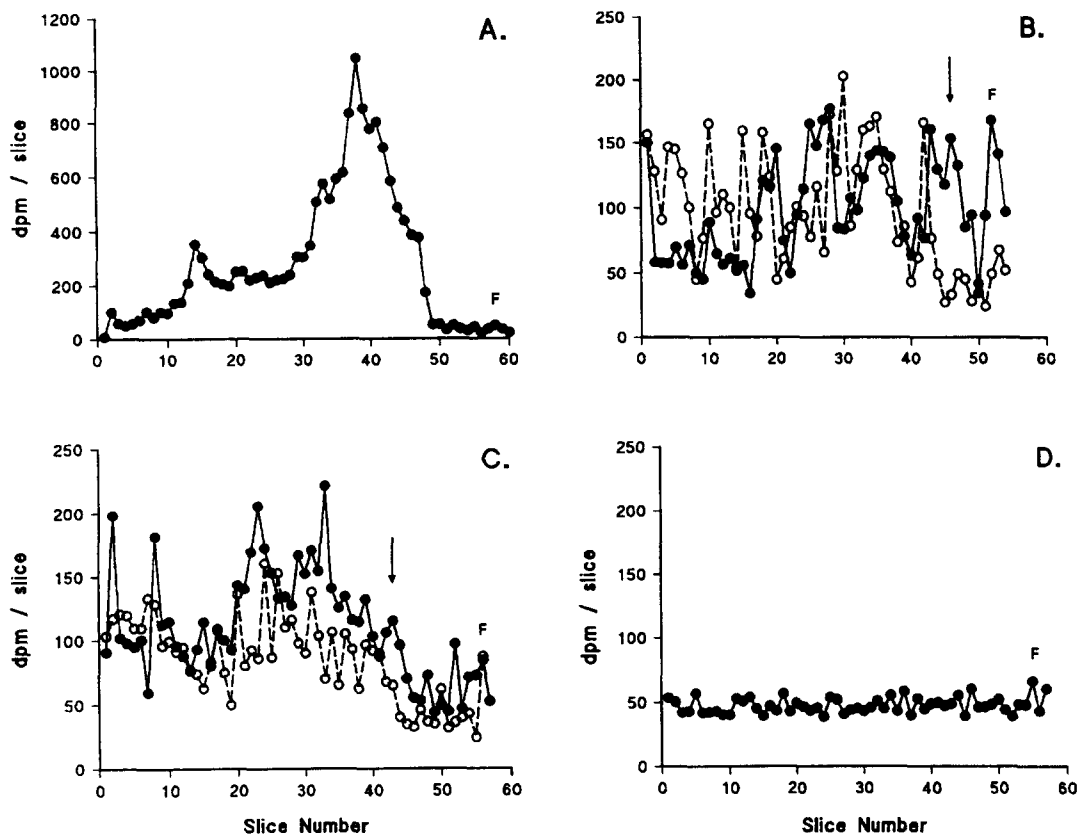

Fig. 4. Identification of ^{32}P-labelled CNBr cleavage products. CNBr digests of polyvinylidene difluoride membrane immobilised α_1 subunit were subjected to immunoprecipitation by anti-α_1 antibodies in the absence (closed symbols) or presence (open symbols) of 1 μg/ml of the corresponding immunising peptide. Peptides eluted from the immunomatrices were separated by SDS-PAGE in 15% acrylamide gels, gels were sliced (2 mm) and slices counted for radioactivity. (A) Profile of ^{32}P-labelled CNBr cleavage products derived from the cardiac α_1 subunit. Peptides immunoprecipitated by anti-α_1 1602–1616 (B), anti-α_1 1681–1694 (C) and anti-E. coli (D) antibodies. Figure shows results representative of at least three experiments. Arrows indicate the position predicted for the complete CNBr digest product containing the consensus cA-PK phosphorylation site. F, ion front.

However, it has been demonstrated in stably transfected cell lines that the activity of the cloned cardiac L-type calcium channel α_1 subunit when expressed alone is subject to cA-PK activity [4,5]. Furthermore, evidence that the C-terminal domain is involved in determining inactivation properties of the cardiac calcium channel [29,30] is consistent with the possibility of regulation of channel function by phosphorylation in this domain. It is noteworthy that the two consensus cA-PK phosphorylation sites Ser^{1627} and Ser^{1700} implicated in this study are conserved in the rabbit skeletal muscle and lung, human β-cell and neuronal D, and rat aorta and brain BI and BII (Ser^{1627} only) α_1 subunit isoforms [11,31–37]. Although these residues are only minor sites for cA-PK phosphorylation in the skeletal muscle isoform [8–10] a participation in L-type channel regulation can not be ruled out. The possibility remains that there is isoform specific differential regulation of calcium channel activity by protein kinases [38,39]. By contrast, consensus sites at Ser^{1575}, Ser^{1848} and Ser^{1928}, for which no evidence for cA-PK-dependent phosphorylation was observed, are unique to the cardiac class α_1 polypeptides [11,31–37]. It is likely that these residues play no part in mediating cA-PK regulation of channel activity.

The identification of CNBr phosphopeptides by specific immunoprecipitation may provide an alternative approach to site-directed mutagenesis and expression of mutants to allow the physiological significance of particular channel phosphorylation events to be addressed. The immunological approach may be particularly instructive should channel function be shown to be sensitive to phosphorylation at multiple cA-PK sites, or should crosstalk between different signalling pathways be shown.

In conclusion, we have shown that the α_1 subunit of the cardiac L-type Ca^{2+} channel is susceptible to phosphorylation by cA-PK. Sites of cA-PK catalysed phosphorylation have been localised to Ser^{1627} and Ser^{1700}. Other putative cA-PK sites in the α_1 subunit appear to be poor substrates.

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