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CELLULAR & MOLECULAR BIOLOGY LETTERS
Volume 12 (2007) pp 621 - 632
http://www.cmbl.org.pl

DOI: 10.2478/s11658-007-0036-8

Received: 26 February 2007
Revised form accepted: 16 July 2007
Published online: 30 August 2007

Research article

CYTOSOLIC PHOSPHOLIPASE A2 REGULATION IN THE HIBERNATING THIRTEEN-LINED GROUND SQUIRREL

ASHLEY K. WOODS and KENNETH B. STOREY*
Institute of Biochemistry and Department of Biology, Carleton University,
1125 Colonel By Drive, Ottawa, Ontario, Canada K1S 5B6

Abstract: Cytosolic calcium-dependent phospholipase A_2 ($cPLA_2$) has multiple roles including production of arachidonic acid (a key player in cellular signaling pathways) and membrane remodeling. Additionally, since catabolism of arachidonic acid generates free radicals, the enzyme is also implicated in ischemic injury to mammalian organs. Regulation of $cPLA_2$ could be important in the suppression and prioritization of cellular pathways in animals that undergo reversible transitions into hypometabolic states. The present study examines the responses and regulation of $cPLA_2$ in skeletal muscle and liver of hibernating thirteen-lined ground squirrels, *Spermophilus tridecemlineatus*. $cPLA_2$ activity decreased significantly by 43% in liver during hibernation, compared with euthermic controls, and K_m values for arachidonoyl thio-PC substrate fell in both organs during hibernation to 61% in liver and 28% in muscle of the corresponding euthermic value. To determine whether these responses were due to a change in the phosphorylation state of the enzyme, Western blotting was employed using antibodies recognizing phospho-Ser^{505} on α-$cPLA_2$. The amount of phosphorylated α-$cPLA_2$ in hibernator liver was just 38% of the value in euthermic liver. Furthermore, incubation of liver extracts under conditions that enhanced protein phosphatase action caused a greater reduction in the detectable amount of phospho-Ser^{505} enzyme content in euthermic, versus hibernator, extracts. The data are consistent with a suppression of $cPLA_2$ function during torpor via enzyme dephosphorylation, an action that may contribute to the well-developed ischemia tolerance and lack of oxidative damage found in hibernating species over cycles of torpor and arousal.

* Author for correspondence; e-mail: kenneth_storey@carleton.ca, tel: 1 613 520-3678, fax: 1 613 520-3745

Abbreviations used: $cPLA_2$ – cytosolic calcium-dependent phospholipase A_2; DTNB – 5,5'-dithio-bis(2-nitrobenzoic acid); MAPK – mitogen-activated protein kinase; PL –phospholipase; PMSF – phenylmethylsulfonyl fluoride

Key words: Metabolic rate depression, Signaling, Arachidonic acid production, Reversible phosphorylation, *Spermophilus tridecemlineatus*

INTRODUCTION

Lipid membranes are responsible for a host of cellular functions including membrane dynamics, protein regulation, signal transduction, modulation of activities of protein kinases, effects on transmembrane movements of nutrients, ions, neurotransmitters and other molecules, endocytosis and vesicular secretion [1, 2]. The composition of lipid membranes relies on a balance between synthesis and catabolism as well as alterations of phospholipid composition to create unique membrane characteristics [2]. Enzymes called phospholipases bring about membrane catabolism and remodeling and can be categorized into five classes: phospholipase A_1 and A_2 (PLA_1, PLA_2) and phospholipases B, C and D (PLB, PLC, PLD). Each differs in the location and type of bond in the phospholipid that they can hydrolyze [3].

Arachidonic acid is a key molecule in cells and the precursor to several metabolic pathways including the production of lipid messengers such as the eicosanoids (e.g. prostaglandins, aromatic hormones, leukotrienes, thromboxanes and platelet-activating factor). Eicosanoids play important cellular roles such as the regulation blood flow, migration, apoptosis, stimulation of the inflammatory response to cell growth, cell differentiation, and the regulation of ion transportation [3-5]. In the resting state, the bulk of arachidonic acid is esterified in membrane phospholipids at the *sn*-2 position [6]. The liberation of arachidonic acid from this position is catalyzed by the activation of the phospholipase A_2 family.

Three large subclasses of PLA_2 can liberate phospholipids at the *sn*-2 position. They differ in their molecular size and their requirement for calcium. Secreted PLA_2 ($sPLA_2$) are small enzymes that are best-known as components of venoms (but are widely distributed in animals), whereas calcium-independent PLA_2 ($iPLA_2$) enzymes are found predominantly in the cytoplasm [7]. The third class of PLA_2 enzymes are the cytosolic calcium-dependent phospholipases ($cPLA_2$). This group preferentially releases arachidonic acid from the *sn*-2 position and requires micromolar amounts of calcium. Three isozymes of $cPLA_2$ are well known: α-$cPLA_2$, β-$cPLA_2$, and γ-$cPLA_2$. All have high sequence homology, but differ in their selectivity for arachidonic acid [5], the alpha isozyme being the most selective. Recent analysis of the mouse genome has also identified the presence of genes for a further three isozymes of $cPLA_2$ although little else is known about them to date [8]. α-$cPLA_2$ is the best-studied and is subject to post-translational modification by phosphorylation on up to four serine residues: S^{437}, S^{454}, S^{505}, and S^{727}. The effects of phosphorylation on S^{437} and S^{454} are unknown since these residues are not well-conserved between species. The source of S^{505} and S^{727} phosphorylation is also controversial but these two residues are conserved and have a direct impact on the activity of α-$cPLA_2$. In different

systems, the source of S^{505} phosphorylation has been linked to the mitogen-activated protein kinase (MAPK) family [8, 9] whereas S^{727} phosphorylation is linked to protein kinases A or C in some systems but this phosphorylation is not necessarily correlated with an increase in activity [6]. Calcium is also required for the translocation of α-$cPLA_2$ to the membrane [10].

Since arachidonic acid is involved in a range of cellular signaling pathways, its regulation could be important in the suppression and prioritization of cellular pathways in animals that undergo reversible transitions into hypometabolic states of torpor or dormancy. Furthermore, recent research has implicated PLA_2 enzymes, and $cPLA_2$ in particular, with ischemic injury due to oxidative stress [11]. Animals that exhibit natural hypometabolism typically maintain well-developed antioxidant defenses that allow them to make transitions between states of high and low oxygen availability/consumption without sustaining oxidative damage [12]. Hence, we had two reasons to suspect that regulation of PLA_2 enzymes could contribute to the control of hypometabolism. Mammalian hibernation is a well-known example of natural hypometabolism [13]. During torpor, metabolic rate can fall by >95% and body temperature is reduced from 37°C to near ambient as a result of coordinated controls on all aspects of cell and organ function. Many enzymes/pathways are regulated during hibernation by mechanisms including reversible protein phosphorylation and differential gene expression [14]. However, the responses of phospholipases during hibernation have received little study to date. Early studies by Brutovetsky and coworkers indicated a role for $cPLA_2$ in the regulation of oxidative activity in liver mitochondria in hibernating ground squirrels and gophers and suggested that the enzyme was important in reversing the inhibition of the respiratory chain during torpor bouts [15, 16].

The present study analyzes $cPLA_2$ from liver and muscle of thirteen-lined ground squirrels, *Spermophilus tridecemlineatus* (Mitchill, 1821), with the goal of determining whether α-$cPLA_2$ activity is regulated between euthermic and hibernating states and whether reversible protein phosphorylation is the mechanism involved.

MATERIALS AND METHODS

Animals and tissue preparation

Thirteen-lined ground squirrels were obtained from TLS Research (Bartlett, IL, USA) and transported to the NIH (Bethesda, MD) where they were held in the animal hibernation facility in the laboratory of Dr. J.M. Hallenbeck (National Institute of Neurological Disorders and Stroke). The squirrels were housed individually in a room with an ambient temperature of 21°C and a 12 h:12 h light:dark cycle, and were fed standard rodent diet and water ad libitum. Once the animals had completed the pre-hibernation phase of hyperphagia that maximized their body lipid reserves (characterized by rapid weight gain from 130-180g up to 220-240g), some animals were placed in a dark room at 5-6ºC to

induce hibernation. After continuous torpor for 2-5 days, with stable core body temperatures near 6°C, hibernators were sacrificed by decapitation. Euthermic controls maintained at 21°C were sampled on the same day. Tissues were rapidly excised, immediately frozen in liquid nitrogen and transported to Ottawa on dry ice where they were stored in a -80ºC freezer until use. Samples of skeletal muscle (hind leg thigh) and liver was crushed under liquid nitrogen using a mortar and pestle and then 150 mg of tissue was mixed with 1 ml of homogenization buffer (50 mmol l^{-1} Tris-HCl, pH 7.4, 1 mmol l^{-1} EDTA, 30 mmol l^{-1} NaF) with a few crystals of phenylmethylsulfonyl fluoride (PMSF) added immediately prior to homogenization using a Polytron homogenizer. Samples were centrifuged at 10,000 x g for 15 min at 4ºC and supernatants were held on ice until use.

$cPLA_2$ assay

The assay kit from Cayman Chemical Co. (Ann Arbor, MI, USA) was used to measure cPLA2 activity. The arachidonoyl thio-PC substrate provided in the assay kit was dissolved in ethanol and solubilized as per manufacturer's instructions. Substrate in ethanol was bubbled under a gentle stream of nitrogen gas and then solubilized in assay buffer (80 mmol l^{-1} HEPES, pH 7.4, 150 mmol l^{-1} NaCl, 10 mmol l^{-1} $CaCl_2$, 4 mmol l^{-1} Triton X-100, 30% v/v glycerol, and 1 g l^{-1} bovine serum albumin) and vortexed until completely dissolved. A solution in 12 ml of assay buffer contained arachidonoyl thio-PC at a final concentration of 1.5 mmol l^{-1}.

Assay specificity for $cPLA_2$ was ensured by two methods: (1) by use of the inhibitor, bromoenol lactone (Sigma Chemical Co., St. Louis, MO, USA; catalogue #B1552), to irreversibly inhibit calcium-independent phospholipase ($iPLA_2$), and (2) by partial purification of samples via gel filtration to separate out low molecular weight proteins including any $sPLA_2$ that was present. Enzyme extracts were layered onto small columns of Sephadex G-50 (equilibrated in homogenization buffer) and then centrifuged for 1 min at high speed in a IEC benchtop centrifuge to remove any thiol contamination that would interfere in the assay as well as low molecular mass (<30 kDa) metabolites and proteins. Samples of eluate were then incubated with the inhibitor bromoenol lactone at a final concentration of 5 μmol l^{-1} for 30 min prior to assay of $cPLA_2$ activity.

The $cPLA_2$ assay was conducted using a 96 well microplate spectrophotometer with a total assay volume of 225 μl. The reaction mixture contained assay buffer, thio-PC substrate and enzyme sample (after incubation with bromoenol lactone inhibitor). Each sample was tested at substrate concentrations varying from 0-1.3 mmol l^{-1} for skeletal muscle and 0-0.665 mmol l^{-1} for liver in different wells; for K_m determinations, velocities were measured at 5-6 suboptimal substrate concentrations. Reactions were started by the addition of enzyme extracts to the wells followed by incubation for 1 h at 23°C. $cPLA_2$ cleaves arachidonoyl thio-PC at the *sn*-2 position and the thiol is released. Assays were stopped by addition

of a mixture of 25 mmol l^{-1} 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) and 475 mmol l^{-1} EGTA. The EGTA chelates the calcium required for phospholipase activity and the DTNB reacts with the thiol product to form a colored 5-thio-2-nitrobenzoic acid complex. Colour was developed for 5 min and then absorbance was measured at 414 nm in a Multiskan Spectrum microplate reader (Thermo Scientific, Waltham, MA, USA). Data were exported to a microplate analysis program [17] and kinetic parameters were analyzed with a non-linear least squares regression program, Kinetics 3.51 [18].

Incubations and Western blotting

Liver extracts were treated under incubation conditions that were designed to alter the phosphorylation state of $cPLA_2$. Tissue samples were homogenized in a simple buffer containing 50 mmol l^{-1} Tris-HCl, pH 7.4 and PMSF added at the time of homogenization and centrifuged as described previously. Supernatants were centrifuged through a Sephadex G-50 column equilibrated in the same buffer and then extracts were divided into two aliquots. To the first aliquot, 1 mmol l^{-1} EDTA and 30 mmol l^{-1} NaF were added to inhibit phosphatases and kinases (referred to as STOP conditions). To the second aliquot, 5 mmol l^{-1} $MgCl_2$ and 5 mmol l^{-1} $CaCl_2$ were added to stimulate endogenous phosphatases. Enzyme extracts were incubated on ice for 4 h (cold incubation provided better long term stability of the enzyme than incubation at room temperature) and then used for Western blot analysis. Protein content of samples was determined by the Coomassie blue dye-binding method. Standard electrophoresis and Western blotting techniques were conducted as previously mentioned [19, 20]. Aliquots containing 60 μg of soluble protein (in sample buffer containing 100 mmol l^{-1} Tris-HCl pH 6.8, 4% w/v SDS, 20% v/v glycerol, 5% v/v β-mercaptoethanol, and 0.2% w/v bromophenol blue) were loaded into each well of 12% SDS-PAGE gels and were separated at 180V for 1 h. Proteins were blotted on to PVDF membranes using wet transfer in cold 25 mmol l^{-1} Tris pH 8.5, 192 mmol l^{-1} glycine and 20% v/v methanol. Transfers were carried out at 300V for 90 min. Membranes were then blocked using 2.5% w/v powdered skim milk in TBST (50 mmol l^{-1} Tris-HCl, pH 6.8, 250 mmol l^{-1} NaCl, 0.05% v/v Tween 20). Membranes were incubated overnight at 4ºC with a peptide antibody (1:1000 v:v dilution) recognizing phosphorylated S^{505} on α-$cPLA_2$ (Cell Signaling Technology, Danvers, MA, USA; catalogue # 2831). Membranes were then incubated with secondary HRP-linked goat anti-rabbit IgG antibody (1:2000 v:v) for 1.5 h at 4ºC and washed three times for 5 min in TBST. Blots were developed using an enhanced chemiluminescence assay using SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology, Rockford, IL, USA). The membrane was scanned using a ChemiGenius Bio Imaging System (Syngene, Frederick, MD, USA) and the resulting image was analyzed with the associated Gene Tools software. To confirm that a consistent amount of protein had been added to each well, membranes were subsequently stained with Coomassie blue and rescanned. Strong bands that showed constant intensities

between samples (and were not close to the molecular weight of $cPLA_2$) were chosen as controls, quantified by densitometry and used to normalize the intensity of the immuno-reacted bands.

RESULTS

Assay and enzyme kinetics for ground squirrel muscle and liver $cPLA_2$

Fig. 1 shows the maximum activity of $cPLA_2$ in skeletal muscle and liver of ground squirrels, assayed at optimal substrate concentrations of 1.33 mmol l^{-1} for muscle and 0.665 mmol l^{-1} for liver. Activity was ~3-fold higher in muscle than in liver; V_{max} values in euthermia were 3.72 ± 0.61 and 1.17 ± 0.42 pmol/min/µg protein for muscle and liver, respectively. Muscle activity was unchanged between euthermic and hibernating states but liver showed a significant decrease in V_{max} activity during hibernation, falling to 43% of the corresponding

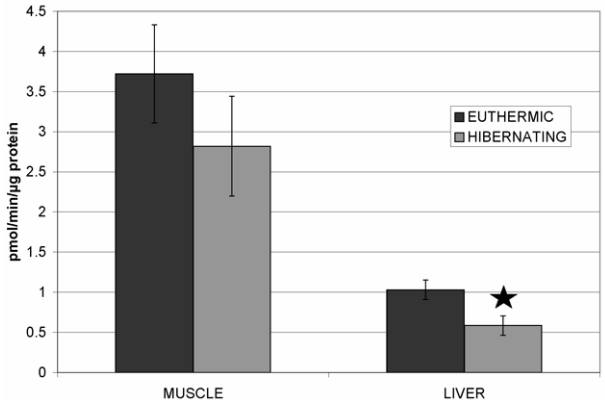

Fig. 1. V_{max} values for $cPLA_2$ activity for muscle and liver from euthermic and hibernating ground squirrels. Assays were carried out at optimal substrate concentrations of 1.33 mmol l^{-1} for muscle and 0.665 mmol l^{-1} for liver. Data are pmol substrate utilized per min per µg soluble protein in Sephadex G50 filtered extracts, means ± SEM, n = 3 for assays conducted at 23ºC. Stars indicate a significant difference between hibernating and euthermic samples as determined by the Student's t-test, $P < 0.05$.

euthermic value. K_m values for arachidonoyl thio-PC are shown in Fig. 2. Substrate concentrations of arachidonoyl thio-PC varying from 0-1.3 mmol l^{-1} for muscle and 0-0.665 mmol l^{-1} for liver were used to generate velocity versus substrate concentration curves; these relationships were hyperbolic for both tissues. Liver $cPLA_2$ showed a markedly lower K_m of 0.056 ± 0.005 mmol l^{-1} than the muscle enzyme which was 0.70 ± 0.016 mmol l^{-1}. For both tissues, the K_m value decreased significantly during hibernation, falling to 61% in liver and 28% in muscle of the corresponding euthermic value.

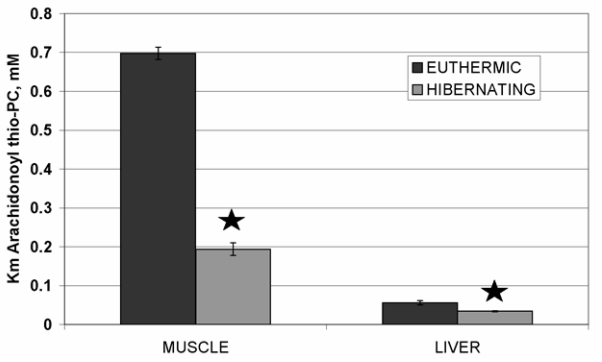

Fig. 2. K_m values for arachidonoyl thio-PC for $cPLA_2$ from ground squirrel skeletal muscle and liver assayed from euthermic and hibernating animals. Other information as in Fig. 1.

Incubations and Western blotting

Preliminary immunoblot analysis showed strong cross-reactivity of the phospho-$cPLA_2$ antibody (detecting phospho-serine residue 505) with a protein of ~110 kDa (the expected molecular mass of $cPLA_2$) in extracts of ground squirrel liver and, therefore, liver samples were used for subsequent incubation studies. The antibody used did not detect a $cPLA_2$ band in muscle extracts, despite multiple trials under different blotting conditions. Fig. 3 shows that the phosphorylated form of the enzyme was readily detected in liver from euthermic animals but the levels were very low under hibernating conditions, about one-third (38%) of the value in euthermic liver. To determine if the change in phospho-$cPLA_2$ band intensity during hibernation was the result of dephosphorylation of the protein, incubation studies were carried out on liver samples from euthermic and hibernating squirrels. Liver extracts were incubated under conditions that preserved the phosphorylation state of the enzyme (STOP conditions included EDTA to inhibit protein kinases and NaF to inhibit phosphatases) versus conditions that stimulated the activities of endogenous protein phosphatases ($MgCl_2$ and $CaCl_2$ added). Fig. 4 shows that the phosphorylated form of $cPLA_2$ was present in substantial amounts in euthermic extracts incubated under STOP conditions, similar to the results seen in Fig. 3. However, when incubated under conditions that stimulated endogenous phosphatases, the intensity of the immunoreactive band decreased significantly by 58% in extracts of euthermic liver. This suggests that incubation conditions that stimulated protein phosphatase activities led to dephosphorylation of $cPLA_2$. Incubations of liver extracts from hibernating animals produced an 40% decrease in phospho-$cPLA_2$ content, but the decrease was not significantly different from the STOP condition. This implies that the enzyme in liver of hibernating animals had a lower initial phosphate content.

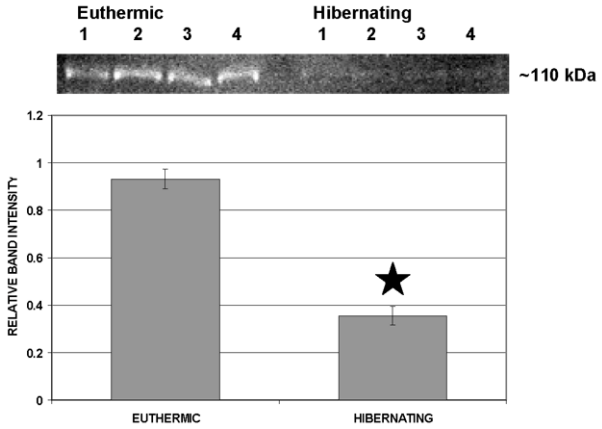

Fig. 3. Western blot analysis of phosphorylated $cPLA_2$ protein levels in liver of euthermic and hibernating ground squirrels. The histogram shows normalized band intensities for euthermic and hibernating samples. Data are means ± SEM, n = 4 determinations on independent tissue extracts. Stars show significant differences in normalized band intensities between the hibernating versus euthermic states, as determined by the Student's t-test $P < 0.05$.

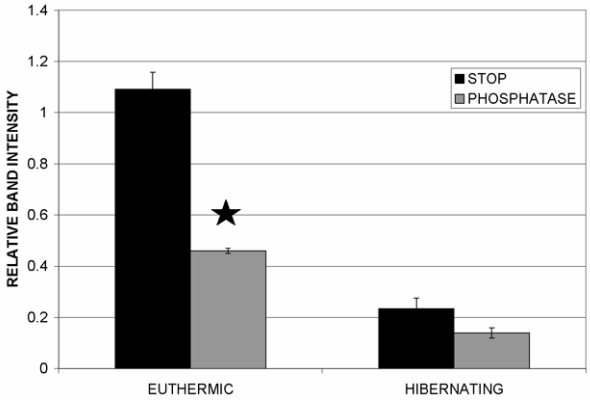

Fig. 4. Western blot analysis of phosphorylated $cPLA_2$ protein levels in liver of euthermic and hibernating squirrels after incubations designed to preserve the natural phosphorylation state of $cPLA_2$ (STOP) or stimulate endogenous phosphatases (PHOSPHATASE). Other information as in Fig. 3.

DISCUSSION

Calcium-dependent cytosolic phospholipase A_2 was assessed in skeletal muscle and liver of euthermic and hibernating ground squirrels to detemine how this enzyme is regulated during torpor. A previous study indicated a decrease in $cPLA_2$ activity in liver mitochondria from the European ground squirrel (*Citellus undulatus*) during hibernation [15]. The authors postulated that the suppression

of $cPLA_2$ in hibernation was linked to the decrease in mitochondrial respiration in hibernation, although the mechanism was unknown. A new review [11] adds another perspective on a possible role for PLA_2 in hibernation by suggesting that PLA_2 enzymes in general, and $cPLA_2$ in particular, are significant players in ischemic injury in mammalian organs. PLA_2 activation increases the production of arachidonic acid and the subsequent catabolism of arachidonic acid by cyclooxygenases and lipoxygenases is one of a number of cellular reactions that are known to generate reactive oxygen species that cause oxidative damage [1]. $cPLA_2$ is activated in multiple models of cell/organ ischemia including both global and focal ischemia in brain and studies using general PLA_2 inhibitors or transgenic mouse strains that are $cPLA_2$ deficient reported smaller infarcts, fewer neurological deficits, and reduced neuronal death after ischemia when the activity of this enzyme was suppressed [11]. Hence, there is strong evidence that $cPLA_2$ has a significant role in ischemic injury in mammals. Notably, hibernators are known to use a number of strategies to minimize oxidative damage both during long-term torpor and during the arousal process when oxygen consumption increases massively to support the tremendous thermogenesis needed to rewarm the body. These include high levels of metabolite antioxidants (e.g. ascorbate) [21] and elevating organ activities of antioxidant enzymes both seasonally and during individual torpor bouts [22-24]. A suppression of activities that produce reactive oxygen species during torpor would be an equally valid way to minimize oxidative damage and is supported by the current data on hibernator $cPLA_2$.

In this study, the properties and phosphorylation state of $cPLA_2$ were investigated in skeletal muscle and liver of euthermic versus hibernating ground squirrels. There were no significant changes in the maximal activity of $cPLA_2$ measured in skeletal muscle under euthermic versus hibernating conditions, but $cPLA_2$ activity decreased significantly in liver during torpor. The K_m for arachidonoyl thio-PC was also significantly reduced for the enzyme from both tissues in torpid animals. These data indicate that differential regulation of $cPLA_2$ occurs during mammalian hibernation. In particular, the change in K_m argues for a stable modification of the enzyme that affects enzyme conformation and thereby influences enzyme kinetic properties. One of the mechanisms that could bring about a hibernation-responsive stable change in $cPLA_2$ activity and K_m is a change in the phosphorylation state of the enzyme.

Alpha-$cPLA_2$ can be phosphorylated by several serine/threonine kinases and tyrosine kinases [8]. In other species, phosphorylation of S^{505} is known to cause an increase in $cPLA_2$ activity [25]. Phosphorylation of this residue is catalyzed by MAPKs, but the specific MAPK isozyme responsible is not known. The antibody used in the present study recognized the peptide sequence containing the phospho-Ser^{505} residue in α-$cPLA_2$. In ground squirrel liver we found high levels phospho-Ser^{505} $cPLA_2$ in the euthermic state and a much lower content during hibernation. Although we lack Western blot data on liver total $cPLA_2$ content in euthermia versus hibenration, the present data show a positive

correlation for liver between phospho-Ser^{505} $cPLA_2$ content and $cPLA_2$ activity. This is consistent with an active $cPLA_2$ during euthermia and a suppression of enzyme activity during hibernation, mediated by reduced phosphorylation of the Ser^{505} site (and possibly changes in the phosphorylation states of other serine residues on α-$cPLA_2$ as well). In order to confirm that a change in the phosphorylation state could be responsible for the change in $cPLA_2$ activity during hibernation, we examined the effects of dephosphorylating $cPLA_2$ in vitro by stimulating the action of endogenous phosphatases. The data in Fig. 4 show that incubation of extracts from euthermic liver under conditions that stimulated protein dephosphorylation resulted in a large decrease in the amount of phospho-Ser^{505} $cPLA_2$ detectable. By contrast, dephosphorylation treatment appeared to reduce the amount of phospho-Ser^{505} $cPLA_2$ in liver extracts although the effect was not significant. This is consistent with the idea that the changes in the activity and K_m of $cPLA_2$ as well as the amount of phospho-Ser^{505} $cPLA_2$ detected by Western blotting between euthermic and hibernating states in ground squirrel liver were due to reduced Ser^{505} phosphorylation during hibernation.

The mechanisms involved in the activation and regulation of $cPLA_2$ are still not fully understood. Initially, it was thought that the activation of $cPLA_2$ was determined by its phosphorylation state and the amount of intracellular calcium [25]. Phosphorylation activates the enzyme but a rise in intracellular Ca^{2+}, acting through the calcium-lipid binding domain, is needed to cause translocation of $cPLA_2$ from the cytosol to the membrane where it gains access to its substrate. However, recent studies have indicated that $cPLA_2$ interaction with other membrane lipids such as cholesterol, phosphatidylinositol bisphosphate, and ceramides, as well as interaction with proteins like annexins, p11 and vimentin, are needed for the complete activity of $cPLA_2$ [26, 27]. Hence, the full story of the mechanisms of regulation of this enzyme during hibernation remains to be be determined as do the full range of metabolic consequences of $cPLA_2$ suppression.

Acknowledgements. We are very grateful Dr. J.M. Hallenbeck (National Institute of Neurological Disorders and Stroke, NIH, Bethesda, MD) for providing samples of ground squirrel tissues and to J.M. Storey for critical review of the manuscript. Research support to KBS is from a discovery grant from the Natural Sciences and Engineering Research Council of Canada and the Canada Research Chairs program.

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