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CALCIUM-INDEPENDENT PHOSPHOLIPASE A_2 IN RABBIT VENTRICULAR MYOCYTES

Caroline S. Beckett and **Jane McHowat***
Department of Pathology, Saint Louis University School of Medicine

Abstract

We have previously reported that the majority of phospholipase A_2 (PLA_2) activity in rabbit ventricular myocytes is membrane-associated, calcium-independent ($iPLA_2$), selective for arachidonylated plasmalogen phospholipids and inhibited by the $iPLA_2$-selective inhibitor bromoenol lactone (BEL). Here, we identified the presence of iPLA2 in rabbit ventricular myocytes, determined the full length sequences for rabbit $iPLA_2\beta$ and $iPLA_2\gamma$ and compared their homology to the human isoforms. Rabbit $iPLA_2\beta$ encoded a protein with a predicated MW of 74 kDa that is 91% identical to the human $iPLA_2\beta$ short isoform. Full length $iPLA_2\gamma$ protein has a predicated MW of 88 kDa and is 88% identical to the human isoform. Immunoblot analysis of $iPLA_2\beta$ and γ in membrane and cytosolic fractions from rabbit and human cardiac myocytes demonstrated a similar pattern of distribution with both isoforms present in the membrane fraction, but no detectable protein in the cytosol. Membrane-associated $iPLA_2$ activity was inhibited preferentially by the R enantiomer of bromoenol lactone (*R*)-BEL, indicating that the majority of activity is due to $iPLA_2\gamma$.

Keywords

bromoenol lactone; phospholipase A_2; myocardium; plasmalogens; arachidonic acid

INTRODUCTION

Phospholipase A_2 catalyzes the hydrolysis of the fatty acid at the *sn*-2 position of membrane phospholipids, resulting in the generation of multiple biologically-active, membrane phospholipid-derived metabolites [for review, see 1]. The family of PLA_2 enzymes has been classified into 15 groups and several subgroups based on the catalytic mechanism, functional and structural features, and sequence homology [1].

We reported previously that the majority of phospholipase A_2 (PLA_2) activity in rabbit ventricular myocytes is membrane-associated, calcium-independent ($iPLA_2$) and selective for arachidonylated plasmalogen phospholipids [2]. Activation of this membrane-associated $iPLA_2$ occurs when ventricular myocytes are incubated with phorbol 12-myristate 13-acetate, suggesting that activation of $iPLA_2$ is regulated by protein kinase C [3]. We have also demonstrated that rabbit ventricular myocyte membrane-associated $iPLA_2$ is activated in response to either thrombin stimulation [4] or the presence of hypoxia [5]. Multiple types of $iPLA_2$ have been identified in cytosolic, microsomal and mitochondrial fractions from mammalian myocardium [6,7], but to date there is no data concerning the specific $iPLA_2$ isoform that may be present and activated in rabbit ventricular myocytes. Our previous data indicate that rabbit ventricular myocyte $iPLA_2$ activity is inhibited by bromoenol lactone (BEL)

*Corresponding Author: Department of Pathology Saint Louis University School of Medicine 1402 S. Grand Blvd. St. Louis, MO 63104 Phone: (314) 977-9295 Fax: (314) 977-8499 jane.mchowat@tenethealth.com.

but not by methyl arachidonyl fluorophosphonate (MAFP) [2], suggesting that it is either iPLA$_2$β or iPLA$_2$γ.

The iPLA$_2$β isoform is predominantly a cytosolic isoform and highly homologous cDNAs have been cloned from the hamster, mouse, rat and human (approximately 95% identity). In addition, several splice variants of human iPLA$_2$β have been identified [8]. However, in COS-7 cells overexpressing iPLA$_2$β, Larsson Forsell et al demonstrated a 5.5-fold increase in membrane-associated iPLA$_2$ activity [8], and membrane-associated iPLA$_2$β has also been found in rat vascular smooth muscle cells [9].

More recently, a novel membrane associated iPLA$_2$ (iPLA$_2$γ) has been identified [7] which possesses several of the properties demonstrated in our previous studies using rabbit ventricular myocytes, including the optimum pH for activity, sensitivity to BEL and a tight association with the membrane fraction. Homology between iPLA$_2$β and iPLA$_2$γ is confined to the ATP binding motif, the serine lipase site and a region of nine amino acids for which, as yet, there is no known functional significance [7]. The human iPLA2γ gene contains 4 possible translation codons that result in proteins of 63, 74, 77 and 88 kDa [10]. When first described, iPLA$_2$γ was proposed to be localized to peroxisomes due to the presence of a C-terminal SKL signal sequence and recent studies have confirmed that the peroxisomal iPLA$_2$γ is the 63 kDa protein [11]. More recent studies have demonstrated higher molecular weight isoforms of iPLA$_2$γ in mitochondria and endoplasmic reticulum [6].

This study was designed to identify the presence of iPLA$_2$β and iPLA$_2$γ in rabbit ventricular myocytes and to determine the similarity of these isoforms to those identified previously.

EXPERIMENTAL PROCEDURES

Rabbit Ventricular Myocyte Isolation and Culture

Adult rabbits of either sex weighing 2 to 3 kg were anesthetized with intravenous pentobarbitone sodium (50 mg/kg) and the heart rapidly removed. The heart was mounted on a Langendorff perfusion apparatus and perfused for 5 minutes with a Tyrode solution containing (mmol/l) NaCl 118, KCl 4.8, $CaCl_2$ 1.2, $MgCl_2$ 1.2, $NaHCO_3$ 24, KH_2PO_4 1.2 and glucose 11; the Tyrode solution was saturated with 95%O_2/5%CO_2 to yield a pH of 7.4. This was followed by a 4 minute perfusion with a Ca free Tyrode solution containing EGTA (100 μM) and a final perfusion for 20 minutes with the Tyrode solution containing 100 μM Ca and 0.033% collagenase. The heart was removed from the perfusion apparatus, the atria were removed and the remaining ventricles were cut into small pieces and incubated in fresh 0.033% collagenase solution at 37°C in a shaking water bath for 4 successive harvests of 20 minutes. Individual myocytes were washed with a HEPES buffer containing (mmol/l): NaCl 133.5, KCl 4.8, $MgCl_2$ 1.2, $CaCl_2$ 0.3, KH_2PO_4 1.2, glucose 10 and HEPES 10 (pH=7.4). Extracellular Ca was increased to 1.2 mM in three stages at intervals of 20 minutes. Myocytes were incubated overnight in M199 medium with 10% fetal calf serum at 37°C and then washed three times with 1.2 mM Ca HEPES solution.

Culture of human cardiac myocytes

Human adult cardiac myocytes (Sciencell, San Diego, CA) were cultured in cardiac myocyte medium consisting of 500 ml of basal medium, 25 ml of fetal bovine serum, 5 ml of cardiac myocyte growth supplement and 5 ml of penicillin/streptomycin solution (Sciencell, San Diego, CA).

Generation of $iPLA_2$ clones

Total RNA for use in all cloning steps was isolated with the Versagene Cell Kit (Gentra Systems, Minneapolis, MN). 5′ and 3′ end sequences of rabbit ventricular myocyte $iPLA_2$ were obtained by rapid amplification of cDNA ends following the manufacturer's instructions for the GeneRacer Kit from Invitrogen (Carlsbad, CA). Briefly, total RNA was dephosphorylated, decapped and ligated to the GeneRacer RNA Oligo to form template for first strand RACE-ready cDNA synthesis. Following cDNA amplification with the appropriate GeneRacer Oligo and gene specific Oligo, 5′ and 3′ end PCR fragments were cloned into the pCR4-TOPO vector and sequenced by automated DNA sequencing with BigDye chemistry. This sequence was conceptually translated and Oligos were designed to yield a full-length amplicon corresponding to the longest open reading frame for each isoform. Oligo dT primed first strand cDNA was generated from total RNA using the Thermoscript RT-PCR System (Invitrogen) and used as template for PCR amplification of full-length $iPLA_2$ clones. These products were cloned into the pCR-XL-TOPO vector and five independent clones of each isoform, −β and −γ, were sequenced. Sequence analysis and assembly was performed using the Vector NTI Suite 9.0.0 (Invitrogen) and the BLAST server at NCBI. Sequence assembly was performed using the ContigExpress program (Invitrogen) and the full-length cDNA sequences were deposited into Genbank ($iPLA_2$-β - Accession # AY744674, $iPLA_2$-γ - Accession # AY738591).

Production of iPLA2γ antibody

A custom-made chicken antibody was obtained from Aves Labs, Inc. (Tigard, OR). Hens were injected four times with a KLH conjugate of the peptide CZSKYIERNEHKMKKVAK and immune eggs were collected for 10 to 15 days after the final injection. The IgY fractions were purified and passed over an affinity column, the column washed and antibody eluted. The final concentration of affinity-purified antibody in the eluant was 0.6 mg/ml.

HPLC Separation of (R)- and (S)-enantiomers of BEL

(*R*)- and (*S*)-enantiomers of BEL were isolated from racemic BEL (Calbiochem) using a chirex 3,5-dinitrobenzoyl-(R)-phenylglycine chiral HPLC column (Phenomenex Inc., Torrance, CA) and previously published methods [17]. The column was equilibrated with hexane/dichlroroethane/ethanol (150:15:1, by vol) and the optical enantiomers eluted isocratically at 2 ml/min. Elution of (*R*)- and (*S*)-BEL was monitored by UV absorbance at 280 nm. Under these conditions the retention times (R_t) for (*R*)- and (*S*)-BEL differ by almost 1 min with the R_t for (S)-BEL being 11.1 min and 12.2 min for (*R*)-BEL [12]. Peaks corresponding to these R_t were collected, dried under N_2, and stored at −20°C. The concentration of each enantiomer was determined spectrophotometrically based on its UV absorbance [12].

Immunoblot analysis of $iPLA_2$ isoforms

Myocytes were suspended in lysis buffer containing (mmol/l) HEPES 20 (pH 7.6), sucrose 250, dithiothreitol 2, EDTA 2, EGTA 2, β-glycerophosphate 10, sodium orthovanadate 1, phenylmethylsulfonyl fluoride 2, leupeptin 20 μg/ml, aprotinin 10 μg/ml and pepstatin A 5 μg/ml (buffer 2). Cells were sonicated on ice for 6 bursts of 10 sec and centrifuged at 10,000 × g at 4°C for 20 min to remove cellular debris and nuclei. Cytosolic and membrane fractions were separated by centrifuging the supernatant at 100,000 × g for 60 min. The pellet was resuspended in lysis buffer and the suspension centrifuged at 100,000 × g for 60 min twice to minimize contamination of the membrane fraction with cytosolic protein. The final pellet was resuspended in lysis buffer containing 0.1% Triton X-100. Protein (cytosol or membrane) was mixed with an equal volume of SDS sample buffer and heated at 95°C for 5 mins prior to loading onto a 10% polyacrylamide gel. Protein was separated by SDS/PAGE at 200 V for 35 mins and electrophoretically transferred to PVDF membranes (Bio-Rad, Richmond, CA) at 100 V for 1 hour. Non-specific sites were blocked with Tris buffered solution containing 0.05%

(v/v) Tween-20 (TBST) and 5% (w/v) nonfat milk for 1 hour at room temperature. The blocked PVDF membrane was incubated with primary antibodies to iPLA$_2$β (1 in 1,000 dilution, Cayman Chemical Company, Ann Arbor, MI) or iPLA2γ (1 in 1,000 dilution, Aves Labs Inc., Tigard, OR), followed by horseradish peroxidase-conjugated secondary antibodies. Regions of antibody binding were detected using enhanced chemiluminescence (Amersham, Arlington Heights, IL) after exposure to film (Hyperfilm, Amersham). Multiple exposures of film to the blots were developed.

Phospholipase A_2 activity

Myocytes were suspended in 1 ml buffer containing (mmol/l): Sucrose 250, KCl 10, imidazole 10, EDTA 5, dithiothreitol (DTT) 2 with 10% glycerol, pH = 7.8 (buffer 1). The suspension was sonicated on ice six times for 10 seconds (using a microtip probe at 20% power output, 500 Sonic Dismembrator, Fisher Scientific) and the sonicate centrifuged at 20,000 × g for 20 minutes to remove cellular debris and nuclei. The supernatant was then centrifuged at 100,000 × g for 60 minutes to separate the membrane fraction (pellet) from the cytosolic fraction (supernatant). The pellet was washed twice to minimize contamination of the membrane fraction with cytosolic protein by resuspending in buffer1, and centrifuging at 100,000 × g for 60 min. The final pellet was resuspended in buffer 1. Phospholipase A_2 activity in cytosolic and membrane fractions was assessed by incubating enzyme (8 μg membrane protein) with 100 μM (16:0, [^{3}H]18:1) plasmenylcholine substrate in assay buffer containing (mmol/l): Tris 10, EGTA 4, 10% glycerol, pH = 7.0 at 37°C for 5 minutes in a total volume of 200 μl. The radiolabeled phospholipid substrate was introduced into the incubation mixture by injection in 5 μl ethanol to initiate the assay. Reactions were terminated by the addition of 100 μl butanol and released radiolabeled fatty acid was isolated by application of 25 μl of the butanol phase to channeled Silica Gel G plates, development in petroleum ether/diethyl ether/acetic acid (70/30/1, v/v) and subsequent quantification by liquid scintillation spectrometry. Protein content of each sample was determined by the Lowry method utilizing freeze dried bovine serum albumin as the protein standard. Radiolabeled (16:0, [^{3}H]18:1) plasmenylcholine was synthesized using [9,10-^{3}H] oleic acid and lysoplasmenylcholine as described in detail previously (4).

Statistical analysis

Statistical comparison of values was performed by Student's t test. All results are expressed as means ± S.E.M. Statistical significance was considered to be $p<0.05$.

RESULTS

iPLA$_2$-β cDNA was amplified, cloned and sequenced from freshly isolated rabbit ventricular myocytes, (GenBank accession No. 744674), and the amino acid sequence was determined (Figure 1). The longest open reading frame of iPLA$_2$β was found to encode a protein of 665 amino acids in length with a predicted MW of 74 kDa that aBLAST analysis of the non-redundant protein database reveals is 91% identical to the human short isoform. The sequences are highly conserved except for a divergent segment at the N-terminus which is 128 amino acids long in the human and 42 amino acids in the rabbit. Immunoblot analysis of cytosolic and membrane fractions isolated from rabbit and human cardiac myocytes demonstrated an iPLA$_2$β immunoreactive band that ran just below the 80 kDa molecular weight marker (Figure 3A). The human and rabbit immunoreactive bands ran at a similar molecular weight and were present in the membrane fraction only. No immunoreactive band was detected with iPLA$_2$β antibody in the cytosolic fraction of human or rabbit myocytes (Figure 3A).

Rabbit ventricular myocyte iPLA$_2$-γ was also cloned and sequenced, and is predicted to encode a full-length protein of 786 amino acids (Figure 2) with a molecular mass of 88 kDa that was

found by BLAST analysis to be 88% identical to the human form. It has been reported previously that the human iPLA$_2$-γ sequence contains multiple alternative start sites for translation, which correspond to protein products of 88, 77, 74 and 63 kDa [10]. We analyzed the amino acid sequence for iPLA$_2$γ in rabbit ventricular myocytes and identified 6 methionine residues upstream of the active site which could encode functional PLA$_2$. In addition to the 88, 77, 74, and 63 kDa isoforms found in the human isoform, we also identified two single amino acid substitutions which could encode alternative start sites for 58 and 49 kDa proteins. Immunoblot analysis of rabbit ventricular myocytes detected immunoreactive bands corresponding to the 88 kDa and 73 kDa isoenzymes in the membrane fraction of isolated rabbit ventricular myocytes. Immunoblot analysis of human cardiac myocytes revealed a single band corresponding to the 73 kDa isoform of iPLA$_2$γ in the membrane fraction (Figure 3B). No bands were detected in the cytosolic fractions from either rabbit or human myocytes (Figure 3B).

Since we detected both iPLA$_2$β and γ in isolated rabbit ventricular myocytes and the majority of iPLA$_2$ activity is membrane-associated, we incubated isolated membrane fractions with (*R*)-BEL, (*S*)-BEL or racemic BEL. Previous studies have indicated that (*S*)-BEL is approximately an order of magnitude more selective for iPLA$_2$β in comparison to iPLA$_2$γ [12]. Conversely, (*R*)-BEL is approximately an order of magnitude more selective for iPLA$_2$γ in comparison to iPLA$_2$β [12]. After pretreatment with BEL for 10 mins, membrane-associated iPLA$_2$ activity was significantly decreased with concentrations of (*R*)-BEL greater than 0.1 μM (Figure 4). At 10 μM, (*R*)-BEL inhibited iPLA$_2$ activity by 90%, whereas (*S*)-BEL inhibited iPLA$_2$ activity by approximately 50% (Figure 4)

DISCUSSION

Myocardial iPLA$_2$ activity in the rabbit was first described by Ford et al in 1991 [13]. They described a ten-fold increase in microsomal, plasmalogen-selective iPLA$_2$ activity during myocardial ischemia [13]. We have demonstrated increases in membrane-associated, plasmalogen-selective iPLA$_2$ activity in isolated rabbit ventricular myocytes in response to thrombin [14] and hypoxia [5]. The activation of iPLA$_2$ and accelerated plasmalogen phospholipid hydrolysis lead to the production of lysoplasmenylcholine [5, 14], an amphipathic metabolite that can have direct effects on the function of integral membrane proteins. We have shown that lysoplasmenylcholine produces action potential derangements in cardiac myocytes at much lower concentrations than that previously demonstrated for lysoplasmenylcholine [5]. The action potential derangements caused by lysoplasmenylcholine occur as a result of its action on multiple membrane currents [15].

Although several studies have described iPLA$_2$ activation in rabbit ventricular myocytes, this is the first study to sequence iPLA$_2$β and iPLA$_2$γ and to demonstrate that iPLA$_2$γ is the major isoform in the rabbit myocardium. Our immunoblot analysis data suggest that the vast majority of iPLA$_2$ is membrane-associated, and this agrees with our previous data measuring iPLA$_2$ activity [2]. In previously reported studies, iPLA$_2$β has been demonstrated to be primarily cytosolic. It was originally purified from the cytosol of the mouse macrophage cell line P388D$_1$ [16] and its cDNA was later identified in P388D$_1$ and Chinese hamster ovary cells [17] and human B lymphocytes [18]. In humans the difference between the membrane-associated and cytosolic isoforms is the presence of 54 amino acid residues that may lead to a membrane-associated protein [8]. While we did not identify a similar sequence in the rabbit isoform, TMpred analysis suggests that rabbit iPLA$_2$β contains a potential transmembrane domain (indicated by the boldface type in Figure 1).

In a previous study, we have identified the presence of multiple iPLA$_2$ isoforms in several tissues and species [6]. The role of myocardial iPLA$_2$ isoforms has been investigated in several

recent studies using mouse models. Calcium-independent PLA_2 activity in murine heart is less than 5% of that in human myocardium, suggesting that the mouse could be a species-specific knockdown model of human ischemia-induced arrhythmogenesis [19]. Accordingly, Mancuso et al generated transgenic mice that expressed amounts of $iPLA_2\beta$ activity comparable to that present in human myocardium and demonstrated malignant ventricular arrhythmias, increased fatty acid release in venous effluents and increased lysophospholipid content in the ischemic zone in response to ischemia [19]. These data demonstrate the role of myocardial $iPLA_2\beta$ in the hydrolysis of membrane phospholipids and arryhthmogenesis in response to ischemia. Transgenic mice expressing $iPLA_2\gamma$ in cardiac myocytes demonstrated a marked loss of mitochondrial phospholipids accompanied by mitochondrial dysfunction [20]. In a subsequent study, mice null for $iPLA_2\gamma$ displayed growth retardation, cold intolerance, reduced exercise endurance and abnormal mitochondrial function [21]. In particular, $iPLA_2\gamma$ knockout mice were more susceptible to sudden death following transverse aortic constriction, indicating the importance of $iPLA_2\gamma$ in the ability of the myocardium to respond to stress [21].

We have previously reported that incubation of isolated membrane fractions from rabbit ventricular myocytes demonstrate increased $iPLA_2$ activity when incubated with phorbol 12-myristate 13-acetate in the absence of Ca [3]. These data suggests that a membrane-associated novel PKC isoform modulates $iPLA_2$ activity. Increased $iPLA_2$ activity required the presence of ATP, suggesting that the enzyme is phosphorylated by PKC [3]. Sequence analysis of rabbit ventricular myocyte $iPLA_2\beta$and γ isoforms reveals the presence of several predicted PKC phosphorylation sites that are indicated by the boxes in Figures 1 and 3.

In conclusion, we have identified and sequenced $iPLA_2\beta$and $iPLA_2\gamma$ from isolated rabbit ventricular myocytes. These isoforms possess a high degree of sequence homology between rabbit and human and there is a similar protein expression pattern when comparing rabbit and human cardiac myocytes. Both isoforms have several putative PKC phosphorylation sites and our previous studies suggest that membrane-associated $iPLA_2$ is activated directly by a membrane-associated novel PKC isoform [3]. Finally, our data obtained using (*R*)- and (*S*)-enantiomers of the $iPLA_2$-selective inhibitor BEL, suggest that the majority of membrane-associated $iPLA_2$ activity is $iPLA_2\gamma$.

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Abbreviations

BEL	bromoenol lactone
$iPLA_2$	calcium-independent phospholipase A_2
MAFP	methyl arachidonyl fluorophosphonate
PKC	protein kinase C
PLA_2	phospholipase A_2

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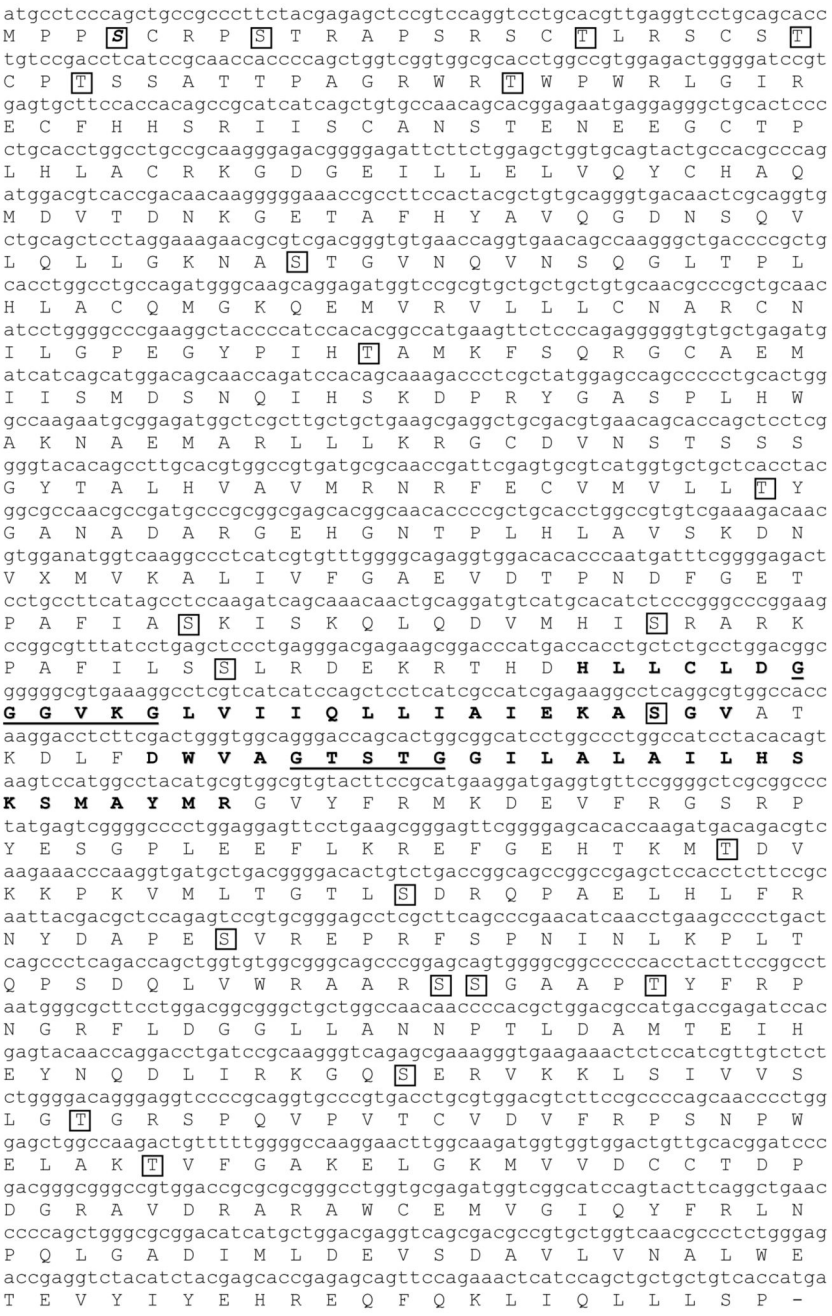

FIGURE 1.

Full-length rabbit iPLA2β cDNA sequence. Underlines indicate the conserved ATP-binding and lipase active sites. Potential transmembrane domains were predicted using the TMpred server and are indicated with boldface type. Potential PKC-phosphorylation sites were predicted using the NetPhosK 1.0 Server and are boxed.

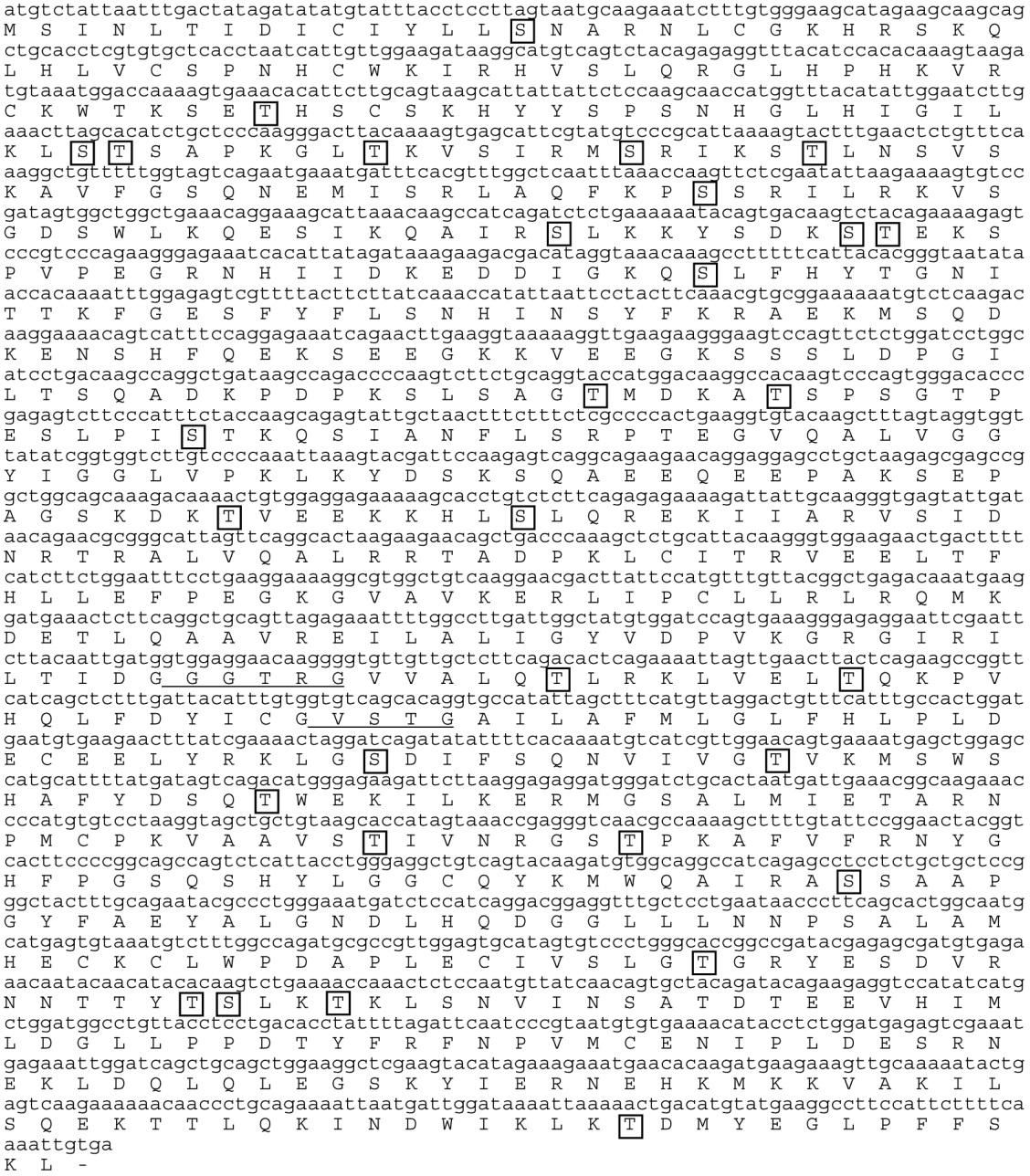

FIGURE 2.

Full-length rabbit iPLA$_2\gamma$ cDNA sequence. Underlines indicate the conserved ATP-binding and lipase active sites. Potential PKC-phosphorylation sites were predicted using the NetPhosK 1.0 Server and are boxed.

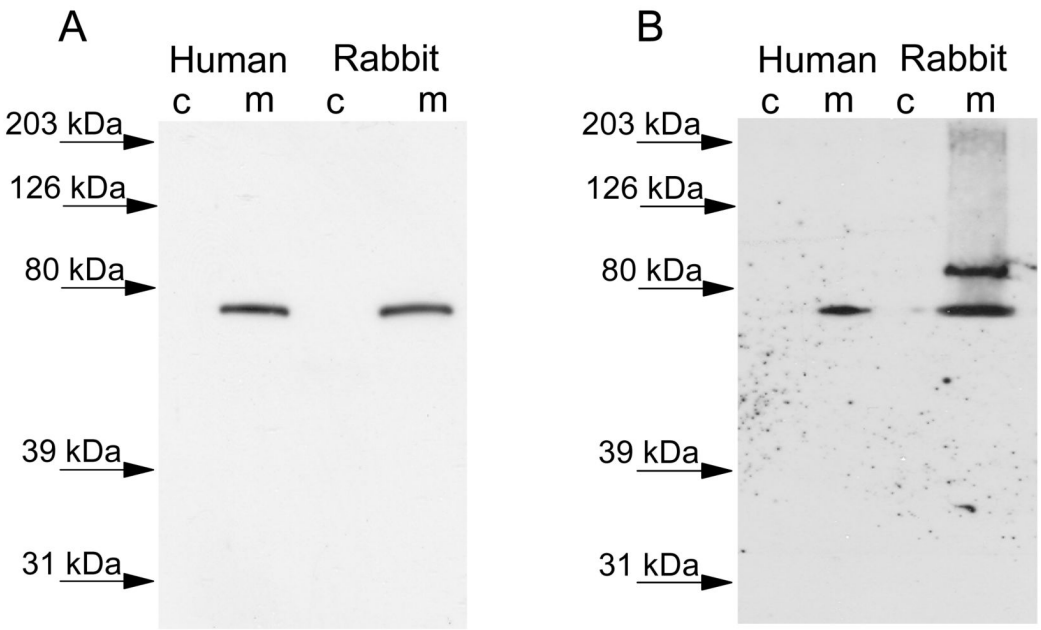

FIGURE 3.

Immunoblot analysis of the localization of iPLA$_2$β (Panel A) and iPLA$_2$γ (Panel B) in the cytosolic (C) and membrane (M) subcellular fractions isolated from rabbit and human cardiac myocytes. Proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. Membranes were probed with anti-iPLA$_2$β or anti-iPLA$_2$γ antibodies (1:1,000 dilution) and incubated with horseradish peroxidase-linked secondary antibody (1:50,000 dilution). Immunoblots were detected with enhanced chemiluminescence and exposure to film.

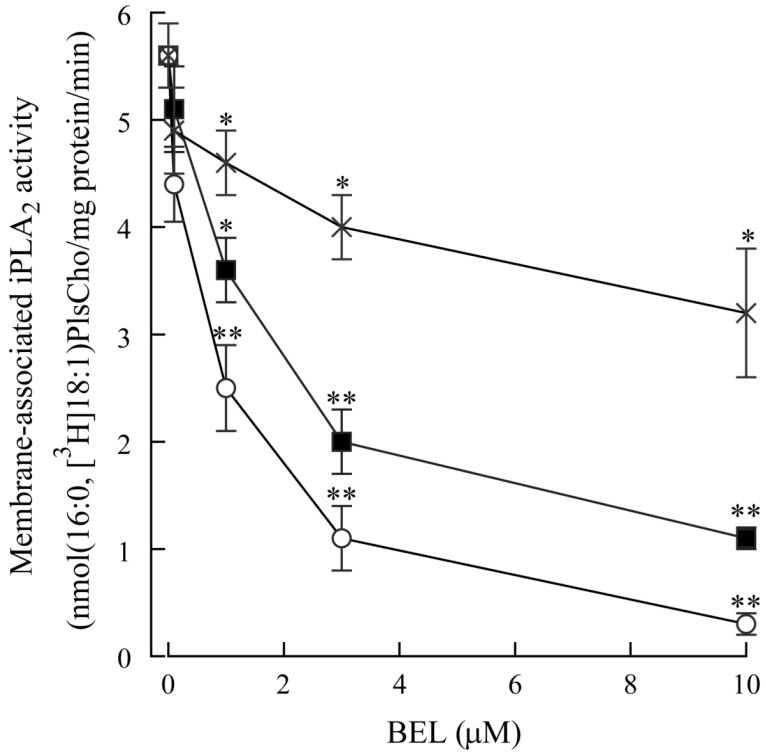

FIGURE 4.

Membrane-associated $iPLA_2$ activity measured in the absence or presence of bromoenol lactone (BEL). Isolated membrane fractions were incubated with increasing concentrations of R-BEL (open circles), S-BEL (X) or racemic BEL (filled squares) for 10 minutes prior to assay of PLA_2 activity. PLA_2 activity was measured using 100 μM (16:0, [^{3}H]18:1) plasmenylcholine (PlsCho) in the absence of Ca (4 mM EGTA). Values are means ± SEM for independent results from 6 separate animals. *$p<0.05$, **$p<0.01$ when compared to activity in the absence of BEL.