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Probes of the Mitochondrial cAMP-dependent Protein Kinase

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Abstract

The development of a fluorescent assay to detect activity of the mitochondrial cAMP-dependent protein kinase (PKA) is described. A peptide-based sensor was utilized to quantify the relative amount of PKA activity present in each compartment of the mitochondria (the outer membrane, the intermembrane space, and the matrix). In the process of validating this assay, we discovered that PKA activity is regulated by the protease calpain. Upon exposure of bovine heart mitochondria to digitonin, Ca^{2+}, and a variety of electron transport chain inhibitors, the regulatory subunits of the PKA holoenzyme (R_2C_2) are digested, releasing active catalytic subunits. This proteolysis is attenuated by calpain inhibitor I (ALLN).

Keywords

signal transduction; sensor; fluorescent peptide; cAMP-dependent protein kinase (PKA); calpain

1. Introduction

Members of the protein kinase family have been implicated in numerous cell functions, ranging from ATP generation to cell growth and division.[1] Kinases catalyze the transfer of a phosphoryl group from ATP to the hydroxyl groups of serine, threonine, or tyrosine residues in proteins. The cAMP-dependent protein kinase (PKA) is a serine/threonine kinase that exists as an inactive tetrameric holoenzyme consisting of two regulatory subunits and two catalytic subunits. The conventional mode of activation of PKA involves the binding of cAMP to the regulatory subunits, causing release of the catalytic subunits, which then phosphorylate a myriad of proteins.[2, 3] PKA is anchored to a variety of intracellular locations via interaction with A-kinase anchoring proteins (AKAPs).

PKA activity at the mitochondria is associated with the regulation of apoptosis, mitochondrial respiration, and ATP synthesis.[4-6] PKA phosphorylates the proapoptotic protein BAD, which prevents cell death.[5] PKA also phosphorylates apoptotic protease-activating factor (Apaf-1), which inhibits the formation of the apoptosome and activation of caspase-9.[7] In addition, PKA increases mitochondrial respiration via phosphorylation of subunits contained within complexes I and IV.[4] Although it is well known that PKA is present at the mitochondria, the relative amount of enzyme present in each compartment

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(outer membrane, intermembrane space, matrix) remains unclear. Orr and colleagues demonstrated that type II PKA is located on the outer membrane of mitochondria in male germ cells.[8] However, PKA has also been shown to be associated with the inner membrane/matrix.[9, 10] Most of these studies employed electron microscopy to pinpoint the suborganelle location of the holoenzyme. However, since the catalytic subunit can diffuse through membranes [11], holoenzyme location as assessed by electron microscopy may not represent the location of the active enzyme. Given this information we sought to develop an assay that would quantify the relative amounts of PKA activity present in each major compartment of the mitochondria.

2. Development of a Fluorescent Sensor for Mitochondrial PKA Activity

Fluorescent sensors of protein kinase activity furnish a direct means to assess catalytic action in a continuous fashion.[12] However, in many instances, the fluorescent response is modest, thereby necessitating the use of large amounts of sensor to ensure a measureable signal. Consequently, we sought to develop a sensor with a large dynamic range, thereby reducing the quantity of sensor required for signal detection and thus the perturbation on the biological system under scrutiny. We employed three coumarin derivatives as the kinase-responsive fluorophores [13]. These fluorophores were appended to the N-terminus of peptides of the general structure coumarin-Aoc-GRTGRRFSYP-amide (**1-3**, Figure 1, Aoc = aminooctanoic acid). We anticipated that negatively charged fluorescent quenchers would interact with the positively charged peptide, resulting in the loss of coumarin fluorescence. However, upon phosphorylation the peptide interacts with a phosphoserine-binding 14-3-3 domain, displacing the quencher, and resulting in a burst of fluorescence (Scheme 1). Peptides **1 - 3** were screened with a variety of negatively charged dyes. Acid green 27 (**4**, Figure 1) furnishes a deep fluorescent quench as well as a dramatic PKA-induced fluorescence increase, with peptide **1** displaying a remarkable 152-fold fluorescence enhancement (Table 1).

We employed the strategy depicted in Figure 2 to assess the location of PKA activity in bovine heart mitochondria. Perhaps the most straightforward way to achieve this would be to subfractionate the mitochondria (i.e. separate the outer membrane, the intermembrane space, and the matrix) and assess the activity of each compartment. However, digitonin is typically used to remove the outer membrane and release the contents of the intermembrane space. Digitonin has been demonstrated to cause leakage of matrix proteins in bovine heart mitochondria,[14] thereby contaminating the intermembrane space fraction, and resulting in an inaccurate assessment of relative amount of protein. Therefore, intact bovine heart mitochondria were treated with cAMP, and PKA activity assessed utilizing the assay described above with peptide **1**. Since the outer membrane of mitochondria is permeant to small molecules less than 5000 Da, including peptide **1**, this measures the PKA activity at both the outer membrane and intermembrane space. Proteolysis of the outer membrane proteins with trypsin prior to assessing PKA activity distinguishes outer membrane activity from intermembrane space activity. Sonication of whole mitochondria followed by treatment with cAMP affords the total PKA activity (outer membrane, intermembrane space, matrix). With information in hand, the relative percentage of PKA activity can be calculated for each major compartment: 85% in the matrix, 6% in the intermembrane space, and 9% on the outer membrane.[14]

3. Proteolytic Regulation of Mitochondrial PKA

While attempting to subfractionate bovine heart mitochondria with digitonin we observed leakage of matrix proteins and proteolysis of both isoforms of the regulatory subunits (RI and RII).[15] By contrast, the catalytic subunit (C) remained intact. Since multiple isoforms

of calpain, a Ca^{2+}-activated protease, have recently been reported to reside in the mitochondria,[16, 17] we investigated whether this protease played a role in the digestion of the PKA R subunits. Indeed, attenuation of digitonin-induced R subunit proteolysis was observed upon the addition of calpain inhibitor I (N-acetyl-Leu-Leu-Nle-al, ALLN) (Figure 3a). Furthermore, addition of Ca^{2+} enhances this proteolysis, consistent with involvement of a Ca^{2+}-dependent protease (Figure 3b). We observed direct activation of mitochondrial calpain by digitonin and Ca^{2+} using a fluorescent methyl-coumarin substrate (Figure 3c).

To further confirm this calpain-mediated selective proteolysis, isolated R and C subunits were subjected to calpain I digestion.[15] Both are prone to digestion; however, the amount of $CaCl_2$ drastically affects selectivity. The R subunit is selectively digested with 10 μM $CaCl_2$; whereas the C subunit also suffers partial proteolysis at Ca^{2+} concentrations ≥ 100 μM (Figure 4a). This suggests that, under physiological Ca^{2+} concentrations (<100 μM), the R subunit can be selectively digested, thereby releasing free C subunit. The holoenzyme R_2C_2 was also subjected to digestion by calpain I. Interestingly, proteolysis of the R subunit, but not the C subunit, even occurs at 100 μM $CaCl_2$, implying that the C subunit is more resistant to proteolysis while in holoenzyme form (Figure 4b). One possible explanation is the formation of an R subunit fragment that protects the C subunit from calpain-mediated proteolysis.

We also investigated whether this selective R subunit proteolysis releases active C subunit from the mitochondria into the supernatant. Mitochondria were treated with either cAMP (2 mM) or Ca^{2+} (100 μM) in the presence of soluble adenylate cyclase inhibitor KH7 (25 μM), centrifuged, and the supernatant examined for release of active C subunit. Western blot analysis of the supernatant shows that C subunit is released into the supernatant upon exposure to either Ca^{2+} or cAMP. Furthermore, the calpain inhibitor ALLN attenuates Ca^{2+}-induced release of C subunit into the supernatant, consistent with a calpain-dependent process (Figure 5). Finally, we confirmed that the released C subunit is catalytically active.

Since Ca^{2+} is a known inducer of the mitochondrial pore transition (MPT)[18], we decided to investigate whether other reagents that induce MPT also activate PKA in a calpain-dependent fashion. Both electron transport chain inhibitors and uncouplers have been linked to induction of MPT[19-22]. Mitochondria were exposed to rotenone (complex I inhibitor), antimycin A (complex III inhibitor), sodium azide (complex IV inhibitor), oligomycin (F_1F_0 ATPase inhibitor) as well as the uncoupler CCCP in the presence and absence of ALLN, centrifuged, and the supernatant examined for released C subunit by Western blot. Exposure to rotenone, azide and CCCP elicit the largest enhancements in PKA activity, all of which are attenuated by ALLN (Figure 6). Antimycin A and oligomycin also induced PKA activity, but to a lesser extent. In addition, the activity elicited by oligomycin proved to be calpain independent (Figure 6).

4. Conclusion

A PKA sensor with a large dynamic range (a phosphorylation-induced 152-fold enhancement in fluorescence) has been used to assess the suborganelle distribution of mitochondrial PKA activity. We found that PKA activity predominantly resides in the matrix (85%), although detectable amounts are present on the outer membrane (9%) and in the intermembrane space (6%).[14] We note that a variety of genetically-encoded protein kinase sensors have been described, most commonly by a mechanism in which phosphorylation induces conformational change and a subsequent florescent readout via a pair of FRET-based green fluorescent proteins.[23] Although a detailed comparison of the relative attributes of peptide-based and genetically-encoded sensors is beyond scope of this brief review, we do note that peptide-based systems can readily be prepared with extremely

bright fluorophores that emit between the long UV and the near IR. Furthermore, the large dynamic range enjoyed by the sensor described in this study exemplifies the possibilities inherent in a system that is essentially non-fluorescent in its unphosphorylated state.

During the validation of this assay, we discovered that PKA activity is regulated in a calpain-dependent fashion.[15] Furthermore, metabolic inhibitors elicit enhanced PKA activity in bovine heart mitochondria by this mechanism. Azide (complex IV inhibitor) and rotenone (complex I inhibitor) are the most robust calpain-mediated activators of PKA identified in this study. Interestingly, treatment of neurons and cadiomyocytes with azide and rotenone is known to result in the activation of calpain. [24, 25] Consequently, it is tempting to speculate that stress-induced MPT and Ca^{2+} release [26] promotes the activation of calpain, the subsequent selective degradation of the R subunits of the PKA holoenzyme, and release of active C subunit. The induction of PKA activity by mitochondrial stress could be a protective mechanism since PKA action is known to inhibit apoptosis [27] and promote enhanced respiration rates [28,29].

Abbreviations

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

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Highlights

- Development of a fluorescent sensor for PKA with a large dynamic range
- Determination of the suborganelle location of mitochondrial PKA activity
- Discovery of a proteolytic regulation of mitochondrial PKA activity

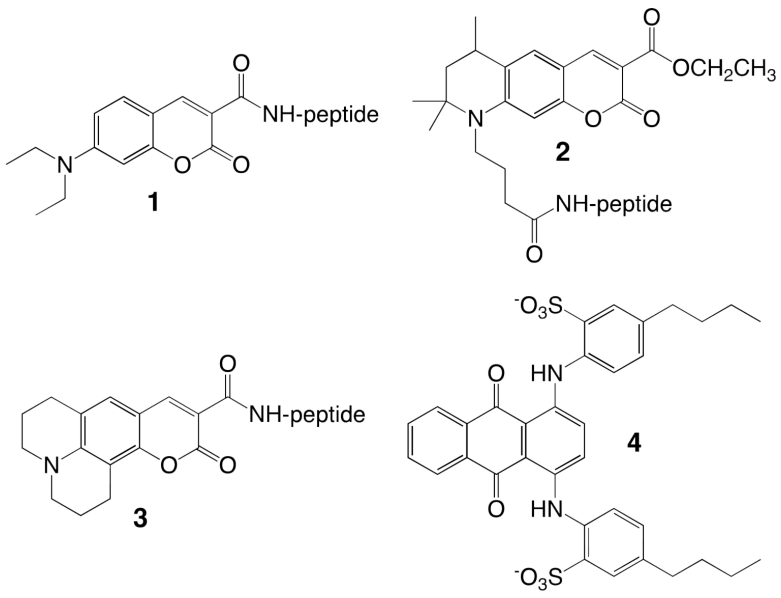
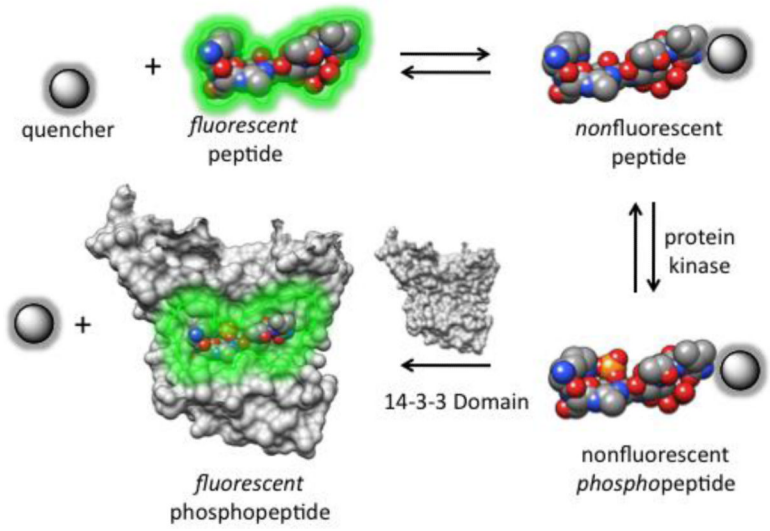


Fig. 1.

Structures of the coumarin derivatives **1 – 3** of the general form fluorophore-Aoc-GRTGRRFSYP-amide. The fluorescent quencher Acid Green 27 (**4**) was identified from a library of forty-seven dyes. Reprinted with permission from [14]. Copyright 2010 American Chemical Society.



Scheme 1.

Protein kinase-catalyzed phosphorylation of a fluorescently quenched peptide generates a fluorescent response in the presence of the phosphoSer-binding 14-3-3 domain. Reprinted with permission from [14]. Copyright 2010 American Chemical Society.

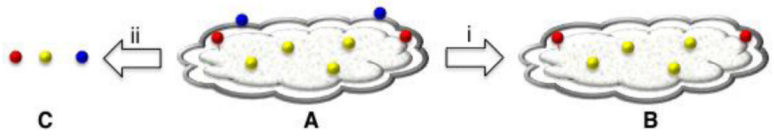


Fig. 2.

Strategy for assessing PKA activity on the outer membrane (blue), in the intermembrane space (red) and in the matrix (yellow). PKA activity of intact mitochondria (**A**) is due to enzyme present on the outer membrane and in the intermembrane space. Trypsinized (i) mitochondria (**B**) lack outer membrane proteins and thus only intermembrane space PKA is present. Sonicated (ii) mitochondria (**C**) furnishes enzyme from all three compartments and thus represents total mitochondrial PKA. Reprinted with permission from [14]. Copyright 2010 American Chemical Society.

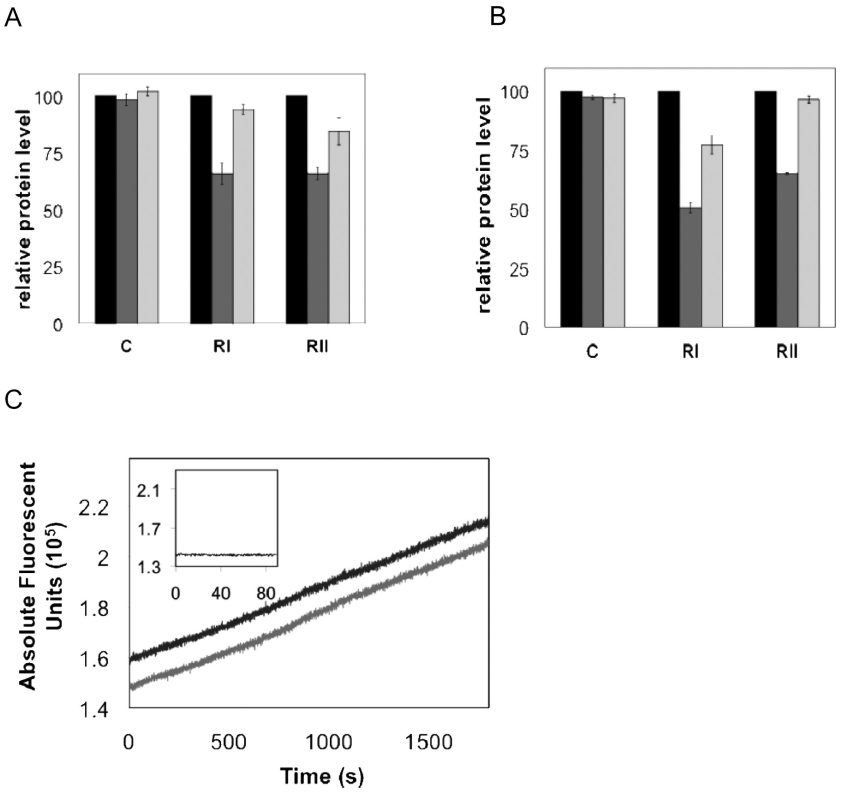


Fig. 3.

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). Reprinted with permission from [15]. Copyright 2012 American Chemical Society.

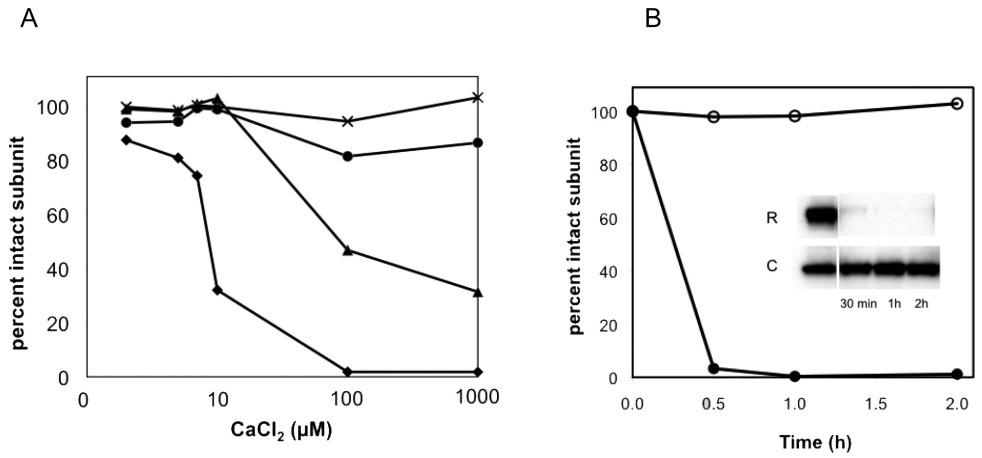


Fig. 4.

Calpain digestion of C, R, and R_2C_2. A. 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 (**X**). B. 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). Adapted with permission from [15]. Copyright 2012 American Chemical Society.

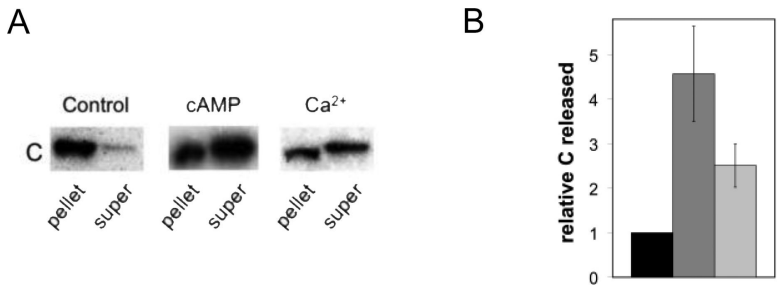


Fig. 5.

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. Quantification of untreated (black bar), Ca^{2+}-exposed (dark gray bar), and Ca^{2+}/ALLN-exposed (light gray bar) mitochondria. Adapted with permission from [15]. Copyright 2012 American Chemical Society.

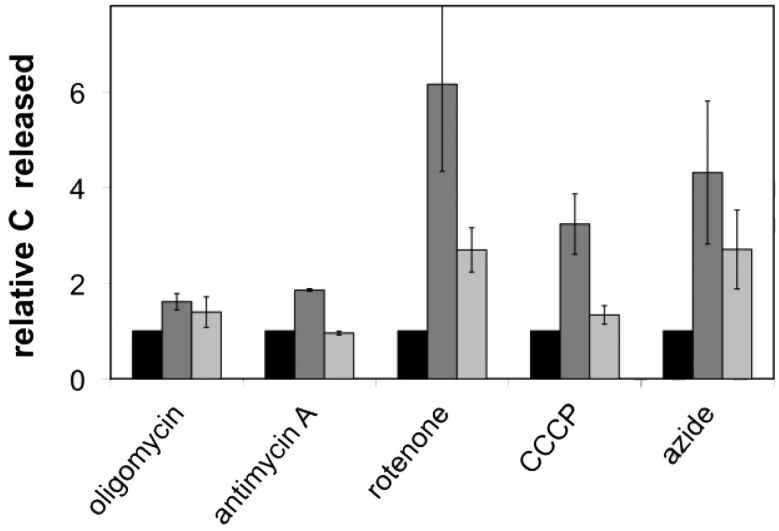


Fig. 6.

Metabolic inhibitors induce activation of PKA in a calpain-dependent fashion. Mitochondria were exposed to oligomycin (5 μM), antimycin A (5 μM), rotenone (10 μM), CCCP (5 μM), or sodium azide (2 mM) in the presence or absence of ALLN (500 μM) 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. Reprinted with permission from [15]. Copyright 2012 American Chemical Society.

Table 1

Photophysical properties, fluorescent fold increase, K_m, and V_{max} for the PKA-catalyzed phosphorylation of sensors **1 – 3** (where sensor = Fluorophore-Aoc-GRTGRRFSYP-amide). Kinetic properties were acquired in the presence of quencher **4** and the 14-3-3 domain. Reprinted with permission from [14]. Copyright 2010 American Chemical Society.

Sensor ($\lambda_{ex}/\lambda_{em}$)	Fluorescent Fold-Increase	K_m (μM)	V_{max} (μmol/min·mg)
1 (420/475 nm)	152	2.2 ± 0.1	0.53 ± 0.03
2 (437/477 nm)	150	1.9 ± 0.1	0.34 ± 0.04
3 (450/490 nm)	28	6.2 ± 0.1	0.20 ± 0.09