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Effects of Collagen and of Aspirin on the Concentration of Guanosine 3′:5′-Cyclic Monophosphate in Human Blood Platelets: Measurement by a Prelabelling Technique

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A method is described for isolating cyclic [^{3}H]GMP from platelets preincubated with [^{3}H]guanine. Collagen increased and aspirin decreased the cyclic [^{3}H]GMP concentration in platelets. The results are consistent with a role for cyclic GMP in the platelet release reaction.

Addition of collagen fibres to platelet-rich plasma causes platelet aggregation mediated by the secretion into the plasma of the aggregating agent ADP, with other platelet granule constituents, in a process known as the platelet release reaction (Holmsen *et al.*, 1969; Mustard & Packham, 1970). Many compounds that inhibit both platelet aggregation and the release reaction act by increasing the cyclic AMP concentration in platelets (Salzman, 1972; Haslam, 1973). Goldberg *et al.* (1973) have pointed out that in several biological systems the cyclic GMP concentration rises in response to stimuli the effects of which are opposed by increases in cyclic AMP. Also there is evidence, from the effects of exogenous cyclic GMP and derivatives, that this compound may play a role in the immunological release of histamine from sensitized human lung tissue (Kaliner *et al.*, 1972) and in the release of lysosomal enzymes during phagocytosis by human leukocytes (Ignarro, 1973), both of which have features in common with the platelet release reaction. We therefore set out to investigate the effect of collagen on the cyclic GMP concentration in platelets to determine if changes occur that can be related to the release and aggregation phenomena. To add an extra dimension to any correlations obtained we also investigated the effect of aspirin (acetylsalicylic acid), a potent inhibitor of the release reaction (Weiss & Aledort, 1967; Evans *et al.*, 1968), on platelet cyclic GMP in the presence and absence of collagen. To surmount the problem of measuring small changes in platelet cyclic GMP against the background of plasma cyclic GMP, we developed a method that involved isolation of cyclic [^{3}H]GMP from platelet-rich plasma that had been preincubated with [^{3}H]guanine.

Experimental

Cyclic GMP, other nucleotides, nucleosides, bases, heparin, aspirin, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid, bovine tendon collagen and cyclic nucleotide phosphodiesterase (0.27 unit/mg) were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. [8-^{3}H]Guanine (5–15Ci/mmol) was obtained from Schwarz/Mann, Orangeburg, N.Y., U.S.A., and [*hydroxymethyl*-^{14}C]inulin (11.4mCi/mmol) from Amersham/Searle Corp., Don Mills, Ont., Canada. Anion-exchange resin (AG 1-X2, 200–400 mesh, Cl^- form) was obtained from Bio-Rad Laboratories, Richmond, Calif., U.S.A., and cellulose powder for t.l.c. (MN 300 HR) was from Macherey, Nagel and Co., Düren, Germany.

Human heparinized platelet-rich plasma containing 4.7×10^8 platelets/ml was prepared from blood of donors who had taken no aspirin for 1 week (Haslam & Rosson, 1972). Less than 10^6 erythrocytes/ml were present. This material was incubated at 37°C with 2μM-[8-^{3}H]guanine (adjusted to 3.0Ci/mmol). To measure the uptake of [8-^{3}H]guanine by the platelets, 0.85ml of labelled platelet-rich plasma was mixed with 0.15ml of 0.154M-NaCl containing 0.05μCi of [*hydroxymethyl*-^{14}C]inulin and then centrifuged (30s, 12000*g*, Eppendorf centrifuge 3200). The platelet pellet was dispersed in 0.5ml of water by sonication and the ^{3}H and ^{14}C radioactivities in it were counted by liquid scintillation. Uptake of [8-^{3}H]guanine was calculated by correcting the total ^{3}H for the ^{3}H in residual plasma (as estimated from the ^{14}C present) and was maximal after 60min incubation, when 3–8% of that added was in the platelets. Experiments were therefore performed 60–90min after addition of [8-^{3}H]guanine. Samples of labelled platelet-rich plasma (0.85ml) were incubated at 37°C with a total of 0.15ml of additions in 0.154M-NaCl. Aspirin was added 10min before collagen. The samples were stirred, from 30s before addition of collagen, in an apparatus for the turbidometric measurement of platelet aggregation (Paynton Associates Ltd., Scarborough, Ont., Canada), so that platelet cyclic GMP could be related to platelet aggregation, measured as change in percentage light transmission (ΔT) in the same samples. Control samples without collagen were stirred for similar time-intervals. Suspensions of collagen fibres were prepared by homo-

genization of collagen at 0°C in 0.154M-NaCl [Willems Polytron; Brinkmann Instruments (Canada) Ltd., Rexdale, Ont., Canada], followed by centrifugation at 100*g* for 5min to remove coarse fibres.

Incubations were terminated by addition of 0.2ml of ice-cold 3M-$HClO_4$ with 0.20μmol of unlabelled cyclic GMP. Precipitated proteins were removed by centrifugation (2min, Eppendorf centrifuge) and the supernatants were neutralized to Methyl Orange with 2M-KOH. After removal of precipitated $KClO_4$ at 0°C the supernatants were added to columns containing 1.5ml of packed AG1 resin, which were eluted with the following sequence of reagents: 6ml of water (shown to elute added guanine, guanosine, hypoxanthine, inosine and some xanthine in preliminary experiments), 15ml of 0.01M-HCl (elutes the remaining xanthine, xanthosine, uric acid, cyclic AMP and AMP), 9ml of 0.05M-HCl (elutes GMP, IMP, XMP and ADP) and finally a further 18ml of 0.05M-HCl (elutes cyclic GMP and GDP), which was collected into silicone-treated flasks (50ml) and freeze-dried. GTP and ATP were not eluted. The residues were dissolved in 0.05ml of water and subjected to t.l.c. in two dimensions on plates (20cm × 20cm) coated with 0.25mm thickness of cellulose. These were developed first with butan-1-ol–acetone–acetic acid–14.8M-NH_3–water (90:30:20:1:60, by vol.) (Turtle & Kipnis, 1967) and then with isobutyric acid–1M-NH_3–0.1M-EDTA (125:75:2, by vol.) (Krebs & Hems, 1953). This procedure separated cyclic GMP from GDP and unidentified radioactive impurities. The cellulose containing cyclic GMP was scraped from the plates and eluted with 2ml of water. Cellulose was removed by centrifugation at 30000*g* for 20min and the extinctions of the supernatants were measured at 252nm. The extinction of material eluted from the same area of a t.l.c. plate derived by processing a sample of platelet-rich plasma without addition of unlabelled cyclic GMP was subtracted (<15%). The recoveries of cyclic GMP through the isolation procedure were calculated and varied from 30 to 50%. The cyclic [^{3}H]GMP in 1.5ml of each eluate from the cellulose was measured by liquid-scintillation counting and corrected for recovery. The significance of changes in platelet cyclic [^{3}H]GMP was assessed by Student's *t* tests.

The purity of the cyclic [^{3}H]GMP isolated was estimated from the specific radioactivity of [^{3}H]GMP formed from it with cyclic nucleotide phosphodiesterase. T.l.c. eluates from identically treated samples of platelet-rich plasma were pooled and freeze-dried. The residue, dissolved in 0.15ml of water, was incubated at 30°C with 0.05ml of 20mM-$MgSO_4$, 0.05ml of 50mM-*N*-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid (adjusted to pH7.5 with NaOH) and 0.1 unit of cyclic nucleotide phosphodiesterase. At intervals 0.05ml samples were removed, heated at 100°C for 2min and centrifuged. The supernatants were chromatographed twice in the same dimension on cellulose plates with the first solvent described above. This separated cyclic [^{3}H]GMP and [^{3}H]GMP, which were eluted with water, measured spectrophometrically and counted for radioactivity by liquid scintillation.

Aqueous solutions containing ^{3}H or ^{3}H and ^{14}C (1.5ml) were counted for radioactivity in a Beckman LS 230 scintillation counter after mixing with a dioxan-based phosphor (10.5ml) (Scales, 1967). With samples containing cyclic [^{3}H]GMP, 2000–10000 counts above background were recorded over 50min (counting efficiency approx. 33%). For ^{3}H with ^{14}C over 10000 counts were recorded in both channels (efficiencies approx. 16 and 50% respectively) and results were corrected for channel cross-over. Quench corrections were applied by the external-standard-ratio method after subtraction of the background (approx. 26c.p.m.).

Results

A small increase in cyclic [^{3}H]GMP occurred during stirring of samples of platelet-rich plasma without any addition other than 0.154M-NaCl. Relative to stirred controls, collagen increased the platelet cyclic [^{3}H]GMP concentration by 72% after 2min ($P<0.02$), when just sufficient was added to cause complete aggregation within this period (Table 1, Expt. 1), whereas a threefold higher collagen concentration causing more rapid aggregation increased the cyclic [^{3}H]GMP concentration by 132% after 1min ($P<0.01$; Expt. 2). The increases in cyclic [^{3}H]GMP were slightly in advance of platelet aggregation. Thus the slight decrease in light-transmission caused by the change in platelet shape that precedes aggregation was associated with a 21% increase in cyclic [^{3}H]GMP relative to the calculated control concentration at that time (Expt. 1). The cyclic [^{3}H]GMP concentration then rose rapidly as aggregation commenced and reached its maximum value when the increase in light-transmission was about one-third maximum.

Incubation of platelet-rich plasma with 0.2mM-aspirin for 10min caused a 45–69% decrease in the basal concentration of cyclic [^{3}H]GMP ($P<0.01$) and prevented the small increase that occurred with stirring (Table 1). Aspirin did not affect the increase in cyclic [^{3}H]GMP concentration caused by either dose of collagen used, although the final concentrations were lower than in its absence, because of the depression of the basal concentration. As the increases in cyclic [^{3}H]GMP caused by collagen depended on the amount of collagen added, aspirin was able to prevent any increase in cyclic [^{3}H]GMP relative to the controls without collagen or aspirin at the lower but not at the higher collagen concentration used. Aspirin inhibited aggregation much more powerfully with the lower dose of collagen.

Table 1. *Effects of collagen and of aspirin on platelet cyclic [^{3}H]GMP in relation to platelet aggregation*

Uptake of [8-^{3}H]guanine by platelets was measured immediately before and after the incubations with collagen etc.: average values per sample were: Expt. 1, 334000d.p.m.: Expt. 2, 500000d.p.m. In all samples labelled platelet-rich plasma was mixed with 0.154M-NaCl with or without aspirin 10min before addition of collagen and the first (0 min) determinations of cyclic [^{3}H]GMP. Incubation period refers to the time after addition of collagen. The final aspirin concentration was 0.2mM. Volumes of collagen suspension added were: Expt. 1, 6.7μl; Expt. 2, 20μl. Cyclic [^{3}H]GMP values and extents of aggregation are means±S.E.M. from three identically treated samples of platelet-rich plasma.

Expt. no.	Additions	Incubation period (min)	Cyclic [^{3}H]GMP (d.p.m./sample)	Extent of aggregation (ΔT)
1	None	0	960± 38	—
		2.0	1100± 69	—
	Aspirin	0	299± 10	—
		2.0	262± 17	—
	Collagen	0.5	1205± 24	−1.3±0.3
		1.0	1800± 88	13.7±1.6
		2.0	1896±172	39.7±0.3
	Collagen+aspirin	0.5	358± 11	−1.8±0.4
		1.0	807± 57	1.8±0.4
		2.0	1051±136	10.7±0.3
2	None	0	561± 29	—
		1.0	781± 30	—
	Aspirin	0	310± 28	—
		1.0	334± 31	—
	Collagen	0.3	645± 23	0
		1.0	1813± 19	26.6±1.2
	Collagen+aspirin	0.3	393±106	0
		1.0	1474±160	19.5±0.4

Cyclic nucleotide phosphodiesterase converted presumed cyclic [^{3}H]GMP, isolated from control or collagen-stimulated platelets, into [^{3}H]GMP with a specific radioactivity not more than 10% lower than the starting material, which was therefore at least 90% pure. Measurement of the cyclic [^{3}H]GMP in platelet-free plasma, prepared by rapid (2min) centrifugation of ^{3}H-labelled platelet-rich plasma in the Eppendorf centrifuge, showed that less than 5% of the basal cyclic [^{3}H]GMP was extracellular.

Discussion

Our method of measuring changes in the concentration of cyclic GMP in platelets was based on the same principle as the determination of labelled cyclic AMP in tissues preincubated with labelled adenine, an approach that has been frequently applied both to platelets (e.g. Ball *et al.*, 1970; Haslam & Taylor, 1971) and other systems (e.g. Kuo & De Renzo, 1969; Shimizu & Daly, 1970). This method should be widely applicable to studies of the roles of cyclic GMP in cell function.

The results show that addition of collagen alone to platelet-rich plasma causes an increase in cyclic [^{3}H]GMP, which slightly precedes and is quantitatively related to the ensuing platelet aggregation. This confirms and extends a recent brief report that collagen can increase platelet cyclic GMP 2–4-fold (White *et al.*, 1973). When aspirin was added before collagen the basal cyclic GMP concentration was depressed but the size of the increase in cyclic GMP above the new control concentration was unaffected, so that the final cyclic GMP concentration with collagen was decreased by the same amount as the basal concentration. Although it is possible that the action of aspirin on the release reaction is unrelated to its effect on cyclic GMP concentrations, the results can be accounted for by postulating that initiation of the release reaction requires an increase in cyclic GMP concentration above approximately the resting concentration observed in the absence of aspirin. This could explain why aspirin powerfully inhibits release and aggregation induced by low but not high collagen concentrations (Zucker & Peterson, 1970; Haslam, 1974), as only the latter increase cyclic GMP well above the normal resting concentration. The failure of aspirin to inhibit the increase in cyclic [^{3}H]GMP with collagen suggests that this increase is unlikely to be secondary to either the release phenomenon or the aggregation phenomenon, both of which are inhibited by aspirin. It follows that cyclic GMP is a better candidate for a role in the release reaction than in the aggregation caused by the ADP released. As a whole the results are consistent with the hypothesis that cyclic GMP mediates or potentiates the platelet re-

lease reaction, which is thus the first secretory process shown to be associated with an increase in intracellular cyclic GMP.

The depression of the basal concentration of cyclic GMP in platelets by aspirin contrasts with the absence of an effect on cyclic AMP (Ball *et al.*, 1970) and represents a previously unknown action of this drug that could be of general importance. As many of the pharmacological actions of aspirin and other non-steroidal anti-inflammatory agents have been attributed to inhibition of prostaglandin synthetase (Vane, 1971) and as prostaglandin $F_{2\alpha}$ has been reported to increase cyclic GMP concentrations in rat uterus (Kuehl *et al.*, 1973), the question arises of whether the action of aspirin on the basal cyclic GMP concentration in platelets could be mediated by an inhibition of prostaglandin synthesis. This seems unlikely for two reasons: first, prostaglandin production by the platelets (Smith & Willis, 1971; Smith *et al.*, 1972), as in other tissues (Ramwell & Shaw, 1970), seems to occur only in response to stimulation, and, secondly, aspirin did not affect the increase in cyclic [^{3}H]GMP induced by collagen, the action of which on platelets is associated with prostaglandin synthesis (Smith *et al.*, 1972). It follows that our results throw some doubt on the proposition that all the effects of aspirin on platelets are mediated by an inhibition of prostaglandin synthetase.

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Ball, G., Brereton, G. G., Fulwood, M., Ireland, D. M. & Yates, P. (1970) *Biochem. J.* **120**, 709–718

Evans, G., Packham, M. A., Nishizawa, E. E., Mustard, J. F. & Murphy, E. A. (1968) *J. Exp. Med.* **128**, 877–894

Goldberg, N. D., Haddox, M. K , Hartle, D. K. & Hadden, J. W. (1973) *Proc. Int. Congr. Pharmacol. 5th*, **5**, 146–169

Haslam, R. J. (1973) *Ser. Haematol.* **6**, 333–350

Haslam, R. J. (1974) in *Anti-inflammatory Agents* (Scherrer, R. A. & Whitehouse, M. W., eds.), vol. 2, Academic Press, New York, in the press

Haslam, R. J. & Rosson, G. M. (1972) *Amer. J. Physiol.* **223**, 958–967

Haslam, R. J. & Taylor, A. (1971) *Biochem. J.* **125**, 377–379

Holmsen, H., Day, H. J. & Stormorken, H. (1969) *Scand. J. Haematol. Suppl.* **8**

Ignarro, L. J. (1973) *Nature (London) New Biol.* **245**, 151–154

Kaliner, M., Orange, R. P. & Austen, K. F. (1972) *J. Exp. Med.* **136**, 556–567

Krebs, H. A. & Hems, R. (1953) *Biochim. Biophys. Acta* **12**, 172–180

Kuehl, F. A., Cirillo, V. J., Ham, E. A. & Humes, J. L. (1973) *Advan. Biosci.* **9**, 155–172

Kuo, J. F. & De Renzo, E. C. (1969) *J. Biol. Chem.* **244**, 2252–2260

Mustard, J. F. & Packham, M. A. (1970) *Pharmacol. Rev.* **22**, 97–187

Ramwell, P. W. & Shaw, J. E. (1970) *Recent Progr. Horm. Res.* **26**, 139–173

Salzman, E. W. (1972) *N. Engl. J. Med.* **286**, 358–363

Scales, B. (1967) *Int. J. Appl. Radiat. Isotop.* **18**, 1–6

Shimizu, H. & Daly, J. (1970) *Biochim. Biophys. Acta* **222**, 465–473

Smith, J. B. & Willis, A. L. (1971) *Nature (London) New Biol.* **231**, 235–237

Smith, J. B., Silver, M. J., Ingerman, C. & Kocsis, J. J. (1972) *Abstr. Congr. Thrombosis 3rd*, 246

Turtle, J. R. & Kipnis, D. M. (1967) *Biochemistry* **6**, 3970–3976

Vane, J. R. (1971) *Nature (London) New Biol.* **231**, 232–235

Weiss, H. J. & Aledort, L. M. (1967) *Lancet* **ii**, 495–497

White, J. G., Goldberg, N. D., Estensen, R. D., Haddox, M. K. & Rao, G. H. R. (1973) *J. Clin. Invest.* **52**, 89a

Zucker, M. B. & Peterson, J. (1970) *J. Lab. Clin. Med.* **76**, 66–75