Effects of Antimycin A and 2-Deoxyglucose on Secretion in Human Platelets

DIFFERENTIAL INHIBITION OF THE SECRETION OF ACID HYDROLASES AND ADENINE NUCLEOTIDES

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1. Shape change, aggregation and secretion of dense-granule constituents in platelets differ in their dependence on cellular energy metabolism. The possibility that such a difference also exists between secretion of dense-granule constituents and acid hydrolases was investigated. 2. Human platelets were incubated with [^{14}C]adenine in plasma, and then washed and resuspended in salt solutions. The effects of incubating the cells with antimycin A and 2-deoxyglucose on the concentrations of [^{14}C]ATP, ADP, AMP, IMP and inosine plus hypoxanthine and on thrombin-induced secretion of ATP plus ADP and acid hydrolases were studied. The metabolic inhibitors only affected ^{14}C -labelled nucleotides, whereas thrombin only liberated unlabelled ATP and ADP. 3. The extent of secretion decreased progressively with time during incubation with the metabolic inhibitors. At any time the secretion of acid hydrolases, β -N-acetylglucosaminidase, β -glucuronidase and β -galactosidase was inhibited to a greater extent than secretion of ATP plus ADP (dense-granule secretion). 4. Incubation with the metabolic inhibitors shifted the log (dose)–response relationship to higher thrombin concentrations, and with a greater shift for acid hydrolase secretion than for dense-granule secretion. 5. Antimycin, when present alone, caused a marked decrease in the rate of acid hydrolase secretion, but had no effect on dense-granule secretion. 6. These results further support the view that acid hydrolase secretion and dense-granule secretion are separate processes with different requirements for ATP energy. Acid hydrolase secretion, but not dense-granule secretion, appears to depend on a simultaneous rapid generation of ATP, which can be accomplished by oxidative, but not by glycolytic, ATP production.

Platelets change shape, aggregate, liberate arachidonate and secrete constituents stored in subcellular organelles upon stimulation with various agonists (Holmsen, 1977). These platelet responses are inhibited when the concentration of ATP available in the cells is lowered by metabolic inhibitors (Mürer *et al.*, 1967; Mürer, 1968; Hagen, 1972; Holmsen *et al.*, 1974; Kattlove, 1974; Rittenhouse-Simmons & Deykin, 1977). Shape change, aggregation and secretion (measured as secondary aggregation) were found to differ in their apparent ATP requirements, with secretion requiring the most energy and shape change the least (Holmsen *et al.*, 1974); the relative ATP requirement for the other responses has not been determined.

Studies on the subcellular localization and the release kinetics of the granule-stored substances suggest that platelet secretion can be subdivided into α -granule secretion, dense-granule secretion and acid hydrolase secretion (Holmsen, 1978). Acid hydrolase secretion appears to be selectively activated by cytochalasin B (Haslam *et al.*, 1975), inhibited by colchicine and vinblastine (Kenney & Chao, 1978)

and requires greater ATP availability than does dense-granule secretion (Hagen, 1972; Fukami *et al.*, 1976; Holmsen & Robkin, 1977; Holmsen, 1978). In the present work the difference in energy requirement of these secretion processes has been studied in more detail. The ATP availability has been varied by incubation of washed human platelets with 2-deoxyglucose and antimycin A, and thrombin-induced secretion was monitored as the release of non-metabolic ATP and ADP (dense-granule secretion) and β -N-acetylglucosaminidase, β -glucuronidase and β -galactosidase (acid hydrolase secretion).

Experimental

Materials

Chemicals and enzymes. Antimycin A in 95% (v/v) ethanol and 2-deoxyglucose in 0.15M-NaCl were stored at -20°C and diluted to desired concentrations before experiments. Both inhibitors, human albumin (fraction V), *p*-nitrophenyl β -D-galactopyranoside, *p*-nitrophenyl *N*-acetyl- β -D-glucosaminide and *p*-nitrophenyl β -D-glucuronide were from Sigma Chemical

Co. (St. Louis, MO, U.S.A.). Thrombin (topical, bovine; Parke Davis and Co., Detroit, MI, U.S.A.) was stored at 100 NIH units/ml of 0.15M-NaCl at -20°C and diluted immediately before the experiments. Other chemicals were reagent grade.

Washed [^{14}C]adenine-labelled platelets. Human blood was collected in 0.11M-sodium citrate (9:1, v/v) and centrifuged at 180g for 15min at room temperature. The supernatant platelet-rich plasma was saved and incubated with 0.4 μM -[U- ^{14}C]adenine (Amersham/Searle, Arlington Heights, IL, U.S.A.; code CFA 436; 250–300mCi/mmol) at room temperature for 1h to label the metabolic adenine nucleotide pool in the platelets (Holmsen *et al.*, 1972). The labelled platelet-rich plasma was diluted with 1 vol. of ice-cold washing solution [0.12M-NaCl/0.03M-Tris/HCl (pH 7.4)/3 mM-sodium EDTA/5 mM-glucose] and centrifuged at 2000g for 15min. The platelets were resuspended in a small volume (20–30ml) of cold washing solution, re-centrifuged and resuspended to 3×10^8 – 6×10^8 cells/ml in cold washing solution with or without glucose. These platelet suspensions were kept on ice (for less than 15min) until the start of the incubations.

Incubation with metabolic inhibitors

All incubations (37°C) were done after 5min temperature equilibration of the platelet suspensions; 33 μl of 2-deoxyglucose in various concentrations or 0.15M-NaCl (control) and 1.67 μl of antimycin A in various concentrations or ethanol (control) were added per ml of platelet suspension. These mixtures are referred to as main incubation mixtures. Samples were removed at the times noted during incubation for the determination of the radioactivity of the [^{14}C]adenine metabolites, the total concentrations of ATP and ADP and thrombin-induced secretion.

Analyses

[^{14}C]Adenine metabolite radioactivity was determined in both the soluble and insoluble parts of EDTA/ethanol extracts of the main incubation mixture after their separation by high-voltage paper electrophoresis (Holmsen *et al.*, 1974). The amounts of ATP and ADP were assayed in the same extracts as described by Holmsen *et al.* (1971).

The extent of secretion was determined by adding 1ml of the main incubation mixture to tubes prewarmed at 37°C and containing 100 μl of thrombin (at various concentrations) or 0.15M-NaCl (control). The tubes were incubated for 60s, chilled in a methanol/ice bath (-30°C) for 5–10s and then placed in a water/ice bath. When all tubes in an experiment had been thus processed, they were centrifuged at 17000g for 10min at 4°C . The supernatants were saved and the cells resuspended in 1.1ml of ice-cold washing solution. Samples (200 μl) of both supernatants and resuspended cells were removed for the

determination of the concentrations of ATP and ADP; 50 μl of 6% Triton X-100 was added to the remainder, and these mixtures were kept at -20°C until used for the determinations of acid hydrolase activity (Holmsen *et al.*, 1975) and protein concentration (Lowry *et al.*, 1951). Bovine serum albumin was used as the standard for protein determination.

The time course of secretion was studied by adding 2ml portions of the main incubation mixture to tubes prewarmed at 37°C . A 250 μl sample, the presample, was immediately transferred to 500 μl of ice-cold washing solution, and 200 μl of a thrombin solution or 0.15M-NaCl was added to the remainder. Samples of 250 μl were then removed, at the times noted, to 500 μl of ice-cold washing solution. All such mixtures, except the presamples, were centrifuged as above and the supernatants saved. Samples of 150 μl were removed from presample supernatants for determination of ATP and ADP; 50 μl of 3.9% Triton X-100 was added to the remainder and the mixture kept at -20°C until used for the determinations of acid hydrolases activity and protein concentration.

The platelet suspensions used contained 189 ± 27 mg of protein/ 10^{11} platelets (mean $\pm$ s.d., $n = 54$). Platelets were counted and sized in a model Z₁ counter (Coulter Electronics, Hialeah, FL, U.S.A.).

Expressions

The sum of radioactivity in ATP, ADP, AMP, IMP and inosine plus hypoxanthine in the platelet suspensions did not change throughout the incubations; the radioactivity of a metabolite is therefore expressed as a percentage of this sum. The adenylate energy charge was calculated according to the formula of Atkinson (1969), except that the total radioactivities of ATP, ADP and AMP were used in place of their concentrations (Mills, 1973).

Secretion of a substance or enzyme (activity) is expressed as percentage of that maximally secretable (Holmsen, 1978):

$$\frac{S_t - S_c}{M_s} \times 100$$

where S_t is the amount (total enzyme activity) in the supernatant from a thrombin-treated sample, S_c is that in the supernatant of the control. M_s , maximally secretable amount, is $S_t - S_c$ for the platelet suspension treated with 5 units of thrombin/ml for 5min. M_s is expressed as a percentage of the total amount (activity) in the platelets, determined as the difference between that in the whole suspension and that in its 17500g supernatant.

Results

Effects of 2-deoxyglucose and antimycin A on [^{14}C]adenine nucleotides in washed platelets

In [^{14}C]adenine-labelled platelet-rich plasma, 2-deoxyglucose causes a fall in [^{14}C]ATP concentra-

tion which is balanced by formation of [^{14}C]hypoxanthine and a decrease in the adenylate energy charge; both decreases increase with the concentration of 2-deoxyglucose and in particular with antimycin A, which has no effect alone. The inhibitors do not affect the unlabelled granule-stored adenine nucleotides (Holmsen *et al.*, 1974). The same effects of 2-deoxyglucose and antimycin A were found with the suspensions of washed platelets used here, except that antimycin A induced an immediate lowering of [^{14}C]ATP concentration, which was independent of the presence of glucose or 2-deoxyglucose, and which lasted for 1–2 min. This effect was abolished by plasma or albumin, showing that its absence from platelet-rich plasma is due to the strong binding of antimycin A to albumin (Slater, 1973). In addition, with the washed platelets in the absence of glucose, the half-maximal rate of [^{14}C]ATP breakdown was obtained at $0.194 \pm 0.043 \text{ mM}$ 2-deoxyglucose ($n = 4$), in contrast with 24 mM in platelet-rich plasma (Holmsen *et al.*, 1974). Glucose counteracted the effect of 2-deoxyglucose with abolition when $[\text{glucose}]/[\text{2-deoxyglucose}] \geq 2$, and the different dose-response relationships for 2-deoxyglucose in the two platelet systems was probably due to the endogenous 5 mM-glucose in platelet-rich plasma.

Effect on secretion of the time of incubation with metabolic inhibitors

A combination of maximal concentrations of antimycin A ($>2.5 \text{ ng/mg}$ of platelet protein) and 2-deoxyglucose ($>1 \text{ mM}$) in the absence of glucose caused abolition of all secretion within 1–2 min. Therefore the experiments were performed with relatively low rates of [^{14}C]ATP breakdown, accomplished by use of glucose together with 2-deoxyglucose and antimycin A.

In experiments ($n = 6$) with slow breakdown of [^{14}C]ATP (antimycin A and [$[\text{2-deoxyglucose}]/[\text{glucose}] = 0.6$], the secretion of β -N-acetylglucosaminidase decreased evenly and was abolished after 25 min. Secretion of ATP plus ADP was little affected during the first 12 min of incubation and was thereafter rapidly inhibited. Thus at all stages the secretion of β -N-acetylglucosaminidase was inhibited to a greater extent than that of ATP plus ADP, and the difference was greatest after 12 min; at this time β -N-acetylglucosaminidase secretion was inhibited by 56%, whereas ATP plus ADP secretion was inhibited by 9%.

Fig. 1 represents experiments ($n = 4$) in which a more rapid lowering of the ATP concentration was obtained (antimycin A and [$[\text{2-deoxyglucose}]/[\text{glucose}] = 4$]). Secretion of β -N-acetylglucosaminidase was inhibited considerably more than that of ATP plus ADP. The difference in inhibition was greater than with slow ATP breakdown throughout the incubation with 95% and 10% inhibition of the

Fig. 1. Effects of time of incubation of platelets with metabolic inhibitors on the extent of secretion: fast breakdown of [^{14}C]ATP

A platelet suspension (5 mM-glucose; 4.1×10^8 cells and 350 000 c.p.m./ml) was incubated with and without 20 mM-2-deoxyglucose and 4 μg of antimycin A/ml. Samples were taken at the times indicated and analysed for [^{14}C]ATP ($\blacktriangle$), adenylate energy charge ($\circ$), secretion of ATP+ADP ($\bullet$) and secretion of β -N-acetylglucosaminidase ($\square$). The platelets contained 9.4 μmol of ATP+ADP/ 10^{11} cells of which 74.7% was maximally secreted, and 12.7 nmol/min per mg of protein of β -N-acetylglucosaminidase, of which 50.7% was maximally secreted.

secretion of β -N-acetylglucosaminidase and ATP plus ADP respectively as the greatest difference at 15 min. Secretion of β -N-acetylglucosaminidase but not that of ATP plus ADP was inhibited immediately after addition of the metabolic inhibitors (zero time; Fig. 1).

Secretion of β -glucuronidase and β -galactosidase was inhibited to the same degree as that of β -N-acetylglucosaminidase. No correlation existed between the degree of inhibition of the secretion of ATP plus ADP or acid hydrolases and the actual concentration of [^{14}C]ATP or the adenylate energy charge.

Increasing inhibition of acid hydrolase secretion occurred only when [^{14}C]ATP and the adenylate energy charge fell progressively. When [^{14}C]ATP was rapidly lowered to a steady value by antimycin A in the presence of glucose, inhibition was immediate and unchanged throughout the 30 min incubation.

Secretion of ATP and ADP was little affected by antimycin A (see below). With only 2-deoxyglucose or a [2-deoxyglucose]/[glucose] ratio of 4, little inhibition of the secretion of ATP plus ADP was seen, and that of acid hydrolases was inhibited to less than 35% after 25 min of incubation.

In the platelet suspensions incubated with and without metabolic inhibitors, 3–5% of the acid hydrolases, ATP and ADP were present extracellularly at any stage of incubation. These nucleotides had the same specific radioactivity as the intracellular ones. The specific radioactivity of the ATP and ADP secreted by thrombin, however, was <2% of that of the intracellular nucleotides throughout the incubations. These observations show that the extracel-

lular platelet constituents had appeared by lysis during the final resuspension of the cells, that metabolic blockade did not cause further lysis, and that thrombin caused secretion of only non-radioactive (granular) adenine nucleotides at any stage of metabolic blockade.

In control platelet suspensions, secretion of ATP plus ADP and acid hydrolases decreased by 10–15% during 30 min of incubation.

Effect of the concentration of thrombin on the secretion by platelets preincubated with 2-deoxyglucose and antimycin A

Platelet suspensions containing 5 mM-glucose were incubated with and without 20 mM-2-deoxyglucose and 4 μ g of antimycin A/ml for 20 min and challenged with various concentrations of thrombin for 3 min. Fig. 2 shows the results from a typical experiment ($n = 4$). In the controls, maximal secretion of ATP and ADP was obtained with 0.8 unit of thrombin/ml, whereas β -N-acetylglucosaminidase secretion required distinctly higher concentrations. In the suspensions incubated with the metabolic inhibitors, no secretion occurred below 1 unit/ml. Above this concentration, secretion of ATP plus ADP increased with increasing thrombin concentrations and was 67% of control at 8.4 units/ml. The secretion of β -N-acetylglucosaminidase was absent below 2.2 units of thrombin/ml, and then rose slowly with increasing thrombin concentration to 26% of control at 8.4 units/ml. The same pattern was observed for secretion of β -galactosidase and β -glucuronidase. Thus the metabolic inhibitors shifted the dose-response relationship to the right with a greater shift for secretion of the acid glycosidases than of ATP plus ADP. When the data in Fig. 2 were plotted as log(dose) against the secretion response (not shown), the slopes of the log(dose)-response plots for each individual secretory process were practically the same with and without metabolic inhibitors.

Fig. 2. Effect of the concentration of thrombin on secretion in platelets preincubated with metabolic inhibitors A platelet suspension (4.8×10^8 cells and 360000 c.p.m./ml), containing 5 mM-glucose, was incubated with (●, ■) and without (○, □) 2 mM-2-deoxyglucose and 4 μ g of antimycin A/ml for 15 min. Secretion of ATP+ADP (○, ●) and β -N-acetylglucosaminidase (■, □) at the thrombin concentrations indicated were measured as described in the Experimental section. The control suspension had 70.6% of total radioactivity in ATP and an adenylate energy charge of 0.906, and the corresponding parameters were 58.7% and 0.872 in the test suspension just before secretion was measured. The platelets contained $8.03 \mu\text{mol}$ of ATP+ADP/ 10^{11} platelets and 11.6 nmol/min per mg of protein of β -N-acetylglucosaminidase, of which 80.3 and 29.2% respectively were maximally secreted.

Effect of preincubation of platelets with antimycin A on the time course of secretion

Antimycin A had little effect on the velocity of secretion of ATP plus ADP (Fig. 3). β -N-Acetylglucosaminidase was secreted more slowly than in controls throughout the incubation period when antimycin A was present (Fig. 3). The time courses for secretion of β -galactosaminidase and β -glucuronidase in the presence of antimycin A appeared to be indistinguishable from that of the control in the first 30 s of the thrombin-platelet interaction, but secretion of these enzymes occurred more slowly thereafter (Fig. 3). The major effect of antimycin A was, however, to decrease the extent of the secretion of the three acid glycosidases.

Fig. 3. Effect of incubation of platelets with metabolic inhibitors on the time course of secretion. A platelet suspension (5.2×10^8 cells and 420000 c.p.m./ml) containing 5 mM-glucose was incubated with (closed symbols) and without (open symbols) $4 \mu\text{g}$ of antimycin A/ml for 10 min followed by determination of the time course of secretion as described in the Experimental section. The platelets contained (a) $8.4 \mu\text{mol}$ of ATP+ADP/ 10^{11} cells (65% maximally secretable), or 5.8 nmol/min per mg of protein of β -N-acetylglucosaminidase (25.3% maximally secretable), (b) 0.945 nmol/min per mg of protein of β -galactosidase (12.3% maximally secretable) or (c) 0.940 nmol/min per mg of protein of β -glucuronidase (13.4% maximally secretable). $\circ$, $\bullet$, ATP+ADP; $\square$, $\blacksquare$, β -acetylglucosaminidase; ∇ , $\blacktriangledown$, β -galactosidase; $\triangle$, $\blacktriangle$, β -glucuronidase.

Discussion

When the ATP concentration and the adenylate energy charge decrease progressively on incubation of platelets with antimycin A and 2-deoxyglucose, the acid hydrolase secretion is inhibited to a greater extent than dense-granule secretion all through the incubation period. Both secretory processes are abolished by prolonged incubation. However, there is no apparent correlation between the degree of inhibition and ATP concentration or the adenylate energy charge when different platelet suspensions are compared. These findings suggest that acid hydrolase secretion depends more on ATP availability than does dense-granule secretion; the degree of their dependence varies, for unknown reasons, among platelet suspensions.

After incubation of platelets with metabolic inhibitors the dose-response relationship shifts to higher agonist concentrations. This shift is greater for acid hydrolase secretion than for dense-granule secretion, which again indicates that acid hydrolase secretion depends more on cellular ATP availability than does dense-granule secretion. This observation also shows that metabolic inhibition of

both secretory processes can be partially overcome by increase in the concentration of inducer, which is also the case for shape change and aggregation (Holmsen *et al.*, 1974). If these platelet responses are supported by ATP-requiring processes, this relation could imply that the priority of ATP usage can be shifted from ongoing processes in the resting cell to responses in the stimulated cell by increase in the strength of stimulus. The slope of the log(dose)-response curves for both secretory processes was not affected by alteration in the ATP availability. Thus this alteration appears not to affect the overall cooperativity in the stimulus-response coupling.

Antimycin A alone had little effect on dense-granule secretion but reduced the extent of the secretion of acid hydrolases. β -N-Acetylglucosaminidase secretion was slower throughout the observation period, and there appeared to be no effect on β -galactosidase and β -glucuronidase secretion within the first 30 s; thereafter, secretion of these enzymes proceeded more slowly. Once inhibition was demonstrable, the secretion of all three enzymes was inhibited to the same degree. Differences between the inhibition of β -N-acetylglucosaminidase and that of β -glucuronidase and β -galactosidase

have been observed in platelets from patients with storage-pool deficiency (Holmsen *et al.*, 1975) and in normal platelets treated with glyoxylate (Holmsen, 1978). It is therefore possible that the secretion and/or binding to storage sites are different for β -N-acetylglucosaminidase and for the other two secreted enzymes.

The above studies show clearly a difference in the energy-dependence for secretion of acid hydrolases and adenine nucleotides and further support our suggestion that dense-granule secretion and acid hydrolase secretion are separate processes (Holmsen, 1978). The connection between energy metabolism and secretion in platelets is unclear. In particular, we do not know whether energy is utilized to support a response after it has been initiated, or whether it is constantly utilized in the resting platelet to maintain responsiveness. However, after ATP production is arrested by antimycin A, 2-deoxyglucose and glucunolactone, so that the only available ATP is the amount present in the platelet, dense-granule secretion is proportional to ATP concentration (Akkerman *et al.*, 1979). This proportionality suggests that ATP is consumed to drive secretion of dense-granule constituents. Similar studies have not yet been performed with acid hydrolase secretion, but, in the light of the present studies, there is reason to assume that ATP is consumed to drive this process also. In this respect the difference in ATP requirement for dense-granule and acid hydrolase secretion is quantitative.

One qualitative difference is apparent: acid hydrolase secretion is inhibited by antimycin A when glucose is present, whereas dense-granule secretion is not. The inhibition is observed immediately after addition of antimycin A and is unchanged unless the ATP availability is altered by 2-deoxyglucose. Since ATP homeostasis is unaltered by antimycin A and glucose, the inhibition could not be due to decreased ATP availability, but apparently to the presence of antimycin A. The findings of Matsui *et al.* (1974) suggest that aerobically generated ATP is required for maximal thrombin-platelet responses, i.e. dense-granule and acid hydrolase secretion. Since mitochondrial generation of ATP is far more rapid (see above) than that in glycolysis, the latter cannot immediately meet a rapid demand for ATP. It is therefore possible that acid hydrolase secretion is driven by processes that consume ATP more rapidly than those underlying dense-granule secretion. Since phosphorylation of myosin light chain precedes acid hydrolase secretion but not dense-granule secretion (Daniel *et al.*, 1977), ATP-consuming contractile processes may be involved in the former but not in the latter secretory process in platelets. This is further supported by the specific effects on acid hydrolase secretion by cytochalasin B and vinca alkaloids (Kenney & Chao, 1978; Haslam *et al.*, 1975).

Involvement of energy metabolism in secretion has been demonstrated for the adrenal medulla (Kirshner & Smith, 1969; Rubin, 1970), mast cells (Yamazaki & Saeki, 1965), pituitary glands (McPherson & Schofield, 1974) and parotid glands (Feinstein & Schramm, 1970). The acid hydrolase secretion from polymorphonuclear leucocytes, as studied by the same techniques as used for platelets, has been thoroughly characterized. It is enhanced by drugs that interfere with microfilaments (Zurier *et al.*, 1973a; Davies *et al.*, 1973; Skossey *et al.*, 1974), inhibited by microtubule disruption (Zurier *et al.*, 1973b, 1974; Hoffstein *et al.*, 1977; Hoffstein & Weissmann, 1978), modified by cyclic nucleotides (Ignarro & George, 1974) and inhibited by non-steroidal anti-inflammatory drugs (Weissmann *et al.*, 1971; Ignarro & Colombo, 1972). The acid hydrolase secretion in platelets also shows these characteristics, as it is enhanced by cytochalasin B (Haslam *et al.*, 1975) and inhibited by colchicine (Kenney & Chao, 1978) and aspirin (Holmsen *et al.*, 1975; Wiley *et al.*, 1976). Although the platelet process depends strongly on rapid ATP regeneration, such a dependence appears not to have been established for the leucocyte system. The involvement of contractile processes in leucocyte secretion was suggested by Hoffstein & Weissmann (1978), which means that secretion should be dependent on energy metabolism in this cell also. Storage granules from leucocytes (Woodin & Wieneke, 1964) and adrenal medulla (Poisner & Trifarò, 1969) contain an ATPase that has been suggested to be activated during secretion.

Besides platelets, both leucocytes (Estensen *et al.*, 1974) and adrenal medulla (Schneider, 1970) secrete acid hydrolases from specific storage sites in addition to constituents of other granules. A different energy requirement for secretion from the various classes of storage sites may also exist in these cell types.

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