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Partial Purification and Characterization of Protein Phosphatases from Bovine Adrenal Medulla

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ABSTRACT

Control of neurosecretion and synthesis of neurotransmitters appears to be regulated through second messengers that change the phosphorylation state of critical enzymes and proteins. Relatively little is known about the role of protein kinases in these processes in neuronal cells and even less is known about the role of protein phosphatases. We have examined the phosphatase activities of the bovine adrenal medulla using chromatographic separation, substrate specificity and inhibitor sensitivity. When fractionated, using an HPLC ion exchange DEAE column, four distinct peaks (peaks I,II,III and IV) of phosphatase activity were observed in the supernatant of homogenized bovine adrenal medulla. These phosphatases have distinctly different specific activities toward different substrates. Peak IV which contains most of the activity toward phosphocasein, showed preferential dephosphorylation of the α subunit of phosphorylase kinase relative to the β subunit and was strongly inhibited by okadaic acid, attributes of the type 2A phosphatase. The apparent molecular weight of phosphatase peak IV is also comparable to the heterotrimeric form of the known protein phosphatase type 2A of mammalian cells.

Key words: Protein phosphatases, okadaic acid, bovine adrenal medulla.

INTRODUCTION

The widespread role of the protein phosphorylation cycle in the regulation of cellular function appears reflected in the large number of proteins phosphorylated or dephosphorylated when the catecholamine-secreting cells of the adrenal medulla (chromaffin cells) are stimulated[1]. We have recently shown evidence, for example, that desensitization of these cells to stimulation involves a phosphorylation-dephosphorylation cycle operating at a post-receptor site[2], possibly the Na^+/Ca^{2+} exchanger[3]. A dephosphorylation process also appears to be important in neurite outgrowth from the related PC12 cells[4,5]. Others have shown that the activity of tyrosine hydroxylase, required for catecholamine synthesis, is regulated by phosphorylation[6].

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Although a great deal of information has been obtained on the structure and function of the protein kinases[7]the phosphatases are less well studied[8], and very little is known about their role in the adrenal medulla. Since protein phosphatases show relatively broad and overlapping substrate specificities *in vitro*[9], it is difficult to purify these enzymes and hard to define their substrates *in vivo* as well. Ingebritsen and Cohen (1983) proposed a well-accepted classification of protein phosphatases based on the criteria of substrate specificity and sensitivity to inhibitors and activators[10]. Type 1 phosphatases are those which dephosphorylate the β-subunit of phosphorylase kinase preferentially and are inhibited by the thermostable proteins, inhibitors 1 and 2. Type 2 phosphatases dephosphorylate the α-subunit of phosphorylase kinase preferentially and are not affected by the inhibitors. The type 2 phosphatases are further subdivided into groups A, B and C according to their catalytic requirements for cations. Protein phosphatase type 2B is Ca^{2+}-dependent and is unaffected by Mg^{2+}[11], whereas protein phosphatase 2C requires Mg^{2+} for activity and is uninfluenced by Ca^{2+}[10]. In contrast, the activities of protein phosphatase 2A and type 1 are independent of divalent cations[12]. Protein phosphatase type 1 and type 2A are inhibited by okadaic acid, a non-phorbol, 12-tetradecanoate-13-acetate-type tumor promoter[13] with I_{50} values of 20 nM and 0.1 nM, respectively[14,15].

As a preliminary step toward understanding the regulatory role of protein phosphatases in the bovine adrenal medulla, we have partly purified and characterized these activities.

MATERIALS AND METHODS

I. *Protein Phosphatase Preparation*

Preparation of protein phosphatases in adrenal medulla was performed largely by the methods described by Chiou (1992)[4]. Fresh bovine adrenal glands were delivered on ice to the laboratory from a local slaughterhouse. All experimental procedures were performed at 4°C. Fat and connective tissues were removed from the glands. The glands were incised about 0.5 cm deep throughout the cortex without damaging the medulla. The glands then were perfused with buffer (154 mM NaCl, 5.6 mM KCl, 3.6 mM $NaHCO_3$, 5.6 mM glucose, 5.0 mM HEPES, 10 % glycerol, pH 7.4) using a 60 ml syringe pressed into the vein of the gland. Perfusion was performed until all blood was cleared from the glands. Then the medullary portion was dissected from the gland, weighed, minced very finely and mixed with 3 volume (w/v) of buffer containing protease inhibitors (10 mM HEPES, 5 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 0.05% PMSF, 4 mg/ml pepstatin A, 4 mg/ml leupeptin, pH 7.4). The mixture was homogenized in a Waring blender for 20 sec, followed by centrifugation at 13,000 x g for 1 hr. The supernatant was centrifuged at 190,000 x g with Beckman airfuge for 20 min, then filtered through a 0.22 μm pore nitrocellulose membrane and then injected into an HPLC ion exchange column (Bio-Gel TSK DEAE-5-PW) equilibrated with buffer (23.5 mM Tris base, 0.1 mM EGTA, 1 mM $MgCl_2$, 0.1% β-mercaptoethanol, 10% glycerol, pH 7.4). The column was washed with buffer until the absorbance of the eluent at 280 nm was lower than 0.07. Subsequently, a linear gradient of 0 to 400 mM KCl in the same buffer was put through the column. The flow rate was 0.8 ml/min and 0.85 ml eluent was collected in each fraction.

II. *Measurement of Protein Phosphatase Activity*

The activity of protein phosphatase was measured by the ability to remove ^{32}P-phosphate from the ^{32}P-labeled casein or phosphorylase a. The assay solution, in 1.5 ml Eppendorf tubes, was composed of 25 μl of enzyme-containing samples, and 75 μl of assay buffer : 45 mM HEPES, 4 mg/ml BSA, 100 mM KCl, 1% β-mercaptoethanol, pH 7.4, containing either 5 mM $MgCl_2$ or 1 mM $MnCl_2$. After thorough vortexing, the tubes were incubated for 10 min at 37°C and 20 μl (50,000 cpm) of ^{32}P-labeled casein or phosphorylase a substrates were added and the

mixture was incubated at 37°C for a period of time short enough so that the released ^{32}P-phosphate count was below 20% of the total counts of the added substrates. The mixture was then precipitated by adding 40 μl of 40% TCA followed by a 2 min centrifugation. After centrifugation, 110 μl of the supernatant was mixed with 5 ml of ScintiVerse E and counted in a Beckman LS 100 scintillation counter. A convenient assay that is not specific for protein phosphatases was also used, which employed para-nitrophenyl phosphate (pNPP) as substrate. The assay was run in buffer containing 50 mM Tris-HCl pH 7.5 , 0.1 mM EGTA, 0.1% β-mercaptoethanol, 5 mM $MgCl_2$ and the yellow color of the released nitrophenol was followed at 405 nm.

III. *Preparation of ^{32}P-casein*

Protein kinase A, 1,200 pmolar units, was dissolved in 1 ml buffer (45 mM HEPES, 100 mM KCl, 5.7 mM $MgCl_2$, 1 mM EDTA, pH 7.4). To this 20 μl of 10 mM cAMP, 8 μl of 10 mM ATP, 1.2 ml of dephosphorylated casein (~1 μg), and 800 μCi [γ-^{32}P]ATP in 1.8 ml of the same buffer was added and the mixture was incubated for 4-5 hr at room temperature. After incubation, the mixture was aliquoted into 4 microcentrifuge tubes (1.5 ml Eppendorff tube), and 40% TCA was added to bring the final concentration of TCA to 10%. After vortexing and incubation on ice for 5 min, the mixture was centrifuged at 12,000 x g for 5 min and the pellet was washed with 1 ml of 10% TCA followed by vigorous vortexing and centrifuging at 12,000 x g for 5 min again. The supernatant was discarded and the pellet was washed repeatedly. Then the pellet was dissolved with 3-4 drops of 10 N NaOH in the presence of one drop of 1% bromophenol blue as an indicator, and the volume brought up to 400 μl by adding an appropriate volume of buffer (45 mM HEPES, 100 mM KCl, 1 mM EDTA, pH 7.4). The solution was dialyzed over a period of several hours at 4°C against a large volume of the same buffer with 4-5 times changes of the buffer.

IV. *Preparation of ^{32}P-phosphorylase a*

Phosphorylase b, 10 μg, dissolved in 1 ml Tris-glycerophosphate buffer (125 mM Tris-base, 125 mM glycerol phosphate, 5 mM DTT, pH 7.0), phosphorylase b kinase 1,000 units, dissolved in 600 μl Tris-glycerophosphate buffer, 200 μl of 60 mM $Mg(CH_3COO)_2$, 200 μl of 18 mM ATP, and 0.5 μCi [γ-^{32}P]ATP were mixed thoroughly and incubated for 3.5 hr at room temperature. After incubation, an equal volume of saturated $(NH_4)_2SO_4$ was added to the mixture. 10 min later the mixture was centrifuged for 7 min at 5,000 x g. The pellet was washed repeatedly and dissolved in Tris-glycerophosphate buffer. The solution was dialyzed against a large volume of the same buffer with 4-5 changes of the buffer over a period of several hours.

V. *Preparation of ^{32}P-phosphorylase Kinase*

8 μg of phosphorylase kinase was dissolved in 1 ml glycerol-phosphate buffer (50 mM glycerol-phosphate, 1 mM DTT, pH 6.8), 100 μl of 0.1 mM cAMP, 500 μl of PKA dissolved in glycerol-phosphate buffer with 0.4 mM EGTA, 50 μl of 10 mM $MgCl_2$ and 2 mM ATP solution, and 0.5 μCi [γ-^{32}P]ATP were mixed thoroughly and incubated for 1.5 hr at room temperature. After incubation, an equal volume of 0.1 M EDTA (pH 7.0, at 0°C) was employed to stop the reaction and then 100 ml of 90% $(NH_4)_2SO_4$ was added. After 10 min, the mixture was centrifuged at 5,000 x g for 5 min. The pellet was washed with 30% $(NH_4)_2SO_4$ twice and then was dissolved in glycerol-phosphate buffer. The solution was dialyzed against a large volume of the same buffer with 4 or 5 changes of the buffer over a period of several hours. The protein solution was centrifuged at 5,000 x g for 3 min to remove any insoluble material and the supernatant was ready to serve as substrate for protein phosphatases.

VI. *Dephosphorylation of α or β Subunit of ^{32}P-phosphorylase Kinase*

Samples of partially purified protein phosphatase fractions were added to 25 μl of ^{32}P-phosphorylase kinase substrate and brought to a final volume of 150 μl in each tube with appropriate addition of column buffer. The mixture was incubated at 37°C for 1 hr then chilled on ice prior to application to an SDS gel. A 6% SDS gel with 4% stacking gel was prepared in advance. The sample was mixed with 2x sample buffer (6% β-mercaptoethanol, 6% SDS, 0.6% bromophenol blue, 20% glycerol buffer) and the mixture was loaded onto the gel at about 25 μl per well and subjected to electrophoresis at a voltage of 50 volt for 2 hr. Subsequent to electrophoresis, the gel was stained with Coomassie Blue and soaked with 2.5% glycerol before drying. The dried gel was transferred to an autoradiograph cassette and exposed at -78°C for 2 to 4 hr depending on the radioactivity. Kodak X-Omat AR 50/13x18 cm XAR 2 film was used and the radiograph was scanned by a MicroScan 1000, 2-D Gel Analysis System (Technology Resources Inc.).

VII. *Measurement of Protein Concentration*

Protein concentration was assayed by Coomassie Blue dye-binding[16]. Bovine serum albumin served as the standard curve.

VIII. *Determination of Enzyme Molecular Weight*

Peak IV enzyme which had been partially purified by HPLC ion exchange on a Bio-Gel TSK DEAE-5-PW column was collected and concentrated using an Amicon Centricon ^{TM}10 microconcentrator. 25 μl (10 μg protein) of the concentrated sample was then added to 30 μl of gel filtration molecular weight standard containing thyroglobulin (670 kDa), γ-globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa) and vitamin B_{12} (1.35 kDa); then the total volume was brought to 200 ml with column buffer. The mixed sample was injected into the HPLC gel filtration column, Bio-Sil SEC-250, 600x7.5 mm, which had been pre-equilibrated with buffer. The fraction size was 1 ml and flow rate was 0.5 ml/min. The molecular weight of phosphatase peak IV was determined by plotting the position of the enzyme activity against the molecular weight standard curve.

IX. *Chemicals*

Dithiothreitol, EGTA (ethylene bis (oxyethylene-nitrilo) tetracetic acid), HEPES (4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid), were purchased from Sigma (St. Louis, MO, U.S.A.). Okadaic acid was purchased from Boehringer Mannheim (Manneheim, F.R.G.). Other salts were from Merck (Darmstadt, F.R.G.). Acrylamide, ammonium persulfate and TEMED (N,N,N',N'-tetra-methylethylenediamine) were obtained from Bio-Rad (Hercules, CA, U.S.A.).

RESULTS

I. *Substrate Specificity of Protein Phosphatases*

As shown in figure 1, the protein phosphatases present in an adrenal medulla homogenate supernatant can be separated on a DEAE-cellulose column into fractions which differ in their activities toward phosphocasein, phosphorylase a, and ρ-nitrophenylphosphate (ρNPP). These protein phosphatases show relatively higher activity toward ρNPP and phosphorylase a as substrates in the early fractions whereas their activity toward phosphocasein increases in the later fractions (Fig.1). Comparison of the phosphatase activities toward phosphocasein, ρNPP and phosphorylase a allowed us to distinguish four distinct peaks of protein phosphatase activity; I, II, III and IV. These four phosphatase peaks were eluted at KCl concentrations of 130 mM, 170 mM, 220 mM, and 280 mM. Peak I is characterized by relative strong phosphorylase a activity. Peak II shows strong activity toward both phosphorylase a and phosphocasein. Peak III is characterized by relative stronger phosphocasein phosphatase activity but little phosphorylase a and ρNPP phosphatase activity. Peak IV is

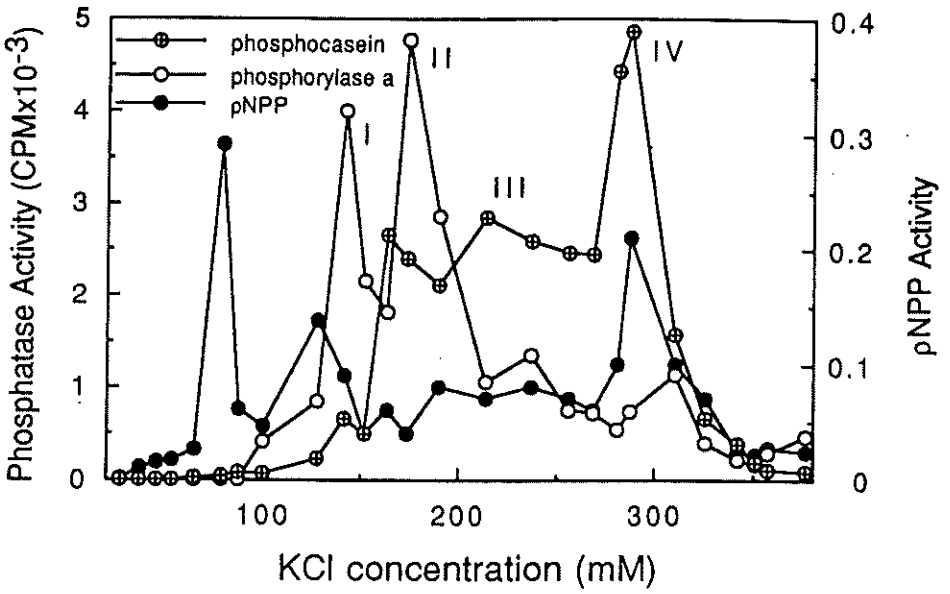

Figure 1. Protein phosphatase activities assayed with phosphocasein, phosphorylase a and pNPP as substrates. Bovine adrenal medulla homogenate was centrifuged at 190,000 x g for 20 minutes and then filtered through a 0.2 μm filter. The filtrate was applied to the HPLC ion exchange Bio-Gel TSK DEAE-5-PW column (75x7.5 mm) and eluted with a linear gradient of 0 to 400 mM KCl. Phosphatase activity was assayed with ^{32}P-phosphocasein, phosphorylase a and pNPP as substrates in the presence of 1 mM Mn^{2+} in the assay buffer.

the major peak of enzyme activity with phosphocasein as substrate and has pNPP phosphatase activity as well. These data demonstrate the multiplicity of protein phosphatases and their overlapping substrate specificities in bovine adrenal medulla. It appears also that there are significant minor phosphatase activities present in addition to the 4 major ones - for example in peak II there appears to be a phosphocasein phosphatase activity that is not coincident with the peak of activity toward phosphorylase a.

Dephosphorylation of α or β subunits of phosphorylase kinase has been shown to be a useful criterion for classifying the protein phosphatases[10]. Activities toward these substrates by the crude fractions from the DEAE column is shown in Fig.2. Peaks II and IV phosphatases preferentially dephosphorylate the α subunit of phosphorylase kinase rather than the β subunit. However, peaks I and III digest both the α and β subunits, but inefficiently. Therefore, by this criterion, peaks II and IV appear to contain chiefly type 2 protein phosphatases, while peaks I and III do not. Table 1 shows the quantitative data for the dephosphorylation of α or β subunits of phosphorylase kinase relative to controls in the absence of phosphatase.

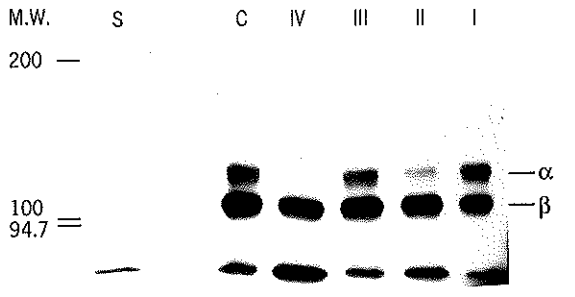

Figure 2. Autoradiograph showing phosphorylation by protein phosphatases of α or β subunits of phosphorylase kinase. Fractions were separated by HPLC using a DEAE-5-PW ion exchange column chromatography as in Fig. 1 and assayed with phosphocasein as substrates. Experiments were performed as described in the Materials and Methods. S: ^{14}C-methylated molecular weight standards, C: control, IV: peak IV enzyme fraction, III: peak III enzyme fraction, II: peak II enzyme fraction, I: peak I enzyme fraction.

II. *Inhibition by Okadaic Acid*

Both the enzyme activities of peaks III and IV in Fig.1 were strongly inhibited by 2 nM and 20 nM okadaic acid. In contrast, the inhibition of peaks I and II was considerably less than 50% at 20 nM okadaic acid (Fig.3), indicating that peaks I and II are insensitive to this potent inhibitor of types 1 and 2A phosphatases[13,14]. Since okadaic acid inhibits phosphatase peaks III and IV at concentrations as low as 2nM it appears that peak IV is a type 2A phosphatase, based on this inhibition and on it's preferential dephosphorylation of the α subunit of phosphorylase kinase.

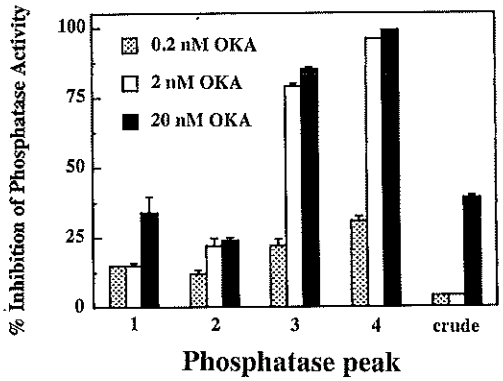

Figure 3 Inhibition of protein phosphatase activity by okadaic acid. Fractions were separated by HPLC using a DEAE-5-PW ion exchange column and assayed with phosphocasein as substrates in the presence of 1 mM Mn^{2+} in the assay buffer.

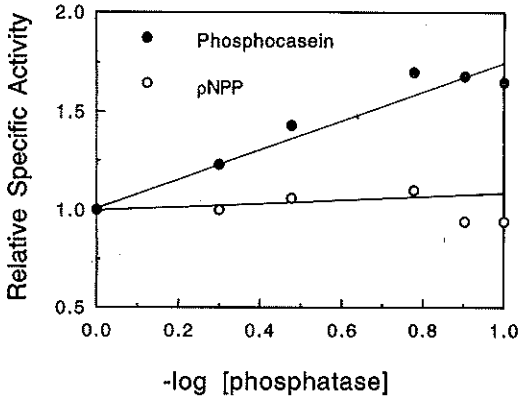

Figure 4. Relative specific activity of protein phosphatases from bovine adrenal medulla extract using pNPP and ^{32}P-phosphocasein as substrates. The eluents from the major enzyme peak fraction were concentrated by using an Amicon Centricon ™ 10 microconcentrator. A series of dilutions was then made from the concentrated sample. Enzyme activity was determined by using pNPP and ^{32}P-phosphocasein as substrates in the presence of 1 mM Mn^{2+} in assay buffer.

III. *Change in Specific Activity upon Dilution of Peak IV Phosphatase*

After separation on a Bio-Gel TSK DEAE-5-PW column, the peak IV phosphatase fractions were collected and concentrated. The specific activity relative to the most concentrated sample increased by 70% with phosphocasein as substrate when the enzyme was diluted 10 times. However, the relative specific activity remained approximately 1.0 with pNPP as substrate during dilution (Fig.4). The relative specific activity became erratic after more than 10 fold dilution, perhaps due to degradation of the enzyme at very low concentration. A possible interpretation of the effect of dilution is that endogenous inhibitors are diluted out during enzyme dilution or there may be dissociation of a regulatory subunit[17].

IV. *Estimation of the Molecular Weight of Peak IV Phosphatase*

Following one step of purification on the

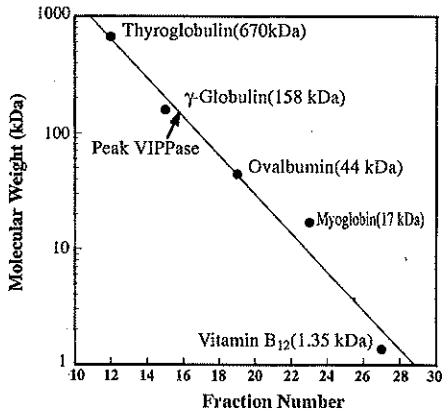

Figure 5 Estimation of the molecular weight of peak VI protein phosphatase by internal standardization. The molecular weight standards were separated on the HPLC gel filtration column and plotted as fraction number vs. molecular weight. The arrow represents the apparent molecular weight of the fraction with the highest phosphatase activity.

DEAE-5PW ion-exchange column, the peak IV phosphatase was concentrated and applied to an HPLC Bio-Sil SEC-250 gel filtration column. The result showed one single peak which exhibited mostly enzyme activity in fractions 15 and 16 (Fig. 5). The apparent molecular weight of the enzyme in peak IV was determined to be 152 kDa by internal standardization which is identical to that of the ABC form of protein phosphatase type 2A[15].

Taken together, the results from substrate specificity and inhibition by okadaic acid allow us consider the relationship of different enzyme peaks from bovine adrenal medulla to the classification scheme proposed by Ingebritsen and Cohen (1983).

Peak I enzyme exhibits low activity with phosphocasein, high activity with phosphorylase a, no preferential dephosphorylation of the α or β subunits of phosphorylase kinase and insensitivity to okadaic acid. Peak I phosphatase enzyme appears to be different from anything in the scheme that Ingebritsen and Cohen (1983) proposed.

Peak II enzyme shows relatively low activity with phosphocasein, high activity with phosphorylase a, preferential dephosphorylation of the α subunit of phosphorylase kinase and insensitivity to okadaic acid. This enzyme peak does not fit the classification scheme of Ingebritsen and Cohen, either.

Peak III appears to have much higher activity with phosphocasein compared with phosphorylase a, no preferential dephosphorylation of the α or β subunits of phosphorylase kinase, is sensitive to okadaic acid (50% inhibition of 0.2 nM okadaic acid). Peak III phosphatase remains to be further characterized. Its specificity toward the β subunit of phosphorylase kinase is more like a type 1 phosphatase, but its sensitivity toward okadaic acid is much greater than that of the typical type 1 phosphatase (I_{50} about 20 nM). Furthermore, appears to be different from any enzyme in the classification scheme of Ingebritsen and Cohen.

Peak IV enzyme shows much higher activity toward phosphocasein than toward phosphorylase a, preferentially dephosphorylates the α subunit of phosphorylase kinase, is sensitive to okadaic acid (35% inhibition at 0.2 nM okadaic acid), exhibits an apparent molecular weight identical to that of ABC form of type 2A phosphatase. It is likely that peak IV phosphatase is a type 2A phosphatase.

DISCUSSION

At least 4 protein phosphatase peaks, termed peaks I, II, III, and IV, were found in the adrenal medulla homogenate separated by DEAE ion-exchange chromatography using ^{32}P-phosphocasein, ρNPP and phosphorylase a as substrates (Fig.1). The major enzyme peak (peak IV) showed a relatively higher phosphocasein activity, lower phosphorylase a and ρNPP activity while the earlier eluted enzyme peaks (peaks I and II) preferentially dephosphorylated phosphorylase a or ρNPP. This suggests that certain protein phosphatases in bovine adrenal medulla exhibit relatively narrow substrate specificities, whereas others, such as peaks I, and II, are broader. The overlapping substrate specificities increase the complexity of the identification of these enzymes. Furthermore, the enzyme peaks may consist of two or even more than two protein phosphatases because one step of ion-exchange chromatography is not sufficient to fractionate the protein phosphatases into individual enzyme peaks. Therefore, although peaks I and II exhibited different characteristics from serine/threoine phosphatases as described in Ingebritsen and Cohen (1983), we cannot exclude the possibility that peaks I and II contain a mixture of different protein phosphatases. It is likely that there are more protein phosphatases present in the bovine adrenal medulla than we have identified. For example, a protein phosphatase requiring activation by MgATP has been reported to be present in the cytosol fraction[15,18]. These inactive enzymes may not be detected in the assay without special activation treatment. Moreover, our study was limited to the cytosolic serine/threonine

Table 1. Dephosphorylation of α or β subunits of phosphorylase kinase by protein phosphatases presented as % dephosphorylation relative to control as determined by densitometry of autoradiographs of Fig.2

Phosphatase peak	% dephosphorylation α	β
I	25.0	33.0
II	83.2	18.6
III	34.5	27.0
IV	100.0	31.0

Fractions were separated by HPLC using a DEAE-5-PW ion exchange column chromatography and assayed with phosphocasein as substrates. Samples from partially purified protein phosphatase fractions with the same enzyme activity were added to 25 μl of ^{32}P-phosphorylase kinase substrate and brought to a final volume of 150 μl in each tube with appropriate addition of column buffer. The mixture was then subjected to 6% SDS electrophoresis and the dried gel transferred to an autoradiograph cassette and exposed at -78°C for 2 to 4 hr depending on the radioactivity. The radiograph was finally scanned by a MicroScan 1000, 2-D Gel Analysis System (The Technology Resources Inc.).

phosphatases and phosphotyrosyl protein phosphatase activity would not have been detected[19,20].

Okadaic acid inhibits PP1 and 2A much more potently than PP2B. PP2C is insensitive to the inhibition by okadaic acid. Although it has been reported that 1% of the total brain protein is PP2B[8], we have not detected PP2B phosphatase activity in bovine adrenal medulla. Crude chromaffin cell extract treated with Ca^{2+} and calmodulin showed no significant activation of phosphatase enzyme activity (data not shown). It is likely that Ca^{2+}/calmodulin dependent phosphatases are present only to a small degree in adrenal medulla cell extract although they are important in central nervous tissue. These results are in agreement with dot immuno-blotting data from Chiou who observed slight cross-reactivity with antibody against calmodulin-dependent phosphatases (PP2B) but not in the areas of the peaks of the phosphatase activities[4]. Moreover, PP1 is generally found to be associated with glycogen or myofilbrils but the entire phosphatase enzyme activity of adrenal medulla was present in soluble cytosol and there is only little glycogen or myofilbrils in adrenal medulla[4]. Therefore, okadaic acid inhibition and preferential dephosphorylation of phosphorylase kinase subunits suggest that most of the enzyme in bovine adrenal medulla is PP2A.

It is well documented by molecular cloning data that multiple isoforms exist in the adrenal medulla. Type 1 phosphatase has been shown to exhibit α, β and γ isoforms. Four different A subunit isoforms have been found originally in type 2A phosphatases, they are designated as $PP2A_o$, $PP2A_1$, $PP2A_2$, $PP2A_c$. Quite recently, additional isoforms with variable subunits have been reported in type 2A phosphatase, such as α or β forms of A subunit, α or β forms of B subunit , B' subunit, B" subunit, and the α or β forms of C subunit. In this study, the molecular weight of peak IV phosphatase is comparable to that of ABC isoform of type 2A phosphatase. This strengthens our conclusion that peak IV enzyme is a type 2A phosphatase. Although multiple isoforms of protein phosphatase have been reported from cloning data, they may not reflect the functional enzyme types present under physiological condition. Zhang et. al. (1992), for example have shown that no pertinent messenger RNA is found in Northern blotting from α and β isoforms of type 1 phosphatase[21].

In conclusion, this study reveals a multiplicity of protein serine-threonine phosphatases present in bovine adrenal medulla. Our data show similarity with the multiplicity of protein serine-threonine phosphatases in PC12 pheochromocytoma cells[22]. In adrenal medulla, type 2A protein phosphatase seems to be the major enzyme. Haavik et al., (1989) have shown that type 2A protein phosphatase is the primary tyrosine

hydroxylase phosphatase in adrenal medulla[23]. Because tyrosine hydroxylase is a rate limiting enzyme in catecholamine biosynthesis, type 2A phosphatase is likely to play a pivotal role in neurosecretory pathway. Quite recently, PP2A has also been shown to play a key role in regulation of adenylyl cyclase during desensitization response in PC12 cells[24]. Chiou (1992) demonstrated that type 2A phosphatase is likely to be involved in the neurite outgrowth in PC12 cells as well[5]. Although type 2A is the major phosphatase, the heterogeneity and multiplicity of phosphatases in adrenal medulla may imply that different phosphatases play different roles in neuronal cell regulation.

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牛腎上腺髓質中蛋白磷酸水解酶之部份純化及其特性研究

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摘　要

調控神經傳導物質的分泌及合成是藉由二級信差改變細胞內重要酵素及蛋白之磷酸化狀態來達成。在神經細胞中對於調控蛋白磷酸化的蛋白激酶所扮演的角色了解不多，對於調控蛋白去磷酸化的蛋白磷酸水解酶所知更少。本文主要是藉由高效液相層析(HPLC)之分離、基質特異性、抑制劑之敏感性等特性來檢測牛腎上腺髓質中蛋白磷酸水解酶之活性。藉由HPLC離子交換DEAE層析柱的分離，均質後之牛腎上腺髓質上清液最少發現有4支不同的蛋白磷酸水解酶活性波峰（波峰I, II, III, IV），這些蛋白磷酸水解酶對不同的基質呈現不同的特異活性。波峰IV對乳酪蛋白基質具有最高酵素活性，進一步分析發現該蛋白磷酸水解酶對磷氧基酶激酶(phosphorylase kinase)基質之α次單位體較β次單位體具有較高之磷酸水解能力，並且該磷酸水解酶可被okadaic acid強烈抑制，分子量的比對亦發現該波峰IV磷酸水解酶與已知哺乳類細胞之異三位體2A型蛋白磷酸水解酶相近，因此該磷酸水解酶可能爲2A型蛋白磷酸水解酶。

關鍵詞：蛋白磷酸水解酶，Okadaic acid，牛腎上腺髓質。