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Phosphorylation of the L-type calcium channel β subunit is involved in β-adrenergic signal transduction in canine myocardium

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Cyclic AMP-mediated phosphorylation of calcium channel subunits was studied in vitro and in vivo in preparations from dog heart. Calcium channels in native cardiac membranes were phosphorylated by cAMP-dependent protein kinase (PKA) solubilized with digitonin and subsequently immunoprecipitated using a polyclonal antibody generated against the deduced carboxy-terminal sequence of the cardiac β subunit. A 62 kDa protein was identified as the major PKA-substrate in the immunoprecipitates. In the intact myocardium, this putative β subunit was found to be phosphorylated in response to cAMP elevating agents. In contrast, no phosphorylation of a protein with an electrophoretic mobility similar to the α_1 subunit was detected, although 1,4-dihydropyridine receptor sites were recovered in the immunoprecipitates. Thus, we suggest that PKA-mediated phosphorylation of the β subunit is the major mechanism for β-adrenergic regulation of cardiac L-type calcium channel activity.

Calcium channel; Canine heart; Immunoprecipitation; Peptide antibody; Protein phosphorylation; β Subunit

1. INTRODUCTION

The dihydropyridine-sensitive L-type calcium channel is causally involved in the modulation of cardiac contractility. Stimulation of β-adrenoceptors leads to an enhanced calcium influx, which is believed to be the result of protein phosphorylation catalyzed by a cAMP-dependent protein kinase [1–3]. However, the target proteins which may be phosphorylated are still unknown. Besides the channel itself [4] distinct regulatory proteins may serve as substrates for phosphorylation [5,6]. If the channel is the target, phosphorylation sites may reside in one of four subunits termed α_1, α_2, β, and δ, which form the oligomeric cardiac calcium channel complex [7–12].

For the skeletal muscle calcium channel, it seems to be clear that the α_1 subunit is the relevant channel component involved in β-adrenergic signal transduction. Although both the α_1 and the β subunits of this channel are substrates for PKA in vitro [13,14], the α_1 subunit is favoured as the primary target of PKA in intact muscle. The phosphorylation of this protein alone leads to increased calcium channel activity in skeletal muscle [15,16].

In cardiac muscle however, the target of PKA is still unclear. Biochemical studies using purified calcium channels gave no indication for a cAMP-dependent phosphorylation of the α_1 subunit [8,9,17]. This is in line with the amino acid sequence deduced from the cloned cardiac α_1 subunit 18 lacking serine-687 which is the major phosphorylation site in the skeletal muscle α_1 subunit [19]. Recently, it has been reported by Yoshida [20] that carboxy-terminal sites within the full-length cardiac α_1 subunit undergo phosphorylation by PKA and may transduce the cAMP effect on channel opening. However, these results have not been confirmed by other laboratories [21].

There is increasing evidence that the β subunit determines some basic properties of the calcium channel α_1 subunit [11,12,21–23]. Recently, cardiac β subunits have been predicted by cDNA cloning. The deduced amino acid sequences show potential PKA phosphorylation sites [11,12,24], but whether phosphorylation occurs in vivo remains to be elucidated.

This study reports on cAMP mediated phosphorylation of the β subunit in intact dog myocardium. Immunoprecipitation with a specific anti-peptide antibody was used to quantify the phosphorylation. The results suggest that the canine cardiac β subunit is a 62 kDa protein which is phosphorylated by PKA in vitro as well as in vivo.

2. MATERIALS AND METHODS

2.1. *Materials*

Drugs and chemicals were obtained from following sources: adjuvant peptide, digitonin, ω-aminohexyl-agarose, 1-methyl-3-isobutylxanthine, Sigma; (+)-[^{3}H]PN200–110 (spec. act. 85 Ci/mmol), [γ-^{32}P]ATP (spec. act. 3000 Ci/mmol), Amersham; [9,21-^{3}H(N)]ryanodine (60 Ci/mmol), 5-methyl-[^{3}H]nitrendipine (spec. act. 79 Ci/mmol) New England Nuclear; protein A-Sepharose CL-4B, Pharmacia; hemocyanin, keyhole limpet (lot 001738), Calbiochem; 2-iminothiolane

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Abbreviations: DHP, dihydropyridine; PKA, cAMP-dependent protein kinase; PN200–110, isopropyl 4-(2,1,3-benzoxadiazol-4-yl)-1,4-dihydro-2,6-dimethyl-5-(methoxy-carbonyl)pyridine-3-carboxylate.

hydrochloride, Fluka; bovine serum albumin (fraction V), Serva; rabbit IgG, Jackson, ImmunoResearch Labs.; pentobarbital, Abbott Labs, North Chicago; DL-isoprenaline Kodak, UK; reserpine, Arzneimittelwerk Dresden. Soluble digitonin was prepared as in [9]. The catalytic subunit of PKA was prepared from bovine heart according to [25].

2.2. *Synthetic peptide*

The peptide P-339 (EWNRDVYIRQ) corresponding to the carboxy-terminal residues 597–606 of CaB2a or residues 623–632 of CaB2b according to the nomenclature given in [11] was synthesized by the solid-phase method [26] and purified by reversed-phase HPLC on a Vydac 214 TP 1010 (10 μm particle size) column. To optimize coupling to SH-activated carriers the peptide was synthesized with an N-terminal chloroacetyl-glycine extension [27]. The identity of the purified peptides was verified by amino acid analysis.

2.3. *Preparation of antibodies*

Keyhole limpet hemocyanin (KLH) was activated with a 10-fold molar excess of 2-iminothiolane hydrochloride (Traut reagent) for 20 min at room temperature. Activated KLH was rapidly separated from the reagent by chromatography on a Sephadex G-25 column. The purified mono-chloroacetyl-glycine peptide P-339 was allowed to couple to activated KLH for 3 h at room temperature. The molar peptide:KLH ratio was 4:1. Aliquots of the KLH-peptide was stored frozen at −20°C until use. Initial immunization was performed using a mixture of KLH-peptide with rabbit antiserum (1 ml) raised against KLH. After 1-week the New Zealand white rabbits were immunized with KLH-peptide (calculated as 200 μg coupled peptide) and 100 μg adjuvant peptide at multiple intradermal sites. Booster injections were performed in the same manner when the titer of antibodies dropped. Antisera were collected after the second injection and tested for their ability to precipitate [^{3}H]PN200–110 labeled cardiac calcium channels. The epitope recognized by pAb-339 was determined by indirect immunophosphatase staining of nitrocellulose transfers of dog heart sarcolemmal membranes as described [28]. To purify the antibodies, the monochloroacetyl-glycine peptide P-339 was immobilized to activated ω-aminohexyl-agarose in the same manner as described for the coupling of the peptide to activated KLH. The antibodies pAb-339 were purified on P-339 resin columns as described [28].

2.4. *Treatment of dogs and tissue sampling*

Experiments were performed in accordance with internationally accepted principles concerning the care and use of laboratory animals. Mongrel dogs of either sex (15–20 kg) were anaesthetized by pentobarbital (35 mg/kg body weight) and artificially respirated with a mixture of nitrous oxide and oxygen (3:1.2). Left thoracotomy was performed. For maximal β-adrenergic stimulation of the heart a group of animals was pretreated with the phosphodiesterase inhibitor 1-methyl-3-isobutylxanthine (IBMX; 2 mg/kg of body weight, i.v.) for 5 min. Then isoprenaline (10 μg/kg of body weight) was introduced into the cavity of the left ventricle of the heart. Tissue samples of the left ventricular wall were rapidly frozen in situ at the maximum response in contractile force development (20 s after drug application) by a Wollenberger clamp precooled in liquid nitrogen. Another group of animals was pretreated with reserpine to deplete hearts of catecholamines as described in [29] for two days before the acute experiment was performed. A third group of open-chest, artificially respirated dogs was left untreated as controls. Tissue samples from the left ventricle were removed as described above. Freeze-clamped heart samples were stored under liquid nitrogen.

2.5. *Phosphorylation and labeling of receptors in cardiac membranes*

Fractions of total cardiac membranes were prepared from frozen tissue samples as described in [30]. Cardiac membranes (3 mg of protein) were phosphorylated, if not stated otherwise, in a final volume of 1.5 ml with 0.63 μM catalytic subunit of PKA and 10 μM [γ-^{32}P]ATP (2–3 nCi/pmol) for 6 min at 30°C in a medium containing 40 mM HEPES/Tris-buffer, pH 7.4, 15 mM $MgCl_2$, 0.1% digitonin, 1 mM EGTA, 5 mM EDTA, 12.5 mM NaF and 25 mM P_i. The phosphorylation reaction was started by addition of [γ-^{32}P]ATP and terminated by addition of 4 ml ice-cold buffer A consisting of 20 mM Tris-HCl buffer, pH 7.4, 10 mM EDTA. Phosphorylated membranes were spun down at 100,000 × g at 4°C, washed with buffer A and pelleted again. The phosphorylated membranes were solubilized in 1% digitonin with a 10:1 (w/w) detergent to protein ratio in 50 mM NaCl, 20 mM Tris-HCl buffer, pH 7.4, and protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride, 1 mM iodoacetamide, 0.1 mM benzamidine, 1 μM pepstatin A) at 0°C for 40 min. Insoluble material was removed by centrifugation at 100,000 × g for 30 min. The DHP receptor of the canine cardiac calcium channel was prelabeled with 10 nM (+)-[^{3}H]PN200–110 as described [9]. The ryanodine receptor of the calcium release channel was prelabeled with 5 nM of [^{3}H]ryanodine according to [31]. Solubilization of the receptors were performed as described for the solubilization of phosphorylated cardiac membranes.

2.6. *Immunoprecipitation*

Antibody beads were produced by incubation of appropriate amounts of pAb-339 rabbit antiserum with preswollen protein A-Sepharose (25 mg) in 1 ml PBS (0.9% NaCl, 50 mM NaH_2PO_4, pH 7.4) for 2 h at room temperature on a rotating wheel. The beads were washed subsequently with PBS and buffer B, consisting of 20 mM Tris-HCl, pH 7.4, 0.1% BSA, 1.3 mM $CaCl_2$, 0.2% digitonin, and protease inhibitors. The antibody beads were mixed with phosphorylated, solubilized membrane proteins (derived from 3 mg membrane protein) in a final volume of 15 ml of buffer B and the incubation continued overnight at 4°C. After precipitation of beads by centrifugation (5 s at 13,000 × g) the supernatants were removed and the beads were washed three times with cold buffer B. SDS sample buffer (5% SDS, 50 mM Tris-HCl, pH 7.5, 0.25 M sucrose, 0.075 M urea, 60 mM β-mercaptoethanol) was then added to the beads and the suspension was heated for 2 min at 94°C. Proteins from the supernatant were subjected to SDS-PAGE.

Immunoprecipitation of the [^{3}H]PN200-110-labeled receptor was performed in the same manner as described for phosphorylated proteins except lower amounts of both membranes and antibodies were employed and 10 μM diltiazem was added to the media. Accordingly, 3–50 μl pAb-339 rabbit antiserum, 0.5–10 μg affinity purified pAb-339 IgG or appropriate amounts of preimmune serum and control rabbit IgG were mixed with preswollen protein A-Sepharose (5 mg/assay) and incubated with 300 μl of solubilized (+)-[^{3}H]PN200-110-labeled receptor (20 fmol). Following a binding and washing procedure, the radioactivity bound to the beads was measured by liquid scintillation counting. The amount of receptor sites remaining in the supernatant was determined by polyethylene glycol precipitation [9]. All procedures were performed under yellow light. Immunoprecipitation of the [^{3}H]ryanodine receptor was performed as described for the DHP receptor except diltiazem and light protection were omitted. Briefly, beads were prepared with 10–50 μl of pAb-339 rabbit antiserum and mixed with 75 fmol of solubilized ryanodine-receptor from dog heart.

2.7. *SDS-PAGE and autoradiography*

SDS-PAGE was performed according to Laemmli [32] using 5–15% gradient polyacrylamide gels. Gels were dried and subjected to autoradiography. Incorporated [^{32}P]P_i was visualized on X-Omat XAR-2 films (Kodak, USA) and quantified by Fujix BAS 2000 (Japan) equipment or bands of interest were cut out from dried gels and counted by liquid scintillation.

2.8. *Other assays*

Tissue levels of cAMP were analysed in neutralized trichloroacetic acid extracts as described in [30]. The activity of cAMP-PK was measured as in [33] and is expressed as the ratio between activities assayed without and with cAMP (−cAMP/+cAMP). Equilibrium binding assays were performed as described in [34]. ATP content was analyzed by HPLC according to [35] using a ProPac PA1 column (Dionex, USA).

3. RESULTS AND DISCUSSION

A polyclonal antibody was generated by immunizing rabbits with a decapeptide, corresponding to the deduced carboxy-terminal amino acid sequence of rabbit cardiac calcium channel β subunit [11]. The antibody, termed pAb-339, cross-reacted with canine cardiac antigens as demonstrated by dose-dependent immunoprecipitation of [^{3}H]PN200-110-labeled calcium channels from digitonin-solubilized dog cardiac membranes (Fig. 1A). Specific immunoprecipitation ranged between 25% and 35% of the DHP-labeled receptors present in the assay. The same immunoprecipitation results were obtained with affinity-purified pAb-339.

In immunoblotting experiments no specific interaction among cardiac antigens derived from different species and pAb-339 was observed, which is probably due to specific epitope recognition. Obviously, pAb-339 reacted with digitonin conformation-dependent epitopes on the β subunit but not with SDS-solubilized cardiac calcium channels. Quite similar behaviour has been reported for monclonal antibodies directed against the cardiac sarcolemmal Na/Ca exchanger [36].

To study whether a component of the calcium channel complex serves as substrate for PKA, dog cardiac membranes were phosphorylated using [γ-^{32}P]ATP and the catalytic subunit of PKA. Two major substrates of PKA were specifically recovered by subsequent immunoprecipitation using pAb-339 (Fig. 1B). The apparent molecular mass of these phosphoproteins were calculated to be about 400 kDa and 61.8 ± 1.3 kDa (mean ± S.D., n = 9). The M_r of the latter phosphoprotein corresponds to the molecular mass of rabbit cardiac β subunits deduced from cDNA cloning [11,12,24]. Since pAb-339 has been raised against the predicted sequence of cardiac β subunit(s), the results strongly suggest that the 62 kDa substrate of PKA represents the native calcium channel β subunit protein in canine heart.

The phosphorylation of the 62 kDa β subunit was found to be absolutely dependent on the amount of exogenously added PKA (Fig. 2). Half-maximal phos-

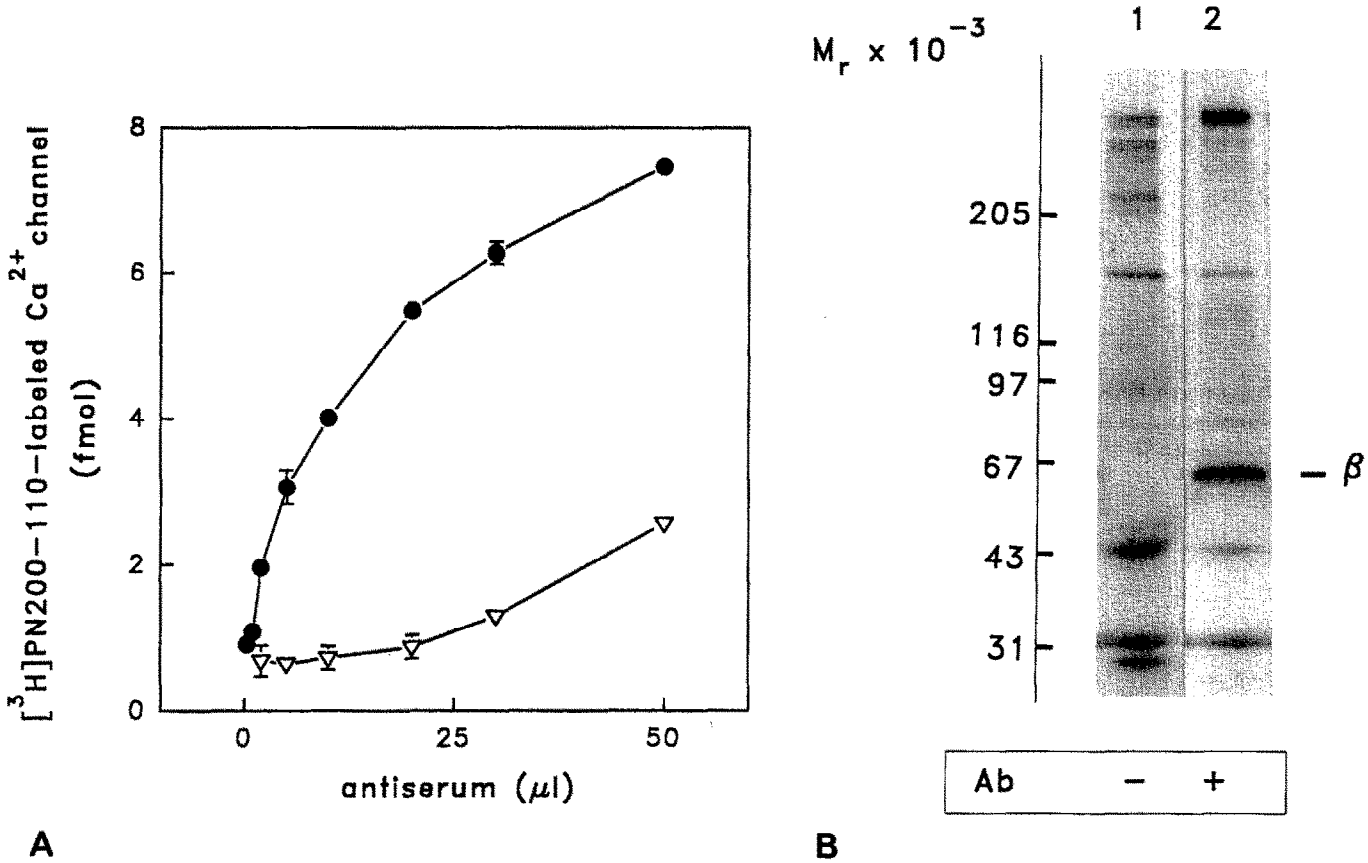

Fig. 1. Immunoprecipitation of labeled dog cardiac calcium channels by the anti-β subunit antibody pAb-339. (A) The indicated amounts of pAb-339 (●) or preimmune serum (▽) were bound to protein A-Sepharose. The antibody-beads were then incubated with 20 fmoles of [^{3}H]PN200-110 labeled calcium channels solubilized from dog cardiac membranes and pelleted. The radioactivity recovered in the precipitate was measured by liquid scintillation counting. Values are means ± S.D. for a typical experiment measured in triplicates. (B) Canine cardiac membranes (3 mg of membrane protein) were phosphorylated using the catalytic subunit of cAMP-dependent protein kinase and 10 μM [γ-^{32}P]ATP, solubilized with 1% of digitonin, immunoprecipitated and subjected to SDS-PAGE followed by autoradiography for 3 days. Lane 1, 200 μl preimmune serum (Ab−); lane 2, 200 μl pAb-P339 (Ab+).

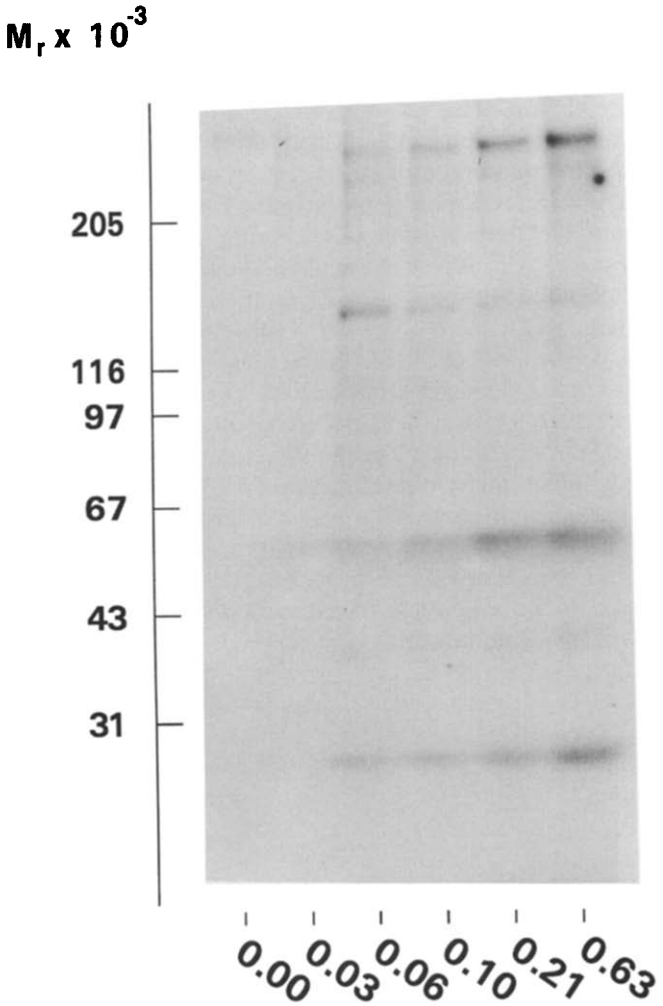

Fig. 2. Phosphorylation of calcium channel β subunit by cAMP-dependent protein kinase. Cardiac membranes from dog were phosphorylated for 1 min either in the absence or in the presence of increasing concentrations of the catalytic subunit of cAMP-dependent protein kinase (0.03–0.63 μM) under standard phosphorylation conditions. Phosphorylated membranes were solubilized, immunoprecipitated with 200 μl of pAb-339 and further processed as described in the legend to Fig. 1.

phorylation was observed at a concentration of about 0.2 μM PKA. Time course experiments with saturating concentrations of PKA revealed that the β subunit is rapidly phosphorylated. Maximal values of $^{32}P_i$ incorporation were reached within 30 s and remained constant for at least 12 min indicating that no dephosphorylation occurred. Furthermore, we have confirmed that the concentration of ATP used was sufficient to saturate the P_i-incorporation into the β subunit and did not drop below a critical level during the phosphorylation reaction.

In addition to the phosphorylated 62 kDa β subunit a high molecular mass protein of about 400 kDa was specifically immunoprecipitated too. Because of similarities in apparent molecular mass with the sarcoplasmic reticulum ryanodine receptor/calcium release channel [37] we tested the possibility whether pAb-339 immunoprecipitates the [^{3}H]ryanodine-labeled receptor from solubilized dog heart membranes. No interaction between the anti-β subunit pAb-339 and the ryanodine receptor was observed. Therefore, the molecular identity of the 400 kDa phosphoprotein and its relation to the calcium channel remains to be elucidated.

To evaluate the phosphorylation of calcium channels in intact heart, we employed the technique of back-phosphorylation. This is based on the in vitro topping-up of phosphorylation of cardiac membrane proteins in the presence of an excess of the catalytic subunit of PKA and [γ-^{32}P]ATP. The induced in vitro $^{32}P_i$ incorporation is inversely related to the in vivo phosphorylation of proteins [15,29,30,38,39]. In this report we have combined the in vitro back-phosphorylation with the immunoprecipitation standardized as described above. Cardiac membranes were isolated from dogs either acutely stimulated by the β-adrenergic agonist isoprenaline or depleted of catecholamines by reserpine as well as from untreated controls. Typical autoradiographs of immunoprecipitates obtained from in vitro back-phosphorylated membranes of the experimental groups are shown in Fig. 3. The $^{32}P_i$ incorporation into the β

Table I

Consequences of β-adrenergic stimulation in intact canine myocardium

Experimental groups	cAMP (pmol per mg wet weight)	PKA activity (−cAMP/+ cAMP)	$^{32}P_i$ incorporation into Ca^{2+} channel β subunit (fmol/mg membrane protein)	DHP-labeling of Ca^{2+} channel α_1 subunit (fmol/mg membrane protein)
Reserpine	0.44 ± 0.02 (6)	0.12 ± 0.01 (7)	8.7 ± 0.9 (11)	12.1
Control	0.49 ± 0.03 (3)	n.d.	4.6 ± 0.5 (3)	n.d.
Isoprenaline	2.57 ± 0.24 (6)*	0.24 ± 0.02 (4)*	1.3 ± 0.3 (5)*	12.9

The data for cAMP, protein kinase activity ratio, and $^{32}P_i$ incorporation are given as means± S.E.M. Numbers in parentheses indicate independent experiments with tissue samples derived from at least 3 different animals. Values of DHP-labeling were calculated from B_{max} taking into consideration the recovery of receptors after solubilization and immunoprecipitation, determined to be 38% and 30%, respectively. The B_{max} was 114 ± 17 fmol/mg membrane protein (n = 3) and 106 ± 20 fmol/mg membrane protein (n = 3) for preparations from the isoprenaline- and reserpine-treated animals, respectively.

*$P < 0.01$ vs. values for control and reserpine.

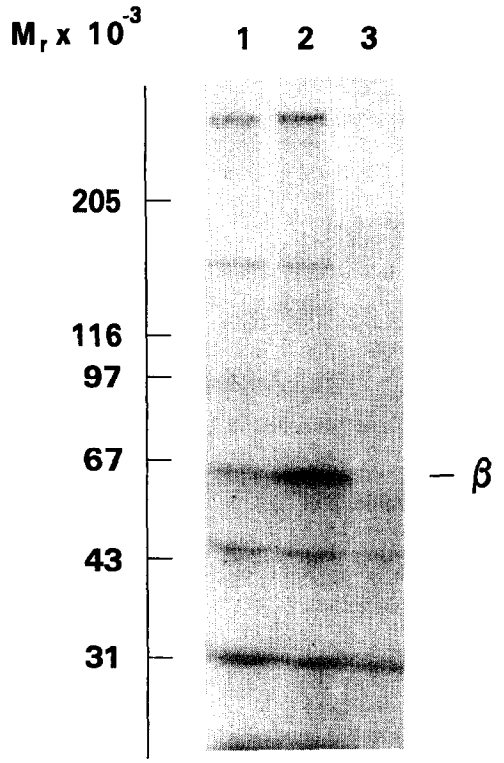

Fig. 3. Autoradiograph of back-phosphorylation of the calcium channel β-subunit prepared from dog hearts. Cardiac membranes were prepared from either control (lane 1), reserpine-treated (lane 2) or isoprenaline-treated (lane 3) dogs. The membranes (3 mg of protein of each preparation) were in vitro back-phosphorylated using the catalytic subunit of the cAMP-dependent protein kinase and 10 μM [γ-^{32}]ATP, solubilized, immunoprecipitated with 200 μl of pAb-339 and autoradiographed as described in the legend to Fig. 1.

protein was most prominent in preparations from reserpine-treated dogs (Fig. 3, lane 2), reduced in controls (lane 1), and nearly completely suppressed in samples from isoprenaline-treated animals (lane 3). Interestingly, comparable changes in back-phosphorylation were observed for the unidentified 400 kDa phosphoprotein (Fig. 3). Another coprecipitating 30 kDa substrate of PKA, however, was not affected by the the β-adrenergic agonist (Fig. 3).

Table I summarizes the quantitative data obtained for the $^{32}P_i$ incorporation into the immunoprecipitated calcium channel β subunit. In controls 4.6 ± 0.5 fmol [^{32}P]phosphate per mg of membrane protein was incorporated into the β subunit. Both isoprenaline- and reserpine-treatment caused significant changes in $^{32}P_i$. From the difference in $^{32}P_i$ incorporation in vitro (isoprenaline versus reserpine) a value of 7.4 ± 0.3 fmol phosphate per mg of membrane protein was calculated for isoprenaline-induced β subunit phosphorylation in vivo. Indeed, isoprenaline treatment increased cAMP content as well as endogenous PKA activity in the same frozen tissue samples used for in vitro back-phosphorylation (Table I). Obviously, the maximal efficient dose of isoprenaline and the presence of phosphodiesterase inhibition caused a rapid and apparently complete phosphorylation of β subunit in response to cAMP elevating drugs.

In order to approximate the molar ratio of β to α_1 subunits, β subunit phosphorylation data were related to the number of DHP binding sites, both recovered by immunoprecipitation. Using data from the reserpine group, for which a low in vivo phosphorylation is expected, the value of the ratio was calculated to be 0.72.

Concerning the DHP receptor α_1 subunit the following merits discussion. Although the data presented in Fig. 1A clearly demonstrate that DHP binding sites were precipitated by pAb-339, no phosphoprotein was detected with electrophoretic mobility similar to the 200 kDa DHP receptor α_1 subunit (Figs. 1,2,3). This result is in close agreement with findings previously obtained by us [9,17] with calcium channels purified from porcine heart as well as by Yoshida et al. [20] who used an anti-α_1 antibody to precipitate phosphorylated rabbit cardiac calcium channels. But, in contrast to the latter authors we did not detect the phosphorylated 250 kDa full-length form of the α_1 subunit. The most likely explanation for this result is the low abundance of the untruncated α_1 form which has been reported not to exceed 10% of the total amount of DHP receptors [40]. Although the data do not exclude the possibility that the full-length form of the cardiac α_1 protein serves as a substrate of PKA, a physiological role of its phosphorylation is questionable.

Taken together, our data suggest that the 62 kDa protein immunoprecipitated by the anti β subunit pAb 339 (i) represents the native calcium channel β subunit in dog heart and (ii) is a physiological target of PKA. These results provide strong evidence for the involvement of the calcium channel β subunit in β-adrenergic signal transduction in myocardium.

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