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CHARACTERIZATION AND PARTIAL PURIFICATION OF A MEMBRANE PROTEIN FROM RABBIT SKELETAL MUSCLE WHICH INHIBITS THE cAMP-DEPENDENT PROTEIN KINASE

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SUMMARY. Rabbit skeletal muscle membranes contain a protein which inhibits the cAMP-dependent protein kinase. The activity of the partially purified membrane protein is characterized by an IC-50 of 10 to 30 nM with respect to the inhibition of the activity of the catalytic subunit of the cAMP-dependent protein kinase and is sensitive to treatment with heat, acid, alkali and trypsin. The active fractions contain proteins ranging from 40 to 120 kDa, analysed by SDS-gel electrophoresis.

PKA is a key enzyme in G-protein mediated signal transduction (1). Phosphorylation of target proteins by PKA can result in multiple metabolic changes or activation of transcription factors that directly interact with corresponding enhancer sequences on eukaryotic DNA (2). PKA activity may be modulated by the level of cAMP (3), the presence of the regulatory subunit (4), counteracting protein phosphatases like calcineurin (5) and inhibitory proteins like the protein kinase inhibitor (6;7). All PKA-inhibitory proteins, known so far, are located in the soluble fraction (6-8). Here we describe the partial purification and characterization of a membrane protein which inhibits the catalytic activity of the PKA.

MATERIAL AND METHODS

Partial purification of the inhibitory protein. Membranes from rabbit skeletal muscle were solubilized using digitonin and then further purified using glycoprotein-affinity chromatography as described previously (9). The glycoprotein fraction was loaded onto

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Abbreviations: FSBA, 5'-[p-(fluorosulfonyl)benzoyladenosine; IC-50, inhibitor concentration, where 50 % inhibition occurs; PKA, cAMP-dependent protein kinase; WGA, wheat germ agglutinin.

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a MonoQ-superose column (1 ml) and washed with 10 volumes of buffer A (0.05 % digitonin, 10 % glycerol, 1 mM $CaCl_2$, 1 mM iodoacetamide, 0.1 mM phenylmethylsulfonyl flouride, 0.1 mM benzamidine, 1 µM pepstatin A, 40 mM Tris-HCl, pH 7.4). Bound proteins were eluted stepwise using 10 volumes of 75, 150, 250, 500 and 1000 mM KCl in buffer A and concentrated as described (9). The 75 mM KCl-eluate of the MonoQ-column was further purified by gel filtration using a 24 ml-column of Superose 12. Elution was performed with buffer A.

Assay for PKA activity. The activity of the PKA was determined according to Bechtel *et al.* (10) in buffer B (40 mM Tris-HCl; 1 mM EGTA; 5 m $MgCl_2$; pH 7.4) containing 30 - 50 µU/ml of the catalytic subunit of PKA (Sigma), Histone IIa (0.3 mg/ml) and the digitonin-extracts or corresponding buffers. The reaction was started by addition of 0.1 mM γ-^{32}P-ATP (1 - 5 Ci/mol) and terminated with 10 % (w/v) trichloroacetic acid. The acid-precipitated protein was collected on nitrocellulose and the ^{32}P-phosphate incorporation was determined by Cerenkov counting.

Determination of ATP-hydrolysis. The ATP-hydrolysis by the inhibitory fractions under the conditions of the protein kinase assay was calculated by measuring the inorganic phosphate liberation (11).

Treatment of the inhibitory fractions with FSBA . FSBA-treatment of inhibitory fractions was performed according to Zoller and Taylor (12). Reactions were terminated by tenfold dilution with 40 mM Tris-HCl, pH 7.4 followed by freezing.

Gel electrophoresis. Gel electrophoresis was performed according to Laemmli (13) using a discontinuous polyacrylamide gel system (5 % stacking gel, 7.5 % running gel). The running gel was silver-stained (14). Protein was determined according to Bradford (15) using bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Partial purification of an inhibitor of PKA. An inhibitory activity, tested against the catalytic subunit of PKA, was purified as described under 'Material and Methods'. The data concerning the recovery and enrichment of the inhibitor are summarized in table 1.

Table 1. *Purification of the protein kinase inhibitor.* Digitonin-extracted skeletal muscle membranes were purified using WGA-affinity and anion-exchange chromatography. The enrichment and recovery of the inhibitory activity, tested against the PKA activity, was determined. The recovery data for protein and inhibitor are given as the mean ± standard deviation of 3 purifications.

Step	Inhibitory activity		Protein
	enrichment	recovery %	recovery %
Digitonin extract	1	100	100
WGA-affinity chromatography	25	31 ± 15	1.2 ± 0.1
Anion-exchange chromatography	167	22 ± 10	0.14 ± 0.03

Table 2. *Treatment of the inhibitory fractions with FSBA*: The 75 mM KCL eluate of the MonoQ-superose column was incubated at room temperature with 2.3 mg/ml FSBA for 3 h . For comparison, the inhibitory fractions were incubated in parallel under the same condition without subtilisin and FSBA, respectively (untreated control). The inhibitory and ATPase activity of the aliquots (4 µg protein) were determined.

Treatment	PKA-inhibition (%)	ATP-hydrolysis (%)
buffer	48	80
FSBA	19	6

The partially purified inhibitory fraction obtained after anion exchange chromatography contained about 20 % of the total inhibitory activity of the digitonin-extracts and showed a 167-fold increase in specific activity. The IC-50 of this fraction was determined to be 1.2 ± 0.1 mg protein/l (mean ± standard deviation from 3 determinations). Preincubation of the partially purified fraction with trypsin, alkali, acid or temperatures above 56°C led to a loss of inhibitory activity (data not shown), indicating that the inhibitor was protenaceous. Since the inhibitory activity bound to the immobilized WGA, we concluded that the it might be a glycoprotein or be associated with a glycoprotein.

Characterization of the nature of the inhibitory activity. Given that the inhibitory activity is due to a membrane protein, we determined whether a PKA regulatory subunit, a PKA antagonizing phosphatase or ATPase, could mimic the observed inhibition of the PKA catalytic subunit. Only neglible amounts of the regulatory subunit of PKA and the phosphatase activity were identified within the inhibitory fraction (data not shown), whereas a substantial ATPase activity was found to be present (buffer-treated fraction, Table2). However, we could not estimate whether the inhibitory activity observed in the preparation is caused by the detected ATPase alone, or is due to the ATPase with an additional inhibitor. Thus, we performed an irreversible inhibition of the ATPase activity with FSBA. Comparison of FSBA- and buffer-treated inhibitory fractions reveals that the ATPase activity was successfully inhibited by FSBA, whereas the inhibitory activity was only 2-fold reduced but not completely abolished. Since the ATP concentration in the assay was about 10-fold higher than the K_M of PKA (16), a 6%-loss of ATP could be viewed as neglible. ATPase- and inhibitory activity, therefore, show different sensitivities to FSBA, indicating that the perceived inhibitory activity is not

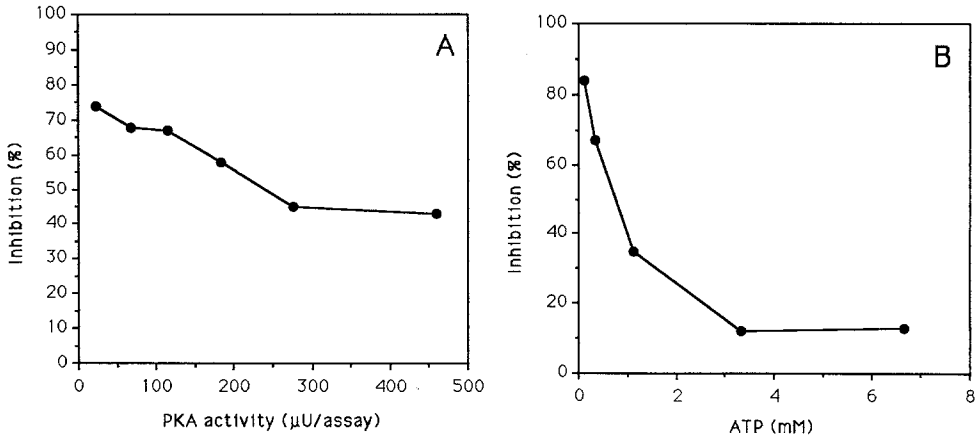

Figure 1. *Dependence of the inhibitory activity on the concentration of ATP and PKA.* PKA assays were performed with the 75 mM eluate of the MonoQ-column or with the corresponding buffer (buffer control) at the indicated concentrations of ATP (see B) or PKA (see A).

simply due to hydrolysis of ATP. This conclusion was further supported by a comparable result using partial subtilisin-digestion which destroy ATPase activity, whereas for the inhibitory activity a 50%-reduction can be observed, indicating that the inhibitory activity is caused by an inhibitory membrane protein in the preparation.

If the FSBA-insensitive inhibitory pool acts directly on PKA then, unlike the ATPase component of the inhibition, it will be sensitive to variation in the concentration of PKA. As shown in Figure 1A the inhibition of the PKA activity decreases to about 40 %, when higher amounts of PKA where used. This indicates that approx. 50 % of the inhibitory activity could not be competed out by the PKA. We, therefore, conclude that approx. 50 % of the measured inhibitory activity is related to the inhibitor, whereas the rest is resulting from ATPase activity. This conclusion is in agreement with the data from the FSBA-treatment. Since the ATPase- and inhibitory activity of the preparation could be competed out using increasing ATP-concentrations (Fig. 1B), we further conclude that the inhibitory activity of the inhibitor is due to its ability to compete with ATP.

Determination of the polypeptide composition of the inhibitor. Since there is strong evidence that the observed inhibiton of PKA was caused in part by a so far undefined protein, we further purified this fraction by gel filtration. Although this procedure was unable to separate the ATPase- and inhibitory activity (data not shown), we could further analyse the eluted proteins from gel

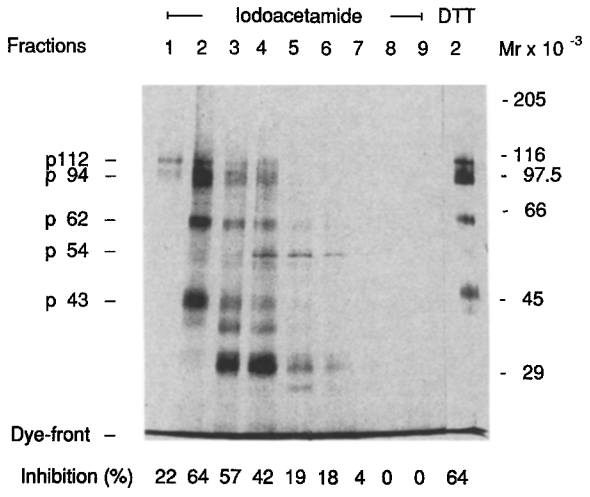

Figure 2. *Gel filtration of the partially purified inhibitory activity.* Identical fractions were used for the assay of inhibitory activity (2 µl/assay) or gel electrophoresis (70 µl/lane). Before electrophoresis the proteins were treated with DTT or iodoacetamide as indicated. After gel electrophoresis the proteins were silver stained.

filtration by SDS-gel electrophoresis. In order to compare the inhibitory activity of the eluted proteins with the silver-stained pattern, we used aliquots from identical fractions for gel electrophoresis and determination of the inhibitory activity. The fraction revealing the highest inhibitory activity (fraction 2; Fig. 2) contained proteins of molecular weights between 40 and 120 kDa. Given the determined IC-50, expressed in mg protein/l, for the MonoQ-eluate (see above) we could estimate the IC-50 to be 10 - 30 nM using these molecular weights. This indicates high affinity binding of the inhibitor to the PKA.

In summary, our results show the presence of a novel inhibitor of the catalytic subunit of the PKA, which is localized in the particulate fraction of rabbit skeletal muscle. Since most protein kinase A inhibitors are heat-resistent, soluble, or are of a low molecular weight (6;17;18), our investigation gives first evidence for a so far unknown mechanism of membrane bound PKA inhibition. This compartimentalized inhibition could be part of a new regulatory mechanism, capable of antagonizing kinase action upon translocation of PKA to the membrane (18).

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