Distribution of protein I in mammalian brain as determined by a detergent-based radioimmunoassay

(protein kinase/protein phosphorylation/synapse/nerve terminals)

SUSAN E. GOELZ*, ERIC J. NESTLER*, BAHRAM CHEHRAZI†, AND PAUL GREENGARD*

*Department of Pharmacology and †Section of Neurosurgery, Yale University School of Medicine, 333 Cedar Street, New Haven, Connecticut 06510

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ABSTRACT A radioimmunoassay has been developed for measuring protein I, a basic, neuron-specific protein associated with nerve terminals. The procedure utilizes the detergents NaDodSO₄ and Nonidet P-40 to prevent nonspecific adsorption of this highly charged protein to various surfaces. By use of this procedure, it has been possible to show that protein I comprises approximately 0.4% of the total protein in cerebral cortex of several mammalian species. In addition, the amount of protein I was determined in about 40 regions of cat brain. The results suggest that measurement of protein I may provide a quantitative method for estimating the density of nerve terminals in various regions of the mammalian nervous system.

Protein I is a neuron-specific phosphoprotein (1, 2) that is concentrated in nerve terminals in apparent association with synaptic vesicles (3-5). Recent studies from this laboratory indicate that protein I may play an important role in neuronal function (2). The phosphorylation of this protein is regulated not only by cyclic AMP-dependent and Ca²⁺-dependent protein kinases *in vitro* (2, 6-8) but also by neurotransmitters (9, 10) and depolarizing agents (9-11) in intact slices of nervous tissue. Although the precise physiological role of protein I remains unclear, it seems likely that it is important in some aspect of the functioning of synaptic vesicles (2).

Many facets of the study of protein I require a rapid and sensitive method for its quantitation, and one of the most sensitive and specific methods for quantitation of the amount of proteins is radioimmunoassay (RIA). However, protein I has a number of properties that make the use of traditional RIAs impossible. Because it is a strongly basic protein (pI ≈ 10.4) (1), it is highly charged at neutral pH and adsorbs nonspecifically to a variety of surfaces (e.g., other proteins, tubes, and pipette tips). Furthermore, protein I is difficult to extract from certain tissues; for example, 1% NaDodSO₄ is required to solubilize protein I from rabbit superior cervical ganglion (unpublished data). In addition, protein I represents a very small percentage of the total protein in some tissues; for example, in the adrenal medulla it constitutes 0.001% of the total protein and, thus, must be assayed in the presence of large amounts of extraneous material to which it adsorbs. It seemed possible that the use of detergents in a RIA might alleviate these problems. Studies (10, 12, 13) have shown that antigen-antibody complexes are not disrupted by the presence of nonionic detergents. In this paper we describe a detergent-based, competitive, nonsolid phase RIA in which NaDodSO₄ and Nonidet P-40 (NP-40) are used to solubilize protein I and to minimize its nonspecific interactions. We have used this assay to quantitate the amount of protein I present in the cerebral cortex of various mammalian species and to determine the regional distribution of protein I in cat brain.

MATERIALS AND METHODS

Materials. [γ -³²P]ATP (5-10 × 10⁷ cpm/nmol) was prepared by the method of Glynn and Chappell (14) from ATP (Sigma) and carrier-free [³²P]orthophosphate (New England Nuclear). Catalytic subunit (purified from bovine heart type II cyclic AMP-dependent protein kinase) was a gift from Angus C. Nairn, and pure protein I from bovine brain was a gift from Louis J. DeGennaro. Protein A-bearing *Staphylococcus aureus* cells (SAC) (Pansorbin) were purchased from Calbiochem/Behring Corp. Bolton-Hunter reagent (4000 Ci/mmol; 1 Ci = 3.7 × 10¹⁰ becquerels) was obtained from New England Nuclear.

Acid Extraction of Protein I from Rat Brain. Rat cerebral cortex (100 mg) was homogenized with a glass/Teflon homogenizer in 1 ml of ice-cold 5 mM 1,4-piperazinediethanesulfonic acid, pH 7.4/5 mM Zn(OAc)₂. The homogenate was centrifuged for 10 min at 3000 × g, and the supernatant was removed. The pellet was rehomogenized in 1 ml of 11 mM ice-cold citric acid (final pH, 2.8-3.0), and the mixture was centrifuged for 3 min in a Beckman microfuge. The supernatant (which contained the protein I) was neutralized with 125 μ l of 0.2 M Na₂HPO₄.

Phosphorylation of Protein I. Protein I was phosphorylated by incubating 3 μ l of the neutralized extract (containing 30 μ g of protein) or 100 ng of pure protein I for 30 min at 30°C with 50 mM 1,4-piperazinediethanesulfonic acid, pH 7.4/10 mM MgCl₂/0.4 mM ethylene glycol bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid/1 mM dithioerythritol/100 nM catalytic subunit/1 μ M [γ -³²P]ATP (10⁸ cpm/nmol) in a final volume of 50 μ l.

Antibody Preparation. A trace amount of ³²P-labeled pure protein I was added to 100-150 μ g of partially purified protein I obtained from bovine brain, and the mixture was subjected to NaDodSO₄/polyacrylamide gel electrophoresis (11). The protein I region of the gel was identified by autoradiography, and homogenized in 3 ml of 150 mM NaCl/10 mM sodium phosphate, pH 7.4, with an Ultra-Turrax. Young adult female New Zealand White rabbits were each injected (subcutaneously and intradermally) with 3 ml of homogenized gel combined with 3 ml of complete Freund's adjuvant. The rabbits were given booster injections (3 ml of homogenized gel without adjuvant) 1, 2, and 3 wk after the first injection. One week after the third booster injection, 100 ml of blood was obtained from each rabbit by cardiac puncture, and the sera were screened for anti-protein I activity by an immunoprecipitation procedure (10). Specificity of the antibody was demonstrated by three different criteria as described (5).

Immunoprecipitation of Protein I. Immunoprecipitation of phosphorylated protein I was performed essentially as described (10). SAC was prepared as described (13), except that (i) 1% NP-40 was used in the buffer (150 mM NaCl/5 mM EDTA/

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Abbreviations: RIA, radioimmunoassay; SAC, protein A-bearing *Staphylococcus aureus*; NP-40, Nonidet P-40.

50 mM Tris, pH 7.4/0.02% sodium azide) and (ii) the cells were finally resuspended in that buffer with 1% NP-40 plus 25 mg of bovine serum albumin per ml. Samples of phosphorylated pure protein I or of phosphorylated rat brain extracts were adjusted to 100- μ l vol with RIA buffer (200 mM NaCl/10 mM EDTA/10 mM NaH_2PO_4 , pH 7.4) containing 0.1% NaDodSO₄/0.5% NP-40 (final concentrations). To this solution, 40 μ l of prepared SAC was added. The mixture was incubated for 30 min at 0°C and centrifuged in a Beckman Microfuge to obtain the "precleared supernatant." To this supernatant, 1 μ l of protein I antiserum was added, and the mixture was incubated for 20 min at 0°C. A 20- μ l aliquot of prepared SAC was then added, and incubation was continued for another 20 min. The solution was centrifuged in a Microfuge for 3 min, and the pellet was washed with the NaCl/EDTA/Tris/ NaN_3 buffer plus 1% NP-40.

The amount of ³²P in the pellet was determined in two ways. First, the pellet was counted by using Cherenkov radiation, and then it was resuspended in 100 μ l of sample buffer, boiled for 5 min, and subjected to NaDodSO₄/polyacrylamide gel electrophoresis as described (8). The protein I bands, located by autoradiography, were cut out, and radioactivity was assayed by liquid scintillation spectrometry.

Iodination of Protein I. Pure protein I (10 μ g) in 25 μ l of 50 mM NaH_2PO_4 (pH 8.0) was added by syringe to 1 mCi of Bolton-Hunter reagent (which had been dried under nitrogen) and was iodinated by incubation for 30 min at 0°C. To stop the reaction, 0.4 ml of 0.2 M glycine in 0.1 M NaH_2PO_4 (pH 8.0) was added, and incubation was continued for 5 min. The mixture was then removed from the vial, the vial was rinsed with 0.1 ml of 0.5% NaDodSO₄/0.25% gelatin/50 mM NaH_2PO_4 , pH 7.2, and the two solutions were combined.

A Sephadex G-50 column (8-ml vol) was equilibrated with 50 mM NaH_2PO_4 (pH 7.2). A 0.5-ml sample of bovine serum albumin (100 mg/ml) was then applied, and the column was re-equilibrated with 0.1% NaDodSO₄/0.25% gelatin/50 mM NaH_2PO_4 , pH 7.2. The iodinated sample was applied to this column and eluted with 0.1% NaDodSO₄/0.25% gelatin/50 mM NaH_2PO_4 , pH 7.2. Fractions (0.25 ml) were collected, and those containing protein I (fractions $\approx$ 17–25) were pooled.

The use of NaDodSO₄ aided in the recovery of the small amounts of protein I used in the iodination procedure. Approximately 5–10% of the total ¹²⁵I was recovered in protein I, as determined by NaDodSO₄/polyacrylamide gel electrophoresis. A typical preparation yielded enough iodinated protein I for 10,000 assay tubes. The ¹²⁵I-labeled protein I was divided into 10- to 30- μ l aliquots and stored at –70°C. Approximately 0.1–0.5 μ l ($5\text{--}10 \times 10^3$ cpm) of the pooled fractions was used for each assay tube. The exact amount of ¹²⁵I-labeled protein I used was such that 50% of the label (0.2–1 ng) was precipitated by the protein I antiserum (generally a final dilution of 1:10,000).

Immediately prior to each RIA, the ¹²⁵I-labeled protein I was precleared with SAC (prepared as described above). This was accomplished by bringing the volume of the ¹²⁵I-labeled protein I to 70 μ l with RIA buffer containing 0.1% NaDodSO₄/0.5% NP-40, adding 10 μ l of SAC, and incubating for 30 min at 0°C. After centrifugation the precleared supernatant was removed and added to the appropriate amount of RIA buffer containing 0.1% NaDodSO₄/0.5% NP-40.

RIA for Protein I. The individual solutions of standard protein I, of precleared ¹²⁵I-labeled protein I, and of protein I antiserum were diluted with RIA buffer containing 0.1% NaDodSO₄/0.5% NP-40. The RIA was performed in 500- μ l Microfuge tubes at 0°C in a final volume of 100 μ l. Concentrations of 0.1%, 0.5%, or 1.0% NaDodSO₄ [with $5 \times$ the percent

(wt/vol) of NP-40] were routinely used in the assay. The samples to be assayed were brought to a final volume of 80 μ l in RIA buffer containing NaDodSO₄/NP-40. At carefully timed intervals, 10 μ l of a 1:1000 dilution of protein I antiserum (diluted immediately before use) was added, and each sample was vortexed. After a 20-min incubation, 10 μ l of ¹²⁵I-labeled protein I was added, and the sample again was vortexed. After a 30-min to 24-hr incubation, a 10- μ l aliquot of SAC was added, and the samples were incubated for a further 20 min and then centrifuged for 3 min in a Beckman Microfuge. The supernatant was carefully removed by aspiration, the pellet was washed once in 200 μ l of NaCl/EDTA/Tris/ NaN_3 buffer plus 1% NP-40, and the radioactivity was measured in a Beckman Biogamma II. The assays were generally performed in triplicate with carefully controlled incubation times.

As a partial test of the specificity of the precipitation step of the RIA procedure, a neutralized extract from rat brain was phosphorylated and then incubated with antiserum and SAC under conditions similar to those used in the RIA procedure (¹²⁵I-labeled protein I was excluded). The suspension was centrifuged, and the resulting pellet was analyzed by NaDodSO₄/polyacrylamide gel electrophoresis. As seen in Fig. 1, protein I was the only ³²P-labeled protein precipitated by the anti-protein I antibody. Preimmune serum did not precipitate any labeled proteins, which suggested that, under the conditions of the assay, only specific precipitation occurred.

Preparation of Samples for the RIA. Tissue samples (1–100 mg wet wt) were homogenized in 100 vol of 1% (wt/wt) NaDodSO₄ in glass/Teflon homogenizers, and the homogenates were boiled for 10 min. The homogenates were diluted 1:40 in RIA buffer containing 0.075% NaDodSO₄/0.5% NP-40 and were assayed directly as described above. At least two concentrations of each sample were assayed. Samples of mammalian cortex could be stored in 1% NaDodSO₄ for up to 3 wk at –20°C without detectable loss of protein I.

Protein concentrations were determined by the method of Lowry *et al.* (15).

RESULTS

The stability of the protein I–antiprotein I IgG interaction was examined in the presence of varying amounts of ionic (NaDodSO₄) and nonionic (NP-40) detergents (Fig. 2). It was found that the complex was stable in NP-40 but not in NaDodSO₄ and that NP-40 partially prevented the disruptive action of NaDodSO₄ on the antigen–antibody complex. This effect of NP-40 is probably due to the formation of mixed micelles composed of the ionic and nonionic detergents (16). Schick and Manning have found that a 5-fold excess of a nonionic detergent was sufficient, by virtue of mixed micelle formation, to remove essentially all NaDodSO₄ from solution. Therefore, in those extractions that required NaDodSO₄, we added a 5-fold excess of NP-40 subsequent to the extraction.

Experiments performed to determine the amount of nonionic detergent necessary to prevent protein I from adhering non-specifically to surfaces revealed that 0.1% NP-40 is sufficient for most materials (polypropylene, polyethylene, glass). Detergent did not prevent adsorption of protein I to polysulfonated or siliconized tubes (data not shown).

Detergent did not appear to adversely affect the RIA procedure for protein I, as concentrations up to 1% NaDodSO₄/5% (wt/vol) NP-40 were used successfully in the assay. Fig. 3 shows a typical assay. This particular experiment illustrates that the phosphorylated and the unphosphorylated forms of protein I were equally effective in competing for the antiserum used. The use of sequential incubations, in which the antiserum was added to the detergent solution containing the unknown sample

FIG. 1. Specificity of the immunoprecipitation of protein I from a mixture of phosphorylated proteins present in an extract from rat brain. A neutralized extract of rat brain was subjected to phosphorylation (lane 3) and then immunoprecipitation with 1 μ l of preimmune serum (lane 1) or with 1 μ l of protein I antiserum (lane 2). Phosphorylated proteins present in the extract and immunoprecipitated pellets were separated on a 10% polyacrylamide/NaDodSO₄ gel and analyzed by autoradiography.

before the ¹²⁵I-labeled protein I was added, increased the sensitivity of the assay and reduced incubation times. Thus, the assay was performed in three steps: (i) the addition of the antiserum to the sample; (ii) the addition of the ¹²⁵I-labeled protein I to the sample containing antiserum; and finally (iii) the addition of the SAC. The incubation times were always 20 min for steps i and iii and varied from 30 min to 24 hr for step ii. The amount of protein I that could be precipitated by the addition of SAC after a 30-min incubation time for step ii was 60% of that obtained after a 24-hr incubation time. Generally, the incubation time for step ii was 3 hr, and, if the time was carefully con-

FIG. 2. Effect of various concentrations of NP-40 and NaDodSO₄ on the stability of the protein I-anti-protein I complex. Pure bovine protein I (100 ng) was phosphorylated and immunoprecipitated as described.

trolled, these nonequilibrium assays were reproducible to within 5%.

The amount of protein I determined by the RIA procedure was directly proportional to the amount of rat brain homogenate assayed (Fig. 4). Identical results were obtained whether the assay was performed in the presence of 0.1%/0.5%, 0.5%/2.5%, or 1%/5% NaDodSO₄/NP40, respectively (data not shown).

The level of protein I in rat cerebral cortex, calculated from the data in Fig. 4, corresponds to about 0.4% of the total protein. Similar values were obtained for protein I concentrations in the cerebral cortex of several other mammalian species (Table 1). The high concentration of protein I in the cortex suggests that protein I is distributed in a large proportion of neurons. That protein I is present at most and possibly at all synapses has, in fact, been indicated by cytochemical studies (17).

By using the RIA procedure, the regional distribution of protein I in cat brain was studied. The amounts of protein I present in various gross and fine brain areas are shown in Table 2.

DISCUSSION

RIA can be an extremely sensitive and specific quantitative technique. It has been used successfully in many endocrino-

FIG. 3. RIA of protein I showing the competition of phosphorylated protein I and of unphosphorylated protein I from rat brain with ¹²⁵I-labeled bovine unphosphorylated protein I for binding to protein I antiserum. □—□, ¹²⁵I-labeled protein I bound in the presence of unphosphorylated protein I; ○—○, ¹²⁵I-labeled protein I bound in the presence of phosphorylated protein I.

FIG. 4. Proportionality of protein I determined by RIA to the amount of rat brain crude homogenate.

logical studies (18) and is rapidly becoming a standard technique in biochemistry and molecular biology. As investigators have attempted to apply RIA to proteins, nucleic acids, and other highly charged molecules, it has become increasingly apparent that standard RIA methodology is not always successful. Basic proteins constitute one class of molecules that is particularly unsuited to the standard competitive RIA. This is due to their highly positive charge at neutral pH, which causes them to adhere nonspecifically to many surfaces. Because detergents often have been used to prevent nonspecific adsorption of proteins, we undertook the development of an RIA in which high concentrations of detergents are routinely used. In addition, the presence of detergent in the RIA makes it possible to analyze the protein I content of tissues from which protein I must be solubilized by 1% NaDodSO₄. Such treatment also may reduce the action of proteases on labile antigens. Thus, the detergent-based RIA may prove useful as a general quantitative tool in biochemistry.

By using this RIA procedure, the amount of protein I was determined in the cerebral cortex of various mammals and in numerous brain regions of the cat. If protein I is indeed highly concentrated at synapses, as is indicated by recent studies (17), then measurement of protein I may provide a procedure for determining the density of nerve terminals in neural tissue. Such studies could be of considerable value because a detailed morphometric examination of the regional distribution of nerve terminals in mammalian brain, a difficult and tedious undertaking, has not been carried out. However, it should be emphasized that the observed differences in the level of protein I amongst the various brain regions may reflect differences not

Table 2. Regional distribution of protein I in various areas of cat brain

Area	Protein I/ total protein, pmol/mg
Cerebrum	
Frontal cortex	51
Occipital cortex	36
Corona radiata	2
Corpus callosum	2
Limbic system	
Hippocampal cortex	54
Amygdala, anterior	32
Amygdala, posterior	37
Olfactory bulb	19
Anterior commissure	3
Basal ganglia and diencephalon	
Caudate	31
Putamen	23
Globus pallidus	32
Internal capsule	<0.2
Thalamic gross section	50
Thalamus, anterior-ventral nucleus	43
Hypothalamus	32
Septal nuclei	30
Preoptic area	24
Pituitary, posterior	9
Pituitary, anterior	<0.2
Pineal	2
Midbrain	
Substantia nigra, anterior	33
Substantia nigra, medial	25
Substantia nigra, posterior	29
Red nucleus	7
Tegmental nuclei, ventral	13
Medial geniculate	30
Inferior colliculus	11
Superior colliculus	15
Periaqueductal grey	25
Cerebral peduncle	8
Pons and medulla	
Pons, dorsal	4
Pons, ventral	1
Raphe nuclei, dorsal	10
Area of locus coeruleus	8
Medulla	7
Cerebellum	
Cortex	28
Peduncle, middle	3
Midline nuclear region	4
Spinal cord	
Cervical segment	<0.2
Grey substance, dorsal	13
Grey substance, ventral	8
Dorsal root ganglia	<0.2

Table 1. Protein I as percentage of cerebral cortex protein in several mammalian species

Species	Protein I, % total protein
Human	0.38
Cow	0.37
Cat	0.40
Rat	0.38
Rabbit	0.36

only in the number of nerve terminals but also in the number of synaptic vesicles per terminal or the amount of protein I per vesicle.

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