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Mitochondrial VDAC can be phosphorylated by cyclic AMP-dependent protein kinase†

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The voltage dependent anion channel (VDAC) of the outer membrane of mitochondria is thought to play a role in transport of metabolites including ATP across mitochondrial membrane and modulate mitochondrial functions such as respiration. However, regulation of this anion channel is only poorly understood. In this paper we demonstrate that VDAC purified from rat liver mitochondria can be phosphorylated by the catalytic subunit of cAMP dependent protein kinase (PKA). PKA phosphorylates VDAC linearly up to fifteenfold in sixty minutes. The level of VDAC phosphorylation increases to twofold and sevenfold of control value after ten and thirty minutes of reaction, respectively. Data presented here suggest the possibilty that voltage dependent anion channel of the outer membrane of mitochondria may be a target of PKA in vivo.

The mitochondrial porin, also known as the mitochondrial megachannel, isolated from a large number of sources exhibit very similar properties such as formation of voltage dependent anion channel (VDAC) in mitoplasts or artificial lipid bilayers. VDAC has been well-characterised biophysically. In rat liver mitochondria, VDAC has a molecular weight of about 35kDa [1], a pore size of approximately 1.7nm with an exclusion limit of 2-8kDa and a high single channel conductance [see refs. 2-4]. Due to its large conductance and its location in the outer membrane bordering the mitochondrial inner membrane and the cytoplasm,VDAC has been suggested to play a role in transmitochondrial metabolite transport [see ref. 5].

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Evidence that VDAC may have a role in respiratory processes reinforces this view [6]. However, there is paucity of data regarding regulation of VDAC through second messenger-mediated systems. The data presented in this paper demonstrate that it is possible to modify VDAC covalently by phosphorylation of purified VDAC molecules using catalytic subunit of cAMP-dependent protein kinase.

Materials and Methods

Chemicals: Hydroxylapatite, all chemicals for gel electrophoresis and molecular weight markers were obtained from BioRad Inc., (USA). Celite-545 was purchased from Loba Chemie (India). Triton X-100, HEPES, ATP, magnesium acetate, bovine serum albumin and catalytic subunit of cAMP dependent protein kinase from bovine heart (PKA) were obtained from Sigma Chemical Co. (USA). All other chemicals were of highest analytical grade commercially available. $\gamma[^{32}P]$-ATP (specific activity 3500 Ci/mMole) was obtained from Bhabha Atomic Research Center (India).

Purification of VDAC: Purification of VDAC was carried out by the method reported in [1]. Briefly, mitochondrial membranes from rat liver were prepared and solubilized by 3% Triton X-100, 1 mM EDTA and 10 mM Tris-HCl, pH 7.0 at a final protein concentration of 5 mg/ml. After 30 minutes at 0°C, the solubilization mixture was centrifuged at 18,000 rpm for 30 minutes and the supernatant was collected. The supernatant was applied on a dry hydroxylapatite/celite (2:1) column (1cm x 10cm). Elution was performed with the solubilization buffer and initial fractions containing the mitochondrial porin (as judged by SDS-polyacrylamide gel electrophoresis) were collected .

Phosphorylation Reaction: The eluate from hydroxylapatite-celite column containing VDAC was mixed with equal volume of cold acetone and kept at -20°C for 1 hour. After centrifugation in a microfuge at 10,000g, the pellet was collected, acetone was evaporated and the pellet was resolubilised in reaction buffer containing 5mM magnesium acetate in 10mM HEPES buffer at pH 7.4 to a final protein concentration of 1.5mg/ml. Each reaction vial (20µl final volume) contained: 6µg VDAC, 6.3 picomolar unit of catalytic subunit of PKA, 10µM Mg^{2+}-ATP, 5mM Mg^{2+} acetate and 10mM HEPES at pH 7.4. The reaction mixture containing VDAC and PKA in HEPES buffer was incubated at 30°C for 15 minutes. The phosphorylation reaction was started by addition of ATP from a stock solution prepared by mixing 4µl cold ATP (1mM), 36µl double distilled water and 20µl $\gamma[^{32}P]$-ATP (specific activity 3500 Ci/mMole). The reaction mixtures were incubated at 30°C for indicated amount of times. The reaction was stopped by adding 7µl sample buffer (4X) for SDS-polyacrylamide gel electrophoresis. The bands corresponding to pure VDAC (33KDa) were cut out from coomassie blue stained gels, dissolved in scintillation fluid and counted in a β counter (LKB, USA). Autoradiograms were produced by exposing X-ray films (INDU, India) to the dried gels in a cassette with intensifying screen at -70°C for various amounts of time.

SDS-Polyacrylamide Gel Electrophoresis: SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to [7] with minor modifications. Samples were denatured by adding appropriate amount of sample buffer (4X) and subsequently incubated at 95°C for three minutes before loading on the gel (12.5%). After the run, the gels were fixed immediately and washed several times with 15% orthophosphoric acid solution to remove free ATP. The gels were then stained with coomassie blue and subsequently destained using standard procedure.

Results and Discussion

VDAC obtained by the method described in ref. [1] was free of any other cntaminant proteins as judged by the single band in the SDS-PAGE profile (Fig. 1, lane b). Other proteins associated with the crude membrane preparation obtained from rat liver mitochondria (fig. 1, lane c) were eliminated. As reported earlier [1], this method yields completely purified VDAC as no other

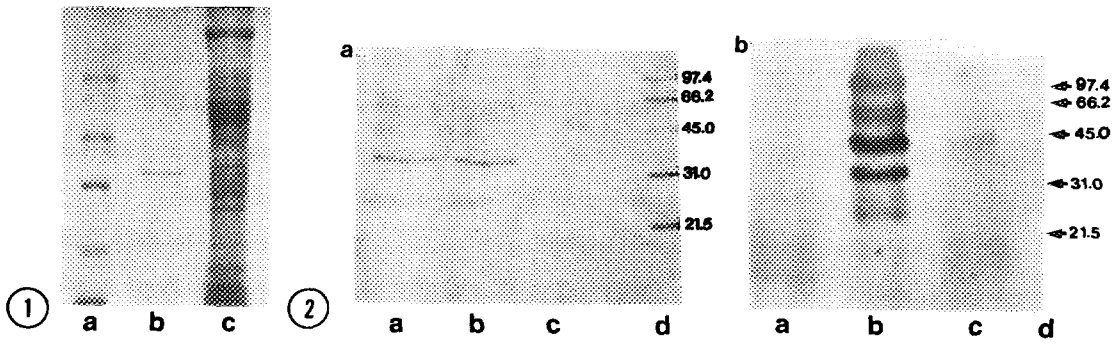

FIG. 1. **SDS-PAGE profile of purified VDAC and crude midochondrial preparation.** Samples were processed as detailed in *materials and methods*. The polyacrylamide gel (12.5%) was stained with coomassie blue and destained using standard procedures. In each lane total protein loaded is mentioned in bracket. *Lane a*: Molecular weight markers, from top to bottom, (M_r in kilodaltons): 94, 67, 43, 30, 20.1 and 14 (6μg each); *Lane b*: Purified VDAC (6μg); *Lane c*: Crude mitochondrial preparation used as starting material for VDAC purification (50μg).

FIG. 2. **Phosphorylation of VDAC by catalytic subunit of PKA.**
a) SDS-PAGE of phosphorylated and unphosphorylated VDAC on 12.5% polyacrylamide gel. b) Autoradiogram of the same gel.
Notice high degree of phosphorylation of the VDAC band (mol. wt. 35kDa approx).
From left to right: *lane a*, Control VDAC (unphosphorylated); *lane b*, VDAC (phosphorylated) with PKA; *lane c*, catalytic subunit of PKA; *lane d*, low molecular weight markers.

contaminant proteins could be detected when high amount of purified protein was loaded on SDS-PAGE gels. When the purified VDAC was phosphorylated using the catalytic subunit of PKA, the band corresponding to purified VDAC was observed to be highly phosphorylated in the autoradiogram (fig. 2, lane b). When the band corresponding to phosphorylated VDAC was cut out from the gel and subjected to limited proteolysis using V-8 protease from S. aureus or pronase, multiple phosphorylated bands were observed (data not shown), confirming covalent attachment of [^{32}P] with VDAC. This indicates that purified VDAC is a substrate for PKA. Under the same conditions, in the control, where no PKA was added (fig. 2, lane a), VDAC phosphorylation was negligible indicating absence of any endogenous protein kinase activity associated with the purified preparation. PKA, however showed some amount of autophosphorylation (band around 42 KD, fig. 2, lane c). Interestingly, several other bands were observed on the autoradiogram (fig. 2, lane b), which are not visible with coomassie blue stain. Some of these bands may correspond to high molecular weight aggregates of phosphorylated VDAC. It may be mentioned here again that this method of purification yields pure mitochondrial porin essentially free of any other contaminant proteins [1].

Radioactivity associated with the band corresponding to VDAC after 30 minutes of starting the phosphorylation reaction by addition of γ[^{32}P]-ATP, was approximately six fold of that of

the control (fig. 3). In this experiment, VDAC at the start of the phosphorylation reaction was taken as the control. On doing a time course of the reaction, it was observed that the amount of phosphorylation increases almost linearly upto sixty minutes (fig. 4) and reaches a plateau between 70 to 90 minutes (data not shown). The increase in phosphorylation with time may be due to presence of multiple phosphorylation sites or due to slow phosphorylation of a single available site.

Data presented above demonstrates phosphorylation of purified VDAC by PKA. There is some evidence that various metabolites e.g. NADH etc may directly affect the single channel properties of VDAC [8]. Recently specific ^{32}P-labelling of purified bovine skeletal muscle VDAC in a stoichiometric ratio (1:1) has been reported [9], although precise mechanism of the phenomenon is not clear. This is the first report that we are aware of, which clearly demonstrates the suitability of purified VDAC as a substrate of a protein kinase which is known to play important role in hormonal regulation of cellular response in many systems. Presently we are investigating the effects of VDAC phosphorylation on its single channel characteristics.

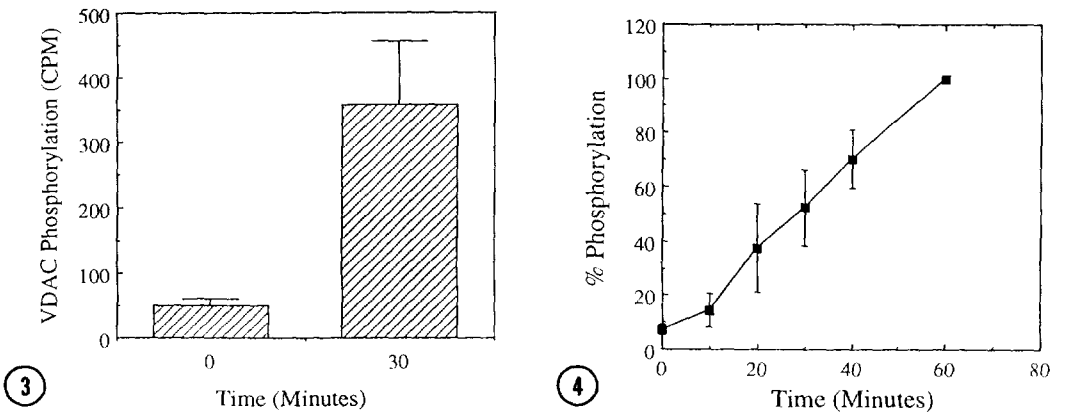

FIG. 3. **VDAC can be phosphorylated by the catalytic subunit of PKA.**
After running the phosphorylation reaction for indicated times, the reaction mixtures were subjected to SDS-PAGE as detailed in *materials and methods*. 6 μg purified VDAC was loaded in each of the lanes *a* and *b* (see fig. 2 legend). The bands corresponding to VDAC were cut out, solubilized in scintillation fluid and counted. The abscissa gives the amount of ^{32}P-radioactivity in counts per minute, associated with the VDAC band at zero and thirty minutes after the start of the reaction. The data indicate approximately sevenfold phosphorylation of the VDAC band in thirty minutes. The vertical bars represent standard deviation of the mean of six independent experiments.

FIG. 4. **Time course of VDAC phosphorylation by the catalytic subunit of cAMP-dependent protein kinase.**
Phosphorylated bands were cut out and counted at various times as detailed in legend of fig. 3 and *materials and methods*. Total phosphorylation after sixty minutes of starting the phosphorylation reaction was taken as 100%. The vertical bars represent standard deviation of the mean of six independent experiments. The curve reaches a plateau between 70 to 90 minutes after starting the reaction (data not shown).

The functional implications of such an event are many. VDAC is thought to play a role in transport of metabolites from mitochondria to cytoplasm [5]. Mitochondrial creatine kinase and hexokinase are known to be associated with VDAC at the contact sites between inner and outer mitochondria [10]. Transport of ATP and/or operation of the creatine/creatine phosphate shuttle through VDAC [5] thus may conceivably be affected directly due to the structural modification of VDAC by phosphorylation.Demonstration of VDAC as a target of phosphorylation by one of the widely present class of protein kinases, i.e. cAMP-dependent protein kinase, therefore points to the possibility of regulation of metabolite transport across mitochondrial membrane by protein kinases in response to intra- or extracellular signals.

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