BIOCHEMISTRY
including biophysical chemistry & molecular biology

Article

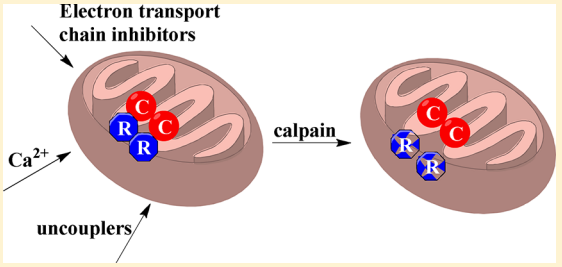
pubs.acs.org/biochemistry

Proteolytic Regulation of the Mitochondrial cAMP-Dependent Protein Kinase

Jennifer R. Shell and David S. Lawrence*

Departments of Chemistry, Chemical Biology and Medicinal Chemistry, and Pharmacology, University of North Carolina, Chapel Hill, North Carolina 27599, United States

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 IκB in an NF-κB-IκB-(C subunit) complex. Stimulation of cells with lipopolysaccharide, endotheln-1, or angiotensin II induces IκB degradation, resulting in the ensuing C subunit-catalyzed phosphorylation of NF-κB p65.[4,5] In addition, it has been recently reported that thrombin and collagen trigger the dissociation of the C subunit from IκB 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 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 to 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.

Received: October 12, 2011
Revised: January 31, 2012
Published: March 3, 2012

   dx.doi.org/10.1021/bi201573k | *Biochemistry* 2012, 51, 2258−2264

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 (3×), PBS (3×), and 0.5% NaCl (3×) 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 nonmitochondrial 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 of 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 11000*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 of whole mitochondria) were pelleted at 9500*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 9500*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 μM KH7 prior to use. Mitochondria were treated with $CaCl_2$ (10 μM, 100 μM), rotenone (10 μM), sodium azide (2 mM), antimycin A (5 μM), oligomycin (5 μM), or CCCP (5 μM) in the presence and absence of ALLN (500 μM) and incubated at 37 °C for 30 min. The samples were then centrifuged at 9500*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 μM) 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 μM $CaCl_2$, whereas the R subunit

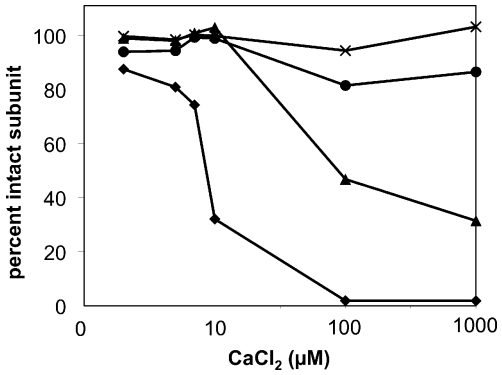


Figure 1. Calpain digestion of bovine PKA R and C subunits. Calpain 1 was added to C or R (I and II) subunit in the presence or absence of ALLN. The reaction was initiated by the addition of variable concentrations of $CaCl_2$ and incubated at 37 °C for 20 min: R subunit (◆), C subunit (▲), R subunit and ALLN (●), and C subunit and ALLN (×).

is ∼70% digested. However, if higher concentrations of Ca^{2+} (≥100 μM) 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

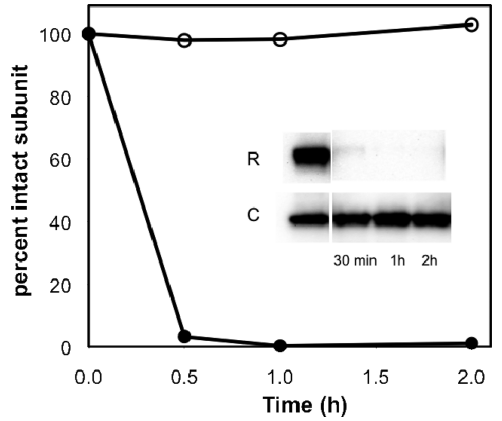


Figure 2. Calpain digestion of intact bovine R_2C_2 holoenzyme. Calpain 1 was added to the R_2C_2 holoenzyme in the presence of 100 μM $CaCl_2$ for 0.5, 1.5, and 2.0 h and the C (○) and R (●) subunits subsequently quantified via Western blot analysis (inset).

the R subunit of the holoenzyme suffers proteolysis. The C subunit remains completely intact, even though the conditions employed (100 μM 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 have 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 (Figure 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

dx.doi.org/10.1021/bi201573k | *Biochemistry* 2012, 51, 2258−2264

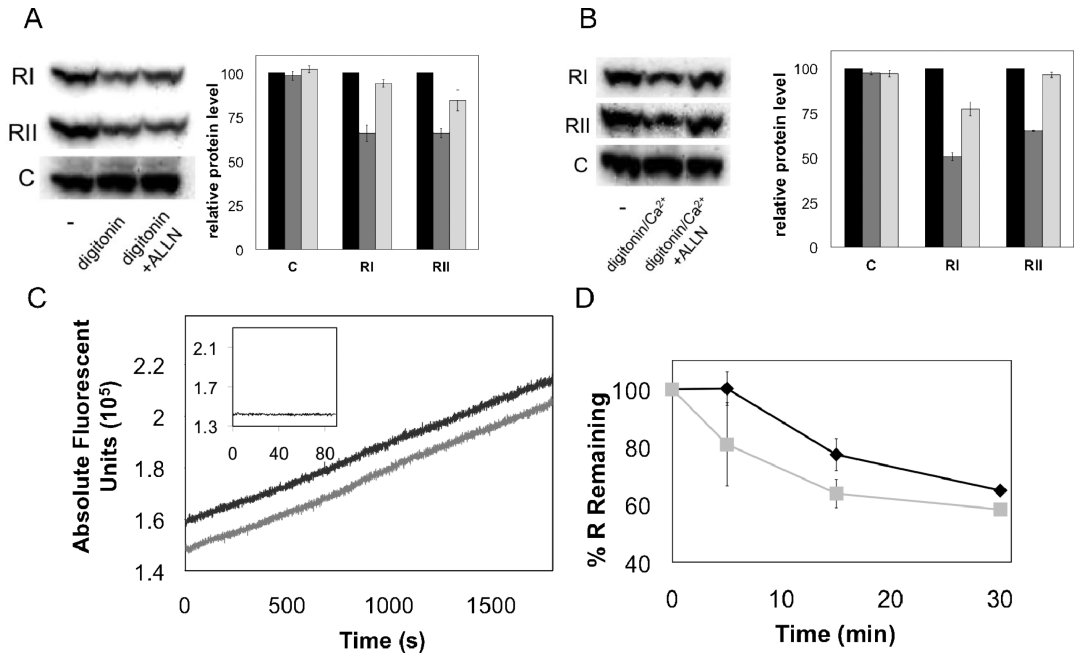


Figure 3. Digitonin and Ca^{2+}-induced digestion of mitochondrial PKA. (A) Bovine heart mitochondria incubated with digitonin in the presence or absence of ALLN where black bar = untreated mitochondria, dark gray bar = digitonin-exposed mitochondria, and the light gray bar = digitonin and ALLN (calpain inhibitor I)-exposed mitochondria. (B) Bovine heart mitochondria incubated with digitonin and $CaCl_2$ in the presence or absence of ALLN where black bar = untreated mitochondria, dark gray bar = digitonin/$CaCl_2$-exposed mitochondria, and the light gray bar = digitonin/$CaCl_2$ 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 $CaCl_2$ (gray trace), or buffer alone (inset). (D) Bovine heart mitochondria incubated with $CaCl_2$ 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.

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: LRRASLG + ATP → LRRApSLG + ADP; (ii) pyruvate kinase: ADP + phosphoenol pyruvate → ATP + pyruvate; (iii) lactate dehydrogenase: pyruvate + NADH → lactate + 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

dx.doi.org/10.1021/bi201573k | *Biochemistry* 2012, 51, 2258−2264

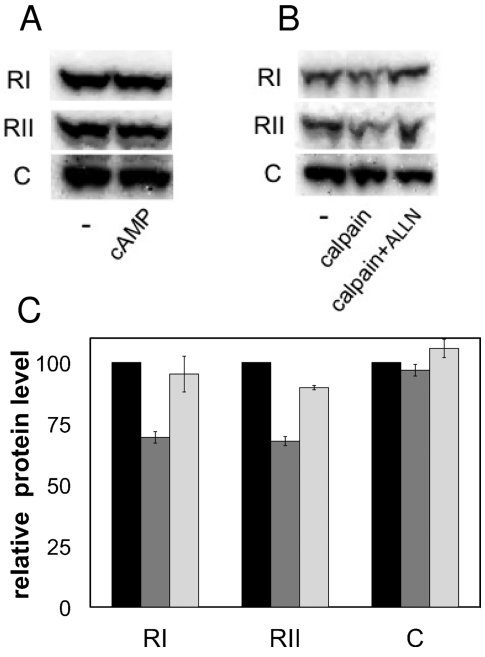


Figure 4. Mitochondrial R and C subunit integrity in response to cAMP and calpain. Mitochondria were treated with (A) cAMP or (B) calpain I and $CaCl_2$. Calpain-exposed mitochondria were examined in the presence or absence of ALLN. Immunoblots were imaged using an AlphaInnotech FC2 and quantified using 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 ± SEM.

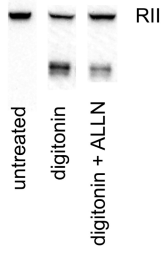


Figure 5. Mitoplast-associated R subunit content from mitochondria treated with digitonin in the absence and presence of ALLN. Mitochondria were treated with digitonin (0.5 mg/mg protein) and phospholipase A in the presence or absence of ALLN (200 μM) and incubated at 37 °C for 2 h. The resultant mitoplasts were then pelleted at 11000*g* for 15 min at 4 °C.

VDAC *in vitro*,[44] which may prevent the leakage of cytochrome *c* and thus inhibit apoptosis. The PKA-catalyzed phoshorylation 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

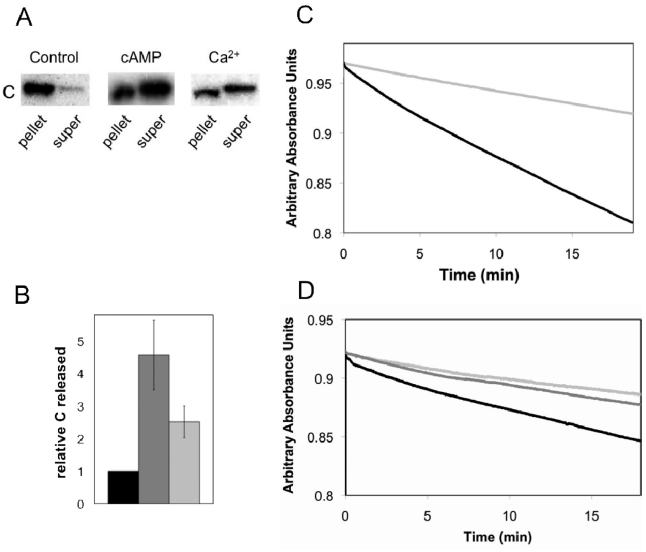


Figure 6. Ca^{2+}-induced PKA activation: release of active C subunit. (A) Mitochondria were treated with cAMP or $CaCl_2$, centrifuged, and the pellet and supernatant processed for Western blot analysis. (B) Mitochondria were incubated with the soluble adenylate cyclase inhibitor KH7 for 30 min, then treated with $CaCl_2$ in the presence or absence of ALLN, centrifuged, and the supernatant analyzed for C subunit by Western blot: untreated (black bar), Ca^{2+}-exposed (dark gray bar), and Ca^{2+}/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 Ca^{2+}-exposed (black trace), Ca^{2+}/ALLN-exposed (light gray trace), and Ca^{2+}/PKI-exposed mitochondria (dark gray trace).

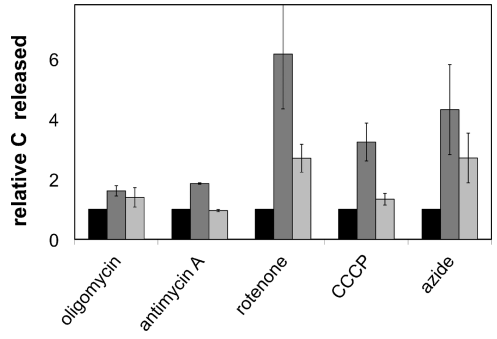


Figure 7. Metabolic inhibitors induce activation of PKA in a calpain-dependent fashion. Mitochondria were exposed to oligomycin, antimycin A, rotenone, CCCP, or sodium azide in the presence or absence of ALLN for 30 min at 37 °C and subsequently centrifuged. The supernatants were analyzed by Western 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 ± SEM.

(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

dx.doi.org/10.1021/bi201573k | *Biochemistry* 2012, 51, 2258–2264

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 proteolyisis 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 IκB.[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 Ca^{2+}. Furthermore, R subunit proteolysis is attenuated by the addition of calpain inhibitor ALLN. Exposure of mitochondria to Ca^{2+} 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.

AUTHOR INFORMATION

Corresponding Author

*E-mail: lawrencd@email.unc.edu.

Author Contributions

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

Funding

This project was supported by the National Institutes of Health [Grant CA79954].

Notes

The authors declare no competing financial interest.

ABBREVIATIONS

ALLN, calpain inhibitor I N-acetyl-Leu-Leu-Nle-CHO; C subunit, catalytic subunit; CCCP, chlorophenylhydrazone; 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; R_2C_2, PKA holoenzyme.

REFERENCES

(1) Manning, G., Whyte, D. B., Martinez, R., Hunter, T., and Sudarsanam, S. (2002) The protein kinase complement of the human genome. *Science 298*, 1912−1934.

(2) Gullingsrud, J., Kim, C., Taylor, S. S., and McCammon, J. A. (2006) Dynamic binding of PKA regulatory subunit RI alpha. *Structure 14*, 141−149.

(3) Kim, C., Xuong, N. H., and Taylor, S. S. (2005) Crystal structure of a complex between the catalytic and regulatory (RIalpha) subunits of PKA. *Science 307*, 690−696.

(4) Zhong, H., SuYang, H., Erdjument-Bromage, H., Tempst, P., and Ghosh, S. (1997) The transcriptional activity of NF-kappaB is regulated by the IkappaB-associated PKAc subunit through a cyclic AMP-independent mechanism. *Cell 89*, 413−424.

(5) Dulin, N. O., Niu, J., Browning, D. D., Ye, R. D., and Voyno-Yasenetskaya, T. (2001) Cyclic AMP-independent activation of protein kinase A by vasoactive peptides. *J. Biol. Chem. 276*, 20827−20830.

(6) Gambaryan, S., Kobsar, A., Rukoyatkina, N., Herterich, S., Geiger, J., Smolenski, A., Lohmann, S. M., and Walter, U. (2010) Thrombin and collagen induce a feedback inhibitory signaling pathway in platelets involving dissociation of the catalytic subunit of protein kinase A from an NFkappaB-IkappaB complex. *J. Biol. Chem. 285*, 18352−18363.

(7) Nguyen, P. V., and Woo, N. H. (2003) Regulation of hippocampal synaptic plasticity by cyclic AMP-dependent protein kinases. *Prog. Neurobiol. 71*, 401−437.

(8) Chain, D. G., Casadio, A., Schacher, S., Hegde, A. N., Valbrun, M., Yamamoto, N., Goldberg, A. L., Bartsch, D., Kandel, E. R., and Schwartz, J. H. (1999) Mechanisms for generating the autonomous cAMP-dependent protein kinase required for long-term facilitation in Aplysia. *Neuron 22*, 147−156.

(9) Liang, Z., Liu, F., Grundke-Iqbal, I., Iqbal, K., and Gong, C. X. (2007) Down-regulation of cAMP-dependent protein kinase by over-activated calpain in Alzheimer disease brain. *J. Neurochem. 103*, 2462−2470.

(10) Chestukhin, A., Muradov, K., Litovchick, L., and Shaltiel, S. (1996) The cleavage of protein kinase A by the kinase-splitting membranal proteinase is reproduced by meprin beta. *J. Biol. Chem. 271*, 30272−30280.

(11) Alhanaty, E., Patinkin, J., Tauber-Finkelstein, M., and Shaltiel, S. (1981) Degradative inactivation of cyclic AMP-dependent protein kinase by a membranal proteinase is restricted to the free catalytic subunit in its native conformation. *Proc. Natl. Acad. Sci. U. S. A. 78*, 3492−3495.

(12) Yan, R., Wang, Z., Yuan, Y., Cheng, H., and Dai, K. (2009) Role of cAMP-dependent protein kinase in the regulation of platelet procoagulant activity. *Arch. Biochem. Biophys. 485*, 41−48.

(13) Lebel, M., Patenaude, C., Allyson, J., Massicotte, G., and Cyr, M. (2009) Dopamine D1 receptor activation induces tau phosphorylation via cdk5 and GSK3 signaling pathways. *Neuropharmacology 57*, 392−402.

(14) Cong, M., Thompson, V. F., Goll, D. E., and Antin, P. B. (1998) The bovine calpastatin gene promoter and a new N-terminal region of the protein are targets for cAMP-dependent protein kinase activity. *J. Biol. Chem. 273*, 660−666.

(15) De Tullio, R., Cantoni, C., Broggio, C., Prato, C., Stifanese, R., Averna, M., Antolini, R., Pontremoli, S., and Melloni, E. (2009) Involvement of exon 6-mediated calpastatin intracellular movements in the modulation of calpain activation. *Biochim. Biophys. Acta 1790*, 182−187.

(16) Arrington, D. D., Van Vleet, T. R., and Schnellmann, R. G. (2006) Calpain 10: a mitochondrial calpain and its role in calcium-induced mitochondrial dysfunction. *Am. J. Physiol. Cell Physiol. 291*, C1159−1171.

(17) Kar, P., Samanta, K., Shaikh, S., Chowdhury, A., Chakraborti, T., and Chakraborti, S. (2010) Mitochondrial calpain system: an overview. *Arch. Biochem. Biophys. 495*, 1−7.

(18) Ozaki, T., Yamashita, T., and Ishiguro, S. (2009) Mitochondrial m-calpain plays a role in the release of truncated apoptosis-inducing factor from the mitochondria. *Biochim. Biophys. Acta 1793*, 1848−1859.
(19) Sardanelli, A. M., Signorile, A., Nuzzi, R., Rasmo, D. D., Technikova-Dobrova, Z., Drahota, Z., Occhiello, A., Pica, A., and Papa, S. (2006) Occurrence of A-kinase anchor protein and associated cAMP-dependent protein kinase in the inner compartment of mammalian mitochondria. *FEBS Lett. 580*, 5690−5696.
(20) Chen, Q., Lin, R. Y., and Rubin, C. S. (1997) Organelle-specific targeting of protein kinase AII (PKAII). Molecular and in situ characterization of murine A kinase anchor proteins that recruit regulatory subunits of PKAII to the cytoplasmic surface of mitochondria. *J. Biol. Chem. 272*, 15247−15257.
(21) Schwoch, G., Trinczek, B., and Bode, C. (1990) Localization of catalytic and regulatory subunits of cyclic AMP-dependent protein kinases in mitochondria from various rat tissues. *Biochem. J. 270*, 181−188.
(22) Agnes, R. S., Jernigan, F., Shell, J. R., Sharma, V., and Lawrence, D. S. (2010) Suborganelle sensing of mitochondrial cAMP-dependent protein kinase activity. *J. Am. Chem. Soc. 132*, 6075−6080.
(23) Cao, G., Xing, J., Xiao, X., Liou, A. K., Gao, Y., Yin, X. M., Clark, R. S., Graham, S. H., and Chen, J. (2007) Critical role of calpain I in mitochondrial release of apoptosis-inducing factor in ischemic neuronal injury. *J. Neurosci. 27*, 9278−9293.
(24) Norberg, E., Gogvadze, V., Ott, M., Horn, M., Uhlen, P., Orrenius, S., and Zhivotovsky, B. (2008) An increase in intracellular Ca2+ is required for the activation of mitochondrial calpain to release AIF during cell death. *Cell Death Differ. 15*, 1857−1864.
(25) Insel, P. A., Zhang, L., Murray, F., Yokouchi, H., and Zambon, A. C. (2011) Cyclic AMP is both a pro-apoptotic and anti-apoptotic second messenger, *Acta Physiol. (Oxford)*, in press.
(26) Rice, J. E., and Lindsay, J. G. (1997) in *Subcellular Fractionation* (Graham, J. M., and Rickwood, D., Eds.) pp 107−142, IRL Press, Oxford, England.
(27) Sasaki, T., Kikuchi, T., Yumoto, N., Yoshimura, N., and Murachi, T. (1984) Comparative specificity and kinetic studies on porcine calpain I and calpain II with naturally occurring peptides and synthetic fluorogenic substrates. *J. Biol. Chem. 259*, 12489−12494.
(28) Cook, P. F., Neville, M. E. Jr., Vrana, K. E., Hartl, F. T., and Roskoski, R. Jr. (1982) Adenosine cyclic 3′,5′-monophosphate dependent protein kinase: kinetic mechanism for the bovine skeletal muscle catalytic subunit. *Biochemistry 21*, 5794−5799.
(29) Jy, W., and Haynes, D. H. (1988) Calcium uptake and release characteristics of the dense tubules of digitonin-permeabilized human platelets. *Biochim. Biophys. Acta 944*, 374−382.
(30) Baines, C. P., Song, C. X., Zheng, Y. T., Wang, G. W., Zhang, J., Wang, O. L., Guo, Y., Bolli, R., Cardwell, E. M., and Ping, P. (2003) Protein kinase Cepsilon interacts with and inhibits the permeability transition pore in cardiac mitochondria. *Circ. Res. 92*, 873−880.
(31) Halestrap, A. P. (2009) What is the mitochondrial permeability transition pore? *J. Mol. Cell Cardiol. 46*, 821−831.
(32) Isenberg, J. S., and Klaunig, J. E. (2000) Role of the mitochondrial membrane permeability transition (MPT) in rotenone-induced apoptosis in liver cells. *Toxicol. Sci. 53*, 340−351.
(33) Pastorino, J. G., Snyder, J. W., Serroni, A., Hoek, J. B., and Farber, J. L. (1993) Cyclosporin and carnitine prevent the anoxic death of cultured hepatocytes by inhibiting the mitochondrial permeability transition. *J. Biol. Chem. 268*, 13791−13798.
(34) Seaton, T. A., Cooper, J. M., and Schapira, A. H. (1998) Cyclosporin inhibition of apoptosis induced by mitochondrial complex I toxins. *Brain Res. 809*, 12−17.
(35) Zoratti, M., and Szabo, I. (1995) The mitochondrial permeability transition. *Biochim. Biophys. Acta 1241*, 139−176.
(36) De Rasmo, D., Panelli, D., Sardanelli, A. M., and Papa, S. (2008) cAMP-dependent protein kinase regulates the mitochondrial import of the nuclear encoded NDUFS4 subunit of complex I. *Cell. Signalling 20*, 989−997.
(37) Technikova-Dobrova, Z., Sardanelli, A. M., Speranza, F., Scacco, S., Signorile, A., Lorusso, V., and Papa, S. (2001) Cyclic adenosine monophosphate-dependent phosphorylation of mammalian mitochondrial proteins: enzyme and substrate characterization and functional role. *Biochemistry 40*, 13941−13947.
(38) Acin-Perez, R., Salazar, E., Kamenetsky, M., Buck, J., Levin, L. R., and Manfredi, G. (2009) Cyclic AMP produced inside mitochondria regulates oxidative phosphorylation. *Cell Metab. 9*, 265−276.
(39) Harada, H., Becknell, B., Wilm, M., Mann, M., Huang, L. J., Taylor, S. S., Scott, J. D., and Korsmeyer, S. J. (1999) Phosphorylation and inactivation of BAD by mitochondria-anchored protein kinase A. *Mol. Cell 3*, 413−422.
(40) Lizcano, J. M., Morrice, N., and Cohen, P. (2000) Regulation of BAD by cAMP-dependent protein kinase is mediated via phosphorylation of a novel site, Ser155. *Biochem. J. 349*, 547−557.
(41) Sastry, K. S., Karpova, Y., Prokopovich, S., Smith, A. J., Essau, B., Gersappe, A., Carson, J. P., Weber, M. J., Register, T. C., Chen, Y. Q., Penn, R. B., and Kulik, G. (2007) Epinephrine protects cancer cells from apoptosis via activation of cAMP-dependent protein kinase and BAD phosphorylation. *J. Biol. Chem. 282*, 14094−14100.
(42) Zhou, X. M., Liu, Y., Payne, G., Lutz, R. J., and Chittenden, T. (2000) Growth factors inactivate the cell death promoter BAD by phosphorylation of its BH3 domain on Ser155. *J. Biol. Chem. 275*, 25046−25051.
(43) Martin, M. C., Allan, L. A., Lickrish, M., Sampson, C., Morrice, N., and Clarke, P. R. (2005) Protein kinase A regulates caspase-9 activation by Apaf-1 downstream of cytochrome c. *J. Biol. Chem. 280*, 15449−15455.
(44) Banerjee, J., and Ghosh, S. (2006) Phosphorylation of rat brain mitochondrial voltage-dependent anion as a potential tool to control leakage of cytochrome c. *J. Neurochem. 98*, 670−676.
(45) Kositprapa, C., Zhang, B., Berger, S., Canty, J. M. Jr., and Lee, T. C. (2000) Calpain-mediated proteolytic cleavage of troponin I induced by hypoxia or metabolic inhibition in cultured neonatal cardiomyocytes. *Mol. Cell. Biochem. 214*, 47−55.
(46) Batra, S. (1976) Mitochondrial calcium release as a mechanism for quinidine contracture in skeletal muscle. *Biochem. Pharmacol. 25*, 2631−2633.
(47) Bernardi, P., and Petronilli, V. (1996) The permeability transition pore as a mitochondrial calcium release channel: a critical appraisal. *J. Bioenerg. Biomembr. 28*, 131−138.
(48) Ogita, M., Ogita, A., Usuki, Y., Fujita, K., and Tanaka, T. (2009) Antimycin A-induced cell death depends on AIF translocation through NO production and PARP activation and is not involved in ROS generation, cytochrome c release and caspase-3 activation in HL-60 cells. *J. Antibiot. (Tokyo) 62*, 145−152.
(49) Crompton, M. (1999) The mitochondrial permeability transition pore and its role in cell death. *Biochem. J. 341* (Pt 2), 233−249.
(50) Muller, U., and Spatz, H. C. (1989) Ca2(+)-dependent proteolytic modification of the cAMP-dependent protein kinase in Drosophila wild-type and dunce memory mutants. *J. Neurogenet. 6*, 95−114.