ISOLATION AND PROPERTIES OF THE BOVINE BRAIN PROTEIN INHIBITOR OF ADENOSINE 3':5'-MONOPHOSPHATE-DEPENDENT PROTEIN KINASES

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1. Introduction

While the heat-stable protein inhibitor (PKI) of the cAMP-dependent protein kinase was first described in skeletal muscle [1] from which it was recently isolated in pure form [2], its greatest concentration occurs in the brain [3]. This is not surprising in view of the very high concentration of protein kinase found in this tissue [4]. It has recently been proposed that cAMP-dependent phosphorylations might be involved in neuronal functions, such as tissue growth, membrane permeability and synaptic transmission (reviewed [5–7]). Another protein kinease inhibitor has also been described in nervous tissue that can block phosphorylations catalyzed by both cyclic nucleotide-dependent and -independent enzymes [8].

It thus appeared desirable to reinvestigate the protein kinase system of the brain. As a first step towards this goal, beef brain protein kinase inhibitor was purified by the procedure established for isolation of the skeletal muscle protein [2]; some of its properties were also investigated.

Abbreviations: PKI, protein kinase inhibitor; cAMP, cyclic adenosine 3':5'-monophosphate; cGMP, cyclic guanosine 3':5'monophosphate; C, catalytic subunit; Tris, tris(hydroxymethyl)amino methane

Enzymes: protein kinase, ATP:protein phosphotransferase (EC 2.7.1.37)

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2. Materials and methods

Histone IIA and protein markers were obtained from Sigma and ^{32}P from New England Nuclear; [γ-^{32}P]ATP, ca. 780 mCi/mmol, was prepared by the procedure in [9]. The catalytic subunit of the cAMP-dependent protein kinase was isolated from bovine heart and assayed as in [2,10]; the protein kinase inhibitor from rabbit skeletal muscle and bovine brain were also assayed as in [2]. The cGMP-dependent protein kinase was purified from fresh bovine lung up to the affinity step and assayed as in [11]. Affinity chromatography was carried out on a cAMP-Sepharose 6B column as in [12].

Protein concentration was determined as in [13]. Molecular weight was estimated by electrophoresis in 0.1% dodecyl sulfate–15% polyacrylamide slab gels, using the discontinuous Tris–glycine buffer system in [14]. For localization of inhibitory activity, gels were sliced (18 μL sections) and immediately extracted with 0.2 ml water at room temperature. The extracts (0.15 ml) were evaporated and assayed without removing contaminating dodecyl sulfate since the C subunit retains full activity in the presence of 1 mM dodecyl sulfate [2]. Polyacrylamide (12%) gel electrophoresis in the absence of denaturing agents was carried out at pH 8.9 as in [15].

Amino acid analyses were performed in a Durrum analyzer (Model D 500) as in [16] after 24 h, 48 h and 72 h hydrolysis in 5.7 N HCl at 110°C. Cysteine was determined as cysteic acid [17]. The NH_2-terminus of the molecule was studied by dansylation [18]. Acid-stable, protein-bound phosphate was measured as in [10] on 5–10 nmol protein.

Incorporation of [^{32}P]phosphate into PKI under the influence of C subunit (19 μM) was studied in the presence of 0.6 mM [γ-^{32}P]ATP, 10 mM magnesium acetate, 50 μM PKI, in 30 mM potassium phosphate, 1 mM dithiothreitol, pH 7.0. After 30 min incubation at 30°C, the proteins were denatured (1 min at 100°C) and separated on a dodecyl sulfate–polyacrylamide gel as described above. Radioactivity was immediately estimated by either slicing and counting the gels or by autoradiography. Samples were never exposed to extremes of pH thus minimizing possible losses of bound phosphate.

3. Results

Brain PKI was purified by the procedure described for the skeletal muscle protein, which includes a heat step, batchwise ion-exchanges on DEAE-cellulose and phosphocellulose, and affinity chromatography on Sepharose 6B to which the catalytic subunit of protein kinase is attached [2]. Two slight modifications were introduced. First, the affinity step was scaled up by the use of a larger column (45 ml) of C-subunit bound to Sepharose 6B (3.25 mg enzyme/ml, i.e., 80 μM) and buffers contained 1 mM dithiothreitol instead of 15 mM 2-mercaptoethanol. Second, after the affinity step, brain PKI was subjected to a final gel filtration through Sephadex G-75 (1.5 × 80 cm column eluted at 7.2 ml/h with 0.2 M acetic acid) to eliminate larger molecular weight contaminants that emerged in the void volume. The material obtained was homogeneous on dodecyl sulfate–polyacrylamide gel electrophoresis, where it migrated with mol. wt 11 000–12 000, identical to that reported for skeletal muscle PKI (fig.1.A,B). It also exhibits a blocked NH_2-terminus, as reported for the muscle protein [2]. Confirmation that this single protein band indeed represents the inhibitor of protein kinase was obtained by assaying individual gel slices. By the same procedure, a similar inhibitory peptide mol. wt 11 000–12 000 could be identified in human brain and in baboon skeletal muscle (J. G. D. and E. H. F., unpublished). There is little doubt, therefore, that a monomeric protein of ca. 100 residues exists in various tissues and species that displays the properties of PKI.

PKI from bovine brain and rabbit skeletal muscle were kinetically indistinguishable. Both inhibited markedly the cAMP-dependent protein kinase by interacting with its catalytic subunit. This interaction occurred as well in the presence or absence of ATP; that is, under the conditions for its assay where ATP is of course present or during the affinity chromatography step which is carried out in the absence of the nucleotide [2]. Both were unable to inhibit the cGMP-dependent protein kinase even at a concentration in excess of 0.1 mM, i.e., at a level 5 orders of magnitude greater than required for a 50% inhibition of the cAMP-dependent enzyme (fig.2). On the contrary, addition of either PKI induced a moderate increase (up to 1.3-fold) in the rate of phosphate incorporation into histones.

While brain and muscle PKI display certain struc-

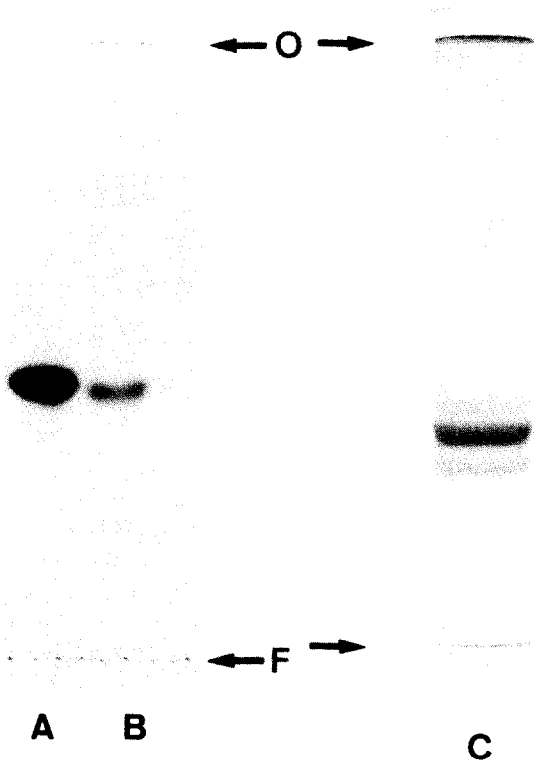

Fig.1.(A,B) Dodecyl sulfate (0.1%)–polyacrylamide (15%) slab-gel electrophoresis of 45 μg (A) and 11 μg (B) bovine brain PKI. R_F for protein markers were, respectively, 0.18 for C subunit (M_r 39 000), 0.28 for carbonic anhydrase (M_r 29 000), 0.45 for β-lactoglobulin (M_r 17 500) and 0.52 for lysozyme (M_r 14 300), relative to dye front. (C) Polyacrylamide (12%) disc gel electrophoresis, at pH 8.9, of brain PKI (40 μg). (O) origin; (F) dye front.

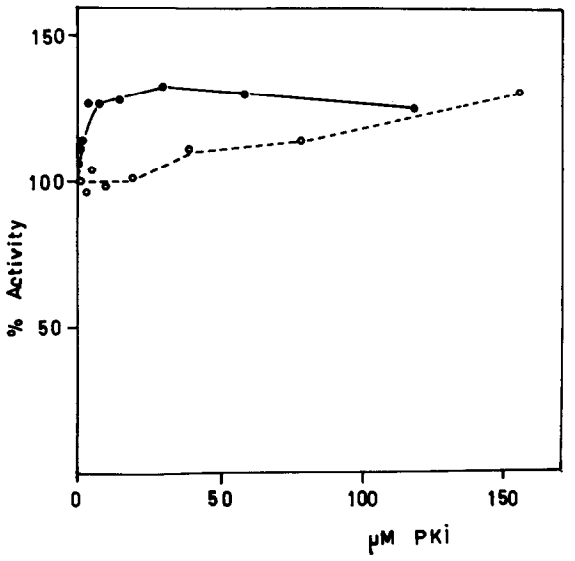

Fig.2. Effect of PKI on beef lung cGMP-dependent protein kinase. Assays were performed as in [11]. In the absence of PKI, the enzyme catalyzed the incorporation of 9 pmol [^{32}P]phosphate/min into mixed histones. (•——•) rabbit skeletal muscle PKI; (o– – –o) bovine brain PKI. For comparison, 100% inhibition of the catalytic subunit of the cAMP-dependent protein kinase (16 nM) would occur at a PKI concentration ⩾ 0.3 μM.

tural characteristics in common (no sulfur-containing amino acid, a single residue of Tyr, Phe and His, a high proportion of acidic and hydroxylated amino acids, less than 1 residue/mol protein-bound phosphate) other differences exist in their primary structure in that the brain protein contains more Glx and Pro residues, perhaps more lysine but less arginine and much less bound phosphate (table 1).

No acid or alkali-stable phosphate could be introduced in skeletal muscle PKI in the presence of catalytic subunit and [γ-^{32}P]ATP. The same experiment could not be repeated with brain PKI because of scarcity of material. Like the skeletal muscle protein, the brain inhibitor is heterogeneous on gel electrophoresis in the absence of denaturing agents [2], in that the major fraction (R_F 0.64, relative to dye front) is flanked by two bands of lower intensities (fig.1C). This excludes the possibility that this microheterogeneity results from variable amounts of bound phosphate, (one of the alternatives suggested for the muscle inhibitor [2]) since the brain protein contains only 0.1 residue/mol. Another explanation would be

Table 1
Amino acid and phosphate analysis of protein kinase inhibitors (residues/mol)

	Skeletal muscle PKI[a]	Bovine brain PKI	
			integer
Asx	12	13.1	13
Thr[b]	8	8.0	8
Ser[b]	11	11.5	11–12
Glx	14	17.8	18
Pro	1	3.5	3–4
Gly	9	8.9	9
Ala	15	13.0	13
Val[c]	3	3.1	3
Met	0	0	0
Ile[c]	5	4.6	4–5
Leu	7	6.5	6–7
Tyr	1	0.9	1
Phe	1	1.1	1
His	1	0.8	1
Lys	4	4.7	5
Trp[d]	0	n.d.	n.d.
Arg	6	3.7.	4
Cys[e]	0	0.3	0
Total	98		100–104
Phosphate[f]	0.7	0.1	

[a] Amino acid composition of skeletal muscle PKI taken from [2] and given for comparison
[b] After extrapolation to zero time of hydrolysis
[c] From the 72 h hydrolysis values
[d] n.d., not determined
[e] As cysteic acid
[f] Acid-stable, protein-bound phosphate (residue/mol)

partial deamidation or, perhaps more likely, the occurrence of 'isoinhibitors' which would account for the non-integral numbers of residues obtained on amino acid analysis (see table 1).

4. Discussion

Results described above indicate that bovine brain contains a protein kinase inhibitor (or isoinhibitors) sharing many properties of the homologous skeletal muscle protein, including acidic character, low molecular weight, similar amino acid composition, ability to interact with, and inhibit, the catalytic subunit of

cAMP-dependent protein kinases and inability to inhibit the cGMP-dependent protein kinase.

This latter feature was already described for crude preparations of PKI [8,12,19]. It also constitutes an additional, and perhaps strongest, piece of evidence for a difference in the catalytic sites of the two cyclic nucleotide-dependent protein kinases [20,21]. This would indicate that PKI must possess other structural features contributing to its remarkable potency and specificity despite the fact that it acts as a competitive inhibitor towards protein substrates [2].

Although this study does not provide data on the cellular and subcellular localization of brain PKI (e.g., whether it is of glial or neuronal origin), its isolation is of physiological importance, since it is able to inhibit not only the cytosolic kinase, but also the neuronal, membrane-bound protein kinase [7]. In this respect, it would be of interest to try to separate the electrophoretic variants of PKI and check whether or not they might interact differently with the various protein kinases.

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