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Multiple Forms of Protein Kinase from Normal Human Brain and Glioblastoma[1]

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ABSTRACT

The biochemical characteristics of the protein kinase (PK; adenosine triphosphate-protein phosphotransferase, EC 2.7.1.37) isozymes in subcellular preparations from normal human brain cortex and glioblastoma were investigated after chromatography on diethylaminoethyl cellulose, and the following results have been obtained.

Two major isozyme forms, eluted by 50 and 200 mM phosphate buffer, are present in both cytosol and membrane-derived preparations from cerebral cortex. Furthermore, these isozyme forms have properties similar to those referred to as type I and type II cyclic adenosine 3':5'-monophosphate-dependent PK. In these chromatographic isozymes, cyclic adenosine 3':5'-monophosphate is more active in stimulating the basal PK enzyme than is cyclic guanosine 3':5'-monophosphate. In glioblastoma, the PK activity from cytosol and particulate preparations is resolved by diethylaminoethyl cellulose in four peaks. In cytosol, the major portion of the enzyme is eluted with a 300 mM buffer (about 50% of the total basal PK activity) and is cyclic nucleotide dependent. On the contrary, in glioblastoma particulate, the PK enzyme is mainly eluted at 50 and 100 mM buffer; neither of these isozymes is cyclic nucleotide dependent. As for cytosol, only the particulate isozyme eluted at 300 mM buffer is strongly activated by cyclic nucleotides.

Finally, in both glioblastoma subcellular preparations, only a type II cyclic adenosine 3':5'-monophosphate-dependent PK is present.

INTRODUCTION

In a previous work we found that the activity and subcellular distribution of cyclic nucleotide-dependent PKs[3] are regulated differently in various human brain tumors. In particular, we observed that the specific activity of the PK enzyme stimulated by cGMP increases significantly in the subcellular particulate fraction obtained from the most malignant tumors (10).

In mammalian tissues, 2 major types of cAMP-dependent PKs (referred to as type I and type II PK) have been differentiated in relation to their elution profile from DEAE-cellulose chromatography and to characteristics of their activation by cAMP (3, 11, 15, 17). It has been suggested that these PK isozymes may subserve different functional roles. Usually, the relative amount of cytosolic type I and type II cAMP PK isozymes changes during mitogenic stimulation and neoplastic transformation, with an increase of the former (1, 2, 4-6, 8, 12, 21). Nevertheless, this biochemical trend is not constant in the different pathological tissues; for example, in C6 glioma cells, the cytoplasm mainly contains the type II cAMP PK (23).

These observations have led us to investigate the distribution and the characteristics of PK isozymes in cytosolic and particulate fractions of normal human brain and glioblastomas.

MATERIALS AND METHODS

Human Material. Pathological tissues were removed during surgical operation from patients with cerebral neoplasia. Control brain cortex was taken from epileptic patients operated on for temporal lobectomy. The samples were taken from areas far from corticographically identified epileptic foci and were histologically normal.

Tissues were immediately frozen in liquid nitrogen and preserved at −80° until use; for the histopathological studies, the tissues were examined as indicated previously (10).

Chemical Material. [γ-^{32}P]ATP (triethylammonium salt, 14.1 Ci/mmol) was purchased from Amersham Radiochemical Centre (Amersham, United Kingdom). Unlabeled cAMP, cGMP, ATP (sodium salt), and calf thymus histone type IIA were obtained from Sigma Chemical Co. (St. Louis, Mo.), and DEAE-cellulose was from Whatman, Ltd. (Springfield Mill, Maidstone, Kent, England). All other reagents were of analytical grade.

Preparation of PKs. All procedures for enzyme preparations were carried out at 4°. The tissues were homogenized with a Teflon-glass pestle homogenizer, with 3 strokes of 10 sec each, in 6 volumes of ice-cold isotonic solution containing 15 mM sodium-potassium phosphate buffer (pH 6.5), 150 mM NaCl, and 1 mM EGTA and then filtered through 5 layers of sterile gauze to remove debris.

The filtrate was centrifuged at 40,000 × *g* (average value) for 60 min. The supernatant solutions were used as the cytosol enzyme source. The original pellets were resuspended in the initial volume of the homogenization buffer, with 10 strokes of a Teflon-glass homogenizer set at 700 rpm, and centrifuged at 40,000 × *g* for 15 min, and the supernatant fluid was removed. This washing step was repeated twice more. After this procedure, the washed particulate subcellular preparation was resuspended in the original volume of homogenization buffer containing 0.10% (w/v) Triton X-100 and was stirred gently at 4° for 30 min. The procedure for solubilization of membrane-associated PKs and the elimination of Triton X-100 was then carried out as indicated by Rubin *et al.* (22). The enzyme activity from this preparation was designed as "membrane-associated" or as "particulate" PK. The PK activity released in the supernatant fluid by Triton X-100 solubilization corresponds to more than 90% of the PK activity present in the original pellets.

DEAE-cellulose Chromatography of PK Subcellular Fraction. DEAE-cellulose chromatography was carried out as indicated by Kuo (17) with minor modifications. Identical columns of DEAE-cellulose (1 x 20 cm) were preequilibrated with 500 ml of sodium-potassium phosphate buffer (5 mM, pH 6.5, containing 1 mM EGTA). The columns were then charged with 2 ml of cytosol or with 2 ml of membrane-associated PK preparations. The columns were eluted with 50 ml of the original buffer and then with 30 ml of 50, 100, and 200 mM sodium-potassium phosphate buffer (pH 6.5, containing 1 mM EGTA); finally, 60 ml of the same buffer at 300 mM were used.

During the elution from DEAE-cellulose columns, proteins were read

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[3] The abbreviations used are: PK, protein kinase; cGMP, cyclic guanosine 3':5'-monophosphate; cAMP, cyclic adenosine 3':5'-monophosphate; EGTA, ethyleneglycol bis(aminoethyl ether)tetraacetic acid.

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at 280 nm. Fractions of 3 ml were collected, and aliquots from each fraction were assayed for PK activity in the absence of cyclic nucleotides. The eluate tube fractions from each gradient of elution exhibiting enzyme activity were pooled and were used as the source of PK activity.

Assay for PK Activity. The assay of PK was based on the phosphorylation of histone and was carried out as described by Kuo (17) with minor modifications deriving from our previous studies on the kinetic analysis of the enzyme obtained from the human brain and cerebral tumors (10).

Enzyme activity was measured in crude cytosol and particulate subcellular preparations and in the DEAE-cellulose fractions. In a final volume of 0.2 ml, standard reaction mixtures contained: 50 μl of incubation medium at pH 6.5 (sodium acetate, 10 μM; magnesium acetate, 2 μM; NaF, 2 μM; EGTA, 0.06 μM; theophylline, 0.5 μM); calf thymus histone, 40 μg; [γ-^{32}P]ATP, 25 μM, containing about 1.2×10^6 cpm; enzyme preparation, 50 to 100 μg of protein. The PK activity was measured in the absence and presence of 5×10^{-6} M cAMP or cGMP. In the absence of cyclic nucleotides, the enzyme activity was referred to as "basal PK activity." All results are expressed as pmol $^{32}P_i$ incorporated (or transferred) to histone per min per 30° per mg protein.

For the evaluation of the enzyme activity dependent upon cyclic nucleotide stimulation, the basal PK activity was subtracted from the total PK activity.

The protein concentration of the different enzyme source was measured as indicated by Lowry *et al.* (19).

Table 1

Specific activities of the different PK isozymes separated by DEAE-cellulose column chromatography in cytosol and particulate subcellular fractions from human brain cortex

After elution with a 300 mM buffer, a protein was collected that was devoid of enzyme activity.

Elution buffer (mM)	PK activity (pmol of P_i transferred to histone/min/mg protein/10^{-1})					
	Cytosol			Particulate		
	Basal	+cAMP	ΔX%[a]	Basal	+cAMP	ΔX%
50	60 ± 5.4[b]	101 ± 7.9	68	64 ± 7.1	203 ± 24.1	214[c]
100	5 ± 0.7	6 ± 0.7	16	48 ±5.9	55 ± 7.2	15
200	39 ± 4.9	208 ± 23.4	429[c]	43 ± 4.7	409 ± 43.2	847[c]
	Basal	+cGMP	ΔX%	Basal	+cGMP	ΔX%
50	60 ± 5.4	69 ± 7.3	14	64 ± 7.1	152 ± 13.5	94
100	5 ±0.7	5 ± 0.5		48 ± 5.9	46 ± 4.0	
200	39 ±4.9	126 ± 17.4	222[c]	43 ± 4.7	90 ± 7.6	110[c]

[a] ΔX%, percentage of change in comparison to basal PK activity.
[b] Mean ± S.E. of 6 tissues obtained from 6 different patients.
[c] $p < 0.01$ (*versus* the basal PK activity). The other differences are not significant. Statistical analysis was performed using Student's paired *t* test.

RESULTS

The subcellular preparations of human brain and glioblastoma were applied to a DEAE-cellulose column, and the adsorbed proteins were eluted with a sodium-potassium phosphate buffer. In the eluted tube fractions, the PK assay was done only in the absence of cyclic nucleotides. The tube fractions from each gradient of elution exhibiting PK activity were pooled and used as an enzyme source for further experiments.

The PK activity from cytosol and membrane-derived human brain was resolved by DEAE-cellulose column in 2 major enzyme peaks, which were eluted at 50 mM (Peak I) and 200 mM (Peak III).

With 25 μM ATP in the reaction mixture, the activity of these isozyme fractions (referred to as basal PK activity) corresponds to about 94% of the total basal PK activity for the cytosol and about 70% for the particulate.

In cytosol, the cAMP stimulation slightly increases the basal activity of Peak I (+68%), while the enzyme of Peak III (which corresponds to about 37% of the total basal activity) is strongly activated by cAMP (430%) and represents 66% of the cAMP-dependent PK from all cytosolic fractions. In brain cytosol, the cGMP stimulates only the basal enzyme activity of Peak III (+222%). According to criteria which characterize the different forms of cAMP-dependent PK, the chromatographic elution profile and the properties of the cyclic nucleotide stimulation indicate that the isozymes of Peaks I and III from brain cytosol have properties very similar to those referred to as type I and type II PK, respectively (3, 11).

As shown in Table 1, the 2 major enzyme fractions of membrane-derived preparations were eluted at the same position as for cytosol (Peaks I and III) and have a basal PK activity similar to those observed in the corresponding peaks of cytosol; the basal activity of these isozymes was enhanced both by cAMP (214 and 847% for Peaks I and III, respectively) and by cGMP stimulation (about 94 and 110%, respectively).

Furthermore, in the brain membrane-derived preparations, one protein is present (eluted at 100 mM buffer and not shown by the chromatographic separation of cytosol) which displays a considerable basal enzyme activity (48 ± 5.9 pmol of P_i transferred per min per mg of protein per 10^{-1}) and is cyclic nucleotide independent.

Table 2

Specific activities of the different PK isozymes separated by DEAE-cellulose column chromatography in cytosol and particulate subcellular fractions from human glioblastoma

Elution buffer (mM)	PK activity (pmol of P_i transferred to histone/min/mg protein/10^{-1})					
	Cytosol			Particulate		
	Basal	+cAMP	ΔX%[a]	Basal	+cAMP	ΔX%
50	216 ± 18.3[b]	270 ± 30.4	24	515 ± 60.2	591 ± 60.8	15
100	96 ± 8.2	111 ± 9.7	16	598 ± 58.4	731 ± 75.2	22
200	292 ± 31.4	356 ± 31.6	22	41 ± 3.7	53 ± 4.8	30
300	576 ± 66.1	1686 ± 171.3	192[c]	121 ± 9.7	262 ± 24.6	117[c]
	Basal	+cGMP	ΔX%	Basal	+cGMP	ΔX%
50	216 ± 18.3	198 ± 23.2		515 ± 60.2	587 ± 65.1	14
100	96 ± 8.2	89 ± 10.8		598 ± 58.4	849 ± 79.8	42
200	292 ± 31.4	208 ± 23.4		41 ± 3.7	40 ± 3.6	
300	576 ± 66.1	1570 ± 146.4	172[c]	121 ± 9.7	294 ± 25.2	143[c]

[a] ΔX%, percentage of change in comparison to basal PK activity.
[b] Mean ± S.E. of 6 tissues obtained from 6 different patients.
[c] $p < 0.01$ (*versus* the basal PK activity). The other differences are not significant. Statistical analysis was performed using Student's paired *t* test.

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The specific PK activities of the chromatographic isozyme peaks from human glioblastoma are summarized in Table 2.

At variance with the cortex, in the pathological tissues, an enzymatic protein is eluted also with a 300 mM buffer (Peak IV); at this salt concentration, glioblastoma cytosol extract displays the greatest PK activity (about 50% of the total basal activity). The enzyme of this peak is enhanced both by cAMP (192%) and by cGMP (172%); on the contrary, all other cytosol isozymes are not significantly activated by cAMP and are completely cGMP independent.

Chromatographic fractionation of glioblastoma particulate showed that the major portion of PK is eluted at 50 mM (Peak I) and 100 mM (Peak II) buffer (about 87% of the total basal activity).

As for cytosol, only the particulate isozyme, which is eluted at 300 mM buffer (Peak IV), is markedly stimulated by cyclic nucleotides.

The level of basal PKs from overall chromatographic fractions is greater in glioblastoma (1180 and 1275 pmol of P_i transferred to histone, respectively, for cytosol and particulate) than in normal brain (104 and 155 pmol of P_i transferred in the same subcellular fractions); on the contrary, the percentage of cAMP stimulation of the overall basal PK is more remarkable in normal tissue (cytosol, 202 and 90%; particulate, 320 and 28%, respectively, for brain cortex and glioblastoma).

Finally, the recovery of the enzyme activity after DEAE-cellulose chromatography was consistently different in the tissues studied. In fact, while the recovery in brain cortex is about 87 and 92% for cytosol and particulate, respectively, in the same preparations deriving from glioblastoma recovery corresponds to 188 and to 245% of the activity charged on the column. Probably, this high recovery is due to a removal during the chromatography purification of some inhibitor factors which are present only in the tumoral tissue (18).

DISCUSSION

Our study indicates that PKs from human brain cortex and glioblastomas submitted to DEAE-cellulose chromatography show marked differences in the proportion and characteristics of the enzyme activities which are eluted at low and high ionic strength.

In human brain, the fractionation of cytosol-prepared PKs demonstrates the presence of 2 major types of PK isozymes which have properties similar to those referred to as type I and type II cAMP-dependent PK, respectively (3, 11, 15, 17). As in other mammalian brains, the Peak III from both subcellular preparations contains the major portion of cAMP-dependent PK. This isozyme is prevalently associated with the membrane-derived preparations, as observed on more differentiated tissues (3, 5, 14, 21).

In human glioblastoma, both cytosol and particulate disclose an enzyme protein (not found in the brain) which is eluted with 300 mM buffer and is activated by both cAMP and cGMP.

On the contrary, in all other cytosol and particulate DEAE-cellulose fractions, the basal PK activity is not activated by cyclic nucleotides.

Since the cAMP-dependent PKs differ in their regulatory subunits but have identical catalytic subunits (9, 15), the phosphorylating activities in Peaks I, II, and III from glioblastoma are probably not dependent (or at least not totally so) upon a free catalytic subunit of the cAMP PK as, in this case, they should elute together.

It should be taken into account that brain tissue contains PKs which are modulated by factors other than cyclic nucleotides as, for instance, Ca^{2+} or the Ca^{2+}-calmodulin complex (15).

The basal level of phosphorylating activities of the overall chromatographic fractions is greater in glioblastoma than in normal brain; this biochemical feature depends chiefly on an increased level of the cyclic nucleotide-independent forms of PKs in pathological preparations. It is interesting to note that a similar biochemical trend has also been shown in other instances of neoplastic growth, as in myeloblastic leukemia, and in virus-transformed cells (7, 12, 16).

In some human and experimental tumors, the ratio of type I to type II cAMP-dependent PK is high in pathological tissues, as a result of the increase of type I PK (1, 4-6, 14). This isozyme is more specifically devoted to cellular proliferating activity (2, 6, 14), while "the activation of type II PK represents a mechanism by which a negative influence can be imposed on the proliferative process" (1).

In contrast with these indications, in both cytosol and particulate of human glioblastoma, there is present only a cAMP-dependent PK isozyme with characteristics of type II PK. On the other hand, an enzymatic profile similar to that of glioblastoma has been detected in some cell lines of rat hepatoma (13, 20) and in particular in the cytosol of the C6 glioma cells (23).

In conclusion, in comparison to normal brain, the most significant changes in the PK system of tumoral tissue are represented by: (*a*) the increase in the overall level of PK enzymes which are scarcely stimulated by cyclic nucleotides; (*b*) the presence of an enzyme peak (Peak IV) not found in the brain subcellular preparations, which is the only PK fraction in glioblastoma stimulated by both cAMP and cGMP. This fraction also shows characteristics of a type II cAMP PK.

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