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PHOSPHOLIPASE A_2 MODULATION OF CYCLIC GMP METABOLISM: CHARACTERISTICS OF GUANYLATE CYCLASE ACTIVATION

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Summary

Characteristics of phospholipase A_2 (PLA_2) modulation of guanylate cyclase were evaluated. Addition of phospholipase A_2 from Vipera russelli venom led to a significant increase in the activity of guanylate cyclase in various rat organs. The activation of the enzyme was selective and was only observed in the particulate fractions of tissue homogenate. The soluble guanylate cyclase from all the tissue tested exhibited lack of stimulation. The treatment of membranes with PLA_2 resulted in solubilization of cyclase activity. The increase in enzyme by PLA_2 was not altered by antioxidants or reducing agents. Addition of calcium ions led to further enhancement in PLA_2-dependent increases in cyclic GMP formation. Peak calcium responses were observed in micromolar concentration ranges. These observations suggest a potential role for PLA_2 and calcium ions in the hormonal regulation of cyclic GMP metabolism.

Guanylate cyclase [GTP pyrophosphate-lyase (cyclizing) EC 4.6.1.2] catalyses the formation of cyclic GMP from GTP and is distributed in cytosolic and particulate fraction of mammalian tissue homogenates (1). Intracellular levels of cyclic GMP are altered by receptor-selective neurotransmitters in the presence of extracellular calcium ions (2). These agonists do not, however, affect the formation of cyclic GMP in the broken cell preparations. The antioxidants such as butylated hydroxyanisol (BHA) or reductants such as dithiothreitol (DTT) which prevent oxidative modulation of cytosolic guanylate cyclase by nitrocompounds (3) and oxygen free radicals (4) also do not modify receptor-mediated increases in cyclic GMP levels. Thus, the mechanism whereby autacoid and neurotransmitter elevate intracellular levels of cyclic GMP remains obscure.

At least two investigators have demonstrated the blockade of autacoid mediated elevations in intracellular levels of cyclic GMP by phospholipase A_2 inhibiting drugs such as mepacrine and quinacrine (5,6). These reports suggest a role for PLA_2 in the regulation of cyclic GMP metabolism by hormones and neurotransmitters. Our laboratory has recently described the activation of guanylate cyclase in rat cerebral cortex membranes by snake venom PLA_2 preparations. This modulation is both selective and specific in nature (7). Here we describe further observations on the nature and characteristics of PLA_2 effect.

Abbreviations: PLA_2, phospholipase A_2; BHA, butylated hydroxanisol; DTT, dithiothreitol; EDTA, ethylenediamine tetraacetic acid

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Material and Methods

Preparation of enzyme: Male Sprague-Dawley rats (weighing 150-200 g) were killed by cervical dislocation. Various organs were quickly removed and immersed in 0.25 M sucrose. Each tissue was blotted, weighed and homogenized in 9 volumes (w/v) of ice-cold homogenizing buffer [0.25 M sucrose, 20 mM Tris-HCl (pH 8.0), 1 mM EDTA and 1 mM DTT] with 15-20 strokes using Dounce glass homogenizer and motor driven Teflon pestle. Homogenates were centrifuged in Beckman L-65 at 105,000 xg for 60 min. Supernatants were collected and the pellets were suspended in the original volume of homogenizing buffer. Samples were centrifuged at 105,000 xg, for 60 min, supernatants were discarded and the pellets were resuspended in homogenizing buffer using the same homogenizer as above. High speed supernatants or pellets were employed as source of soluble or particulate guanylate cyclase.

Determination of Guanylate Cyclase Activity: Guanylate cyclase activity was determined by procedure as described previously (8). 100 µl incubation medium contained 50 mM Tris-HCl (pH 7.6), 10 mM theophylline, 10 mM creatine phosphate, 20 µg creatine phosphokinase, 4 mM $MgCl_2$, 1 mM GTP, enzyme preparation (20 to 200 µg protein) and other additions as desired. Some incubation included PLA_2 (0.1 mg/ml) as described in the text. Guanylate cyclase reactions were initiated with the addition of metal-GTP and continued for 10 min at 37°C. Reactions were terminated with ice-cold 50 mM sodium acetate buffer (pH 4.0). Tubes were heated at 90°C for 3 min and centrifuged at 2000 rpm to remove the denatured protein. Cyclic GMP was determined in the clear supernatant by radioimmunoassay (RIA) as described (9). Prior to RIA, samples were acetylated by the procedure of Harper and Brooker (10) to increase the sensitivity of cyclic GMP determination in RIA.

Protein was measured by the procedure of Lowry *et al.* (11) using bovine albumin as standard. Specific activity of guanylate cyclase was expressed as pmole cyclic GMP formed per mg protein per min. Value reported here represent the means of triplicate determinations from representative experiments.

Phospholipase A_2 (*Vipera russelli*) and other rare chemicals were procured from Sigma Chemical Co as described previously (7).

Results

Addition of snake venom (PLA_2) to guanylate cyclase incubations led to a significant increase in cyclic GMP formation. The stimulation of guanylate cyclase occurred in preparations from various rat organs (Table I). However, the PLA_2 stimulation of cyclic GMP formation exhibited selectivity. Guanylate cyclase in particulate fractions of all tissue homogenates was activated. The increase in enzyme activity varied from 2 to 42 fold depending upon the organ. The enzyme activity in the cytosolic fraction, however, remained unaltered by PLA_2. In the presence of Mn^{2+} cation cofactor increase guanylate cyclase activity did not exceed 50% (data not shown).

Because of known ability of PLA_2 to solubilize erythrocyte membranes, its effects on brain particulate guanylate cyclase preparations was studied. Treatment of brain membranes with PLA_2 (0.1 mg/ml) at 37°C led to significant loss of proteins from the particulate material to the soluble fraction. As shown in Table II, the effect of PLA_2 treatment on guanylate cyclase was to cause both activation as well as solubilization of activity. When expressed on protein basis, guanylate cyclase activity was almost identical in membranes and the solubilized fraction following PLA_2 treatment. Appearance of higher total activity also suggests unmasking of guanylate cyclase in brain membranes by PLA_2 treatment.

TABLE I

Effect of Phospholipase A_2 on Guanylate Cyclase Activity from Various Rat Organs

Tissue	pmole Cyclic GMP/mg Protein/min					
	Particulate			Cytosolic		
	$-PLA_2$	$+PLA_2$	Fold ↑	$-PLA_2$	$+PLA_2$	Fold ↑
Liver	0.8	6.3	7.9	4.8	4.3	0.9
Lung	1.6	14.5	9.0	14.8	16.8	1.1
Heart	0.2	2.1	10.6	5.4	5.8	1.1
Cerebral Cortex	0.6	2.4	3.7	3.0	3.2	1.1
Kidney	0.1	0.5	5.0	10.7	8.4	0.8
Testes	0.8	1.8	2.3	3.0	2.6	0.9
Pancreas	0.4	4.0	10.0	0.4	0.7	1.7
Fat Pad	1.4	58.8	42.0	13.3	15.7	1.2

Enzyme preparations from different tissues (50-230 µg protein) were incubated with and without PLA_2 (0.1 mg/ml). Guanylate cyclase activity was determined using 4 mM $MgCl_2$ and 1 mM GTP as described in 'Material and Methods'. Values reported here are the mean of a triplicate determination from the representive experiment.

TABLE II

Effect of Phospholipase A_2 on Solubilization of Membranes

Treatment	Fraction	Guanylate Cyclase Activity		
		Protein mg	Activity (pmole/10 min)	Specific Activity (pmole/mg/min)
Control	P	18.0	132.0	0.72
	S	N.D.	N.D.	N.D.
PLA_2 (0.1 mg/ml)	P	10.7	310.0	2.9
	S	8.8	210.0	2.4

105,000 x g pellet from rat cerebral cortex (total activity 140.0 pmole Cyclic GMP/10 min) containing 20 mg protein was treated with Phospholipase A_2 (0.1 mg/ml) at 37°C for 30 min. Parallel controls were incubated in the absence of PLA_2 Following the treatment, samples were centrifuged at 105,000 x g for 60 min at 4°C. Supernatants (S) were collected and the pellets (P) were suspended in the 5 volumes of 'homogenizing buffer' and recentrifuged at 105,000 x g for 60 min. Supernatants were discarded and the pellets were reconstituted in the original volume of 'homogenizing media' and used as enzyme source. Guanylate cyclase activities were determined in 50-100 µg protein using 4 mM $MgCl_2$ and 1 mM GTP. N.D., not detected.

In view of known requirement of calcium ions for optimal expression of PLA_2 activity (12) as well as for hormone-dependent increase in intracellular levels of cyclic GMP, effects of Ca^{2+} concentration were investigated on basal and PLA_2-stimulated guanylate cyclase in rat liver membranes. Figure 1

shows that PLA_2 activation of guanylate cyclase was enhanced by increase in calcium concentrations. The values of Ca^{2+} shown here represent concentrations above 100 μM EDTA introduced through enzyme preparations. Up to 10 μM calcium there was no significant effect on the activation. However, the peak enhancement was observed at 50-100 μM Ca^{2+} which came down with further increase in the cation. This pattern of calcium effect was not altered with preincubation of the membranes. The observed effects of calcium on PLA_2 stimulation of guanylate cyclase were similar to the known effect of Ca^{2+} on PLA_2 activity (12).

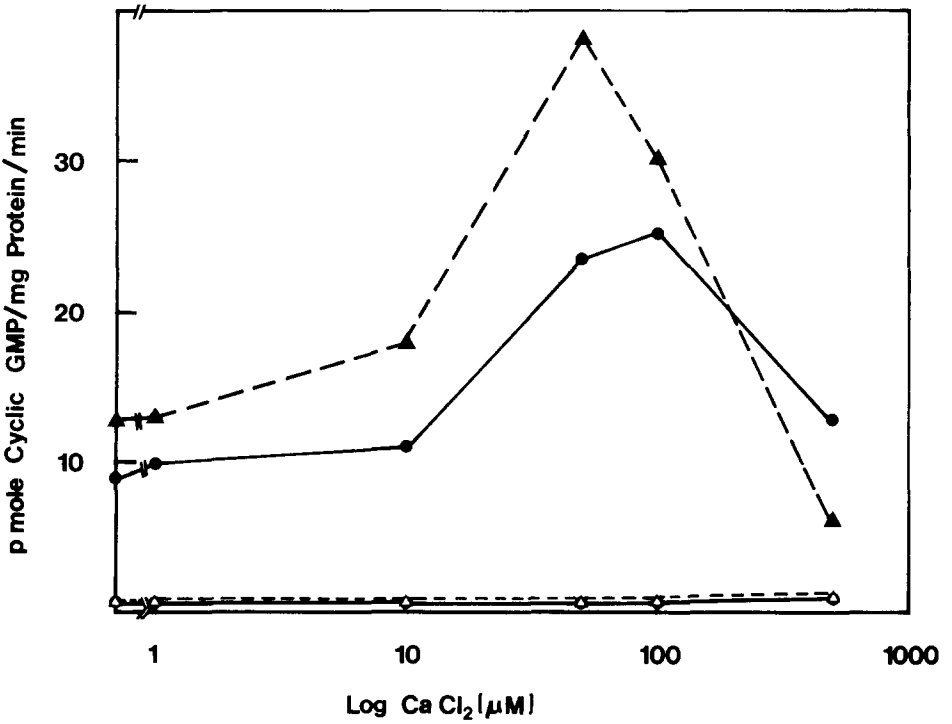

Fig. 1

Effect of Ca^{2+} ions on Phospholipase A_2 activation of guanylate cyclase. Rat liver membranes (100-200 μg proteins) were preincubated at 0°C (○,●) and 37°C (△, ▲) for 20 min without (open symbols) and with (closed symbols) PLA_2 (0.1 mg/ml) in the presence of various concentrations of calcium chloride. Reaction was initiated with the addition of 4 mM $MgCl_2$ and 1 mM GTP.

Cytosolic guanylate cyclase from mammalian tissues has been shown to be oxidatively modulated by a variety of hydrophilic and hydrophobic substances (2). Various agents were included in the incubations to test if redox phenomenon was involved in the PLA_2 activation of guanylate cyclase. Reducing agents glutathione and ascorbic acid or antioxidant butylated hydroxyanisol did not alter the basal or PLA_2 stimulated activities of liver guanylate cyclase as shown in Table III. Similar observations were made with oxidized glutathione or dehydroascorbic acid. Addition of hydroxylamine, an oxidant and known activator of soluble guanylate cyclase, did enhance the activity of particulate enzyme as reported previously (3). It, however, did not affect PLA_2 response. In fact, PLA_2 prevented the activation of guanylate cyclase with hydroxylamine. The

TABLE III

Effect of Redox Agents on Phospholipase A_2 Activation of Guanylate Cyclase

Addition (10^{-3}M)	pmole Cyclic GMP/mg Protein/min.	
	$-PLA_2$	$+PLA_2$
None	0.9	17.3
Hydroxylamine	34.2	17.4
Methylhydroxylamine	2.4	14.9
Glutathione	1.0	16.4
Ascorbic Acid	0.9	15.0
Oxidized Glutathione	0.8	16.5
Dehydroascorbic Acid	0.9	15.7
Butylated Hydroxyanisol	0.7	18.9

Rat liver membranes (24 µg Protein) were incubated in the absence and presence of Phospholipase A_2 (0.1 mg/ml) using 4 mM $MgCl_2$ and 1 mM GTP.

data summarized in Table III suggested independent modulation of guanylate cyclase with hydroxylamine and phospholipase A_2. The nucleophil perhaps oxidatively modulates the particulate enzyme whereas the PLA_2-dependent activation may bear a different mode. Some reports have speculated on the PLA_2-dependent release of unsaturated fatty acid and lysophospholipid which are known activators of guanylate cyclase (2). However, in our experiments we did not observe activation of soluble enzyme with PLA_2.

Discussion

Intracellular levels of cyclic GMP are altered by receptor-selective agonists in the presence of extracellular calcium ions (2). However, the mechanism of this phenomenon is unknown. Guanylate cyclase, which catalyses formation of cyclic GMP from GTP, is distributed in soluble and particulate compartment (1), and is not affected by these agonists. While the cytosolic and particulate enzymes are oxidatively modulated by nitro-compounds (3,18), not much information is available on the physiological regulation of particulate guanylate cyclase in mammalian systems. We have earlier described a selective and specific activation of guanylate cyclase by snake venom phospholipase A_2 (7)

This report describes further observations on the modulation of guanylate cyclase by phospholipase A_2. Inclusion of PLA_2 in guanylate cyclase incubation mixture led to significant increases in the catalysis of Mg^{2+}-dependent cyclic GMP formation in the tissues examined. Although the degree of activation varied with organs, no tissue specificity was observed. The PLA_2 effect exhibited definite selectivity in that only the particulate guanylate cyclase was stimulated. The lack of PLA_2 responsiveness in cytosolic enzyme was not attributable to the presence of any inhibitory component in the cytosolic compartment since homogenates and reconstituted systems were activated by PLA_2 (data not shown). Similar observations on particulate guanylate cyclase activation by atrial natriuretic factor (ANF) have been recently reported from this and other laboratories (13,14). ANF, however, exhibited tissue specificity and did not

activate guanylate cyclase from all tissues. The treatment of membranes with PLA_2 resulted in solubilization of guanylate cyclase activity. However, the detergent action of PLA_2 could not solely account for increase in activity since the activity in the post-treatment membrane was equally high. In addition, our previous investigations with PLA_2 (7) have shown that PLA_2 is able to stimulate the Triton X-100 solubilized guanylate cyclase preparations. These observations suggest a more specific effect of PLA_2 on the enzyme. In contrast to the nitro-drugs and oxygen radicals which modulate guanylate cyclase by oxidative mechanisms (3), the PLA_2-dependent activation is not affected by antioxidants or reductants suggesting a different mode of action.

The effect of calcium ions on PLA_2 stimulation of guanylate cyclase as shown in Fig. 1 is of considerable significance and pertinence. The sensitivity of PLA_2-dependent guanylate cyclase activation to micromolar concentrations (50-100 μM) of calcium ions reflects the potential regulatory influence this cation can exert in physiological environment over cyclic GMP metabolism. Similar calcium-dependence and sensitivity to calcium ions is known to occur for phospholipase A_2 itself in mammalian cell membranes (12). The receptor-selective autacoids and neurotransmitters which raise intracellular levels of cyclic GMP have also been suggested to raise intracellular calcium concentrations according to the 'gating theory' of Michell (15). Earlier observation from this laboratory have demonstrated the blockade of guanylate cyclase activation by phospholipase A_2 inhibitor, quinacrine (7). Reports in the literature on blockade of hormone-dependent elevations of cyclic GMP by mepacrine (5) and calcium channel blockers (16) strongly points to a role for PLA_2 in the process. The opening of calcium channels in the plasma membranes and calcium inward movement coupled with turnover of phosphoinositides may be triggered by the activation of such receptors as adrenergic (α_1) or muscarinic-cholinergic (17), and precede the activation of membrane-bound guanylate cyclase by phospholipase A_2 in the complex cascade of events.

The reasons for variations in PLA_2 response in different tissues is not clear at the present time. Whether the observed differences in the degree of PLA_2-dependent activation of cyclase in broken cell preparations from different tissues are reflective of general variations which also occur in elevations of cyclic GMP levels in intact cells in response to calcium-dependent agonists (2), or if a direct correlation exists between the effect of PLA_2 and receptor-selective agonists on cyclic GMP, is difficult to establish since the precise mechanism(s) underlying the PLA_2-dependent activation is not understood at this time. However, to some extent certain tissue-specific cellular components involved in the multi-step hormone- or autacoid- dependent modulation of cyclic GMP metabolism, may be responsible for these variations. PLA_2 is an intrinsic protein embedded in the phospholipid matrix of cell membranes and catalyses the hydrolysis of phospholipids to lysophospholipids and unsaturated fatty acids which are known to stimulate guanylate cyclase activity (2). However, the data obtained in our investigations do not seem to support the involvement of phospholipid metabolites since these metabolites are known to affect both forms of guanylate cyclase. Further investigations are currently underway in this laboratory to get better insight into the PLA_2 modulation of cyclic GMP metabolism.

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