EFFECT OF SEROTONIN ON CYCLIC NUCLEOTIDES OF HUMAN PLATELETS*

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SUMMARY. Serotonin produced a 6 to 10 fold increase of cyclic GMP over baseline levels of this nucleotide in platelets. Maximum stimulation was reached within 30 sec to 1 min after addition of serotonin and was dependent upon its concentration in the medium. Inhibition of serotonin uptake by methysergide, dihydroergotamine and chloroimipramine did not influence the serotonin-induced stimulation of cyclic GMP but glutaraldehyde and formaldehyde blocked it completely. Cyclic AMP levels in platelets were not affected by serotonin. The serotonin-induced stimulation of cyclic GMP is independent of the uptake of this biogenic amine by platelets and is not due to platelet aggregation.

Serotonin which can be taken up by platelets against high concentration gradients (1) is stored in electron dense subcellular organelles and released during platelet aggregation. Serotonin itself has been shown capable of inducing weak platelet aggregation (2,3). Specific receptors for this substance have been suggested in the central nervous system (4,5). In platelets, a ganglioside has been identified with serotonin-binding capacity (6). There is good evidence that the reversible attachment of biologically active agents to specific sites of macromolecular structural elements may result in changes of their functional ability to synthesize substances which in turn may mediate cellular functions. Serotonin and cyclic nucleotides qualify in this context which prompted us to investigate their relationship in platelets.

MATERIALS AND METHODS. Platelets were isolated from fresh human whole blood obtained from non-fasting, healthy, male volunteers by a method previously described (7). The platelets were washed once in 25 mM Tris-HCl, pH 7.2 containing 116 mM NaCl, 4.17 mM KCl, 1.8 mM KH_2PO_4, 1.18 mM $MgCl_2$ and 5 mM β-D glucose, were then suspended in the same medium and

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their concentration adjusted to 1 x 10^9 platelets/ml. Up to this point all procedures were carried out at room temperature.

Aliquots of the platelet suspension (2 ml) were equilibrated at 37°C for 5-10 min. Incubations were started with the addition of 0.1 ml of the above Tris-HCl medium. In experimental samples this addition contained serotonin (5-hydroxytryptamine creatinine sulfate) of the desired concentration. The incubations were stopped by rapid admixture of 1 ml ice-cold 6% perchloric acid containing 2000-3000 cpm [^{3}H] cGMP (<1 pmol; sp act 9.92 Ci/mmol) and centrifugation at 4°C. The resultant supernatant was neutralized with 5N KOH. Precipitated $KClO_4$ was removed by centrifugation and the supernatant stored at -20°C until cyclic nucleotides could be purified and assayed.

Determination of cyclic nucleotides was preceded by purification of the neutralized perchloric acid extracts essentially according to the method of Schultz et al (8). The eluates were lyophilized and the contents dissolved in 50 mM Na-acetate buffer, pH 6.2. An aliquot of each sample was used for determination of [^{3}H] guanosine 3':5'-cyclic monophosphate (cyclic GMP) recovery which ranged from 50 to 80%. Adenosine 3':5'-cyclic monophosphate (cyclic AMP) and cyclic GMP were measured by radioimmunoassay (9) utilizing the cyclic nucleotide antibodies and [^{125}I] antigens of Collaborative Research, Inc. (Waltham, Mass.). With each assay, standards of authentic cyclic GMP or cyclic AMP were run. The antigen-antibody complexes were precipitated with sheep or rabbit IgG after addition of normal carrier rabbit serum diluted 1:200. Aliquots of neutralized perchloric acid extracts of several representative experiments were incubated with phosphodiesterase (EC 3.1.4.17) resulting in hydrolysis of 90-95% of the cyclic nucleotides. All incubations were carried out in duplicate. The results were corrected for the recovery of [^{3}H] cyclic nucleotide. Because platelet preparations showed at times considerable variation in their cyclic nucleotide contents, individual experiments shown in results were replicated at least 3 or more times.

To determine the uptake of serotonin, platelets suspended in the medium and at the concentration described above were incubated at 37°C with 2.5 µg [^{14}C]5-hydroxytryptamine/ml (sp act 13.5 mCi/mmol). At various time intervals aliquots of the suspensions were removed, added to 25 volumes of ice-cold 0.154 M NaCl and centrifuged immediately at 4°C. The pellet was dissolved in 0.3 ml of 0.3 N KOH and the radioactivity counted as described previously (10).

Platelet aggregation was determined with an aggregometer of Chrono-Log Corp. (Broomal, Pa.), the platelet suspension being stirred at 1000 rpm in a well heated to 37°C. Changes in light transmission were registered on a 10 mV recorder (11).

RESULTS. The basal levels of cyclic GMP in washed platelets varied between 0.6 and 1.8 pmols/1 x 10^9 cells with a mean of 1.2 ± 0.6 (SEM; n=8). The addition of serotonin at a final concentration of 5.9 µM produced a sharp increase in cyclic GMP which reached its maximum within 1 min (Fig. 1). The serotonin-induced stimulation peaked at about 6 times the baseline level of cyclic GMP and returned within 10 min to the original cyclic GMP concentration of platelets. A second addition of serotonin resulted in a renewed increase of cyclic GMP which reached a maximum only slightly below that of the first peak. The decline of cyclic GMP to baseline

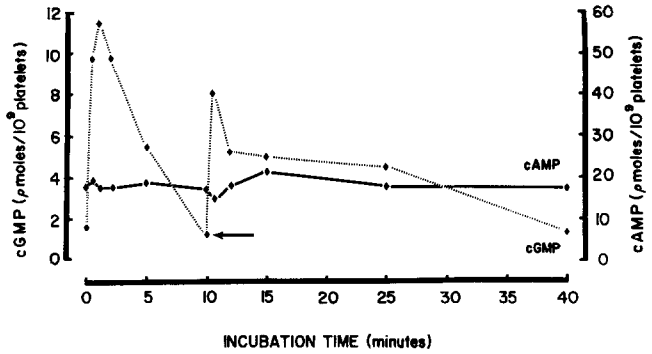
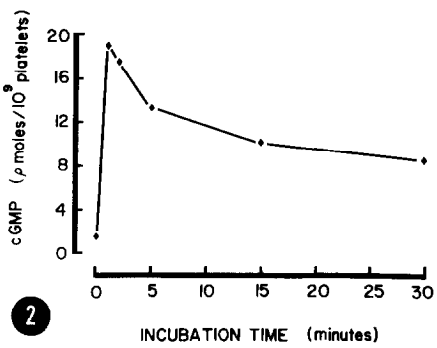


Figure 1:

Effect of serotonin on cyclic AMP and cyclic GMP levels of human platelets. Serotonin 2.5 µg per ml (5.9 µM) was added to the platelet suspensions (1 x 10^9/ml) which were incubated in a water-bath at 37°C. After 10 min (indicated by arrow), additional serotonin 2.5 µg per ml was added. Aliquots of the incubation mixture were removed at various times after the first and second addition of serotonin and added to perchloric acid. Extraction, purification and assays for cyclic nucleotides were carried out as described in METHODS.

levels after the second stimulation took much longer, reaching the concentration observed in non-serotonin-incubated platelets only after 30 min. No change was noted in the concentration of cyclic AMP after the first or second addition of serotonin. When serotonin was added in a higher concentration (23.6 µM), the cyclic GMP stimulation became more effective, resulting in a 10 fold increase in cyclic GMP levels within 1 min (Fig. 2). However, this increase declined slowly. Even after 30 min, baseline values of cyclic GMP had not been reached. The stimulation of cyclic GMP which was about 6 fold the baseline value at 1.2 µM increased to 10 fold the basal value at 23.6 µM serotonin (Fig. 3). Cyclic AMP levels were not affected by increasing serotonin concentrations in the medium.

A comparison of the time relation between uptake of serotonin by platelets (Fig. 4) and stimulation of cyclic GMP makes it apparent that the latter precedes the accumulation of the amine in platelets. These findings suggest that the rise of cyclic GMP levels was related to the up-



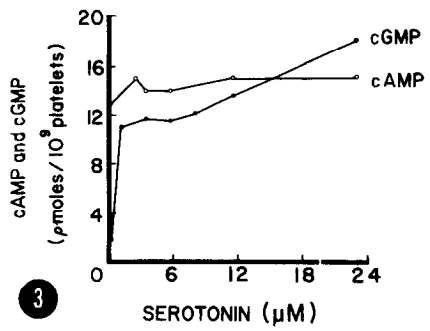


Figure 2:

Effect of serotonin on cyclic GMP levels of human platelets. Serotonin, 10 μg per ml (23.6 μM) was added to platelet suspensions (1 x 10^9/ml) which were incubated in a water-bath at 37°C. Aliquots were removed after indicated period of incubation, and added to perchloric acid. Extraction, purification and assays for cyclic GMP were carried out as described in METHODS.

Figure 3:

Effect of various concentrations of serotonin on cyclic nucleotide levels of human platelets. Platelet suspensions were equilibrated at 37°C for 5-10 min and then serotonin in concentrations ranging from 1.2 to 23.6 μM was added. After 1 min of incubation, perchloric acid was added and extracts were made. Cyclic AMP and cyclic GMP were purified and assayed as described in METHODS. Before addition of serotonin, cyclic AMP and cyclic GMP concentration per 10^9 platelets was 16 ± 3 and 1.2 ± 0.6 pmoles, respectively.

take of serotonin by platelets. But serotonin antagonists such as methysergide, dihydroergotamine and chloroimipramine (12,13), the potent inhibitors of serotonin uptake were ineffective in preventing or reducing the serotonin-induced rise of cyclic GMP (Table I). Incubation of platelets with these agents did not show very marked changes in their basal cyclic GMP levels. Glutaraldehyde and formaldehyde which have recently been reported to stop serotonin uptake within seconds (14) were able to completely inhibit the serotonin-induced increase of cyclic GMP and also depressed severely the baseline level of this cyclic nucleotide in platelets. Control experiments proved that these aldehydes in the concentrations used in our experiments did not interfere with the antigen-

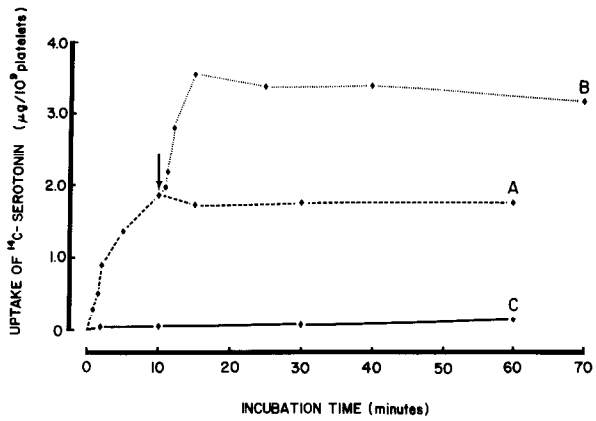


Figure 4:

Uptake of [^{14}C]serotonin by human platelets. (A) Platelet suspensions (1 x 10^9 platelets per ml) were incubated with serotonin, 2.5 μg/ml. (B) After incubation for 10 min as indicated by arrow, additional serotonin 2.5 μg/ml was added to the platelet suspensions. (C) Platelet suspension was incubated with chloroimipramine (5 μM) for 5 min and then serotonin 2.5 μg per ml was added. Aliquots were removed after various time periods, added to 25 volumes of ice-cold 0.9% NaCl and centrifuged immediately at 4°C. The pellet was dissolved in 0.3 ml of 0.3 N KOH and the radioactivity was measured.

antibody reaction of the radioimmunoassay. The effect of serotonin on cyclic GMP was independent of platelet aggregation as demonstrated by the complete absence even of the primary wave of aggregation in the washed platelet suspensions.

DISCUSSION. Serotonin has been reported to increase the cyclic GMP content of uterine smooth muscle (15) and to induce accumulation of this cyclic nucleotide in adherent mononuclear cells of human blood (16). These tissues or cells are not known to concentrate serotonin as blood platelets do. Because of the extraordinarily steep electrochemical gradient against which this biogenic amine can be accumulated by platelets (1) and stored in their subcellular granules we anticipated a direct dependence of the cyclic GMP stimulation on the uptake and storage of serotonin by platelets. However, our studies clearly refute such a hypothesis. The inability even of specific inhibitors of serotonin uptake

TABLE I

EFFECT OF VARIOUS INHIBITORS OF SEROTONIN UPTAKE ON CYCLIC GMP LEVELS IN HUMAN PLATELETS

Inhibitors	Serotonin (5.9 μM)	Cyclic GMP (p-moles/10^9 platelets)
----	----	1.3
----	+	7.6
Chloroimipramine (10 μM)	+	10.1
Dihydroergotamine - mesylate (15 μM)	+	8.4
Methysergide - maleate (21 μM)	+	9.4
Glutaraldehyde (0.25%)	+	<0.3
Formaldehyde (0.37%)	+	<0.3

The inhibitor in a required concentration was added to the washed platelet suspension and the suspension was incubated in a shaking water-bath at 37°C for 5 min. Serotonin (5.9 μM) was added and after 1 min of incubation an equal volume of ice-cold perchloric acid containing [^{3}H] cyclic GMP as marker was added. Perchloric acid extraction, cyclic GMP purification and the assay were carried out as described in METHODS.

to block the intracellular rise in cyclic GMP suggests that the binding site for serotonin on the regulatory subunit of guanylate cyclase may have different structural characteristics from the receptor site for serotonin on platelet membranes. The strong inhibitory action of glutaraldehyde and formaldehyde is probably related to their ability to establish covalent crosslinks between certain membrane proteins (17). Direct inhibition of platelet guanylate cyclase activity by glutaraldehyde has recently been observed (L. Cutler, G. Rodan and M. Feinstein, personal

communication). As our studies show, the serotonin-induced increase in cyclic GMP is independent of platelet aggregation. However, aggregation induced by epinephrine (18), collagen (19) or arachidonic acid (20) have recently been shown to be associated with an intracellular rise of this cyclic nucleotide. The secretory phenomenon which renders aggregation irreversible may be mediated by cyclic GMP (19). The role of cyclic GMP is not specific for platelet aggregation. Cyclic GMP may have a role in cell mobility as studies with polymorphonuclear leukocytes (21) and monocytes (16) have suggested. Enhancement of monocyte movement by serotonin in response to endotoxin-treated serum has been reported (22). Platelets also possess mobility (23,24). Whether their migration is influenced by serotonin is not known as yet. It is of interest, however, that both cellular functions associated with an elevation of cyclic GMP seem to have a contractile phenomenon as their basis.

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