Protein kinase C in the wood frog, *Rana sylvatica*: reassessing the tissue-specific regulation of PKC isozymes 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_3 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 isozymes, 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 isozymes and specific phosphorylation sites detected by immunoblotting decreased in phosphorylation levels in hind leg skeletal muscle and hearts of frozen frogs. Most PKC isozymes and specific phosphorylation sites detected in livers and kidneys also declined; the only exceptions were the levels of isozymes/phosphorylation sites detected by the phospho-PKCα/βII (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 isozymes and/or specific phosphorylation sites detected by immunoblotting. Rather, increases were observed for the levels of isozymes/phosphorylation sites detected by the phospho-PKCα/βII (Thr638/641), phospho-PKCδ (Thr505), and phospho-PKCθ (Thr538) antibodies; all other isozymes/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. PKCδ).

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Introduction

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

24   Dieni et al., 2012; Zhang and Storey, 2013; do Amaral et al., 2013), and; 4) suppression of

25   metabolic pathways that would otherwise divert glucose away from cryoprotection (e.g. pentose

26   phosphate pathway, glycolysis; Dieni and Storey, 2010; 2011), among others. Following the

27   return of warmer temperatures and the arrival of spring, frogs thaw and resume their natural life

28   cycle with no apparent debilitating results of the freeze-thaw process.

29   Given the scope of these necessary adaptations, it is likely- and has in fact already been

30   demonstrated- that altered signaling comprises a major facet of the mechanisms behind the

31   biochemical outcomes facilitating survival. In addition to those signaling enzymes already

32   referenced (i.e. Akt, AMPK, glycogen synthase kinase-3 or GSK3, protein kinase A or PKA),

33   additional kinases and phosphatases have been shown to play a role in wood frog freeze-

34   tolerance. For instance, mitogen activated protein kinases (MAPKs) are activated in various

35   tissues and are suggested as having a role in regulating metabolic or gene expression responses

36   that would facilitate survival in the freezing and/or thawing processes (Greenway and Storey,

37   2000). Past studies have also suggested a potential role for protein kinase C (PKC) in freezing,

38   anoxia, and dehydration, based on patterns of inositol 1,4,5-trisphosphate (IP_3), a second

39   messenger associated with cytosolic calcium increases and a co-product of diacylglycerol (DAG;

40   Holden and Storey 1996; 1997). Increases in cytosolic calcium and DAG both lead to PKC

41   activation.

42   PKC in fact consists of a family of 15 serine/threonine-protein kinase isozymes in

43   humans, divided into subfamilies with specific second messenger requirements and upstream

44   regulators (Mellor and Parker, 1998). Our lab has previously demonstrated *in vivo* roles for PKC

45   in various forms of animal stress physiology, including: 1) reptilian anaerobiosis (Mehrani and

46   Storey, 1996); 2) mammalian hibernation (Mehrani and Storey, 1997), and; 3) fish exercise and

47   bioenergetics (Brooks and Storey, 1998). Meanwhile, *in vitro* stimulation of endogenous PKC

48   has been shown to significantly affect the kinetic properties of glucose-6-phosphate

49   dehydrogenase (G6PDH; Dieni and Storey, 2010), and hexokinase (Dieni and Storey, 2011)

50   from wood frog tissue extracts. Given the potential importance of PKC in wood frog freeze-

51   tolerance, the present study further explores the regulation of this family of kinases *in vivo*, using

52   phospho-specific immunoblotting to establish tissue-specific phosphorylation states of the PKC

53   isozymes in control and frozen frogs.

54

55   **Materials and methods**

56

57   *Animals*

58

59   Conditions for animal care, experimentation, and euthanasia were approved by the

60   Carleton University Animal Care Committee (B09-22) in accordance with guidelines set down

61   by the Canadian Council on Animal Care. Male wood frogs were captured from spring breeding

62   ponds in the Ottawa, Ontario area. Animals were washed in a tetracycline bath, and placed in

63   plastic containers with damp sphagnum moss at 5ºC for two weeks prior to experimentation.

64   Control frogs were sampled from this condition. For freezing exposure, frogs were placed in

65   closed plastic containers with damp paper toweling on the bottom, and put in an incubator set

66   at -3ºC. A 45 min cooling period was allowed during which body temperature of the frogs cooled

67   to below -0.5ºC and nucleation was triggered due to skin contact with ice crystals formed on the

68   paper toweling (Storey and Storey, 1985). Subsequently, timing of a 24 h freeze exposure began.

69   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 -80ºC 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 -80ºC. 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_3VO_4, and 10 mM ß-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 4ºC 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 -20ºC until

93   use.

94

95   *SDS-PAGE and polyvinylidene difluoride membrane transfer*

96

97   Aliquots of thawed samples containing 20 μg of protein were loaded into wells of SDS-

98   polyacrylamide gels (8% resolving gel, 5% stacking gel, made from a 30% acrylamide and bis-

99   acrylamide solution, 37.5:1; 161-0158, Bio-Rad, Hercules, CA, USA), along with Kaleidoscope

100   prestained markers (161-0324, Bio-Rad, Hercules, CA, USA) to estimate molecular weight of

101   PKC isozymes. Samples were electrophoresed at 180 V in a Mini-PROTEAN III apparatus (Bio-

102   Rad, Hercules, CA, USA) using 1x running buffer (5x running buffer contained 15.1 g Tris-base,

103   94 g glycine, and 5 g SDS per litre, pH 8.3). Proteins were then wet-transferred to polyvinylidene

104   difluoride (PVDF) membrane (Millipore, Bedford, MA, USA) using a current of 300 mA for 1.5

105   h at 4ºC in a Bio-Rad Mini Trans-Blot Cell apparatus (Bio-Rad, Hercules, CA, USA). Transfer

106   buffer contained 25 mM Tris-base pH 8.8, 192 mM glycine, and 20% v:v methanol, chilled to

107   4ºC.

108

109   *Immunoblotting of PVDF membranes and analysis*

110

111   Primary antibodies (Cell Signalling Technology, Danvers, MA, USA) were the

112   following: phospho-PKC (pan) (βII Ser660) antibody (9371), which detects all of PKCα, βI, βII,

113   δ, ε and η isoforms only when phosphorylated at a carboxy-terminal residue homologous to

114   Ser660 of PKCβII; phospho-PKCδ/θ (Ser643/676) antibody (9376), which detects both PKCδ

115   when phosphorylated at Ser643 and PKCθ when phosphorylated at Ser676; phospho-PKCα/βII

116 (Thr638/641) antibody (9375); phospho-PKCδ (Thr505) antibody (9374; this antibody has since

117 become unavailable after this work was carried out); phospho-PKCθ (Thr538) antibody (9377);

118 phospho-PKCζ/λ (Thr410/403) antibody (9378); PKD/PKCμ antibody (2052); phospho-

119 PKD/PKCμ (Ser916) antibody (2051); phospho-PKD/PKCμ (Ser744/748) antibody (2054). All

120 primary IgG antibodies were raised in rabbit. These were purchased together as the Phospho-

121 PKC Antibody Sampler Kit (9921; this kit has since become unavailable after this work was

122 carried out). Stock primary antibodies were diluted between 1:5000 to 1:10,000 in Tris-buffered

123 saline supplemented with Tween-20 (TBST; 20 mM Tris pH 7.5, 150 mM NaCl, 0.05% v:v

124 Tween-20). Secondary antibody used was the anti-rabbit IgG, HRP-linked antibody (7074; also

125 supplied within the Phospho-PKC Antibody Sampler Kit). Stock secondary antibodies were

126 diluted 1:2000 in TBST.

127   After transfer was complete, PVDF membranes were quickly equilibrated in TBST and

128 then blocked with 5% w:v nonfat milk dissolved in TBST for 15 min at room temperature. The

129 blot was rinsed with TBST and then incubated with primary antibody in TBST on a shaking

130 platform overnight at 4°C. Blots were washed twice with TBST and incubated with secondary

131 antibody for 1.5 h at room temperature. Immunoreactive bands were visualized using enhanced

132 chemiluminescence (ECL; RPN2108, GE Healthcare Life Sciences, Baie d'Urfé, QC, Canada)

133 following the manufacturer's protocol. The luminol and oxidizing reagents were mixed 1:1 v:v

134 on the membrane for 1 min and the ECL signal was detected using a ChemiGenius (SynGene,

135 Frederick, MD, USA).

136   Total protein was then visualized on the PVDF membrane by staining for 30 min with

137 Coomassie blue staining solution (0.25% w:v Coomassie Brilliant Blue R, 50% v:v methanol,

138 7.5% v:v acetic acid) followed by destaining with destain solution (25% v:v methanol, 10% v:v

139   acetic acid). Three Coomassie-stained bands that did not differ in intensity between active and

140   frozen conditions were used to normalize the corresponding intensity of the immunoreactive

141   band in each lane to correct for any unequal protein loading, as described previously (Dieni et

142   al., 2012).

143   Intensities of ECL-visualized and Coomassie-stained bands were quantified using the

144   associated Gene Tools program (v. 3.00.02). Data were analyzed by one-way ANOVA followed

145   by Tukey's test; a statistically-significant difference was accepted with values of $p < 0.05$ or

146   smaller.

147

148   **Results and Discussion**

149

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

151

152   The generally-accepted model for activation of PKC isozymes has been reviewed (Mellor

153   and Parker, 1998; Parker and Murray-Rust, 2004; Gomperts et al., 2009) and follows here. PKCs

154   are biosynthesized as catalytically inactive, and must first bind to the intracellular face of the

155   plasma membrane in order to be unfolded, and rendered competent. A number of upstream

156   signals can activate phospholipases, hydrolysing inositol phospholipids to various combinations

157   of diacylglycerols and IP_3; IP_3 will in turn trigger calcium efflux from the endoplasmic

158   reticulum, which then propagates further calcium influx from the extracellular environment.

159   Conventional PKC isozymes (cPKC; α, β, γ) bind to the membrane via two specific bridging

160   interactions: C1 domains that bind to DAG and C2 domains that bind to calcium-phospholipid

161   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; δ, ε, η, θ) and atypical PKC isozymes (aPKC; ζ, λ,

173 μ). 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

185   (http://www.cellsignal.com/pdf/9921.pdf). A summary of changes between control and frozen

186   frogs is presented in Table 1. In general, levels of phosphorylated PKC isozymes (and non-

187   phosphorylated PKD/PKCμ) tended to globally decrease during wood frog freezing in hind leg

188   skeletal muscle, liver, kidney, and heart; the only tissue in which increases in phospho-PKC were

189   observed was the brain.

190   PDK1 itself, and its targets, have been shown to change in phosphorylation state during

191   wood frog freezing. For instance, levels of phospho-Thr308-Akt (a phosphorylation site of

192   PDK1) decrease in muscle and heart during freezing, suggesting decreased action of PDK1 in

193   these tissues. By contrast, levels of both phospho-Ser241-PDK1 and phospho-Thr308-Akt

194   increase in liver, suggesting increased PDK1 action in livers of frozen frogs (Zhang and Storey,

195   2013). For optimal clarity, specific changes in PKC isozymes will be further described and

196   discussed on a tissue-by-tissue basis, and compared to previously-established changes in PDK1,

197   PKD1 targets, or previously-assessed targets downstream of PKC isozymes.

198

199   *Muscle*

200

201   Only 2 primary antibodies were immunoreactive to frog muscle extracts: phospho-

202   PKCα/βII (Thr638/641), and phospho-PKCθ (Thr538). Bands detected by both these antibodies

203   decreased in intensity in frozen frogs (Fig. 1; Table 1). Thr638/641 is in the turn motif of

204   PKCα/βII, and is autophosphorylated after initial phosphorylation of the activation loop by

205   PDK1 (Ron and Kazanietz, 1999). Whether phospho-Thr638/641 is necessary for PKCα/βII

206   activity is debatable; rather, it is more recognized for its importance in duration of PKC

207   activation, and slowing the rate of PKC active loop dephosphorylation (Bornancin and Parker,

208   1996). Meanwhile, Thr538 is in the activation loop of PKCθ, and is directly phosphorylated by

209   PDK1; as such, it is unequivocally needed for PKCθ activity (Liu et al., 2002). Taken together,

210   these results suggest a combination of lower activity, a shorter duration of activation, and a

211   higher rate of dephosphorylation of these PKC isozymes in muscle during freezing.

212   These decreases in the phosphorylation levels of PKC muscle isozymes correlate well

213   with recently-presented decreases in phospho-Thr308-Akt (a target that, along with PKC, is also

214   a direct substrate of PDK1; Zhang and Storey, 2013). By contrast, past studies would suggest a

215   need for PKC to remain active in muscle; these have shown that in response to freezing, IP_3

216   levels rose moderately, by 55%, in skeletal muscle (Holden and Storey, 1996; 1997). Moreover,

217   calcium binding and uptake into the sarcoplasmic reticulum were strongly decreased in skeletal

218   muscle of frozen frogs, leading to increased cytosolic calcium levels (Hemmings and Storey,

219   2001). However, in yet other studies, muscle IP_3 levels remained constant in frogs subjected to

220   short-term anoxia at 5ºC, and then fell by 40% after 2 days of anoxic exposure. The contrasting

221   past and present findings of increased IP_3 and calcium levels in frozen frogs, yet decreased

222   PKCα/βII and PKCθ phosphorylation in this same physiological state, along with decreased IP_3

223   levels in frogs subjected to long-term anoxia, leaves us with a very uncertain role for muscle

224   PKC in the adaptation to these stresses.

225

226   *Liver*

227

228   8 of the 9 primary antibodies were immunoreactive to frog liver extracts; only the

229   phospho-PKCζ/λ (Thr410/403) antibody failed to reveal any bands. Overall, band intensities

230   again tended to decrease in frozen frogs (Fig. 2; Table 1). As in muscle, phospho-Thr538-PKCθ

231 levels decreased, but to such an extent where they were non-quantifiable in frozen frogs.

232 Similarly, phospho-Thr505-PKCδ levels also decreased to an extent where they were non-

233 quantifiable. Interestingly, in contrast to Thr538, an activation loop phosphorylation of PKCθ

234 which is unequivocally needed for activity, Thr505 is also an activation loop residue of PKCδ,

235 but one which is at best only debatably necessary for activity, and is autophosphorylated in

236 addition to being phosphorylated by PDK1 (Le Good et al., 1998; Liu et al., 2002; Steinberg,

237 2004; Liu et al., 2006). Furthermore, phospho-Thr676/643-PKCδ/θ levels (a turn motif

238 phosphorylation) decreased by over 50%. While the role of this turn motif phosphorylation is

239 inconclusive in PKCδ or PKCθ (Li et al., 1997; Liu et al. 2002), decreases in turn motif phospho-

240 Thr676/643 will potentially compound the decreases in activation loop phospho-Thr505/538,

241 further depressing PKCδ and PKCθ activities.

242   Additional decreases are observed in non-phosphorylated levels of PKD/PKCμ, of

243 phospho-Ser744/748-PKD/PKCμ, and of phospho-Ser916-PKD/PKCμ. Ser916 is an

244 autophosphorylation site that correlates with catalytic activity in PKD/PKCμ (Matthews et al.,

245 1999), whereas Ser744 and possibly Ser748 are activation loop phosphorylation sites, also

246 critical to activity (Waldron et al., 2001; Waldron and Rozengurt, 2003). Interestingly, while

247 PKD/PKCμ was originally classified as a member of the PKC family (and is still very much

248 considered as such), Ser744/748 is in fact phosphorylated by other PKC isozymes upstream of

249 PKD/PKCμ, most notably PKCδ (Waldron et al., 2001; Waldron and Rozengurt, 2003). Given

250 that total and phospho-levels of PKD/PKCμ and upstream PKCδ all decreased, these suggest that

251 PKD/PKCμ will also be inactive in frozen frogs.

252   Wood frog liver is quite possibly the best-characterized tissue in terms of proteins and

253 genes that may have potential relationships with PKC; by virtue of their decreased

254   phosphorylation, the potential decline of PKCδ, PKCθ, and PKD/PKCμ activities contrast with

255   previous findings. One early study demonstrated a progressive rise in IP_3 levels over the course

256   of the freezing process, ultimately rising 11-fold higher than control values after 24 h of freezing

257   (Holden and Storey, 1996). Based on this finding, important roles were suggested for PKC,

258   including: i) overriding of normal cellular metabolic controls; ii) enabling "acceptance" of

259   prolonged and extreme reductions in cell volume, along with accompanying hyperosmolality and

260   elevated ionic strength, and; iii) exerting tight control over glycogen phosphorylase, in order to

261   drive glycogenolysis to the degree necessary for extreme the hyperglycemia observed during

262   freezing. A follow-up study, exploring second messenger changes in frogs subjected to

263   dehydration and anoxia, noted increased IP_3 levels in both dehydrated and anoxic frogs (Holden

264   and Storey, 1997). Because of the response to both dehydration and anoxia, the importance of

265   PKC in freezing was reaffirmed; indeed, both dehydration and anoxia result from the freezing of

266   extracellular water in wood frogs.

267   Upon the discovery of *fr47*, a novel gene associated with freezing survival, it was noted

268   that its expression pattern paralleled that of IP_3 accumulation, suggesting that PKC may activate

269   freeze-response genes (McNally et al., 2003). Later, NFκB, a transcription factor crucial in

270   cellular stress response and survival, was found to have increased DNA-binding affinity in

271   frozen frogs; its sequestering binding partner, IκB, was also found to increase in phosphorylation

272   during freezing (Storey, 2008) IκB is a substrate of the IκB kinase (IKK), which is in turn a

273   substrate of PKC isozymes (Lallena et al., 1998; Diaz-Meco and Mostat, 2012). Recently, it was

274   shown that Nrf2, a transcription factor activated during oxidative stress, has increased DNA-

275   binding affinity in frozen frogs; moreover, transcription of *gsta*, a gene under Nrf2 control, was

276   elevated in frozen frogs (Zhang, 2013). It was reiterated in this study that PKC phosphorylates

277 Keap1, a sequestering binding partner of Nrf2, inducing dissociation and activation of Nrf2 and

278 the transcription of antioxidant response genes. Lastly, and possibly most conflicting with the

279 presently-observed decrease in liver phospho-PKC levels, are the rise of both PDK1 and Akt

280 phosphorylation levels in frozen frogs (Zhang and Storey, 2013). Given these collective findings,

281 the presently-suggested decline in liver PKC activities is at odds with the previously-inferred

282 importance of PKC in freezing survival mechanisms.

283   A possible reconciliation is that although decreases are observed in the phosphorylation

284 states of PKCδ, PKCμ, and PKCθ, levels detected by the phospho-Thr638/641-PKCα/βII

285 antibody remain apparently unchanged in frozen frogs. Thr638, a turn motif phosphorylation

286 site, is not critical to PKCα catalytic function but rather controls the duration of its activation by

287 regulating the rate of dephosphorylation and inactivation (Bornancin and Parker, 1996; Li et al.,

288 1997; Ron and Kazanietz, 1999). By contrast, Thr641 is also a turn motif phosphorylation site

289 but is fundamental to the activity of PKCβI and PCKβII (Zhang et al., 1993; Ron and Kazanietz,

290 1999; Leonard et al., 2011). Taken together, these suggest that some cPKCs will continue to be

291 active and/or remain active for longer in frozen frogs. It should be noted however, that while

292 levels of phospho-Thr638/641-PKCα/βII remained unchanged, levels detected by the phospho-

293 PKC (pan) (βII Ser660) antibody decreased in frozen frogs. This antibody is specific for PKCα,

294 βI, βII, δ, ε and η when autophosphorylated at a carboxy-terminal residue homologous to Ser660

295 in the hydrophobic motif of PKCβII, following initial phosphorylation of Thr500 by PDK1;

296 phospho-Ser660 plays important roles in correct folding, as well as the binding of protein

297 substrate, ATP, and calcium (Zhang et al., 1993; Ron and Kazanietz, 1999; Leonard et al., 2011).

298 Another possible reconciliation is that while much of the literature supports an activation model

299 where phosphorylation is necessary for PKC activity, there are exceptions; as indicated earlier

300   for instance, the active loop phosphorylation at Thr505-PKCδ is not required for activity

301   (Steinberg, 2004; Liu et al., 2007). As an nPKC, PKCδ- and others that behave similarly- may

302   therefore be active in presence of elevated DAG regardless of phosphorylation state.

303

304   *Kidney*

305

306   6 antibodies revealed bands in frog kidney extracts. As in liver, levels of phospho-

307   Thr638/641-PKCα/βII remain unchanged between control and frozen frogs (Fig. 3; Table 1).

308   However, significant decreases were observed in the levels bands detected by phospho-Thr505-

309   PKCδ, phospho-Thr676/643-PKCδ/θ, non-phosphorylated PKD/PKCμ, and phospho-PKC (pan)

310   (βII Ser660) antibodies. Moreover, decreases were also observed in the intensities of the bands

311   detected by the phospho-PKCζ/λ (Thr410/403) antibody. Thr410 is an activation loop residue in

312   PKCζ, as is Thr403 in PKCλ (also known as PKCι in mammals), and these are directly

313   phosphorylated by PDK1 and are critical to activity (Le Good et al., 1998; Le Good and

314   Brindley, 2004). Together, these results would suggest a decreased overall activity for PKC

315   isozymes in kidney of frozen frogs. As with muscle and liver, however, kidney IP_3 levels were

316   shown to increase in previous studies (Holden and Storey, 1997). In frogs dehydrated by 40%,

317   IP_3 levels rose by 60%; levels remained unchanged in frogs exposed to anoxia (Holden and

318   Storey, 1997) and in frozen frogs (Holden and Storey, 1996). Again, this presents us with

319   potentially contrasting results between second messengers, specific stresses, and PKC

320   phosphorylation state; second messengers responsible for PKC activation increase in kidney (but

321   only in response to dehydration, and not to anoxia or freezing), while PKC phosphorylation itself

322   does not increase during freezing.

323

Heart

325

326   6 antibodies revealed bands in frog kidney extracts, and all of these decreased in frozen

327 frogs (Fig. 4; Table 1). Levels of bands detected by phospho-Thr638/641-PKCα/βII, phospho-

328 Thr676/643-PKCδ/θ, non-phosphorylated PKD/PKCμ, phospho-PKC (pan) (βII Ser660),

329 phospho-Thr538-PKCθ, and phospho-Thr410/403-PKCζ/λ antibodies all decreased significantly;

330 indeed, phospho-Thr676/643-PKCδ/θ, non-phosphorylated PKD/PKCμ, and phospho-

331 Thr410/403-PKCζ/λ levels all decreased to an extent where they were no longer detectable in

332 frozen frogs.

333   With decreases observed in the phosphorylation levels of all PKC isozymes detected in

334 heart, this would suggest overall decreased PKC activity in this tissue. The decreases in

335 phosphorylation levels of these PKC isozymes also correlate with decreases in phospho-Thr308-

336 Akt in frog heart during freezing (Zhang and Storey, 2013). Previous studies showed no

337 significant changes in IP$_3$ levels in response to freezing (Holden and Storey, 1996), yet

338 significant increases were observed after only 1 h of enduring anoxia (Holden and Storey, 1997).

339 Thus, our present results in heart are in agreement with decreased phosphorylation of other

340 PDK1 targets and unchanged second messenger levels in freezing, but not with increased second

341 messenger levels in response to anoxia.

342

Brain

344

345   8 antibodies revealed bands in frog brain extracts. Of the five tissues investigated in the

346   present study, the results obtained in the brain were the most unique; changes (or lack thereof) in

347   phospho-PKC levels in brain during frog freezing contrasted strongly with those observed in

348   muscle, liver, heart, and kidney (Fig. 5; Table 1). No changes were observed in the levels of

349   bands detected by phospho-Thr676/643-PKCδ/θ, phospho-Ser744/748-PKD/PKCμ, non-

350   phosphorylated PKD/PKCμ, and phospho-PKC (pan) (βII Ser660) antibodies; each of these were

351   detectable in both control and frozen frogs to approximately the same extent. Interestingly,

352   phospho-Thr638/641-PKCα/βII levels increased by 121.3% in brains of frozen frogs. Moreover,

353   phospho-Thr505-PKCδ and phospho-Thr538-PKCθ, which were not detectable in control frogs,

354   were detectable (albeit faintly) in frozen frogs. Overall, whereas phospho-PKC levels generally

355   tended to decline in other organs of frozen frogs, phospho-PKC levels largely remained

356   unchanged or even increased in brain.

357   The increase in brain PKC phosphorylation during freezing is of great interest. Previous

358   studies have shown that brain IP_3 levels rose significantly after 4 h of freezing (Holden and

359   Storey, 1996), and so this is one frog tissue in which the overall increases in PKC

360   phosphorylation state correlate with this rise in IP_3. Numerous other adaptive "activations" occur

361   in frog brains in response to freezing, including: 1) moderately-increased expression of *fr10* (Cai

362   and Storey, 1997) and *li16* (Sullivan and Storey, 2012), novel genes with putative roles in

363   freezing protection; 2) increased levels of c-Fos (Greenway and Storey, 2000), and; 3) up-

364   regulation of genes for ribosomal proteins, including the acidic ribosomal phosphoprotein P0

365   (Wu et al., 2005) and the ribosomal large subunit protein 7 (Wu et al., 2008). At present,

366   however, we cannot conclusive identify any of the proteins listed here as being substrates of

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

368 factors downstream of PKC.

369 Conceivably, the phosphorylation and activation of PKC (along with other proteins) in

370 brain- contrasted against the decreased or unchanged phosphorylation of PKC in other tissues-

371 suggests a unique and vital role for brain PKC during freezing. Indeed, the importance of PKC in

372 cerebral protection has been demonstrated well-beyond the niche of wood frog freezing (Sun et

373 al., 2013; Thompson et al., 2013). Future efforts will be required to establish the catalytic

374 competence of PKC isozymes in frozen frog brains, to identify the targets being phosphorylated

375 by PKC, and to determine their most likely role in frog cerebral/neuroprotection during freezing

376 based on our current understanding of other models.

377

378 **Conclusion**

379

380 The present study investigated the phosphorylation state of conventional, novel, and

381 atypical PKC isozymes in five tissues of freeze-tolerant frogs, as well as non-phosphorylated

382 PKCμ/PKD. Broadly, phospho-PKC levels and non-phosphorylated PKCμ/PKD decreased in

383 muscle, livers, kidneys, and hearts of frozen frogs; the only exception was protein detected by

384 the phospho-Thr638/641-PKCα/βII (turn motif), which showed no change in livers or kidneys

385 between the control and frozen states. This protein alone would partially support the findings of

386 past studies, where IP_3 was shown to dramatically increase in livers of frozen frogs and thus an

387 important role was suggested for PKC in the freezing process; however, even the steady

388 phosphorylation state of Thr638/641 is seemingly contradicted by the decreased phosphorylation

389 of Ser660 (hydrophobic motif) in both livers and kidneys of frozen frogs. A particularly

390   interesting finding in this study is PKC would seem to play an important role in the brains of

391   frozen frogs, as the phospho-levels of all isozymes detected remain either remain unchanged or

392   even increase in the frozen state.

393   The results of this study succeed in answering some questions of past studies pertaining

394   to the state of PKC in freeze-tolerance, but raise many others and indeed pose some

395   contradictions. Whereas our lab has previously asserted that based on second messenger patterns,

396   PKC would play an important role (particularly in liver and muscle), our present findings would

397   seem to instead suggest a diminished role for PKC in most tissues, based on our current

398   understanding of PKC isozymes and their generally-accepted activation model. This in itself,

399   however, can be debated by pointing to studies which demonstrate that activation loop

400   phosphorylation is, in fact, not needed for PKC activity (e.g. PKCδ). To clarify the role of PKC

401   in wood frog freezing, it is now evident that catalytic assays are necessary in order to

402   unequivocally establish the actual activities of each of these isozymes in the control and frozen

403   states. Moreover, there is an additional difficulty in contextualizing the role of PKC due to the

404   fact that very few downstream targets of PKC have been assessed in wood frogs. Those that were

405   discussed in this report are either only speculative (e.g. transcription factors that might control

406   expression of *li16*, *fr10* and *fr47*), or are several degrees removed from being direct PKC

407   substrates and/or are under the control of multiple kinases (e.g. NFκB/IκB via IKK, Nrf2 for

408   which Keap1 can be modified via several pathways, etc.) There is a clear need to assess the

409   phosphorylation state of direct PKC substrates (e.g. the MARCKS family) in order to better

410   determine the activity and role of PKC in freeze-tolerance.

411

412   **Acknowledgements**

413

416

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418

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531

Tables

Table 1. Summary of changes in phosphorylation levels of PKC isozymes (or non-phosphorylated PKD/PKCμ) 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-PKCα/βII (Thr638/641)	-41.8% [c]	=	=	-28.6% [a]	+121.3% [b]
Phospho-PKCδ (Thr505)	ND	--	-76.6% [d]	ND	++
Phospho-PKCδ/θ (Ser643/676)	ND	-54.8% [a]	-75.7% [c]	--	=
Phospho-PKD/PKCμ (Ser744/748)	ND	--	ND	ND	=
Phospho-PKD/PKCμ (Ser916)	ND	--	ND	ND	ND
PKD/PKCμ	ND	-76.4% [d]	-66.7% [d]	--	=
Phospho-PKC (pan) (βII Ser660)	ND	-82.7% [c]	-74.6% [a]	-38.6% [c]	=
Phospho-PKCθ (Thr538)	-50.4% [d]	--	ND	-35.8% [b]	++
Phospho-PKCζ/λ (Thr410/403)	ND	ND	-66.8% [d]	--	ND

545 **Figures**

546

547 **Fig. 1.**

548

Control   Frozen

Phospho-PKCα/β II (Thr638/641)

Phospho-PKCθ (Thr538)
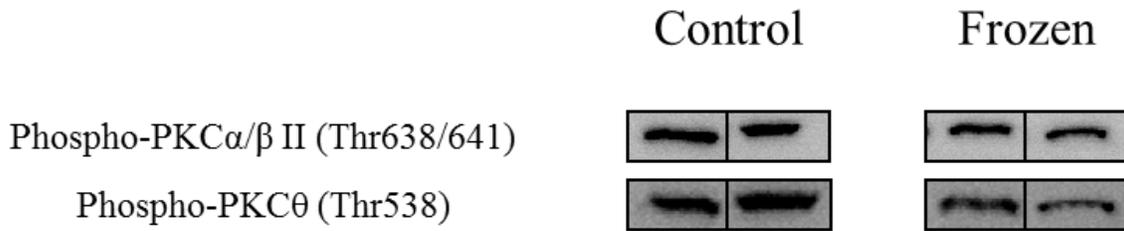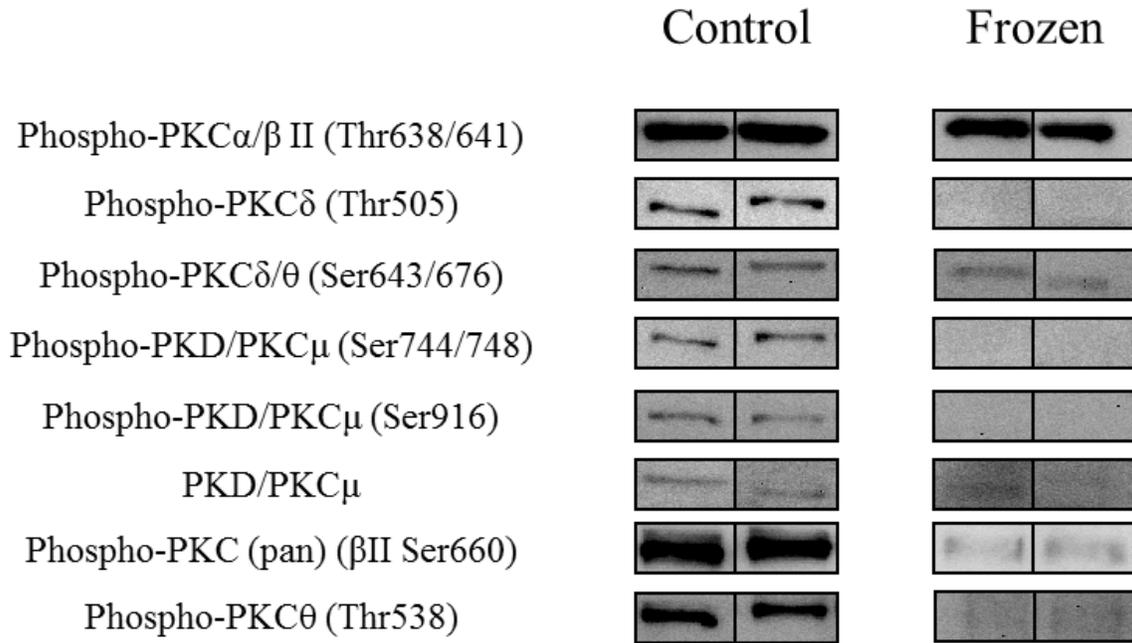
549
550

551 **Fig. 1.** Changes in phosphorylation levels of PKC isozymes in frog hind leg skeletal muscle

552 during freezing. Relative levels were determined from immunoblots of $n = 5$ independently-

553 prepared tissue homogenates from pooled tissues of either control frogs, or frogs frozen for 24 h.

554 2 representative bands out of the 5 total bands for both control and frozen frogs are included in

555 this figure.

556

557

558 **Fig. 2.**

559

560

561

562 **Fig. 2.** Changes in phosphorylation levels of PKC isozymes in frog liver during freezing.

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

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

565 representative bands out of the 5 total bands for both control and frozen frogs are included in this
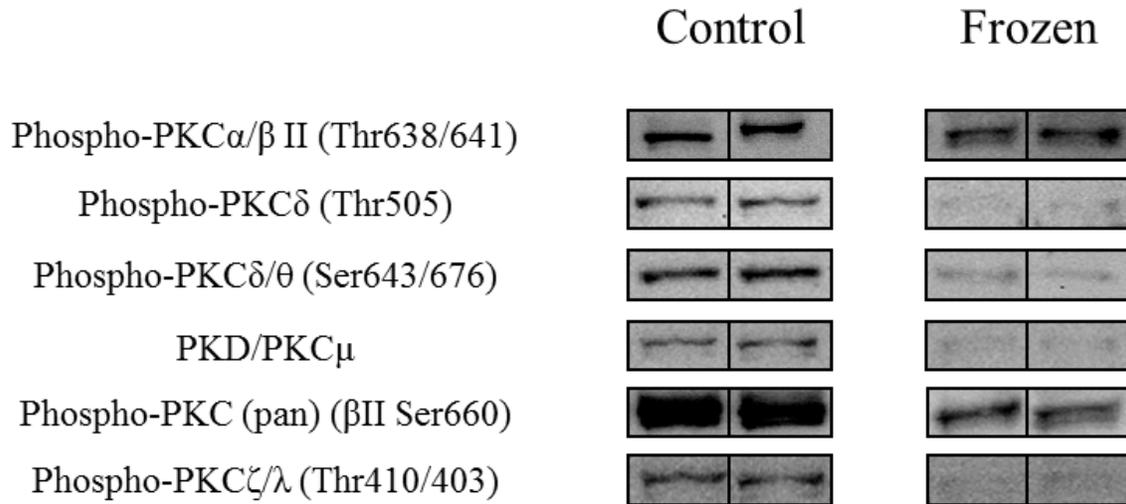
566 figure.

567

568

569　　**Fig. 3.**

570

Control　　　Frozen

Phospho-PKCα/β II (Thr638/641)

Phospho-PKCδ (Thr505)

Phospho-PKCδ/θ (Ser643/676)

PKD/PKCμ

Phospho-PKC (pan) (βII Ser660)

Phospho-PKCζ/λ (Thr410/403)

571

572

573　　**Fig. 3.** Changes in phosphorylation levels of PKC isozymes in frog kidney during freezing.

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

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

576　　representative bands out of the 5 total bands for both control and frozen frogs are included in this
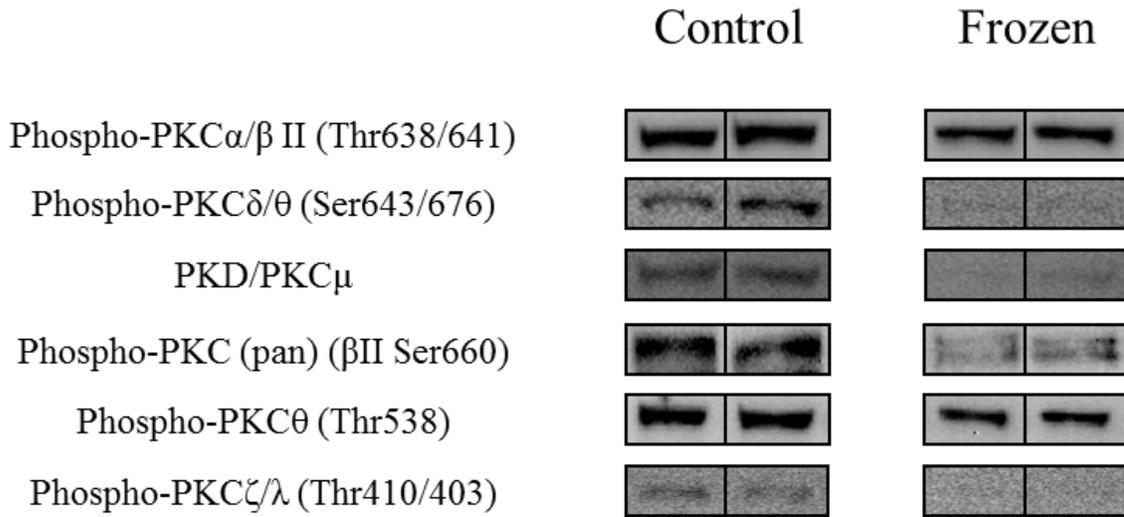
577　　figure.

578

579

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
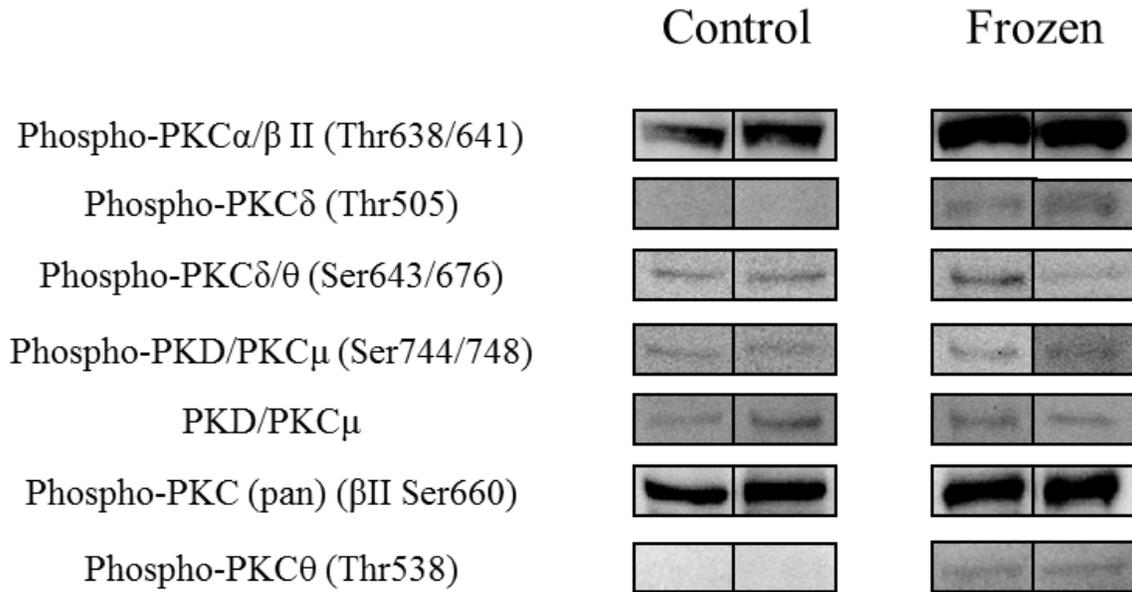
588　figure.

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.