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EFFECT OF THIO REAGENTS ON PLATELET TRANSPORT PROCESSES AND RESPONSES TO STIMULI

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Abstract—*N*-ethylmaleimide at submillimolar concentrations inhibited platelet aggregation and shape-change induced by ADP, collagen-induced aggregation, and the active transport of adenosine (but not of adenine or serotonin). *p*-Chloromercuribenzene sulphonate had broadly similar activity but was much less potent. Iodoacetate, which at 0.3 mM caused leakage of ^{3}H from the cytoplasm of platelets prelabelled with [^{3}H]adenine, had no inhibitory effects at lower concentrations, and 5′5′-dithiobisnitrobenzoic acid was inactive up to 3 mM. Dithiothreitol, which slightly inhibited aggregation at 0.5 mM, induced aggregation directly at concentrations above 1 mM, and 2-amino(4-isothioureylmethylene)-thiazol di HCl was a potent and selective inhibitor of serotonin transport, and also inhibited collagen-induced aggregation. Ecto-SH groups are apparently not involved in regulating platelet active transport processes or responses to stimuli, but intracellular thio groups are important in platelet secretion, aggregation, and shape-change, and also in the transport of serotonin and adenosine, but not of adenine.

Previous studies of the effects of thio reagents on blood platelets have been mainly concerned with platelet aggregation. Agents which block sulphydryl groups inhibit platelet aggregation, provided they can penetrate the cell membrane [1–3], whereas agents which reduce disulphide bonds exert a dual effect: low concentrations inhibit aggregation, but higher concentrations can directly induce aggregation [4, 5]. There have been no detailed studies of the effects of thio reagents on other platelet responses, such as shape-change (the initial response to interaction of stimuli with platelet membrane receptors), or active transport processes. Membrane receptors for stimuli on other cells, however, are known to contain functionally important thio groups [6–8] and sulphydryl group blockers inhibit active transport in erythrocytes [9, 10]. In this study we investigated the effects of six thio reagents (with different actions) on platelet shape-change and aggregation induced by ADP, on collagen-induced aggregation, and on the active transport of serotonin, adenine and adenosine.

MATERIALS AND METHODS

Platelet aggregation and shape change. Platelet aggregation induced by 10 μM ADP or 10 μg/ml connective tissue suspension [11] was measured photometrically in 0.1 ml volumes of human citrated platelet-rich plasma (PRP) as described previously [12]. Platelet shape-change (rate of decrease in light transmission induced by 10 μM ADP) was measured in 0.1 ml samples of PRP containing 3 mM EDTA.

Uptake of serotonin, adenosine and adenine. Samples of PRP (1 ml) were added to 0.1 ml iso-osmotic saline or drugs, and incubated at 37° for 5 min. Replicate subsamples of 0.1 ml were taken for the measurement of platelet aggregation and shape change (see above). Further 0.1 ml subsamples were added to 10 μl of 11.0 μM [^{14}C]5HT, 5.5 μM [^{14}C]adenosine, or 1.1 μM [^{3}H]adenine in microcentrifuge tubes, vortex-mixed for 5 sec, then incubated at 37°. After 3 min (5HT and adenine) or 12 min (adenosine), uptake was terminated by adding 5 vol of ice-cold iso-osmotic saline containing 0.4% w/v EDTA, 10 μM 5HT, 5 μM adenosine and 1 μM adenine. Samples were then immediately centrifuged (14,700 *g*: 30 sec) in a Quickfit microcentrifuge, and 0.25 ml of supernatant was transferred to a vial containing 10 ml of scintillant (toluene with 0.33% w/v 5-(4-biphenyl)-2-(4-*t*-butylphenyl)-1-oxa-3,4 diazole (Butyl PBD) plus 30% v/v ethoxyethanol). Radioactivity was measured in a Nuclear Chicago Mk 2 liquid scintillation counter.

Platelet lysis. Samples of PRP were incubated at 37° for 45 min with [^{3}H]adenine (final concentration 0.1 μM), which is incorporated into the platelet cytoplasmic pool of adenine nucleotides, and hence serves as a cytoplasmic marker. Subsamples were then incubated for 8 min at 37° with drug or an equivalent volume of saline. After adding 0.5 ml ice-cold iso-osmotic saline containing 0.4% w/v EDTA, samples were centrifuged (14,700 *g*: 30 sec), and 0.25-ml volumes of supernatant transferred to vials containing scintillant (see above). Increases in the amount of ^{3}H in the supernatant of samples containing drugs (compared with control samples) indicated cell lysis.

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Abbreviations used in this paper: Ag 307 (2-amino(4-iso thioureylemethylene)-thiazol diHCl), DTNB (5′5′-dithio-bis nitrobenzoic acid), DTT (dithiothreitol), 5HT (5-hydroxytryptamine), IAA (iodoacetate), NEM (*N*-ethylmaleimide), PCMBS (*p*-chloromercuribenzene sulphonate), PRP (platelet-rich plasma).

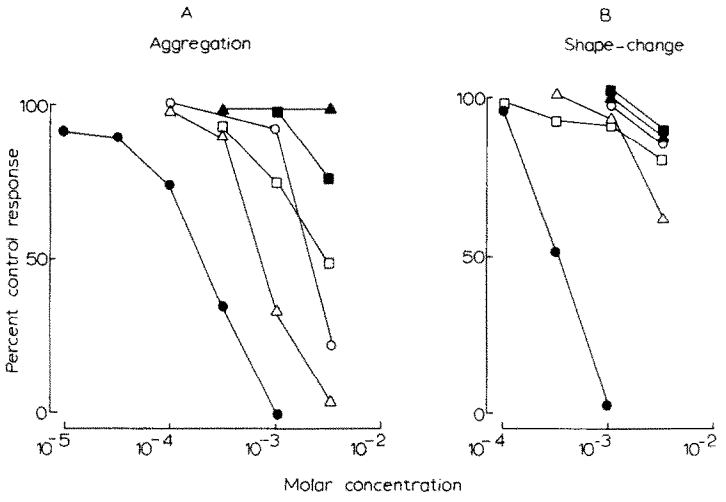

Fig. 1. Effects of thio reagents on platelet aggregation and shape-change induced by ADP. Drugs were incubated for 5 min at 37° in human citrated PRP before aggregation was induced by 10 μM ADP. Shape change was measured in replicate samples containing 3 mM EDTA. Responses were measured photometrically (see Methods). Points shown are means of duplicate determinations, and results are expressed as percentage of the control response. Twelve replicate controls were performed in each experiment. ●——● NEM; ○——○ DTT; ▲——▲ DTNB; △——△ PCMBS; ■——■ Ag307; □——□ IAA.

Isotopes. All isotopes were obtained from The Radiochemical Centre, Amersham, U.K. 5-Hydroxy [side chain-2-^{14}C] tryptamine creatinine sulphate (58 mCi/m-mole) was dissolved in 0.1 M sodium acetate-acetic acid buffer (pH 5.5), and [8-^{3}H]adenine (24 Ci/m-mole) was dissolved in iso-osmotic saline, each isotope at a concentration of 20 μM. [U-^{14}C]adenosine (57 mCi/m-mole) was dissolved in 0.05 M Tris-buffered saline (pH 7.4) at a concentration of 88 μM. All isotopes were stored at −20° in 0.2 ml vol, and fresh samples were thawed just before each experiment.

Drugs and reagents. ADP (disodium salt), adenosine, adenine, 5HT-creatinine sulphate, EDTA (disodium salt), Tris (hydroxy-methyl) amino methane, dithiothreitol (DTT), *p*-chloro-mercuribenzene sulphonate (PCMBS) and iodoacetate (IAA) were obtained from Sigma, U.K. *N*-ethylmaleimide (NEM) and 5′,5′-dithio-bis nitrobenzoic acid (DTNB) were obtained from Koch–Light, Colnbrook, U.K. The sodium salt of DTNB was prepared as described by Boyne and Ellman [13]. 2-amino(4-isothioureylmethylene)-thiazol dihydrochloride (Ag 307) was kindly donated by Dr H. Laborit, CEPBEPE, Paris.

RESULTS

Control aggregation and shape-change responses to ADP and collagen did not change significantly during the course of each experiment. All experiments were performed on plasma samples from the same subject, but as there were minor differences in control responses in different experiments, results obtained in test samples were expressed as percentages of the corresponding mean control response.

ADP-induced aggregation and shape change. The compounds under investigation were tested at concentrations up to 3 mM, and all except DTNB inhibited aggregation (Fig. 1a). NEM was the most potent inhibitor (IC_{50} = 0.2 mM) followed by PCMBS (IC_{50} = 0.8 mM), DTT (IC_{50} = 2 mM) and IAA (IC_{50} = 3 mM). The effect of DTT was complicated by the fact that at concentrations above 1 mM it produced a slow, direct aggregation response [5]. The ADP-induced shape-change response was less susceptible to inhibition by thio reagents than was the aggregation response (Fig. 1b). NEM was again the most potent inhibitor (IC_{50} = 0.3 mM), and PCMBS inhibited by 40 per cent at 3 mM, but all the other reagents had little effect even at this concentration.

Collagen-induced platelet aggregation. Collagen-induced platelet aggregation was more readily inhibited by the thio reagents than was aggregation induced by ADP. The most potent inhibitor was NEM (IC_{50} = 0.1 mM), followed by IAA (IC_{50} = 0.3 mM), PCMBS (IC_{50} = 4 mM), Ag 307 and DTT (IC_{50} = 0.5 mM). DTNB was without effect at concentrations up to 3 mM.

Active transport. The experimental conditions in these studies were adjusted so that less than 50 per cent of the labelled adenine or 5HT was incorporated into platelets in control samples. Since adenosine is deaminated in plasma, experimental conditions were arranged such that only about 25 per cent of the added adenosine was incorporated into control samples, thereby reducing the influence of deamination on the results. As in the aggregation studies, values obtained in test samples are expressed as percentages of the corresponding mean control response.

5HT. The mean amount of 5HT taken up was 29.9 ± 6.3 S.E.M. pmole/10^8 cells/min in control samples (n = 12). The most potent inhibitor of 5HT uptake was Ag 307 (IC_{50} = 0.15 mM), followed by PCMBS (IC_{50} = 0.5 mM) and NEM (IC_{50} = 3 mM). The other reagents tested had little effect on 5HT uptake even at 3 mM (Fig. 2a).

Adenine. The amount of adenine taken up by control samples was 4.1 ± 0.09 S.E.M. pmole/10^8 cells/min

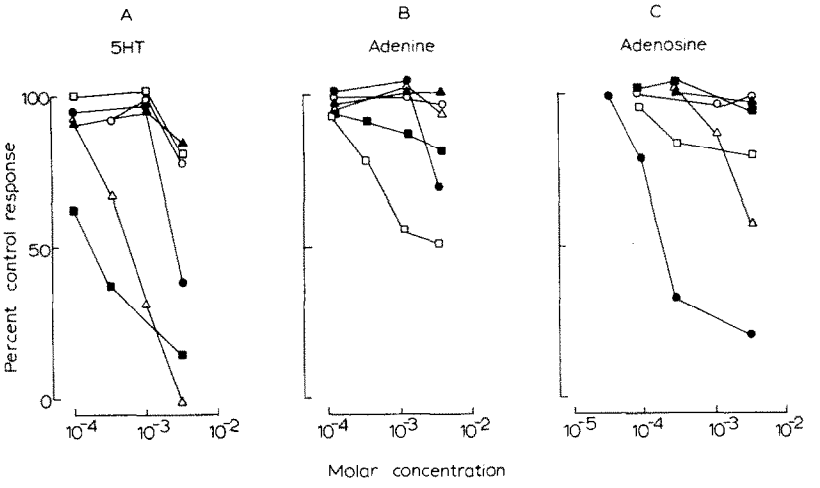

Fig. 2. Effects of thio reagents on platelet active transport processes. Uptake of [^{14}C]5HT, [^{3}H]adenine and [^{14}C]adenosine by human platelets was measured as described in Methods. Uptake in the presence of drugs is expressed as percentage of the control response. Points shown are means of duplicate determinations. Twelve replicate controls were performed in each experiment. ●——● NEM; ○——○ DTT; ▲——▲ DTNB; △——△ PCMBS; ■——■ Ag307; □——□ IAA.

(n = 12). None of the compounds tested was a powerful inhibitor of adenine uptake: the most potent was IAA, which inhibited uptake by almost 50 per cent at 1 mM (Fig. 2b).

Adenosine. The mean amount of adenosine transported by control samples was 11.67 ± 0.2 S.E.M. pmole/10^8 cells/min (n = 12). NEM was the most potent inhibitor of adenosine uptake (IC_{50} = 0.2 mM). None of the other compounds tested inhibited uptake by 50 per cent (Fig. 2c).

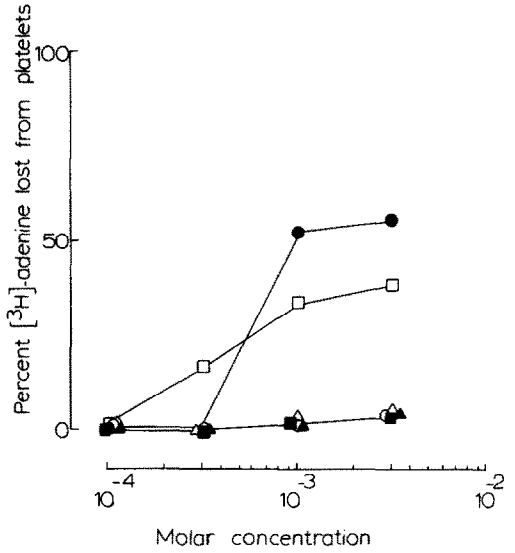

Fig. 3. Platelet lysis induced by thio reagents. Human platelets were relabelled with [^{3}H]adenine (see Methods) and then incubated with drugs for 8 min at 37°. The amount of ^{3}H in the supernatant of control samples was subtracted from the values in samples containing drugs, and the difference expressed as a percentage of the [^{3}H]adenine content in control platelets. Points shown are means of triplicate determinations. Six replicate controls were performed in each experiment. ●——● NEM; ○——○ DTT; ▲——▲ DTNB; △——△ PCMBS; ■——■ Ag307; □——□ IAA.

Platelet lysis. The effects of the thio reagents on platelet membrane integrity was assessed by measuring the leakage of radioactivity from platelets prelabelled with [^{3}H]adenine.

Only IAA (≥0.3 mM) and NEM (≥1 mM) caused significant leakage of this cytoplasmic marker (Fig. 3).

DISCUSSION

The aim of these experiments was to investigate the effects of several thio reagents with different actions on platelet active transport processes and responses to stimuli, and thus to determine the nature and localisation of thio groups which might be functionally important in these platelet reactions. The main findings are summarised in Table 1.

Perhaps the most striking observation was the lack of effectiveness of DTNB, which blocks-SH groups by thiol–disulphide exchange but does not penetrate cell membranes [10, 14]. This implies that there are no ecto-sulphydryl groups on the platelet which regulate responses to stimuli or active transport processes. In contrast, NEM, which alkylates SH groups and rapidly penetrates cell membranes [15], was a potent inhibitor of aggregation, shape-change and adenosine transport: at sub-millimolar concentrations, adenine and 5HT transport was unaffected. PCMBS, which converts SH groups to mercaptides and penetrates cell membranes only slowly [9], exhibited a similar spectrum of activity to NEM, but was much less potent. One anomalous finding was that PCMBS inhibited 5HT transport more effectively than NEM, but since PCMBS can selectively release 5HT from platelets [16; MacIntyre, unpublished], it is likely that the observed inhibition of 5HT uptake was due to leakage of endogenous 5HT. IAA, which also alkylates SH groups (although by a different mechanism from NEM) and penetrates membranes rapidly [10, 14] was much less effective than NEM, but at concentrations below 1 mM released ^{3}H from the cytoplasm

Table 1.

Drug	Membrane penetration	Action	IC_{50} value (mM) Collagen aggregation	ADP aggregation	ADP shape change	5HT transport	Adenosine transport	Adenine transport	Lysis ($> 10\%$ leakage)
DTNB	None	SH → disulphide	⩾3	⩾3	⩾3	⩾3	⩾3	⩾3	⩾3
NEM	Rapid	SH alkylated (nucleophilic addition)	0.1	0.2	0.3	2	0.2	⩾3	1
PCMBS	Slow	SH → mercaptide	0.4	0.8	3	0.5	3	⩾3	⩾3
IAA	Rapid	SH alkylated (alkyl substitution)	0.3	3	⩾3	⩾3	⩾3	3	0.3
DTT	?	S − S reduced	0.5	2	>3	>3	⩾3	⩾3	⩾3
Ag 307	Rapid	SH donated	0.5	>3	⩾3	0.2	⩾3	⩾3	⩾3

of platelets preloaded with [^{3}H]adenine. The differential effects of IAA and NEM could arise because of differences in their reactivity with intracellular SH groups [17].

The only effect observed with DTT at sub-millimolar concentrations was inhibition of collagen-induced aggregation: there was no significant effect on active transport, and although ADP-induced aggregation could be inhibited by 1–2 mM DTT, previous studies [5, 18] have shown that under these conditions DTT induces aggregation directly.

Ag 307, which penetrates membranes rapidly and apparently competes with glutathione by donating SH groups [19] has been shown previously to stabilise lysosomal membranes [20]. In our experiments it was a potent inhibitor of 5HT transport, and to a lesser extent of collagen-induced aggregation, but had no effect on other platelet reactions at sub-millimolar concentrations. The results obtained in the present study indicate that intracellular SH groups play an important role in platelet aggregation, confirming the earlier report of Harbury and Schrier [3]. Collagen-induced aggregation, which depends on the release of platelet constituents, was more easily inhibited than ADP-induced aggregation implying that intracellular SH groups are more important in the platelet release reaction than in primary aggregation. The platelet shape change was less susceptible to inhibition than the aggregation response to ADP. Since collagen-induced aggregation was also inhibited by Ag 307 and DTT, it is possible that the platelet release reaction depends on the maintenance of a critical SH–SS equilibrium. We cannot be certain, however, whether Ag 307 and DTT act at the same site as the SH blockers, and, indeed, it is not known whether DTT can penetrate cell membranes.

The studies of platelet active transport processes revealed that 5HT uptake was markedly reduced by Ag 307, and adenosine uptake by NEM, at concentrations which did not affect the other transport processes. This implies that the adenosine transport carrier contains a functionally important free SH group which is presumably on the inside of the plasma membrane, since PCMBS had little effect and DTNB was inactive. The effect of Ag 307 (which donates SH groups intracellularly) suggests that an intracellular SH–SS equilibrium may be important in 5HT uptake. Also, since Ag 307 stabilises granule membranes [20] and since granule uptake of 5HT probably contributes to measured [^{14}C]5HT uptake [21, 22] the inhibitory effect of Ag 307 may be on granule uptake rather than at the plasma membrane.

Previous studies with erythrocytes, polymorphonuclear leukocytes and cultured cells [23–25] have shown that a partial characterisation of "receptors" involved in transport and cell functions (e.g. phagocytosis, cell motility, cell adhesion) can be achieved through differential effects of thio reagents. The results of the present study indicate that a similar discrimination can be made in the platelet, and further investigations of this type should lead to a better understanding of the nature of specialised membrane and intracellular sites involved in active transport and cellular responses to stimuli.

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