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INHIBITORY ACTION OF GUANOSINE 3',5'-MONOPHOSPHATE ON THROMBIN-INDUCED PHOSPHATIDYLINOSITOL TURNOVER AND PROTEIN PHOSPHORYLATION IN HUMAN PLATELETS*

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SUMMARY: Human platelets activated by thrombin rapidly produce diglyceride from phosphatidylinositol (PI). Concomitantly, a protein having a molecular weight of about 40,000 is heavily phosphorylated and serotonin is released. These reactions are inhibited in parallel manners by sodium nitroprusside which elevates cyclic GMP. 8-Bromocyclic GMP also inhibits the reactions. Instead, both agents stimulate the phosphorylation of a distinct protein having a molecular weight of about 50,000. Since stimulants of platelets such as thrombin normally increase cyclic GMP, the results imply that this cyclic nucleotide may be involved in a circuit leading to the feedback inhibition of PI hydrolysis presumably through protein phosphorylation.

Cyclic GMP has been often presumed to be a second messenger for various hormones and neurotransmitters (for reviews see Refs. 1,2), but its definitive role has not yet been fully understood. Recently, Schultz et al. (3) have proposed that cyclic GMP may be a feedback inhibitor rather than a positive messenger of muscarinic cholinergic transmitter for smooth muscle contraction. This assumption has been

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The abbreviations used are: SNP, sodium nitroprusside; 8bcGMP, 8-bromo-cyclic GMP; PI, phosphatidylinositol; DG, diglyceride.

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based on the observation that cyclic GMP-elevating agents such as sodium nitroprusside (SNP) and 8-bromo-cyclic GMP (8bcGMP) cause muscle relaxation, although muscarinic cholinergic stimulators enhance cyclic GMP levels as well as phosphatidylinositol (PI) hydrolysis upon muscle contraction. Analogously, Haslam et al. (4) have proposed more recently that, although cyclic GMP is increased in human platelets stimulated by thrombin and collagen, this cyclic nucleotide probably inhibits platelet activation, since SNP known as a potent platelet inhibitor markedly increases cyclic GMP. These authors have also noted that both SNP and 8bcGMP enhance the phosphorylation of specific proteins having molecular weights of about 50,000 (50K protein) and 49,000 (49K protein) in intact platelets. Extending these observations, we wish to propose in this communication that cyclic GMP blocks PI turnover presumably at the level of diglyceride (DG) formation, and thereby counteracts platelet activation. Preceding reports from this laboratory (5,6) have described that the signal-induced PI hydrolysis to produce DG is directly coupled to the activation of Ca^{2+}-activated, phospholipid-dependent protein kinase, which may play a role in the platelet release reaction probably through phosphorylation of a protein having a molecular weight of 40,000 (40K protein). This species of protein kinase will be referred to as protein kinase C in this communication.

EXPERIMENTAL PROCEDURES

Washed human platelets were prepared by the method of Baenziger and Majerus (7). Bovine thrombin, SNP, and 8bcGMP were products of Mochida Pharmaceutical Co., Nakarai Chemicals, and Baehringer Mannheim, respectively. Other chemicals and materials were prepared as described in earlier reports (5,6). The platelets were prelabelled with either [^{3}H]arachidonic acid, ^{32}Pi, or [2-^{14}C]serotonin under the conditions described by Rittenhouse-Simmons (8), Haslam et al. (4), and Haslam et al. (9), respectively. The radioactive platelets were then activated by bovine thrombin. The radioactive DG and PI were directly extracted from the platelets with chloroform-methanol (2:1), subjected to silicic acid column chromatography followed by silica plate thin

Table I

Inhibition of thrombin-induced PI hydrolysis, DG formation, 40K protein phosphorylation, and serotonin release

Platelets, which were prelabelled with either [^{3}H]arachidonic acid, ^{32}Pi, or [2-^{14}C]serotonin, were suspended in 15 mM Tris/HCl at pH 7.5 containing 0.14 M NaCl, 5.5 mM glucose, and 2 mM $CaCl_2$. The suspension was incubated with 0.1 mM SNP or 1 mM 8bcGMP for 10 min at 37°C, and then stimulated by thrombin (0.05 unit/1 x 10^8 cells) for 15 s at 37°C. Other detailed conditions were similar to those described in an earlier report (6).

Addition	PI hydrolysis*/	DG formation	40K protein phosphorylation**/	Serotonin release
	(Δ cpm)	(cpm)	(Δ O.D. at 430 nm)	(cpm)
None	0	210	0	700
Thrombin	18,200	1,160	0.40	6,050
Thrombin + SNP	11,000	720	0.19	3,600
Thrombin + 8bcGMP	10,500	560	0.17	2,040
SNP	100	230	0	650
8bcGMP	200	210	0	720

*/ The basal level of radioactive PI in resting platelets was 43,000 cpm. The numbers given in this table were obtained by subtracting the radioactivity of PI in each experiment from this basal value.

**/ The background of O.D. at 430 nm for this autoradiograph was 0.19 and this value was subtracted from each experimental value.

layer chromatography, and quantitated as described by Rittenhouse-Simmons (8). Radioactive platelet proteins were directly subjected to sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis, stained, dried on a filter paper, and then exposed to an X-ray film to prepare an autoradiograph. The electrophoresis was carried out under the conditions specified by Laemmli (10). The relative intensity of each band was quantitated by densitometric tracing of each band using a Shimadzu chromatogram scanner, Model CS-910. The release of radioactive serotonin from activated platelets was determined by the method of Costa and Murphy (11). Cyclic GMP was determined by radioimmunoassay by the method of Steiner et al. (12).

RESULTS AND DISCUSSION

When platelets were stimulated by thrombin under the conditions given in Table I, a significant quantity of PI rapidly disappeared with the concomitant formation of DG. Although this DG was probably derived from PI hydrolysis as reported from several laboratories in-

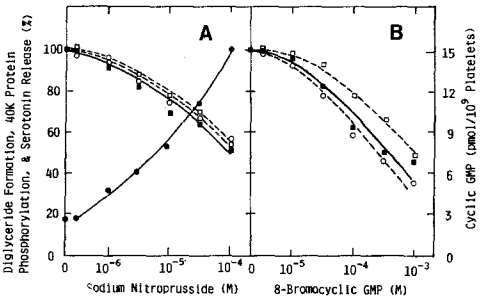

Fig. 1. Effect of various concentrations of SNP and 8bcGMP on thrombin-induced DG formation, 40K protein phosphorylation, and serotonin release. DG formation, protein phosphorylation, and serotonin release were allowed to proceed under the conditions given in the legend to Table I except that SNP and 8bcGMP were added as indicated. Cyclic GMP was measured after preincubation with various concentrations of SNP for 10 min at 37°C. A, with SNP; B, with 8bcGMP. (■——■), DG formation; (□-----□), 40K protein phosphorylation; (o-----o) serotonin release; (●——●), cyclic GMP formation.

cluding our own (6,8,13), the amount of DG produced was far less than that of PI disappeared. It is likely that this difference was due to rapid conversion of DG back to PI by way of phosphatidic acid (PI turnover) (8,14,15), and also to further degradation of DG to monoglyceride and free fatty acid by the action of DG lipase recently found in platelets (16). In any case, the thrombin-induced formation of DG was always accompanied with 40K protein phosphorylation and serotonin release, and these reactions were parallel with one another.

The results presented in Table I also show that the disappearance of PI as well as the three reactions mentioned above were simultaneously inhibited to significant extent by the addition of SNP and 8bcGMP. Apparently, both agents showed practically no effect on resting platelets. Such inhibitory effects of SNP and 8bcGMP on platelet activation are more quantitatively given in Fig. 1. DG formation, 40K protein phosphorylation, and serotonin release were progressively inhibited in parallel manners by increasing amounts of these agents. It was noted that cyclic GMP was increased by the addition of SNP. A typical

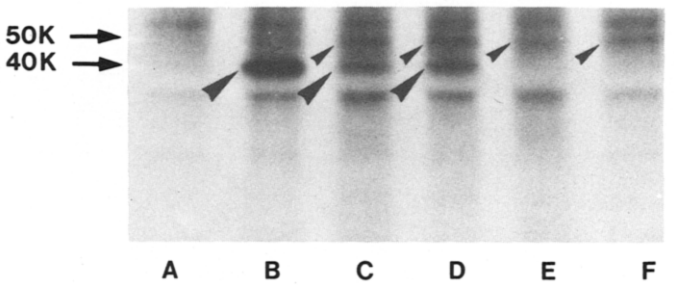

Fig. 2. Effect of thrombin, SNP, and 8bcGMP on phosphorylation of platelet proteins. Protein phosphorylation was allowed to proceed as described in the legend to Table I and an autoradiograph was prepared. A, with saline alone; B, with thrombin alone; C, with thrombin plus SNP; D, with thrombin plus 8bcGMP; E, with SNP alone; F, with 8bcGMP alone. The locations of 50K and 40K proteins are indicated by small and large arrowheads, respectively.

example of autoradiograph illustrated in Fig. 2 shows that both SNP and 8bcGMP inhibited the thrombin-induced phosphorylation of 40K protein. It is also evident that both SNP and 8bcGMP stimulated the phosphorylation of a distinct protein having a molecular weight of around 50,000 (50K protein). The phosphorylation of this particular protein was enhanced by increasing amounts of SNP and 8bcGMP, and this protein phosphorylation was inversely proportional to the formation of DG as shown in Fig. 3.

Previous reports from this laboratory (5,6) have described that DG produced by PI hydrolysis may serve as an initiator for the selective activation of protein kinase C, which is responsible for 40K protein phosphorylation eventually leading to release reaction. Thus, it is possible to assume that SNP inhibits platelet activation, at least in part, by decreasing DG which accumulates during thrombin stimu lation. In addition, since SNP has been known as a powerful inducer of intracellular cyclic GMP, the inhibitory effect of SNP may presumably be mediated through this cyclic nucleotide. This assumption seems to be consistent with the recent proposal by Haslam et al. (4) that cyc-

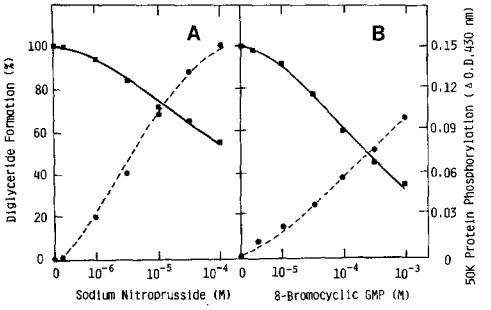

Fig. 3. Effect of various concentrations of SNP and 8bcGMP on 50K protein phosphorylation and DG formation. Protein phosphorylation and DG formation were allowed to proceed as described in the legend to Table I. A background for 50K protein phosphorylation, which was obtained in the absence of SNP and 8bcGMP, was 0.01 at O.D. 340 nm. This value was subtracted from each experimental value. A, with SNP; B, with 8bcGMP. (●-----●), 50K protein phosphorylation; (■——■), DG formation.

lic GMP may serve as a feedback inhibitor rather than a positive stimulator for platelet activation, although this cyclic nucleotide is increased by thrombin and collagen (17,18). These authors have also shown that SNP and 8bcGMP enhance the phosphorylation of 50K protein as well as 49K protein, but these two proteins were not resolved under the conditions employed for the present studies. Nevertheless, a line of evidence available at present implies that in a receptor cascade system occurring in activated platelets cyclic GMP may be involved in an intracellular circuit leading to the feedback inhibition of PI turnover presumably through protein phosphorylation.

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