ISOLATION OF STIMULATORY MODULATOR FROM RAT BRAIN AND ITS SPECIFIC EFFECT ON GUANOSINE 3':5'-MONOPHOSPHATE-DEPENDENT PROTEIN KINASE FROM CEREBELLUM AND OTHER TISSUES

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SUMMARY: The stimulatory and the inhibitory activities of protein kinase modulator originally present in the crude modulator preparation obtained from rat brain was separated by Sephadex G-100 gel filteration. The isolated stimulatory modulator, as the crude modulator, augmented the activity of guanosine 3':5'-monophosphate (cGMP)-dependent protein kinase from both the mammalian tissues (cerebellum, heart and lung) and the lobster tail muscle. The purified cGMP-dependent protein kinase was not activated by cGMP unless the stimulatory modulator, or the crude modulator, was added. The isolated inhibitory modulator, as the crude modulator, on the other hand, depressed the activity of adenosine 3':5'-monophosphate (cAMP)-dependent protein kinase. It neither inhibited nor stimulated the activity of cGMP-dependent protein kinase.

Protein inhibitor of cAMP-dependent protein kinase was reported to occur in many mammalian tissues (1). This factor was purified from rabbit skeletal muscle and its properties were studied (2,3). The crude preparations of the factor obtained from various sources, with a possible exception of the skeletal muscle, was subsequently found not only to inhibit cAMP-dependent protein kinase but also to stimulate cGMP-dependent class protein kinase purified from lobster tail muscle (4-6). Due to the dual activities of the crude preparations, it has been suggested that the factors be called protein kinase modulators (5). It was shown earlier that the homogeneous preparation of protein kinase modulator from lobster tail muscle possesses both inhibitory and stimulatory activities as in its crude state (5). It remains to be seen whether the two distinct activities of the crude protein kinase modulator from mammalian tissues are due to the same protein as a single entity, as in the case of the modulator from the lobster muscle.

EXPERIMENTAL PROCEDURE

Preparation of inhibitory and stimulatory modulators: Fresh whole rat brain (20-30 g) were homogenized in 3 volumes of ice-cold 5 mM potassium phosphate buffer, pH 7.0, containing 1 mM EDTA (extraction buffer) for 1 min at maximum speed in a Sorvall Omni-Mixer. The homogenate was centrifuged at 40,000 x g for 10 min, and the supernatant was collected. The brain extract was boiled for 10 min, cooled in ice, followed by centrifugation to remove the precipitates. One-ninth volume of 50% trichloroacetic acid was added to the supernatant, and the resultant precipitate was collected by centrifugation. The precipitate was then suspended in appropriate volumes (5-8 ml) of water, and the pH was adjusted to 7.5 with 1 N NaOH. The solution was dialyzed overnight against the extraction buffer. The dialysate was centrifuged and the clear supernatant solution (crude protein kinase modulator) was collected. The procedure to this point was essentially the same as that described for rabbit skeletal muscle (1,7) and lobster tail muscle (5). Solid ammonium sulfate (35 g/100 ml) was added to the crude modulator solution. After stirring for 30 min, the precipitate was collected by centrifugation, and was dissolved in 5 to 8 ml of the extraction buffer. Next, it was loaded onto a Sephadex G-100 column (3 x 43 cm) which has been previously equilibrated with the same buffer. This gel filtration step, using 50 mM phosphate buffer (pH 7.0) for elution, separated the crude protein kinase modulator into the stimulatory and inhibitory modulator components.

Assay for protein kinase and protein kinase modulator: The standard system (8,9) for protein kinases contained, in a final volume of 0.20 - 0.25 ml, potassium phosphate buffer, pH 7.0, 10 μmoles; theophylline, 0.5 μmole; arginine-rich histone (HA, Worthington), 40 μg; $MgCl_2$, 2 μmoles; [γ-^{32}P]ATP (New England Nuclear), 1 nmole, containing about 0.9 x 10^6 cpm; with or without appropriate amounts of protein kinase modulator preparations, cAMP or cGMP, as indicated. The reaction was carried out for 10 min at 30°. One unit of the enzyme activity is defined as that amount of enzyme that transferred 1 pmole of ^{32}P from [γ-^{32}P]ATP to recovered histone under the assay conditions. One unit of the inhibitory modulator is defined as that amount of the factor that depressed 20 units of cAMP-dependent protein kinase 20% in the presence of 0.4 μM cAMP; one unit of the stimulatory modulator is that amount of the factor that augmented 20 units of cGMP-dependent protein kinase 20% in the presence of 0.4 μM cGMP(5,10).

Other methods: cGMP-dependent protein kinase from guinea pig cerebellum and lung (adult and fetus), from rat cerebellum and from dog heart atrium was purified through the hydroxylapatite step as described recently for the enzyme from guinea pig fetal lung (11). The cGMP-dependent enzyme from lobster tail muscle was either prepared through the DEAE-cellulose step (crude preparation) (12) or further purified employing additional Sephadex G-200 and hydroxylapatite steps (purified preparation) as described for the mammalian enzyme (11). cAMP-dependent protein kinase from guinea pig cerebellum was purified through the DEAE-cellulose step (13). Tissue contents of cAMP and cGMP were determined by the protein kinase catalytic methods (14).

RESULTS AND DISCUSSION

The protein kinase modulator prepared from whole rat brain exhibited abilities to stimulate cerebella cGMP-dependent protein kinase in the

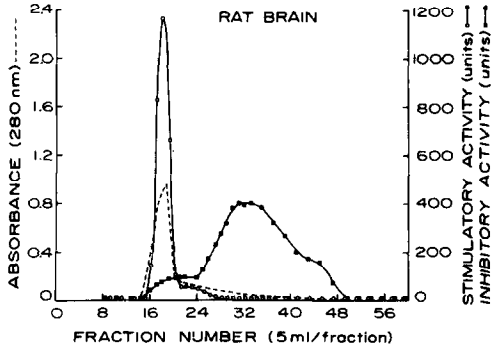

Fig. 1. Chromatography of crude protein kinase modulator from rat whole brain (27 g) on Sephadex G-100 column (3 x 43 cm). Potassium phosphate buffer (50 mM, pH 7.0) was used for elution.

presence of cGMP and to inhibit cerebella cAMP-dependent protein kinase in the presence of cAMP (data not shown), as reported earlier for the effects of crude modulator preparations on the two classes of protein kinases, both prepared from a wide variety of sources (4-6,8-11). The stimulatory component of the crude modulator was found to be separated from the inhibitory component by Sephadex G-100 chromatography (Fig. 1). The isolated stimulatory modulator progressively augmented the cerebella cGMP-dependent enzyme in a dose-dependent manner (Fig. 2). The stimulatory modulator, however, was without effect on cerebella cAMP-dependent enzyme, which would have been inhibited had the crude modulator (containing both the inhibitory and stimulatory components) been used instead. The isolated inhibitory modulator, presumably the same as the protein inhibitor of cAMP-dependent protein kinase reported by Walsh et al. (1), progressively inhibited cAMP-dependent protein kinase; it was without a significant effect on the cGMP-dependent class of enzyme, which would have been stimulated had the crude modulator preparation been used instead (Fig. 3).

A tissue-, species- or phylum-specificity appeared to be lacking in interactions between the stimulatory modulator and cGMP-dependent protein kinase. Thus, the modulator isolated from rat brain stimulated the enzyme

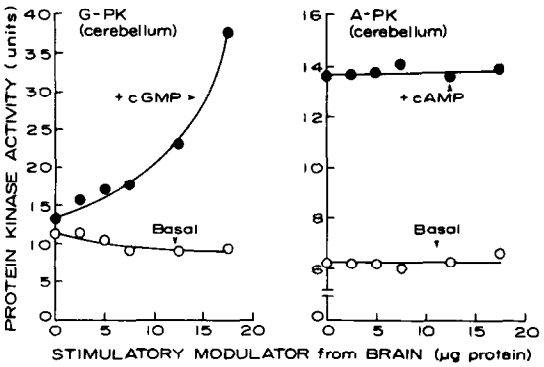

Fig. 2. Comparison of the effects of varying amounts of the isolated stimulatory modulator from rat brain on the activities of cGMP-dependent protein kinase (G-PK, 6.4 μg) and cAMP-dependent protein kinase (A-PK, 11.5 μg) purified from guinea pig cerebellum. When present, the concentration of either cyclic nucleotide was 0.4 μM.

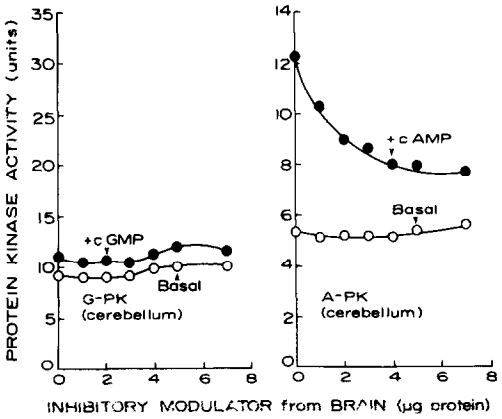

Fig. 3. Comparison of the effects of varying amounts of the isolated inhibitory modulator from rat brain on the activities of cGMP-dependent protein kinase (G-PK, 6.4 μg) and cAMP-dependent protein kinase (A-PK, 11.5 μg) purified from guinea pig cerebellum. When present, the concentration of either cyclic nucleotide was 0.4 μM.

purified from either dog atrium, guinea pig cerebellum and lung or lobster tail muscle, as it stimulated the enzyme from rat cerebellum (Table 1). It should be mentioned that the purified cGMP-dependent protein kianse was not stimulated by cGMP in the absence of stimulatory modulator, which was added to the incubation system as an isolated form (Table 1) or as a mixture with inhibitory modulator in the crude form (11).

TABLE 1. Effects of the isolated stimulatory modulator from whole rat brain on cGMP-dependent protein kinase prepared from various sources

Source and amount of cGMP-dependent protein kinase	Protein kinase activity					
	- Stimul. modulator			+ Stimul. modulator		
	None	+cGMP	+cAMP	None	+cGMP	+cAMP
	units					
None (control)	0.0	0.0	0.0	0.1	0.0	0.1
Guinea pig cerebellum (6.4 µg)	11.8	12.6	12.0	10.6	46.4	15.2
Rat cerebellum (5.8 µg)	8.3	9.2	8.6	7.6	43.2	14.5
Dog heart atrium (8.0 µg)	1.2	1.3	1.3	1.1	18.2	3.1
Guinea pig lung, adult (5.2 µg)	2.2	2.3	2.1	2.0	24.4	6.0
Guinea pig lung, fetus (0.8 µg)	1.8	1.9	1.8	1.4	25.0	4.6
Lobster tail muscle, crude (310 µg)	5.2	16.8	7.2	4.8	31.5	11.6
Lobster tail muscle, purified (36 µg)	3.0	3.8	3.1	2.9	26.1	6.0

When present, the amount of stimulatory modulator was 35 µg protein, and the concentration of either cyclic nucleotide was 0.4 µM.

TABLE 2. Correlation among levels of cyclic nucleotides, protein kinases and protein kinase modulators in different parts of guinea pig brain

Parameter	Cerebellum	Cerebral cortex	Midbrain
Cyclic nucleotides (pmoles/mg protein)			
cGMP	4.6	0.4	0.2
cAMP	7.5	10.8	12.5
Ratio	0.61	0.04	0.02
Protein kinases (units/mg tissue)			
cGMP-dependent	20	6	4
cAMP-dependent	17	21	30
Ratio	1.3	0.3	0.1
Protein kinase modulator (units/mg tissue)			
Stimulatory	2.6	1.6	1.5
Inhibitory	1.0	1.1	1.1
Ratio	2.6	1.4	1.4

For estimation of tissue levels of protein kinases in extracts, the cGMP-dependent enzyme was assayed in the presence of 0.4 µM of cGMP and stimulatory modulator (20 µg); the cAMP-dependent enzyme was assayed in the presence of 0.4 µM cAMP but in the absence of either the stimulatory or inhibitory modulator. The tissue levels of modulators were assessed using the crude protein kinase modulator preparations.

The activity of the stimulatory modulator was destroyed by trypsin, but was not affected by phospholipase C nor by lysozyme (data not shown), indicating that the factor was a protein. The modulator was extremely stable to heat, retaining total activity after boiling for 3 hours.

The data presented in Table 2 demonstrate a correlation among the cyclic nucleotide related parameters in different parts of the brain. Thus, the cerebellum, compared to cerebral cortex and midbrain, had a higher content of cGMP, higher tissue levels of the cGMP-dependent enzyme and stimulatory modulator that regulates the activity of this cGMP target enzyme. It has been shown that cGMP may mediate cholinergic transmission in the brain (15-17). It is conceivable, therefore, that high levels of cGMP-dependent protein kinase and stimulatory modulator in cerebellum may have some functional significance. Possible biological roles of the cGMP-dependent enzyme were suggested by recent observations that its levels in the brain was the highest in the adult, but its levels in the heart and lung were the highest in the fetus of guinea pigs (9). Moreover, the tissue levels of the stimulatory modulator were shown to alter in the adipose tissue and the pancreas of alloxan-induced diabetic rats (18).

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