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# Protein kinase C in the wood frog, *Rana sylvatica*: reassessing the tissue-specific regulation of PKC isozyms during freezing

The wood frog, *Rana sylvatica*, survives whole-body freezing and thawing each winter. The extensive adaptations required at the biochemical level are facilitated by alterations to signaling pathways, including the insulin/Akt and AMPK pathways. Past studies investigating changing tissue-specific patterns of the second messenger IP<sub>3</sub> in adapted frogs have suggested important roles for protein kinase C (PKC) in response to stress. In addition to their dependence on second messengers, phosphorylation of three PKC sites by upstream kinases (most notably PDK1) is needed for full PKC activation, according to current generally-accepted models. The present study uses phospho-specific immunoblotting to investigate phosphorylation states of PKC- as they relate to distinct tissues, PKC isozyms, and phosphorylation sites- in control and frozen frogs. In contrast to past studies where second messengers of PKC increased during the freezing process, phosphorylation of PKC tended to generally decline in most tissues of frozen frogs. All PKC isozyms and specific phosphorylation sites detected by immunoblotting decreased in phosphorylation levels in hind leg skeletal muscle and hearts of frozen frogs. Most PKC isozyms and specific phosphorylation sites detected in livers and kidneys also declined; the only exceptions were the levels of isozyms/phosphorylation sites detected by the phospho-PKC $\alpha/\beta$ II (Thr638/641) antibody, which remained unchanged from control to frozen frogs. Changes in brains of frozen frogs were unique; no decreases were observed in the phosphorylation levels of any of the PKC isozyms and/or specific phosphorylation sites detected by immunoblotting. Rather, increases were observed for the levels of isozyms/phosphorylation sites detected by the phospho-PKC $\alpha/\beta$ II (Thr638/641), phospho-PKC $\delta$  (Thr505), and phospho-PKC $\theta$  (Thr538) antibodies; all other isozyms/phosphorylation sites detected in brain remained unchanged from control to frozen frogs. The results of this study indicate a potential important role for PKC in cerebral protection during wood frog freezing. Our findings also call for a

reassessment of the previously-inferred importance of PKC in other tissues, particularly in liver; a more thorough investigation is required to determine whether PKC activity in this physiological situation is indeed dependent on phosphorylation, or whether it deviates from the generally-accepted model and can be “overridden” by exceedingly high levels of second messengers, as has been demonstrated with certain PKC isozymes (e.g. PKC $\delta$ ).

**Protein kinase C in the wood frog, *Rana sylvatica*: reassessing the tissue-specific regulation  
of PKC isozymes during freezing**

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## 1 Introduction

2  
3 For animals living in boreal climates, cold temperatures- in particular, sustained periods  
4 of subzero temperatures for months at a time- present a challenge to survival. For many of these  
5 animals, the solution is migration- retreating to warmer zones until temperatures in their boreal  
6 homes rise once again. For other animals, however, migration of this scope is not possible, and  
7 unique arrays of adaptive mechanisms are utilized to endure the prolonged cold. One such  
8 animal is the wood frog, *Rana sylvatica* (reviewed in Storey and Storey, 1996). Each winter, this  
9 anuran endures whole-body freezing; approximately 65-70% of extracellular and extra-organ  
10 water freezes in the form of nucleated ice, via the actions of ice-nucleating proteins or ice-  
11 structuring proteins. During this time, cerebral and cardiovascular activities are undetectable by  
12 conventional means. Intracellular freezing and any resulting irreparable damage to cellular  
13 contents is prevented by natural cryoprotection; liver glycogen stores undergo extensive  
14 hydrolysis (causing a decrease in liver mass by approximately 45%), and glucose is exported and  
15 systemically distributed, accumulating in some tissues at levels up to 40-60 times higher than  
16 euglycemic levels (Storey and Storey 1985; Costanzo et al., 1993). Such a broad reorganization  
17 requires numerous modulations at several levels of the signaling and metabolic hierarchy of  
18 glucose metabolism, including: 1) phosphorylation and sustained activation of liver glycogen  
19 phosphorylase (Crerar et al., 1988; Mommsen and Storey, 1992); 2) adaptations to plasma  
20 membranes in order to facilitate glucose transport and distribution (King et al., 1993); 3) tissue-  
21 specific adjustment of anabolic and catabolic signaling pathways (e.g. the insulin/Akt pathway,  
22 and the adenosine monophosphate-activated protein kinase or AMPK pathway) to optimize  
23 glucose production, distribution, uptake, and utilization as a cryoprotectant (Rider et al., 2006;

Dieni et al., 2012; Zhang and Storey, 2013; do Amaral et al., 2013), and; 4) suppression of metabolic pathways that would otherwise divert glucose away from cryoprotection (e.g. pentose phosphate pathway, glycolysis; Dieni and Storey, 2010; 2011), among others. Following the return of warmer temperatures and the arrival of spring, frogs thaw and resume their natural life cycle with no apparent debilitating results of the freeze-thaw process.

Given the scope of these necessary adaptations, it is likely- and has in fact already been demonstrated- that altered signaling comprises a major facet of the mechanisms behind the biochemical outcomes facilitating survival. In addition to those signaling enzymes already referenced (i.e. Akt, AMPK, glycogen synthase kinase-3 or GSK3, protein kinase A or PKA), additional kinases and phosphatases have been shown to play a role in wood frog freeze-tolerance. For instance, mitogen activated protein kinases (MAPKs) are activated in various tissues and are suggested as having a role in regulating metabolic or gene expression responses that would facilitate survival in the freezing and/or thawing processes (Greenway and Storey, 2000). Past studies have also suggested a potential role for protein kinase C (PKC) in freezing, anoxia, and dehydration, based on patterns of inositol 1,4,5-trisphosphate (IP<sub>3</sub>), a second messenger associated with cytosolic calcium increases and a co-product of diacylglycerol (DAG; Holden and Storey 1996; 1997). Increases in cytosolic calcium and DAG both lead to PKC activation.

PKC in fact consists of a family of 15 serine/threonine-protein kinase isozymes in humans, divided into subfamilies with specific second messenger requirements and upstream regulators (Mellor and Parker, 1998). Our lab has previously demonstrated *in vivo* roles for PKC in various forms of animal stress physiology, including: 1) reptilian anaerobiosis (Mehrani and Storey, 1996); 2) mammalian hibernation (Mehrani and Storey, 1997), and; 3) fish exercise and

bioenergetics (Brooks and Storey, 1998). Meanwhile, *in vitro* stimulation of endogenous PKC has been shown to significantly affect the kinetic properties of glucose-6-phosphate dehydrogenase (G6PDH; Dieni and Storey, 2010), and hexokinase (Dieni and Storey, 2011) from wood frog tissue extracts. Given the potential importance of PKC in wood frog freeze-tolerance, the present study further explores the regulation of this family of kinases *in vivo*, using phospho-specific immunoblotting to establish tissue-specific phosphorylation states of the PKC isozymes in control and frozen frogs.

## Materials and methods

### *Animals*

Conditions for animal care, experimentation, and euthanasia were approved by the Carleton University Animal Care Committee (B09-22) in accordance with guidelines set down by the Canadian Council on Animal Care. Male wood frogs were captured from spring breeding ponds in the Ottawa, Ontario area. Animals were washed in a tetracycline bath, and placed in plastic containers with damp sphagnum moss at 5°C for two weeks prior to experimentation. Control frogs were sampled from this condition. For freezing exposure, frogs were placed in closed plastic containers with damp paper toweling on the bottom, and put in an incubator set at -3°C. A 45 min cooling period was allowed during which body temperature of the frogs cooled to below -0.5°C and nucleation was triggered due to skin contact with ice crystals formed on the paper toweling (Storey and Storey, 1985). Subsequently, timing of a 24 h freeze exposure began. All frogs were sacrificed by pithing, followed by rapid dissection, and flash-freezing of tissue

70 samples in liquid nitrogen. Tissues were then stored at -80°C until use.

71  
72 *Tissue extract preparation for SDS-PAGE and immunoblotting*

73  
74 Soluble protein extracts were prepared from tissues that had been previously stored  
75 at -80°C. Briefly, samples of frozen tissues were weighed and then quickly homogenized using a  
76 Polytron PT1000 homogenizer (Brinkmann Instruments, Rexdale, ON, Canada) at 50% of full  
77 power in a 1:5 w:v ratio with ice-cold buffer A (20 mM Hepes, 200 mM NaCl, 0.1 mM EDTA,  
78 10 mM NaF, 1 mM Na<sub>3</sub>VO<sub>4</sub>, and 10 mM β-glycerophosphate). Protease and phosphatase  
79 inhibitors were added just prior to homogenization: 1:1000 v:v protease inhibitor cocktail  
80 (P8340, Sigma, St. Louis, MO, USA), 1:1000 v:v phosphatase inhibitor cocktail 1 (P2850,  
81 Sigma, St. Louis, MO, USA), and a few crystals of phenylmethylsulfonyl fluoride (PMSF).  
82 Samples were centrifuged at 10,000 x g for 15 min at 4°C and then supernatants were removed  
83 and held on ice.

84 Soluble protein concentration was quantified by the Bradford assay (Bradford, 1976)  
85 using the Bio-Rad Protein Assay Dye Reagent Concentrate (500-0006, Bio-Rad, Hercules, CA,  
86 USA), according to the manufacturer's instructions, and a Dynatech MR5000 microplate reader  
87 (DYNEX Technologies Inc., Chantilly, VA) set at 595 nm. Samples were then adjusted to equal  
88 soluble protein concentrations by the addition of small volumes of buffer A; this compensates for  
89 differences in the wet:dry ratio of tissues from control versus frozen frogs. Aliquots were mixed  
90 1:1 v:v with SDS-PAGE sample buffer containing: 100 mM Tris-HCl (pH 6.8), 4% w:v sodium  
91 dodecyl sulfate (SDS), 20% v:v glycerol, 5% v:v 2-mercaptoethanol and 0.2% w:v bromophenol  
92 blue. Following boiling for 5 min, samples were cold-snapped on ice, and stored at -20°C until



use.

#### *SDS-PAGE and polyvinylidene difluoride membrane transfer*

Aliquots of thawed samples containing 20 µg of protein were loaded into wells of SDS-polyacrylamide gels (8% resolving gel, 5% stacking gel, made from a 30% acrylamide and bis-acrylamide solution, 37.5:1; 161-0158, Bio-Rad, Hercules, CA, USA), along with Kaleidoscope prestained markers (161-0324, Bio-Rad, Hercules, CA, USA) to estimate molecular weight of PKC isozymes. Samples were electrophoresed at 180 V in a Mini-PROTEAN III apparatus (Bio-Rad, Hercules, CA, USA) using 1x running buffer (5x running buffer contained 15.1 g Tris-base, 94 g glycine, and 5 g SDS per litre, pH 8.3). Proteins were then wet-transferred to polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, USA) using a current of 300 mA for 1.5 h at 4°C in a Bio-Rad Mini Trans-Blot Cell apparatus (Bio-Rad, Hercules, CA, USA). Transfer buffer contained 25 mM Tris-base pH 8.8, 192 mM glycine, and 20% v:v methanol, chilled to 4°C.

#### *Immunoblotting of PVDF membranes and analysis*

Primary antibodies (Cell Signalling Technology, Danvers, MA, USA) were the following: phospho-PKC (pan) (βII Ser660) antibody (9371), which detects all of PKCα, βI, βII, δ, ε and η isoforms only when phosphorylated at a carboxy-terminal residue homologous to Ser660 of PKCβII; phospho-PKCδ/θ (Ser643/676) antibody (9376), which detects both PKCδ when phosphorylated at Ser643 and PKCθ when phosphorylated at Ser676; phospho-PKCα/βII

(Thr638/641) antibody (9375); phospho-PKC $\delta$  (Thr505) antibody (9374; this antibody has since become unavailable after this work was carried out); phospho-PKC $\theta$  (Thr538) antibody (9377); phospho-PKC $\zeta/\lambda$  (Thr410/403) antibody (9378); PKD/PKC $\mu$  antibody (2052); phospho-PKD/PKC $\mu$  (Ser916) antibody (2051); phospho-PKD/PKC $\mu$  (Ser744/748) antibody (2054). All primary IgG antibodies were raised in rabbit. These were purchased together as the Phospho-PKC Antibody Sampler Kit (9921; this kit has since become unavailable after this work was carried out). Stock primary antibodies were diluted between 1:5000 to 1:10,000 in Tris-buffered saline supplemented with Tween-20 (TBST; 20 mM Tris pH 7.5, 150 mM NaCl, 0.05% v:v Tween-20). Secondary antibody used was the anti-rabbit IgG, HRP-linked antibody (7074; also supplied within the Phospho-PKC Antibody Sampler Kit). Stock secondary antibodies were diluted 1:2000 in TBST.

After transfer was complete, PVDF membranes were quickly equilibrated in TBST and then blocked with 5% w:v nonfat milk dissolved in TBST for 15 min at room temperature. The blot was rinsed with TBST and then incubated with primary antibody in TBST on a shaking platform overnight at 4°C. Blots were washed twice with TBST and incubated with secondary antibody for 1.5 h at room temperature. Immunoreactive bands were visualized using enhanced chemiluminescence (ECL; RPN2108, GE Healthcare Life Sciences, Baie d'Urfé, QC, Canada) following the manufacturer's protocol. The luminol and oxidizing reagents were mixed 1:1 v:v on the membrane for 1 min and the ECL signal was detected using a ChemiGenius (SynGene, Frederick, MD, USA).

Total protein was then visualized on the PVDF membrane by staining for 30 min with Coomassie blue staining solution (0.25% w:v Coomassie Brilliant Blue R, 50% v:v methanol, 7.5% v:v acetic acid) followed by destaining with destain solution (25% v:v methanol, 10% v:v

acetic acid). Three Coomassie-stained bands that did not differ in intensity between active and frozen conditions were used to normalize the corresponding intensity of the immunoreactive band in each lane to correct for any unequal protein loading, as described previously (Dieni et al., 2012).

Intensities of ECL-visualized and Coomassie-stained bands were quantified using the associated Gene Tools program (v. 3.00.02). Data were analyzed by one-way ANOVA followed by Tukey's test; a statistically-significant difference was accepted with values of  $p < 0.05$  or smaller.

## Results and Discussion

### *Overall scope of phospho-PKC levels and changes in freezing*

The generally-accepted model for activation of PKC isozymes has been reviewed (Mellor and Parker, 1998; Parker and Murray-Rust, 2004; Gomperts et al., 2009) and follows here. PKCs are biosynthesized as catalytically inactive, and must first bind to the intracellular face of the plasma membrane in order to be unfolded, and rendered competent. A number of upstream signals can activate phospholipases, hydrolysing inositol phospholipids to various combinations of diacylglycerols and IP<sub>3</sub>; IP<sub>3</sub> will in turn trigger calcium efflux from the endoplasmic reticulum, which then propagates further calcium influx from the extracellular environment. Conventional PKC isozymes (cPKC;  $\alpha$ ,  $\beta$ ,  $\gamma$ ) bind to the membrane via two specific bridging interactions: C1 domains that bind to DAG and C2 domains that bind to calcium-phospholipid complexes. Once bound, cPKCs unfold such that their hydrophobic motifs interact with and

162 activate 3-phosphoinositide dependent protein kinase-1 (PDK1), and the PKC pseudosubstrate  
163 motif is withdrawn from its catalytic core. PDK1, currently the single conclusive upstream  
164 kinase responsible for phosphorylation of the PKC activation loop (Le Good et al., 1998; Ron  
165 and Kazanietz, 1999), phosphorylates this loop and triggers two successive  
166 autophosphorylations- one on the PKC turn motif, and one on the hydrophobic motif. Only once  
167 at this stage- phosphorylated at three sites and bound to both DAG and calcium- are cPKCs are  
168 recognized as fully-active. Depletion of DAG and calcium will induce cPKC refolding and  
169 inactivation; however, as long as the aforementioned sites remain phosphorylated, cPKCs can be  
170 instantly reactivated upon reintroduction of DAG and calcium. Thus, for cPKCs, all three criteria  
171 of DAG, calcium, and upstream phosphorylation are necessary for full activity; this is  
172 complicated for novel PKC isozymes (nPKC;  $\delta$ ,  $\epsilon$ ,  $\eta$ ,  $\theta$ ) and atypical PKC isozymes (aPKC;  $\zeta$ ,  $\lambda$ ,  
173  $\mu$ ). nPKCs are calcium-independent, but still rely on DAG for activity; aPKCs rely on neither  
174 calcium nor DAG, or any other phospholipids, though they possess other unique domains such as  
175 the phox-bem1 domain, which suggests that protein-protein interactions with cytosolic partners  
176 may be necessary for activity (Gomperts et al., 2009). In both these PKC subfamilies however,  
177 phosphorylation is nonetheless a prerequisite for catalytic competence.

178       Extracts of hind leg skeletal muscle, liver, heart, kidney, and brain from control and  
179 frozen frogs were probed with all 9 primary antibodies of the Phospho-PKC Antibody Sampler  
180 kit; however, not all antibodies revealed the presence of immunoreactive bands in each tissue  
181 extract (e.g. only 2 out of the 9 primary antibodies revealed bands in muscle homogenates). In  
182 each case where antibodies detected bands, only a single and distinct band appeared;  
183 immunoreactive bands were confirmed to be PKC isozymes by comparing their approximate  
184 molecular weights to those listed on the manufacturer's datasheet provided

(<http://www.cellsignal.com/pdf/9921.pdf>). A summary of changes between control and frozen frogs is presented in Table 1. In general, levels of phosphorylated PKC isozymes (and non-phosphorylated PKD/PKC $\mu$ ) tended to globally decrease during wood frog freezing in hind leg skeletal muscle, liver, kidney, and heart; the only tissue in which increases in phospho-PKC were observed was the brain.

PDK1 itself, and its targets, have been shown to change in phosphorylation state during wood frog freezing. For instance, levels of phospho-Thr308-Akt (a phosphorylation site of PDK1) decrease in muscle and heart during freezing, suggesting decreased action of PDK1 in these tissues. By contrast, levels of both phospho-Ser241-PDK1 and phospho-Thr308-Akt increase in liver, suggesting increased PDK1 action in livers of frozen frogs (Zhang and Storey, 2013). For optimal clarity, specific changes in PKC isozymes will be further described and discussed on a tissue-by-tissue basis, and compared to previously-established changes in PDK1, PKD1 targets, or previously-assessed targets downstream of PKC isozymes.

### *Muscle*

Only 2 primary antibodies were immunoreactive to frog muscle extracts: phospho-PKC $\alpha/\beta$ II (Thr638/641), and phospho-PKC $\theta$  (Thr538). Bands detected by both these antibodies decreased in intensity in frozen frogs (Fig. 1; Table 1). Thr638/641 is in the turn motif of PKC $\alpha/\beta$ II, and is autophosphorylated after initial phosphorylation of the activation loop by PDK1 (Ron and Kazanietz, 1999). Whether phospho-Thr638/641 is necessary for PKC $\alpha/\beta$ II activity is debatable; rather, it is more recognized for its importance in duration of PKC activation, and slowing the rate of PKC active loop dephosphorylation (Bornancin and Parker,

1996). Meanwhile, Thr538 is in the activation loop of PKC $\theta$ , and is directly phosphorylated by PDK1; as such, it is unequivocally needed for PKC $\theta$  activity (Liu et al., 2002). Taken together, these results suggest a combination of lower activity, a shorter duration of activation, and a higher rate of dephosphorylation of these PKC isozymes in muscle during freezing.

These decreases in the phosphorylation levels of PKC muscle isozymes correlate well with recently-presented decreases in phospho-Thr308-Akt (a target that, along with PKC, is also a direct substrate of PDK1; Zhang and Storey, 2013). By contrast, past studies would suggest a need for PKC to remain active in muscle; these have shown that in response to freezing, IP<sub>3</sub> levels rose moderately, by 55%, in skeletal muscle (Holden and Storey, 1996; 1997). Moreover, calcium binding and uptake into the sarcoplasmic reticulum were strongly decreased in skeletal muscle of frozen frogs, leading to increased cytosolic calcium levels (Hemmings and Storey, 2001). However, in yet other studies, muscle IP<sub>3</sub> levels remained constant in frogs subjected to short-term anoxia at 5°C, and then fell by 40% after 2 days of anoxic exposure. The contrasting past and present findings of increased IP<sub>3</sub> and calcium levels in frozen frogs, yet decreased PKC $\alpha/\beta$ II and PKC $\theta$  phosphorylation in this same physiological state, along with decreased IP<sub>3</sub> levels in frogs subjected to long-term anoxia, leaves us with a very uncertain role for muscle PKC in the adaptation to these stresses.

#### *Liver*

8 of the 9 primary antibodies were immunoreactive to frog liver extracts; only the phospho-PKC $\zeta/\lambda$  (Thr410/403) antibody failed to reveal any bands. Overall, band intensities again tended to decrease in frozen frogs (Fig. 2; Table 1). As in muscle, phospho-Thr538-PKC $\theta$

levels decreased, but to such an extent where they were non-quantifiable in frozen frogs. Similarly, phospho-Thr505-PKC $\delta$  levels also decreased to an extent where they were non-quantifiable. Interestingly, in contrast to Thr538, an activation loop phosphorylation of PKC $\theta$  which is unequivocally needed for activity, Thr505 is also an activation loop residue of PKC $\delta$ , but one which is at best only debatably necessary for activity, and is autophosphorylated in addition to being phosphorylated by PDK1 (Le Good et al., 1998; Liu et al., 2002; Steinberg, 2004; Liu et al., 2006). Furthermore, phospho-Thr676/643-PKC $\delta/\theta$  levels (a turn motif phosphorylation) decreased by over 50%. While the role of this turn motif phosphorylation is inconclusive in PKC $\delta$  or PKC $\theta$  (Li et al., 1997; Liu et al. 2002), decreases in turn motif phospho-Thr676/643 will potentially compound the decreases in activation loop phospho-Thr505/538, further depressing PKC $\delta$  and PKC $\theta$  activities.

Additional decreases are observed in non-phosphorylated levels of PKD/PKC $\mu$ , of phospho-Ser744/748-PKD/PKC $\mu$ , and of phospho-Ser916-PKD/PKC $\mu$ . Ser916 is an autophosphorylation site that correlates with catalytic activity in PKD/PKC $\mu$  (Matthews et al., 1999), whereas Ser744 and possibly Ser748 are activation loop phosphorylation sites, also critical to activity (Waldron et al., 2001; Waldron and Rozengurt, 2003). Interestingly, while PKD/PKC $\mu$  was originally classified as a member of the PKC family (and is still very much considered as such), Ser744/748 is in fact phosphorylated by other PKC isozymes upstream of PKD/PKC $\mu$ , most notably PKC $\delta$  (Waldron et al., 2001; Waldron and Rozengurt, 2003). Given that total and phospho-levels of PKD/PKC $\mu$  and upstream PKC $\delta$  all decreased, these suggest that PKD/PKC $\mu$  will also be inactive in frozen frogs.

Wood frog liver is quite possibly the best-characterized tissue in terms of proteins and genes that may have potential relationships with PKC; by virtue of their decreased

phosphorylation, the potential decline of PKC $\delta$ , PKC $\theta$ , and PKD/PKC $\mu$  activities contrast with previous findings. One early study demonstrated a progressive rise in IP<sub>3</sub> levels over the course of the freezing process, ultimately rising 11-fold higher than control values after 24 h of freezing (Holden and Storey, 1996). Based on this finding, important roles were suggested for PKC, including: i) overriding of normal cellular metabolic controls; ii) enabling “acceptance” of prolonged and extreme reductions in cell volume, along with accompanying hyperosmolality and elevated ionic strength, and; iii) exerting tight control over glycogen phosphorylase, in order to drive glycogenolysis to the degree necessary for extreme the hyperglycemia observed during freezing. A follow-up study, exploring second messenger changes in frogs subjected to dehydration and anoxia, noted increased IP<sub>3</sub> levels in both dehydrated and anoxic frogs (Holden and Storey, 1997). Because of the response to both dehydration and anoxia, the importance of PKC in freezing was reaffirmed; indeed, both dehydration and anoxia result from the freezing of extracellular water in wood frogs.

Upon the discovery of *fr47*, a novel gene associated with freezing survival, it was noted that its expression pattern paralleled that of IP<sub>3</sub> accumulation, suggesting that PKC may activate freeze-response genes (McNally et al., 2003). Later, NF $\kappa$ B, a transcription factor crucial in cellular stress response and survival, was found to have increased DNA-binding affinity in frozen frogs; its sequestering binding partner, I $\kappa$ B, was also found to increase in phosphorylation during freezing (Storey, 2008) I $\kappa$ B is a substrate of the I $\kappa$ B kinase (IKK), which is in turn a substrate of PKC isozymes (Lallena et al., 1998; Diaz-Meco and Mostat, 2012). Recently, it was shown that Nrf2, a transcription factor activated during oxidative stress, has increased DNA-binding affinity in frozen frogs; moreover, transcription of *gsta*, a gene under Nrf2 control, was elevated in frozen frogs (Zhang, 2013). It was reiterated in this study that PKC phosphorylates



Keap1, a sequestering binding partner of Nrf2, inducing dissociation and activation of Nrf2 and the transcription of antioxidant response genes. Lastly, and possibly most conflicting with the presently-observed decrease in liver phospho-PKC levels, are the rise of both PDK1 and Akt phosphorylation levels in frozen frogs (Zhang and Storey, 2013). Given these collective findings, the presently-suggested decline in liver PKC activities is at odds with the previously-inferred importance of PKC in freezing survival mechanisms.

A possible reconciliation is that although decreases are observed in the phosphorylation states of PKC $\delta$ , PKC $\mu$ , and PKC $\theta$ , levels detected by the phospho-Thr638/641-PKC $\alpha$ / $\beta$ II antibody remain apparently unchanged in frozen frogs. Thr638, a turn motif phosphorylation site, is not critical to PKC $\alpha$  catalytic function but rather controls the duration of its activation by regulating the rate of dephosphorylation and inactivation (Bornancin and Parker, 1996; Li et al., 1997; Ron and Kazanietz, 1999). By contrast, Thr641 is also a turn motif phosphorylation site but is fundamental to the activity of PKC $\beta$ I and PKC $\beta$ II (Zhang et al., 1993; Ron and Kazanietz, 1999; Leonard et al., 2011). Taken together, these suggest that some cPKCs will continue to be active and/or remain active for longer in frozen frogs. It should be noted however, that while levels of phospho-Thr638/641-PKC $\alpha$ / $\beta$ II remained unchanged, levels detected by the phospho-PKC (pan) ( $\beta$ II Ser660) antibody decreased in frozen frogs. This antibody is specific for PKC $\alpha$ ,  $\beta$ I,  $\beta$ II,  $\delta$ ,  $\epsilon$  and  $\eta$  when autophosphorylated at a carboxy-terminal residue homologous to Ser660 in the hydrophobic motif of PKC $\beta$ II, following initial phosphorylation of Thr500 by PDK1; phospho-Ser660 plays important roles in correct folding, as well as the binding of protein substrate, ATP, and calcium (Zhang et al., 1993; Ron and Kazanietz, 1999; Leonard et al., 2011). Another possible reconciliation is that while much of the literature supports an activation model where phosphorylation is necessary for PKC activity, there are exceptions; as indicated earlier

for instance, the active loop phosphorylation at Thr505-PKC $\delta$  is not required for activity (Steinberg, 2004; Liu et al., 2007). As an nPKC, PKC $\delta$ - and others that behave similarly- may therefore be active in presence of elevated DAG regardless of phosphorylation state.

#### *Kidney*

6 antibodies revealed bands in frog kidney extracts. As in liver, levels of phospho-Thr638/641-PKC $\alpha$ / $\beta$ II remain unchanged between control and frozen frogs (Fig. 3; Table 1). However, significant decreases were observed in the levels bands detected by phospho-Thr505-PKC $\delta$ , phospho-Thr676/643-PKC $\delta$ / $\theta$ , non-phosphorylated PKD/PKC $\mu$ , and phospho-PKC (pan) ( $\beta$ II Ser660) antibodies. Moreover, decreases were also observed in the intensities of the bands detected by the phospho-PKC $\zeta$ / $\lambda$  (Thr410/403) antibody. Thr410 is an activation loop residue in PKC $\zeta$ , as is Thr403 in PKC $\lambda$  (also known as PKC $\iota$  in mammals), and these are directly phosphorylated by PDK1 and are critical to activity (Le Good et al., 1998; Le Good and Brindley, 2004). Together, these results would suggest a decreased overall activity for PKC isozymes in kidney of frozen frogs. As with muscle and liver, however, kidney IP<sub>3</sub> levels were shown to increase in previous studies (Holden and Storey, 1997). In frogs dehydrated by 40%, IP<sub>3</sub> levels rose by 60%; levels remained unchanged in frogs exposed to anoxia (Holden and Storey, 1997) and in frozen frogs (Holden and Storey, 1996). Again, this presents us with potentially contrasting results between second messengers, specific stresses, and PKC phosphorylation state; second messengers responsible for PKC activation increase in kidney (but only in response to dehydration, and not to anoxia or freezing), while PKC phosphorylation itself does not increase during freezing.

## Heart

6 antibodies revealed bands in frog kidney extracts, and all of these decreased in frozen frogs (Fig. 4; Table 1). Levels of bands detected by phospho-Thr638/641-PKC $\alpha$ / $\beta$ II, phospho-Thr676/643-PKC $\delta$ / $\theta$ , non-phosphorylated PKD/PKC $\mu$ , phospho-PKC (pan) ( $\beta$ II Ser660), phospho-Thr538-PKC $\theta$ , and phospho-Thr410/403-PKC $\zeta$ / $\lambda$  antibodies all decreased significantly; indeed, phospho-Thr676/643-PKC $\delta$ / $\theta$ , non-phosphorylated PKD/PKC $\mu$ , and phospho-Thr410/403-PKC $\zeta$ / $\lambda$  levels all decreased to an extent where they were no longer detectable in frozen frogs.

With decreases observed in the phosphorylation levels of all PKC isozymes detected in heart, this would suggest overall decreased PKC activity in this tissue. The decreases in phosphorylation levels of these PKC isozymes also correlate with decreases in phospho-Thr308-Akt in frog heart during freezing (Zhang and Storey, 2013). Previous studies showed no significant changes in IP<sub>3</sub> levels in response to freezing (Holden and Storey, 1996), yet significant increases were observed after only 1 h of enduring anoxia (Holden and Storey, 1997). Thus, our present results in heart are in agreement with decreased phosphorylation of other PDK1 targets and unchanged second messenger levels in freezing, but not with increased second messenger levels in response to anoxia.

## Brain

8 antibodies revealed bands in frog brain extracts. Of the five tissues investigated in the present study, the results obtained in the brain were the most unique; changes (or lack thereof) in phospho-PKC levels in brain during frog freezing contrasted strongly with those observed in muscle, liver, heart, and kidney (Fig. 5; Table 1). No changes were observed in the levels of bands detected by phospho-Thr676/643-PKC $\delta$ / $\theta$ , phospho-Ser744/748-PKD/PKC $\mu$ , non-phosphorylated PKD/PKC $\mu$ , and phospho-PKC (pan) ( $\beta$ II Ser660) antibodies; each of these were detectable in both control and frozen frogs to approximately the same extent. Interestingly, phospho-Thr638/641-PKC $\alpha$ / $\beta$ II levels increased by 121.3% in brains of frozen frogs. Moreover, phospho-Thr505-PKC $\delta$  and phospho-Thr538-PKC $\theta$ , which were not detectable in control frogs, were detectable (albeit faintly) in frozen frogs. Overall, whereas phospho-PKC levels generally tended to decline in other organs of frozen frogs, phospho-PKC levels largely remained unchanged or even increased in brain.

The increase in brain PKC phosphorylation during freezing is of great interest. Previous studies have shown that brain IP<sub>3</sub> levels rose significantly after 4 h of freezing (Holden and Storey, 1996), and so this is one frog tissue in which the overall increases in PKC phosphorylation state correlate with this rise in IP<sub>3</sub>. Numerous other adaptive “activations” occur in frog brains in response to freezing, including: 1) moderately-increased expression of *fr10* (Cai and Storey, 1997) and *li16* (Sullivan and Storey, 2012), novel genes with putative roles in freezing protection; 2) increased levels of c-Fos (Greenway and Storey, 2000), and; 3) up-regulation of genes for ribosomal proteins, including the acidic ribosomal phosphoprotein P0 (Wu et al., 2005) and the ribosomal large subunit protein 7 (Wu et al., 2008). At present, however, we cannot conclusively identify any of the proteins listed here as being substrates of

PKC, nor can we confirm that any of the upregulated-genes are facilitated by transcription factors downstream of PKC.

Conceivably, the phosphorylation and activation of PKC (along with other proteins) in brain- contrasted against the decreased or unchanged phosphorylation of PKC in other tissues- suggests a unique and vital role for brain PKC during freezing. Indeed, the importance of PKC in cerebral protection has been demonstrated well-beyond the niche of wood frog freezing (Sun et al., 2013; Thompson et al., 2013). Future efforts will be required to establish the catalytic competence of PKC isozymes in frozen frog brains, to identify the targets being phosphorylated by PKC, and to determine their most likely role in frog cerebral/neuroprotection during freezing based on our current understanding of other models.

## Conclusion

The present study investigated the phosphorylation state of conventional, novel, and atypical PKC isozymes in five tissues of freeze-tolerant frogs, as well as non-phosphorylated PKC $\mu$ /PKD. Broadly, phospho-PKC levels and non-phosphorylated PKC $\mu$ /PKD decreased in muscle, livers, kidneys, and hearts of frozen frogs; the only exception was protein detected by the phospho-Thr638/641-PKC $\alpha$ / $\beta$ II (turn motif), which showed no change in livers or kidneys between the control and frozen states. This protein alone would partially support the findings of past studies, where IP<sub>3</sub> was shown to dramatically increase in livers of frozen frogs and thus an important role was suggested for PKC in the freezing process; however, even the steady phosphorylation state of Thr638/641 is seemingly contradicted by the decreased phosphorylation of Ser660 (hydrophobic motif) in both livers and kidneys of frozen frogs. A particularly

interesting finding in this study is PKC would seem to play an important role in the brains of frozen frogs, as the phospho-levels of all isozymes detected remain either remain unchanged or even increase in the frozen state.

The results of this study succeed in answering some questions of past studies pertaining to the state of PKC in freeze-tolerance, but raise many others and indeed pose some contradictions. Whereas our lab has previously asserted that based on second messenger patterns, PKC would play an important role (particularly in liver and muscle), our present findings would seem to instead suggest a diminished role for PKC in most tissues, based on our current understanding of PKC isozymes and their generally-accepted activation model. This in itself, however, can be debated by pointing to studies which demonstrate that activation loop phosphorylation is, in fact, not needed for PKC activity (e.g. PKC $\delta$ ). To clarify the role of PKC in wood frog freezing, it is now evident that catalytic assays are necessary in order to unequivocally establish the actual activities of each of these isozymes in the control and frozen states. Moreover, there is an additional difficulty in contextualizing the role of PKC due to the fact that very few downstream targets of PKC have been assessed in wood frogs. Those that were discussed in this report are either only speculative (e.g. transcription factors that might control expression of *li16*, *fr10* and *fr47*), or are several degrees removed from being direct PKC substrates and/or are under the control of multiple kinases (e.g. NF $\kappa$ B/I $\kappa$ B via IKK, Nrf2 for which Keap1 can be modified via several pathways, etc.) There is a clear need to assess the phosphorylation state of direct PKC substrates (e.g. the MARCKS family) in order to better determine the activity and role of PKC in freeze-tolerance.

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## References

- Bornancin F, Parker PJ. 1996. Phosphorylation of threonine 638 critically controls the dephosphorylation and inactivation of protein kinase Ca. *Curr. Biol.* 6:1114-1123,
- Bradford MM. 1976. Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248-254.
- Brooks SP, Storey KB. 1998. Protein kinase C from rainbow trout brain: identification and characterization of three isozymes. *Biochem. Mol. Biol. Int.* 44:259-267.
- Cai Q, Storey KB. 1997. Upregulation of a novel gene by freezing exposure in the freeze-tolerant wood frog (*Rana sylvatica*). *Gene* 198:305-312.
- Costanzo JP, Lee RE, Lortz PH. 1993. Glucose concentration regulates freeze tolerance in the wood frog *Rana sylvatica*. *J. Exp. Biol.* 181:245-255.
- Crerar MM, David ES, Storey KB. 1988. Electrophoretic analysis of liver glycogen phosphorylase activation in the freeze-tolerant wood frog. *Biochim. Biophys. Acta. Mol. Cell Res.* 971:72-84.
- Diaz-Meco MT, Mostat J. 2012. The atypical PKCs in inflammation: NF- $\kappa$ B and beyond. *Immunol. Rev.* 246:154-167.
- Dieni CA, Bouffard MC, Storey KB. 2012. Glycogen synthase kinase-3: cryoprotection and glycogen metabolism in the freeze-tolerant wood frog. *J. Exp. Biol.* 215:543-551.

- 436 Dieni CA, Storey KB. 2010. Regulation of glucose-6-phosphate dehydrogenase by reversible  
437 phosphorylation in liver of a freeze tolerant frog. *J. Comp. Physiol. B* 180:1133-1142.
- 438 Dieni CA, Storey KB. 2011. Regulation of hexokinase by reversible phosphorylation in skeletal  
439 muscle of a freeze-tolerant frog. *Comp. Biochem. Physiol. B* 159:236-243.
- 440 do Amaral MC, Lee RE Jr, Costanzo JP. 2013. Enzymatic regulation of glycogenolysis in a  
441 subarctic population of the wood frog: implications for extreme freeze tolerance. *PLoS*  
442 *ONE* 8:e79169.
- 443 Gomperts BD, Kramer IM, Tatham PER. 2009. Phosphorylation and dephosphorylation: protein  
444 kinases A and C. In: *Signal Transduction*. London: Academic Press, 243-272.
- 445 Greenway SC, Storey KB. 2000. Activation of mitogen-activated protein kinases during natural  
446 freezing and thawing in the wood frog. *Mol. Cell. Biochem.* 209:29-37.
- 447 Hemmings SJ, Storey KB. 2001. Characterization of sarcolemma and sarcoplasmic reticulum  
448 isolated from skeletal muscle of the freeze tolerant wood frog, *Rana sylvatica*: the  $\beta_2$ -  
449 adrenergic receptor and calcium transport systems in control, frozen and thawed states.  
450 *Cell Biochem. Funct.* 19:143-152.
- 451 Holden CP, Storey KB. 1996. Signal transduction, second messenger, and protein kinase  
452 responses during freezing exposures in wood frogs. *Am. J. Physiol.* 271:R1205-R1211.
- 453 Holden CP, Storey KB. 1997. Second messenger and cAMP-dependent protein kinase responses  
454 to dehydration and anoxia stresses in frogs. *J. Comp. Physiol. B* 167:305-312.
- 455 King PA, Rosholt MN, Storey KB. 1993. Adaptations of plasma membrane glucose transport  
456 facilitate cryoprotectant distribution in freeze-tolerant frogs. *Am. J. Physiol.* 265:R1036-  
457 R1042.
- 458 Lallena MJ, Diaz-Meco MT, Bren G, Payá CV, Moscat J. 1999. Activation of I $\kappa$ B kinase by



459 protein kinase C isoforms. *Mol. Cell. Biol.* 19:2180-2188.

460 Le Good JA, Brindley DN. 2004. Molecular mechanisms regulating protein kinase C $\zeta$  turnover  
461 and cellular transformation. *Biochem. J.* 378:83-92.

462 Le Good JA, Ziegler WH, Parekh DB, Alessi DR, Cohen P, Parker PJ. 1998. Protein kinase C  
463 isotypes controlled by phosphoinositide 3-kinase through the protein kinase PDK1.  
464 *Science* 281:2042-2045.

465 Leonard TA, Różycki B, Saidi LF, Hummer G, Hurley JH. 2011. Crystal structure and allosteric  
466 activation of protein kinase C  $\beta$ II. *Cell* 144:55-66.

467 Li W, Zhang J, Bottaro DP, Li W, Pierce JH. 1997. Identification of serine 643 of protein kinase  
468 C- $\delta$  as an important autophosphorylation site for its enzymatic activity. *J. Biol. Chem.*  
469 272:24550-24555.

470 Liu Y, Belkina NV, Graham C, Shaw S. 2006. Independence of protein kinase C- $\delta$  activity from  
471 activation loop phosphorylation: structural basis and altered functions in cells. *J. Biol.*  
472 *Chem.* 281:12102-12111.

473 Liu Y, Graham C, Li A, Fisher RJ, Shaw S. 2002. Phosphorylation of the protein kinase C-theta  
474 activation loop and hydrophobic motif regulates its kinase activity, but only activation  
475 loop phosphorylation is critical to *in vivo* nuclear-factor- $\kappa$ B induction. *Biochem. J.*  
476 361:255-265.

477 Matthews SA, Rozengurt E, Cantrell D. 1999. Characterization of serine 916 as an *in vivo*  
478 autophosphorylation site for protein kinase D/protein kinase C $\mu$ . *J. Biol. Chem.*  
479 37:26543-26549.

480 McNally JD, Sturgeon CM, Storey KB. 2003. Freeze-induced expression of a novel gene, *fr47*,  
481 in the liver of the freeze-tolerant wood frog, *Rana sylvatica*. *Biochim. Biophys. Acta*

1635:183-191.

Mehrani H, Storey KB. 1996. Liver protein kinase C isozymes: properties and enzyme role in a vertebrate facultative anaerobe. *Int. J. Biochem. Cell Biol.* 28: 1257-1269.

Mehrani H, Storey KB. 1997. Protein kinase C from bat brain: the enzyme from a hibernating mammal. *Neurochem. Int.* 31:139-150.

Mellor H, Parker PJ. 1998. The extended protein kinase C superfamily. *Biochem J.* 332:281-292.

Mommsen TP, Storey KB. 1992. Hormonal effects on glycogen metabolism in isolated hepatocytes of a freeze-tolerant frog. *Gen. Comp. Endocrinol.* 87:44-53.

Parker PJ, Murray-Rust J. 2004. PKC at a glance. *J. Cell Science* 117:131-132.

Rider MH, Hussain N, Horman S, Dilworth SM, Storey KB. 2006. Stress-induced activation of the AMP-activated protein kinase in the freeze-tolerant wood frog *Rana sylvatica*. *Cryobiology* 53:297-309.

Ron D, Kazanietz MC. 1999. New insights into the regulation of protein kinase C and novel phorbol ester receptors. *FASEB J.* 13:1658-1676.

Steinberg SF. 2004. Distinctive activation mechanisms and functions of protein kinase C $\delta$ . *Biochem. J.* 384:449-459.

Storey JM, Storey KB. 1985. Triggering of cryoprotectant synthesis by the initiation of ice nucleation in the freeze tolerant frog, *Rana sylvatica*. *J. Comp. Physiol. B* 156:91-195.

Storey KB, Storey JM. 1996. Natural freezing survival in animals. *Annu. Rev. Ecol. Syst.* 27:365-386.

Storey KB. 2008. Beyond gene chips: transcription factor profiling in freeze tolerance. In: Lovegrove BG, McKechnie AE, eds. *Hypometabolism in Animals: Hibernation, Torpor and Cryobiology*. Pietermaritzburg: University of KwaZulu-Natal, 101-108.

- 505 Sullivan KJ, Storey KB. 2012. Environmental stress responsive expression of the gene *li16* in  
506 *Rana sylvatica*, the freeze-tolerant wood frog. *Cryobiology* 64:192-200.
- 507 Sun X, Budas GR, Xu L, Barreto GE, Mochly-Rosen D, Giffard RG. 2013. Selective activation  
508 of protein kinase C $\epsilon$  in mitochondria is neuroprotective *in vitro* and reduces focal  
509 ischemic brain injury in mice. *J. Neurosci. Res.* 91:799-807.
- 510 Thompson JW, Dave KR, Saul I, Narayanan SV, Perez-Pinzon MA. 2013. Epsilon PKC  
511 increases brain mitochondrial SIRT1 protein levels via heat shock protein 90 following  
512 ischemic preconditioning in rats. *PLoS ONE* 8:e75753.
- 513 Waldron RT, Rey O, Iglesias T, Tugal T, Cantrell D, Rozengurt E. 2001. Activation loop Ser744  
514 and Ser748 in protein kinase D are transphosphorylated *in vivo*. *J. Biol. Chem.* 35:32606-  
515 32615.
- 516 Waldron RT, Rozengurt E. 2003. Protein kinase C phosphorylates protein kinase D activation  
517 loop Ser744 and Ser748 and releases autoinhibition by the Pleckstrin Homology domain.  
518 *J. Biol. Chem.* 278:154-163.
- 519 Wu S, De Croos JN, Storey KB. 2008. Cold acclimation-induced up-regulation of the ribosomal  
520 protein L7 gene in the freeze tolerant wood frog, *Rana sylvatica*. *Gene* 424:48-55.
- 521 Wu S, Storey KB. 2005. Up-regulation of acidic ribosomal phosphoprotein P0 in response to  
522 freezing or anoxia in the freeze tolerant wood frog, *Rana sylvatica*. *Cryobiology* 50:71-  
523 82.
- 524 Zhang J, Storey KB. 2013. Akt signaling and freezing survival in the wood frog, *Rana sylvatica*.  
525 *Biochim. Biophys. Acta* 1830:4828-4837.
- 526 Zhang J, Wang L, Petrin J, Bishop R, Bond RW. 1993. Characterization of site-specific mutants  
527 altered at protein kinase C $\beta$ 1 isozyme autophosphorylation sites. *Proc. Acad Natl. Sci.*

528            *USA* 90:6130-6134.

529    Zhang J. 2013. Roles of Akt signaling and its downstream pathways in wood frog freeze

530            tolerance. Ph.D. Thesis, Carleton University.

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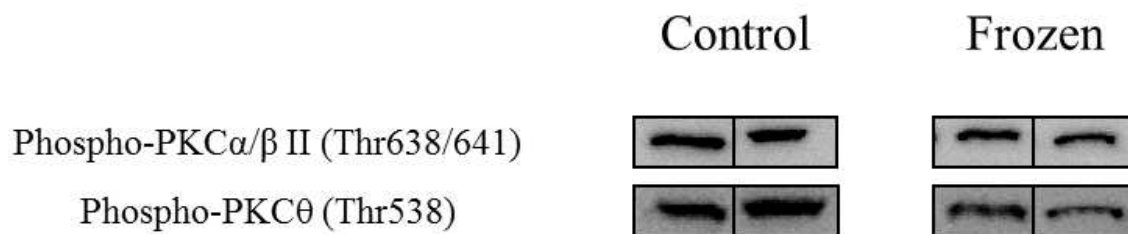
## Tables

**Table 1.** Summary of changes in phosphorylation levels of PKC isozymes (or non-phosphorylated PKD/PKC $\mu$ ) during wood frog freezing. Relative levels were determined from immunoblots of  $n = 5$  independently-prepared tissue homogenates from pooled tissues of control or frozen frogs. Quantifiable decreases (-) or increases (+) are presented numerically. An equal sign (=) indicates no significant change. In some instances, bands were detectable in control frogs but were undetectable or too faint to be accurately quantified in frozen frogs (--), or vice-versa (++) . ND indicates that bands for that isozyme or phosphorylation site were not detected in that tissue. Statistical significance as determined by one-way ANOVA followed by Tukey's test is as follows: *a*,  $p < 0.05$ ; *b*,  $p < 0.01$ ; *c*,  $p < 0.005$ ; *d*,  $p < 0.001$ .

	Muscle	Liver	Kidney	Heart	Brain
Phospho-PKC $\alpha$ / $\beta$ II (Thr638/641)	-41.8% <sup>c</sup>	=	=	-28.6% <sup>a</sup>	+121.3% <sup>b</sup>
Phospho-PKC $\delta$ (Thr505)	ND	--	-76.6% <sup>d</sup>	ND	++
Phospho-PKC $\delta$ / $\theta$ (Ser643/676)	ND	-54.8% <sup>a</sup>	-75.7% <sup>c</sup>	--	=
Phospho-PKD/PKC $\mu$ (Ser744/748)	ND	--	ND	ND	=
Phospho-PKD/PKC $\mu$ (Ser916)	ND	--	ND	ND	ND
PKD/PKC $\mu$	ND	-76.4% <sup>d</sup>	-66.7% <sup>d</sup>	--	=
Phospho-PKC (pan) ( $\beta$ II Ser660)	ND	-82.7% <sup>c</sup>	-74.6% <sup>a</sup>	-38.6% <sup>c</sup>	=
Phospho-PKC $\theta$ (Thr538)	-50.4% <sup>d</sup>	--	ND	-35.8% <sup>b</sup>	++
Phospho-PKC $\zeta$ / $\lambda$ (Thr410/403)	ND	ND	-66.8% <sup>d</sup>	--	ND

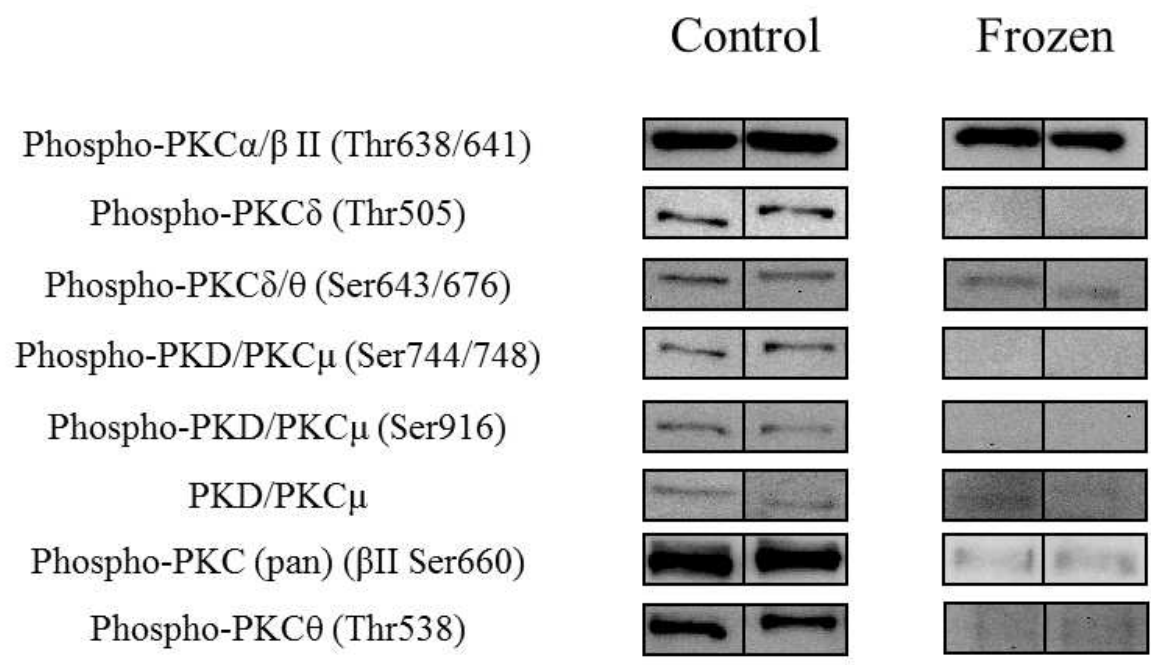
## Figures

**Fig. 1.**



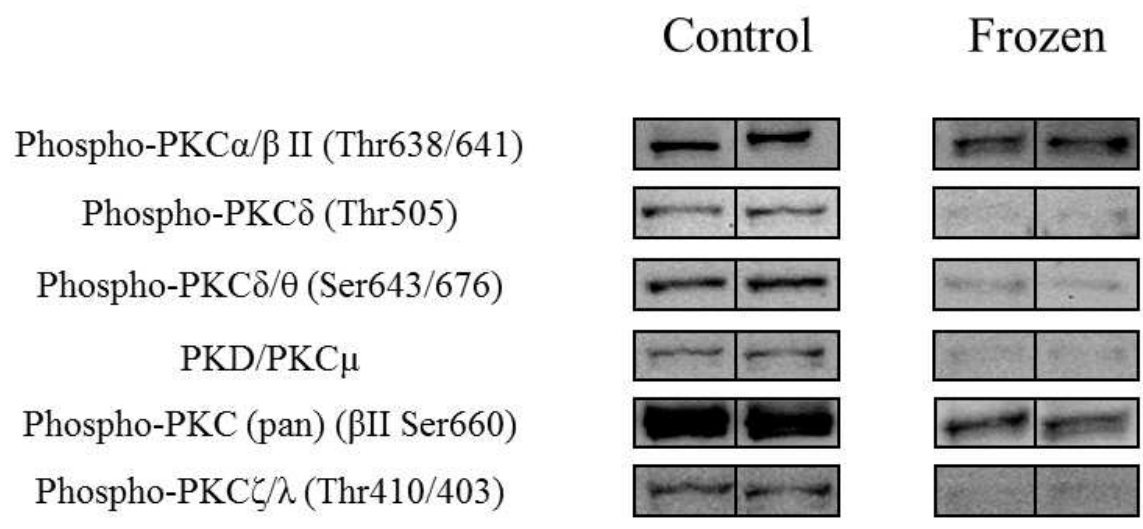
**Fig. 1.** Changes in phosphorylation levels of PKC isozymes in frog hind leg skeletal muscle during freezing. Relative levels were determined from immunoblots of  $n = 5$  independently-prepared tissue homogenates from pooled tissues of either control frogs, or frogs frozen for 24 h. 2 representative bands out of the 5 total bands for both control and frozen frogs are included in this figure.

**Fig. 2.**



**Fig. 2.** Changes in phosphorylation levels of PKC isozymes in frog liver during freezing. Relative levels were determined from immunoblots of  $n = 5$  independently-prepared tissue homogenates from pooled tissues of either control frogs, or frogs frozen for 24 h. 2 representative bands out of the 5 total bands for both control and frozen frogs are included in this figure.

**Fig. 3.**

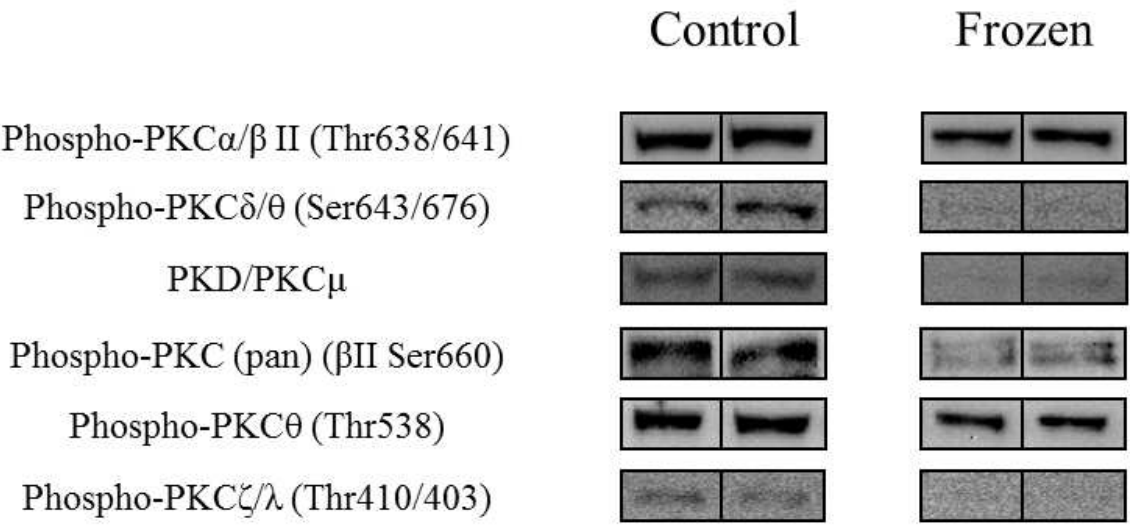


**Fig. 3.** Changes in phosphorylation levels of PKC isozymes in frog kidney during freezing. Relative levels were determined from immunoblots of  $n = 5$  independently-prepared tissue homogenates from pooled tissues of either control frogs, or frogs frozen for 24 h. 2 representative bands out of the 5 total bands for both control and frozen frogs are included in this figure.



580 **Fig. 4.**

581



582

583

584 **Fig. 4.** Changes in phosphorylation levels of PKC isozymes in frog heart during freezing.

585 Relative levels were determined from immunoblots of  $n = 5$  independently-prepared tissue

586 homogenates from pooled tissues of either control frogs, or frogs frozen for 24 h. 2

587 representative bands out of the 5 total bands for both control and frozen frogs are included in this

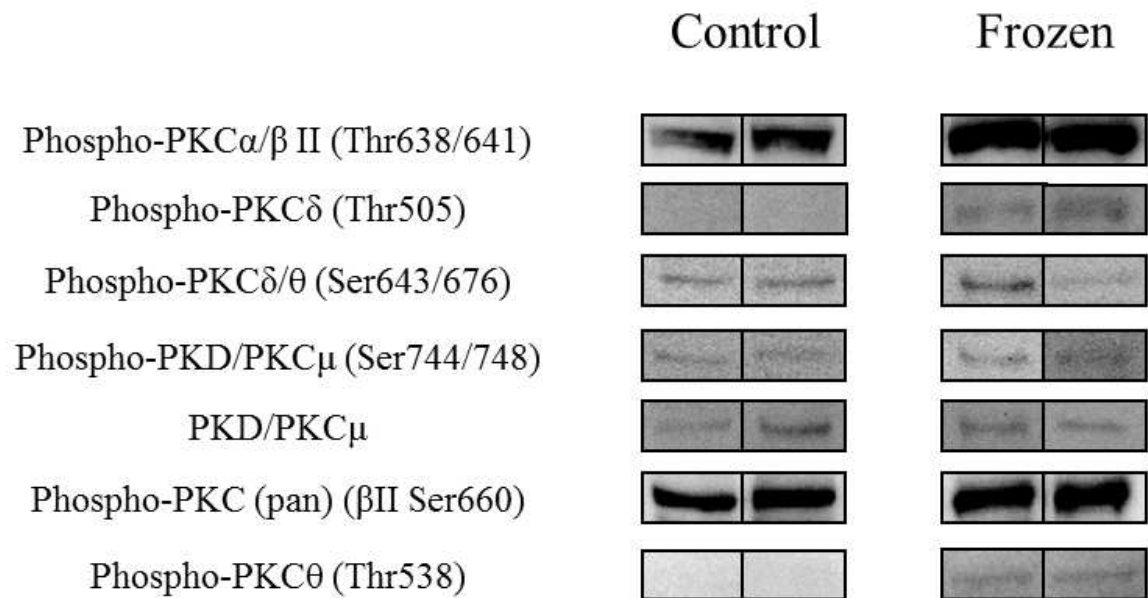
588 figure.

589

590

591 **Fig. 5.**

592



593

594

595 **Fig. 5.** Changes in phosphorylation levels of PKC isozymes in frog brain during freezing.

596 Relative levels were determined from immunoblots of  $n = 5$  independently-prepared tissue

597 homogenates from pooled tissues of either control frogs, or frogs frozen for 24 h. 2

598 representative bands out of the 5 total bands for both control and frozen frogs are included in this

599 figure.