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Proteolytic Regulation of the Mitochondrial cAMP-Dependent Protein Kinase

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Abstract

The mitochondrial cAMP-dependent protein kinase (PKA) is activatable in a cAMP-independent fashion. The regulatory (R) subunits of the PKA holoenzyme (R_2C_2), but not the catalytic (C) subunits, suffer proteolysis upon exposure of bovine heart mitochondria to digitonin, Ca^{2+} , and a myriad of electron transport inhibitors. Selective loss of both the RI- and RII-type subunits was demonstrated via western blot analysis and activation of the C subunit was revealed by phosphorylation of a validated PKA peptide substrate. Selective proteolysis transpires in a calpain-dependent fashion as demonstrated by exposure of the R and C subunits of PKA to calpain and by attenuation of R and C subunit proteolysis in the presence of calpain inhibitor I. By contrast, exposure of mitochondria to cAMP fails to promote R subunit degradation, although it does result in enhanced C subunit catalytic activity. Treatment of mitochondria with electron transport chain inhibitors rotenone, antimycin A, sodium azide, and oligomycin, as well as an uncoupler of oxidative phosphorylation, also elicits enhanced C subunit activity. These results are consistent with the notion that signals, originating from cAMP-independent sources, elicit enhanced mitochondrial PKA activity.

Members of the protein kinase family have been implicated in a myriad of processes, from ATP generation to unrestrained growth and division.(1) Although the cAMP-dependent protein kinase (PKA) is commonly held as the prototypical protein kinase, its mechanism of activation is atypical relative to nearly all other protein kinase family members. PKA is inactive in its holoenzyme form, a tetrameric species consisting of two regulatory (R) and two catalytic (C) subunits. Upon binding of cAMP to the R subunits, the C subunits are released, and are thus free to catalyze the phosphorylation of an array of proteins.(2, 3) However, it is now recognized that PKA can be activated in a cAMP-independent fashion as well. For example, the C subunit can exist in an inactive state via association with I κ B in an NF- κ B-I κ B-(C subunit) complex. Stimulation of cells with lipopolysaccharide, endothelin-1, or angiotensin II induces I κ B degradation, resulting in the ensuing C subunit-catalyzed phosphorylation of NF- κ B p65.(4, 5) In addition, it has been recently reported that thrombin and collagen trigger the dissociation of the C subunit from I κ B via a PI3K-dependent pathway.(6) A somewhat analogous proteasome-mediated R-subunit degradation/C-subunit activation mechanism has been reported as well.(7) However, in this case, ubiquitination of the R subunit is cAMP-dependent.(8) The active C subunit generated via this process has been linked to the regulation of hippocampal synaptic plasticity. Proteolysis has not only been shown to promote PKA activity, but recently C subunit degradation has been observed in a pathological state as well. Specifically, overactive calpain has been identified in

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AUTHOR CONTRIBUTIONS

Jennifer Shell performed the experiments and, in collaboration with David Lawrence, designed the experiments and wrote the manuscript.

diseased neurons from Alzheimer's patients, where it induces the proteolysis of both the R and C subunits of PKA.(9) Shaltiel and his collaborators demonstrated, in a series of papers, that the C subunit is inactivated by Zn^{2+} -metalloprotease-mediated proteolysis.(10, 11)

There have been several reports describing the concerted action of PKA and calpain, including the regulation of platelet procoagulant activity(12), D1 receptor-mediated phosphorylation of tau(13), and the PKA-catalyzed phosphorylation of the calpain inhibitor calpastatin(14, 15), to name but a few. Calpains are a class of ubiquitously expressed Ca^{2+} -activated cysteine proteases. Recent studies have shown that calpain-1 (μ -calpain), calpain-2 (m-calpain), and calpain-10 are present in the mitochondria (16–18), an organelle with significant PKA activity(19–22). Both calpain-1, which requires micromolar levels of Ca^{2+} , and calpain-2, which requires millimolar levels of Ca^{2+} , are located in the mitochondrial intermembrane space, while calpain-10 is embedded within the matrix. PKA, like the calpains, is interspersed throughout the major mitochondrial compartments.(22) Finally, the calpains(23, 24) and PKA(25) are known play key roles in mediating (and blocking) apoptosis.

We have examined the relationship between mitochondrial calpain and PKA activity, and have discovered that calpain activates the R_2C_2 holoenzyme in a cAMP-independent fashion. A variety of agents, including inhibitors of the electron transport chain, activate calpain in a Ca^{2+} -dependent manner, which in turn, catalyzes the proteolysis of the R subunit, thereby releasing the C subunit in its active form.

MATERIALS AND METHODS

Reagents and antibodies

PKA R and C subunits (from bovine heart), PKA holoenzyme, Leu-Arg-Arg-Ala-Ser-Leu-Gly (kemptide), rotenone, sodium azide, antimycin A, oligomycin, carbonyl cyanide 3-chlorophenylhydrazone (CCCP), calpain inhibitor I N-acetyl-Leu-Leu-Nle-CHO (ALLN), antibodies against calpain-1, calpain-2, and calpain-10 were purchased from Sigma. Calpain-1 was purchased from Calbiochem, and phospholipase A2 was purchased from Worthington Biochemical. Bovine heart mitochondria and MitoProfile® Membrane Integrity Western blot antibody cocktail were purchased from Mitosciences. The antibodies against the PKA C subunit, RI subunit, RII subunit, calnexin, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and cytochrome *c* were purchased from BD Biosciences while the goat anti-mouse HRP-conjugated secondary antibody was purchased from Santa Cruz Biotechnology. Protein quantification was accomplished using the BCA protein assay (Pierce). Immunoblots were performed using Snap i.d. (Millipore), and detection was accomplished using SuperSignal West Pico chemiluminescent substrate (Pierce). The resulting images were visualized and quantified using an AlphaInnotech FluorChem FC 2 imager. The intactness of the isolated mitochondria was assessed via a previously described protocol.(26)

Western blot analyses

25 μg of total protein was loaded onto 4 - 12% bis-Tris polyacrylamide gels, separated by electrophoresis, and electroblotted onto PVDF membranes. The membranes were then blocked in 0.5% nonfat dry milk followed by incubation with the appropriate primary antibody (C subunit 1:1000, RI: 1:1000, RII: 1:1000, calnexin 1:4000, GAPDH 1:5000, cytochrome *c* 1:1000, Na^+/K^+ ATPase 1:2000, calpain-1 1:1000, calpain-2 1:1000, calpain-10 1:1000) for 10 min (C subunit, calnexin, GAPDH, cytochrome *c*, Na^+/K^+ ATPase) or 2×20 min (RI and RII). The membranes were then washed 3 times with 0.1% Tween-20 in PBS followed by incubation with a goat anti-mouse secondary antibody

(1:2000) for 10 min. Subsequent washes with 0.1% Tween-20 in PBS (3x), PBS (3x), and 0.5 % NaCl (3x) were performed prior to detection.

Assessment of Mitochondrial Purity and Integrity

The purity of bovine heart mitochondria isolated was assessed via western blot analysis using antibodies against proteins localized to mitochondrial (cytochrome *c*) and non-mitochondrial sites, including the endoplasmic reticulum (calnexin), the plasma membrane (Na^+/K^+ ATPase), and the cytosol (GAPDH) (data not shown). The mitochondrial preparation is not contaminated with proteins from other membranes or the ER, and displays only minimal contamination from the cytoplasm. Mitochondrial structural integrity (>90%) was assessed via measurement of cytochrome *c* oxidase activity.(26)The mitochondria were also probed via western blot for the presence of calpain and were found to contain calpain-1 and calpain-10, but not calpain-2 (data not shown).

Calpain Digestion of Isolated R and C Subunits of PKA and the PKA Holoenzyme

0.4 μg of calpain 1 was added to 0.9 μg of C or R subunit in the presence or absence of calpain inhibitor I (200 μM). The reaction was initiated by the addition of 1 mM, 100 μM , 10 μM , 7 μM , 5 μM , or 2 μM CaCl_2 , and incubated at 37 °C for 20 min. For digestion of the holoenzyme, 0.8 μg calpain 1 was added to 5 μg of holoenzyme, and the reaction was initiated by the addition of 100 μM CaCl_2 . The reaction was allowed to incubate at 37 °C for 30 min, 1 h, or 2 h. Reactions were stopped by the addition of loading buffer (106 mM Tris HCl, 141 mM Tris base, 2% LDS, 10% glycerol, 0.51 mM EDTA, 0.22 mM SERVA® Blue G250, 0.175 mM phenol red, pH 8.5), and the samples were run on a 4–12% bis-tris polyacrylamide gel, and the resulting gel was stained with Coomassie blue.

Digitonin/ Ca^{2+} Treatment of Bovine Heart Mitochondria

Mitochondria were suspended in a hypotonic buffer (0.08 M sucrose, 0.1 mM EDTA, 10 mM Tris-HCl, pH 7.4) and allowed to incubate for 30 min on ice in the presence or absence of 200 μM calpain inhibitor I. The samples were then treated in the presence or absence of digitonin (0.5 mg/mg mitochondrial protein), phospholipase A (4.4 U/mg protein), in the presence or absence of CaCl_2 (100 μM), and allowed to incubate at 37 °C for 2 h (in the presence of digitonin) or 5 min – 2 h (in the absence of digitonin). For mitoplast preparation, digitonin-treated samples were pelleted at $11\,000 \times g$ for 15 min.

Calpain Digestion of Bovine Heart Mitochondria

All samples containing calpain inhibitor I were incubated for 30 min prior to calpain treatment. 0.4 μg of calpain 1 was added to the mitochondria in the presence and absence of calpain inhibitor I (200 μM) followed by the addition of CaCl_2 (10 μM). The samples were allowed to incubate at 37 °C for 20 min. The reaction was stopped by the addition of loading buffer (106 mM Tris HCl, 141 mM Tris base, 2% LDS, 10% glycerol, 0.51 mM EDTA, 0.22 mM SERVA® Blue G250, 0.175 mM phenol red, pH 8.5).

Calpain Activity Assay

Calpain activity was assessed spectrophotometrically using the calpain substrate N-succinyl-Leu-Tyr-7-amido-4-methylcoumarin as previously described.(27) Mitochondria (275 μg whole mitochondria) were pelleted at $9,500 \times g$ for 10 min at 4 °C, resuspended in a hypotonic buffer (0.08 M sucrose, 0.1 mM EDTA, 10 mM Tris-HCl, pH 7.4) for digitonin treatment, or in an isotonic buffer (0.25 M sucrose, 0.1 mM EDTA, 10 mM Tris HCl, pH 7.4) for CaCl_2 treatment, and incubated for 30 min on ice. The samples were then treated with digitonin (0.5 mg/mg mitochondrial protein) and phospholipase A (4.4 U/mg protein) or CaCl_2 (100 μM) in the presence of 100 μM N-succinyl-Leu-Tyr-7-amido-4-

methylcoumarin. Activity was measured using excitation and emission wavelengths of 355 and 444 nm, respectively.

Mitochondria Release Assays

All mitochondrial samples were centrifuged at $9,500 \times g$ for 10 min at 4 °C, rinsed 3 times, and resuspended in isotonic buffer (0.25 M sucrose, 0.1 mM EDTA, 10 mM Tris HCl, pH 7.4) containing 25 μ M KH7 prior to use. Mitochondria were treated with CaCl_2 (10 μ M, 100 μ M), rotenone (10 μ M), sodium azide (2 mM), antimycin A (5 μ M), oligomycin (5 μ M), or CCCP (5 μ M) in the presence and absence of ALLN (500 μ M) and incubated at 37 °C for 30 min. The samples were then centrifuged at $9,500 \times g$ for 10 min at 4 °C, and the supernatant was either analyzed by western blot or used in an enzyme-coupled assay in the presence or absence of PKI (100 μ M) via a previously described protocol(28) to detect PKA activity.

RESULTS AND DISCUSSION

Our initial experiments focused on purified bovine heart C and R subunits. Both were subjected to proteolysis by calpain I (Figure 1). Although both are prone to calpain-mediated digestion, the amount of Ca^{2+} profoundly dictates proteolytic selectivity. The C subunit remains intact after treatment with calpain in the presence of 10 μ M CaCl_2 , whereas the R subunit is ~70% digested. However, if higher concentrations of Ca^{2+} ($\geq 100 \mu\text{M}$) are used, some C subunit digestion occurs as well. The calpain inhibitor ALLN blocks the proteolysis of the R subunit at all Ca^{2+} concentrations and of the C subunit at high Ca^{2+} levels. We subsequently evaluated the calpain-mediated digestion of the R_2C_2 holoenzyme (Figure 2). Curiously, only the R subunit of the holoenzyme suffers proteolysis. The C subunit remains completely intact, even though the conditions employed (100 μ M Ca^{2+}) result in the partial digestion of isolated C subunit (i.e. in the absence of R subunit). One possible explanation is that calpain-catalyzed proteolysis in an *in vitro* setting generates R subunit fragments that retain the ability to bind to and protect the C subunit from calpain. These results merit further analysis. Most importantly, however, the selective sensitivity of the R subunit to proteolysis suggests that an analogous phenomenon, in a physiological setting, could result in the calpain-dependent activation of PKA. We explored the latter possibility using isolated mitochondria from bovine heart.

Digitonin is known to trigger the release of Ca^{2+} from Ca^{2+} stores.(29) Given the fact that calpains are Ca^{2+} -dependent, we decided to explore whether digitonin and/or Ca^{2+} influence mitochondrial PKA activity. Both RI and RII isoforms of PKA, but not the C subunit, are partially digested upon exposure of mitochondria to digitonin (Figure 3A). Degradation is prevented by the addition of the calpain inhibitor ALLN, implying a calpain-dependent process. Furthermore, R subunit proteolysis is enhanced by the addition of Ca^{2+} , likewise consistent with the involvement of calpain, a Ca^{2+} -dependent protease (Figure 3B). We also employed the fluorescent calpain substrate, *N*-succinyl-Leu-Tyr-7-amido-4-methylcoumarin, to examine mitochondrial calpain activity. Untreated mitochondria do not display calpain activity (Figure 3C inset), whereas mitochondria exposed to either digitonin or Ca^{2+} exhibit a time-dependent hydrolysis of the methylcoumarin-based fluorescent calpain substrate (Figure 3C). These experimental results are all consistent with the notion that digitonin-induced R subunit loss is mediated by calpain. A time course study of Ca^{2+} -induced digestion revealed that the maximum extent of R subunit proteolysis is approximately 25 – 35% (Figure 3D), indicating that some, but not all mitochondrial R subunit, is subject to calpain-mediated digestion.

In contrast to Ca^{2+} and digitonin-exposure, R subunit digestion is not observed in mitochondria treated with cAMP (Figure 4A). As a control, calpain I was added to

mitochondria, and the relative amounts of RI, RII, and C subunits assessed by western blot analysis. Once again, degradation of both RI and RII, but not C subunit, was observed (Figure 4B). Approximately 30% of R subunit content is lost upon treatment with calpain (Figure 4C). We've previously shown that the majority of PKA in bovine heart mitochondria is contained within the matrix(22) and one might predict that the R subunits in this compartment would be impervious to externally added calpain. The fact that the amount of R subunit loss is similar, whether induced by added calpain or by digitonin, suggests that only a fraction of the total mitochondrial PKA content is sensitive to calpain and that it is likely this fraction is present outside of the matrix. However, given the structural nature of mitochondria, it is challenging to assess the proteolytic sensitivity of R in the various mitochondrial compartments. For example, we prepared mitoplasts in the conventional digitonin-mediated fashion, but in the absence and presence of calpain inhibitor ALLN (Fig. 5). Mitoplasts prepared in the presence of ALLN display a 15% greater amount of intact R than those prepared in the absence of inhibitor. It is tempting to speculate that R subunit associated with the outer leaflet, as opposed to matrix R, is susceptible to digitonin-induced calpain-catalyzed proteolysis. Of additional note is the formation of an R subunit fragment of ~36 kDa, which is not observed when isolated R subunit is treated with calpain.

We subsequently examined whether digestion of the R subunit leads to the release of active C subunit. Mitochondria were exposed to either cAMP or CaCl_2 , centrifuged, and the supernatant analyzed for active released C subunit. The experiments were performed in the presence of KH7, a soluble adenylyl cyclase inhibitor, which was used to block the possible generation of mitochondrial cAMP. As expected, mitochondrial exposure to cAMP promotes the release of C subunit into the supernatant as evidenced by western blot analysis (Figure 6A). We note that there is also C subunit associated with the pellet, an observation consistent with our recent demonstration of PKA activity in the matrix.(22) As a control, we found very little C subunit present in the supernatant when untreated mitochondria are centrifuged (Figure 6A). In addition, Ca^{2+} promotes mitochondrial release of C subunit into the supernatant (Figure 6A), and the latter is ameliorated by addition of the calpain inhibitor ALLN (Figure 6B), consistent with the notion that this phenomenon is calpain-dependent. The supernatant in these experiments was also examined for PKA activity, using a previously described enzyme-coupled assay(28): (i) PKA: $\text{LRRASLG} + \text{ATP} \rightarrow \text{LRRApSLG} + \text{ADP}$ (ii) pyruvate kinase: $\text{ADP} + \text{phosphoenolpyruvate} \rightarrow \text{ATP} + \text{pyruvate}$ (iii) lactate dehydrogenase: $\text{pyruvate} + \text{NADH} \rightarrow \text{lactate} + \text{NAD}^+$. The supernatant from untreated mitochondria displays a small amount of background kinase activity in the enzyme-coupled assay. This may be due to the presence of mitochondrial ADP or pyruvate, which would generate a response in the enzyme-coupled assay. Nonetheless, PKA activity from the supernatant of Ca^{2+} -treated mitochondria is 2.7 times greater than that of the untreated control. Furthermore, in the presence of PKI, a selective PKA inhibitor, or in the presence of ALLN (Figure 6D), no increase in supernatant PKA activity is observed. In short, a calpain-mediated process liberates C subunit from the mitochondrial milieu as an active enzyme.

Given that Ca^{2+} is an inducer of mitochondrial membrane permeability transition (MPT)(30, 31), we decided to investigate whether other agents known to cause MPT also activate PKA in a calpain-dependent fashion. Electron transport chain inhibitors are widely linked to induction of MPT due to depolarization of the mitochondrial membrane, loss of membrane potential, and mitochondrial swelling.(32–35) In addition, uncouplers of oxidative phosphorylation produce similar effects.(32–35) Therefore, we explored whether exposure of mitochondria to electron transport chain inhibitors rotenone (complex I), antimycin A (complex III), sodium azide (complex IV), and oligomycin (F_1F_0 ATPase), as well as the uncoupler CCCP, elicits enhanced PKA activity. Mitochondria were treated with each of these reagents in the presence and absence of calpain inhibitor ALLN, pelleted, and the

supernatants analyzed for C subunit. Exposure of mitochondria to rotenone, azide, and CCCP induced release of the C subunit into the supernatant, whereas the presence of ALLN blocks release (Figure 7). The amount of C subunit released upon exposure to rotenone, azide, and CCCP is 6.2, 4.3, and 3.2 times greater, respectively, than that of the untreated control.

Antimycin A and oligomycin also stimulate C subunit release from the mitochondria, but to a lesser extent (1.9 and 1.6 times greater, respectively, than that of an untreated control). Release of C subunit in the antimycin A-treated sample is attenuated by ALLN, while release is not affected by ALLN in the oligomycin-treated sample (Figure 7). A recent study has shown that antimycin A induces calpain activation.(48) However, to the best of our knowledge, oligomycin has not been demonstrated to activate calpain. Finally, the supernatants in all of the mitochondria exposed to electron transport inhibitors were assayed for PKA activity and, as expected, increased C subunit release (as revealed by western blot analysis) parallels enhanced kinase activity (data not shown).

What are the possible consequences of stress-induced calpain-mediated PKA activation? The activation of PKA by reagents that inflict mitochondrial stress may represent a protective mechanism. Indeed, PKA activation is known to be cytoprotective. PKA activity is associated with increased cell survival via phosphorylation of proapoptotic BAD protein. (39–42) In addition, PKA phosphorylates apoptotic protease-activating factor 1 (Apaf-1), preventing formation of the apoptosome and activation of caspase-9.(43) Furthermore, PKA phosphorylates VDAC *in vitro* (44), which may prevent the leakage of cytochrome c and thus inhibit apoptosis. The PKA-catalyzed phosphorylation of complex I increases mitochondrial respiration.(36, 37) One possible explanation for rotenone's induction of PKA activity is that inhibition of complex I may trigger a feedback response (PKA-catalyzed phosphorylation of complex I) to restore respiration. Indeed, this may be the case for azide-induced (complex IV inhibition) activation of PKA as well since PKA phosphorylates subunits of cytochrome c oxidase, which likewise promotes enhanced respiration rates.(38) Consistent with our observation of the azide-triggered calpain-dependent activation of PKA is a previous report that azide treatment of neonatal cardiomyocytes initiates calpain activation.(45) Indeed, the more pronounced induction of the C subunit by rotenone (complex I) and azide (complex IV) than either antimycin A (complex III) or oligomycin (F_1F_0 ATPase) may be a consequence of the demonstrated PKA-catalyzed phosphorylation of complexes I (36, 37) and IV (38). The latter may be consistent with a tight regulatory feedback loop (*vide infra*) between PKA and complexes I and IV.

Finally, as noted above, mitochondria treated with CCCP also display enhanced C-subunit activity. This is consistent with the fact that uncouplers of oxidative phosphorylation induce Ca^{2+} release(46), a prerequisite for calpain activation. These results suggest the following sequence of events in response to mitochondrial stress: MPT induction(32), release of matrix Ca^{2+} (47), activation of mitochondrial calpain, subsequent proteolysis of the R subunits, and release of active C subunits.

Given the observation that high Ca^{2+} concentrations can lead to calpain-induced C subunit digestion (Figure 1), it is possible that a delicate balance exists between PKA activation and inactivation. In particular, dysregulation of intracellular Ca^{2+} homeostasis is implicated in many disease models. Mitochondria buffer cytosolic Ca^{2+} to a certain extent, but mitochondrial Ca^{2+} overload leads to apoptotic and necrotic cell death.(31, 49) Calpains are activated in Ca^{2+} -induced apoptotic cell death and have been demonstrated to be involved in the induction of MPT.(17) Indeed, PKA activity is downregulated in diseased neurons in the presence of 2.5 mM Ca^{2+} . A decrease in PKA activity is attributed to calpain digestion of both R and C subunits.(9)

Proteolytic regulation of PKA activity appears to be both an alternative to and synergistic with cAMP activation. Examples of cAMP-independent regulation of PKA activity have been reported involving activation of the C subunit via degradation of I κ B.(4–6) In some instances, such as with proteosomal regulation of the PKA holoenzyme and with calpain regulation of PKA activity in *Drosophila*, elevation of cAMP levels is required prior to proteolysis of R subunit.(8, 50) The calpain-dependent activation of mitochondrial C subunit originates from non-cAMP-dependent sources, since activation occurs in the presence of KH7 (see Figure 6), a soluble adenylate cyclase inhibitor.

In summary, we have demonstrated the digestion of the R subunits, but not the C subunits, of PKA in bovine heart mitochondria in the presence of digitonin and/or Ca²⁺. Furthermore, R subunit proteolysis is attenuated by the addition of calpain inhibitor ALLN. Exposure the mitochondria to Ca²⁺ induces cAMP independent PKA activity, which is blocked by calpain inhibitor I or by the PKA-specific inhibitor PKI. Metabolic inhibitors rotenone, sodium azide, antimycin A, and CCCP also elicit enhanced C subunit activity in a calpain-dependent fashion. Further studies are underway to elucidate the downstream effects of calpain-mediated PKA activation.

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Abbreviations

ALLN	calpain inhibitor I N-acetyl-Leu-Leu-Nle-CHO
C subunit	catalytic subunit
CCCP	chlorophenylhydrazine
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
kemptide	Gly-Arg-Arg-Ala-Ser-Leu-Gly
PKA	cAMP-dependent protein kinase
PKI	protein kinase inhibitor
R subunit	regulatory subunit
R₂C₂	PKA holoenzyme

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Figure 1. Calpain Digestion of Bovine PKA R and C Subunits

Calpain 1 was added to C or R (I and II) subunit in the presence or absence of ALLN. The reaction was initiated by the addition of variable concentrations of CaCl₂, and incubated at 37 °C for 20 min: R subunit (♦), C subunit (▲), R subunit and ALLN (●), and C subunit and ALLN (X).

Figure 2. Calpain Digestion of Intact Bovine R_2C_2 Holoenzyme

Calpain 1 was added to the R_2C_2 holoenzyme in the presence of 100 μ M $CaCl_2$ for 0.5, 1.5, and 2.0 h and the C ($\circ$) and R ($\bullet$) subunits subsequently quantified via western blot analysis (inset).

Figure 3. Digitonin and Ca²⁺-induced digestion of mitochondrial PKA

A. Bovine heart mitochondria incubated with digitonin in the presence or absence of ALLN where black bar = untreated mitochondria, dark gray bar = digitonin-exposed mitochondria, and the light gray bar = digitonin and ALLN (calpain inhibitor I)-exposed mitochondria. B. Bovine heart mitochondria incubated with digitonin and CaCl₂ in the presence or absence of ALLN where black bar = untreated mitochondria, dark gray bar = digitonin/CaCl₂-exposed mitochondria, and the light gray bar = digitonin/CaCl₂ and ALLN (calpain inhibitor I)-exposed mitochondria. C. Calpain activity (assessed utilizing the fluorescent substrate N-succinyl-Leu-Tyr-7-amido-4-methylcoumarin) from bovine heart mitochondria incubated with digitonin (black trace), digitonin and CaCl₂ (gray trace), or buffer alone (inset). D. Bovine heart mitochondria incubated with CaCl₂ in the presence or absence of ALLN as a function of time where the black trace is the rate of proteolysis of RI and the gray trace the rate of proteolysis of RII. Immunoblots were imaged using an AlphaInnotech FC2, and quantified using Fluorchem FC2 software. The data represents the mean of 3 experiments ± SEM.

Figure 4. Mitochondrial R and C subunit integrity in response to cAMP and calpain
Mitochondria were treated with (A) cAMP or (B) calpain I and CaCl_2 . Calpain-exposed mitochondria were examined in the presence or absence of ALLN. Immunoblots were imaged using an AlphaInnotech FC2, and quantified using Fluorchem FC2 software. Quantification of untreated (black bar), calpain I-exposed (dark gray bar), and calpain I/ALLN-exposed (light gray bar) mitochondria are shown in C. The data represents the mean of three experiments $\pm$ SEM.

Figure 5. Mitoplast-associated R subunit content from mitochondria treated with digitonin in the absence and presence of ALLN

Mitochondria were treated with digitonin (0.5 mg/mg protein) and phospholipase A in the presence or absence of ALLN (200 μ M) and incubated at 37°C for 2 h. The resultant mitoplasts were then pelleted at 11,000 $\times$ g for 15 min at 4 °C.

Figure 6. Ca²⁺-induced PKA activation: release of active C subunit

A. Mitochondria were treated with cAMP or CaCl₂, centrifuged, and the pellet and supernatant processed for western blot analysis. B. Mitochondria were incubated with the soluble adenylate cyclase inhibitor KH7 for 30 min, then treated with CaCl₂ in the presence or absence of ALLN, centrifuged, and the supernatant analyzed for C subunit by western blot: untreated (black bar), Ca²⁺-exposed (dark gray bar), and Ca²⁺/ALLN-exposed (light gray bar) mitochondria. C. PKA activity, using a previously described enzyme-coupled assay(21), in untreated (light gray trace) and cAMP-exposed (black trace) mitochondria. D. PKA activity in Ca²⁺-exposed (black trace), Ca²⁺/ALLN-exposed (light gray trace), and Ca²⁺/PKI-exposed mitochondria (dark gray trace).

Figure 7. Metabolic inhibitors induce activation of PKA in a calpain-dependent fashion

Mitochondria were exposed to oligomycin, antimycin A, rotenone, CCCP, or sodium azide in the presence or absence of ALLN for 30 min at 37 °C and subsequently centrifuged. The supernatants were analyzed by western blot for the presence of the C subunit. The relative amount of C subunit released from untreated (black bar), metabolic inhibitor-exposed (gray bar), and metabolic inhibitor/ALLN-exposed mitochondria. The data represents the mean of three experiments $\pm$ SEM.