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Human Blood Platelet Secretion: Optical Multichannel Analyzer Measurements Using Acriflavine as a Release Indicator†

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ABSTRACT: Blood platelets preloaded with the fluorescent amine acriflavine release the trapped fluorophore after stimulation with thrombin or the divalent cation ionophore A23187. Release was detected by an increase in acriflavine fluorescence, which is otherwise strongly quenched in the platelet, by using an optical multichannel analyzer to monitor the spectral and temporal reaction parameters. The secretion of [^{14}C]serotonin and acriflavine is well correlated, suggesting that acriflavine, like serotonin and the closely related fluorescent drugs mepacrine and acridine orange, is accumulated in and released from platelet dense bodies. Acriflavine secretion at 37 °C in the absence of external calcium is characterized by a short delay, followed by a rapid biphasic increase in fluorescence that implies at least a three-stage secretory process. For saturating levels of thrombin the delay was 1.5 s and release was 90% complete within 6-7 s. The delay could not be shortened by prestimulation under conditions that induce shape changes but not release, i.e., with ADP, arachidonic acid, or low levels of thrombin or A23187. Acriflavine secretion induced by A23187 was similar but less effective; the reaction was slower, the yield was smaller, and, in contrast to thrombin, the longer lag period could be significantly shortened by prestimulation.

Blood platelets are activated by agents of diverse structure, responding in a characteristic pattern of shape changes, secretion, and aggregation. The actual mechanism of secretion has not been elucidated in detail, but it is generally assumed that it includes fusion of the organelle and plasma membranes, followed by extrusion of the organelle contents (Skaer, 1981). The best characterized secretory granules of human platelets are the dense bodies, which contain and selectively release amines (mainly serotonin), nucleotides (mainly ATP and ADP), and calcium ions (Pletscher et al., 1971). Virtually all the serotonin in whole blood is contained in the platelet dense bodies and platelets possess a highly efficient system to transport serotonin and other amines across the plasma and granule membranes (Rudnick et al., 1980). This physiological mechanism has been widely used to load dense bodies with radiolabeled serotonin for studying the release reaction. Furthermore, it has been established that fluorescent amines such as mepacrine and acridine orange selectively accumulate in dense bodies, allowing a precise visualization of these granules with microscopic techniques (Skaer, 1981; Lorez et al., 1975; Skaer et al., 1981).

The present report indicates that the fluorescent dye acriflavine is, like other amines, specifically accumulated in platelet dense bodies. The acriflavine fluorescence is efficiently quenched during incorporation of the dye into the platelet, and the apparent quantum yield increases 5-fold or more upon secretion. We have used this system to investigate the rapid release induced by thrombin and the ionophore A23187 in real time, focusing our interest on the common and different features of secretion induced by the two stimuli under physiological conditions.

Experimental Procedures

Preparation of Gel-Filtered Human Blood Platelets. Citrated blood was obtained from the Central Laboratory of the Blood Transfusion Service of the Swiss Red Cross within 20 h of collection, and platelet-rich plasma (PRP)[1] was prepared by standard techniques (Bettex-Galland & Lüscher, 1960). All further operations and all experiments (except where noted) were carried out with platelets continuously maintained at 37 °C. The PRP was titrated with acid citrate dextrose (ACD) to pH 6.5, diluted to 1×10^9 platelets/mL with citrate buffer (30 mM sodium citrate, 100 mM NaCl, 4.8 mM glucose, 3 mM KCl, pH 6.5), and incubated for 20 min to obtain platelets with a flat disk shape (Patscheke, 1981). Ten milliliters of this ACD-PRP was applied on a thermostated Sepharose 2B column (35 mL of Sepharose) equilibrated with the elution buffer (147 mM NaCl, 4.8 mM glucose, 3 mM KCl, 3 mM MES/NaOH, pH 6.5). The platelets, collected at a rate of 0.5 mL/min, retained their discoid shape on the column. The pH of the gel-filtered platelets was adjusted to

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[1] Abbreviations: ACD, acid citrate dextrose (NIH formula A: 0.8% citric acid, 2.2% sodium citrate, 2.45% hydrous glucose); ISIT, intensified silicon intensified target; MES, 2-(*N*-morpholino)ethanesulfonic acid; OMA, optical multichannel analyzer; PMS, phenylmethanesulfonyl; PRP, platelet-rich plasma; TES, 2-[[tris(hydroxymethyl)methyl]-2-amino]ethanesulfonic acid.

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7.4 with 154 mM TES/NaOH (end concentration 30 mM TES), and the suspension was diluted with test medium to a final concentration of 4×10^8 platelets/mL. The test medium contained 30 mM TES/NaOH, pH 7.4, 118 mM NaCl, 3 mM KCl, 60 μM $CaCl_2$, and 10 μg/mL potato apyrase (final concentrations).

Labeling Procedures. Platelets were loaded with acriflavine by incubating ACD-PRP (1×10^9 platelets/mL, pH 6.5) in the presence of 10 μM dye (stock solution: 5 mM acriflavine in dimethyl sulfoxide) for 20 min, followed by gel filtration, pH adjustment, and dilution with test medium as described above. As judged from releasable fluorescence, about 50% of the total acriflavine added was taken up by the platelets; increasing the incubation time to 1 h did not further increase the uptake. In some experiments other fluorophores such as mepacrine (20 or 50 μM) or acridine orange (20 μM) were used instead of acriflavine. For serotonin labeling, gel-filtered platelets (4×10^8/mL, pH 7.4) were incubated with 0.5 μM [^{14}C]serotonin (10 nCi/mL) for 1 h. On the average, 89% of the labeled serotonin was taken up by the platelets.

Release Measurements. The increase in fluorescence resulting from acriflavine secretion was recorded with an optical multichannel analyzer (OMA) interfaced to a minicomputer (D. Deranleau et al., unpublished). Fluorescence spectra are repetitively acquired by the current version of this apparatus at rates of up to 100 spectra/s (500 digitized channels/spectrum, dispersion 1.6 nm/channel) via an ISIT videcon detector whose sensitivity approaches that of a photon counter. In the presence report, an acquisition rate of two spectra/s was used. A 500-W high-pressure mercury arc lamp in combination with a 405-nm (10-nm band-pass) interference filter was used for excitation. A cutoff filter was interposed between the sample and the polychromator to attenuate the intense exciting light scattered by the sample, allowing simultaneous acquisition of both 90° scattering and fluorescence data. The sample (0.5 mL of the relevant platelet suspension in a thermostated round plastic cuvette) was stirred at 13 rps with a Teflon-covered magnetic bar. Light transmitted by the sample and impinging on a fast photodiode allowed for simultaneous recording of the apparent light transmission, thereby affording a means of following shape changes, secretion in general, and aggregation when present. The changes in apparent light transmission observed during activation were similar to those that are obtained with so-called "aggregometers" (simple turbidimeters). In addition, switching the stirrer mechanism on and off allowed us to estimate the "discoidity" of the cells (Latimer et al., 1976; Patscheke & Wörner, 1978) before and during sequential recording of the fluorescence spectra. Addition of stimulators was done by rapid injection of 5–10 μL of concentrated stock solution to the stirred sample. For stock solution, thrombin was dissolved in buffered saline and A23187 in dimethyl sulfoxide. Mixing time as determined by fluorophore injection was less than 0.5 s. *Unless otherwise specified, the experiments were done in the presence of 2.5 mM EDTA added 2 min prior to stimulation.* This (a) reduces calcium in the external medium to negligible levels and assures an exclusively endogenous mobilization of calcium upon stimulation, (b) precludes interference with ionophore action by tying up other divalent cations that bind even more strongly than calcium to A23187, (c) prevents aggregation (Born, 1970), which could interfere with fluorescence measurements, and (d) excludes possible reinforcement of the release reaction by secreted calcium or by aggregation-related effects. At relatively high levels of thrombin (>0.1 unit/mL), however, allowing aggregation to occur by replacing the EDTA with 1 mM calcium

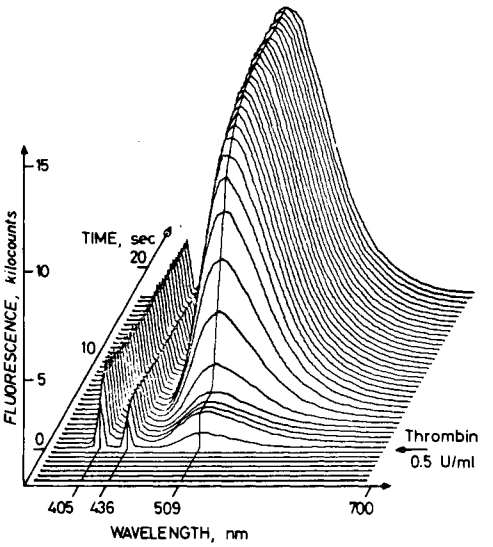

FIGURE 1: Thrombin-induced release of acriflavine from platelets. Forty uncorrected fluorescence emission spectra, range 360–700 nm, recorded in 20 s. After seven spectra thrombin was injected and the excitation shutter opened. The final thrombin concentration was 0.5 unit/mL. The two small peaks at 405 and 436 nm are due to scattered exciting light and the 509-nm peak represents acriflavine fluorescence.

did not affect the fluorescence resulting from acriflavine release.

For [^{14}C]serotonin release experiments, stimulation and fluorescence measurements were done in the OMA as described, and the release reaction was stopped by injection of 40 μL of 8% formaldehyde at various times after stimulation (final concentration 0.6%). Stopping was complete within about 0.5 s under these conditions, but required 1.5 s if 0.5% glutaraldehyde was used instead. This confirms work by Costa & Murphy (1975) demonstrating the superiority of formaldehyde over glutaraldehyde as a fixative of the platelet release reaction. The stopped reaction mixtures were transferred to an ice bath and, when cold, were centrifuged at 3500*g* for 5 min. Four hundred microliters of the supernatant was added to 4 mL of scintillation fluid (3 mg/mL Packard Permablend in 2 volumes of xylene plus 1 volume of Triton X-100), and the radioactivity was measured in a liquid scintillation counter. Release was expressed as percentage of radioactivity released from total platelet-associated radioactivity (Massini & Näf, 1979). Acriflavine fluorescence was also determined in the supernatants, and the measured recoveries indicate that it, like serotonin, is in fact secreted into the external medium.

Biochemicals. Bovine thrombin (50 NIH units/mg) was obtained from Hoffmann-La Roche, Basel, Switzerland, A23187 from Calbiochem, Lucerne, Switzerland, acriflavine from Aldrich, Beerse, Belgium, and [^{14}C]serotonin from Amersham, Great Britain. Arachidonic acid, quinacrine dihydrochloride (mepacrine), and acridine orange were all obtained from Sigma, Munich, Federal Republic of Germany. Potato apyrase was isolated according to Traverso-Cori et al. (1965), and formaldehyde was prepared from paraformaldehyde (Karnovsky, 1965).

Results and Discussion

Assessment of the Method. A typical data set obtained with the optical multichannel analyzer is shown in Figure 1, which illustrates the time-dependent effect of 0.5 unit/mL thrombin on the fluorescence spectra of acriflavine-labeled platelets. Of immediate concern in the present work is the large peak at around 509 nm, which is due to acriflavine released in increasing amounts from the platelet at various times following

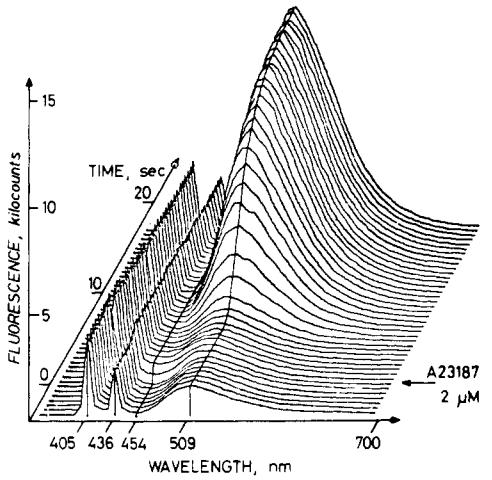

FIGURE 2: A23187-induced release of acriflavine from platelets. Conditions similar to those of Figure 1, except that A23187 (final concentration 2 μM) was injected after seven spectra and the excitation shutter was open continuously. Note the appearance of A23187 fluorescence at 454 nm, signaling the start of the experiment.

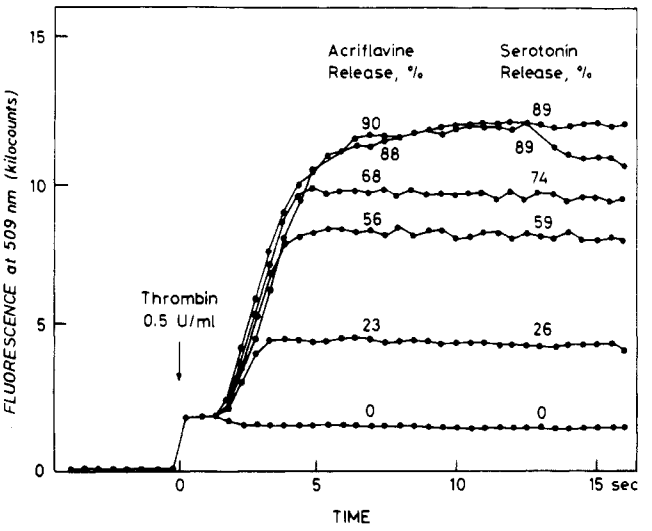

FIGURE 3: Correlation of thrombin-induced acriflavine and [^{14}C]-serotonin release in doubly labeled platelets. Secretion was stopped with 0.6% formaldehyde at 1, 3, 4, 6, 14, and 55 s (from bottom to top) after addition of 0.5 unit/mL thrombin. Each curve represents the intensity of acriflavine fluorescence at the apparent fluorescence maximum (509 nm; see Figure 1). Percentages of acriflavine and [^{14}C]serotonin released prior to fixation are indicated on the curves.

secretion. The spectra are separated by 0.5-s intervals, and actual secretion is preceded by a delay or lag period of 1–2 s after injection of the stimulant. The fluorescence then increases rapidly and, although it is not easy to see in the pseudo-three-dimensional presentation, levels off in about 10 s. No shifts in the apparent wavelength of the intensity maximum were observed during secretion. The apparent wavelength of the intensity maximum is the same as that of acriflavine in buffer alone, indicating that the fluorophore is released into an aqueous environment and is not associated with a lipophilic (membrane) component. A set of data similar to the one just discussed, but using the divalent cation ionophore A23187 instead of thrombin to stimulate platelet secretion, is presented in Figure 2. In this case one can also see a small amount of A23187 fluorescence, which remains constant as time increases.

In both cases scattered exciting light, highly attenuated by the emission cutoff filter, is responsible for the small peak at 405 nm. The other small peak at 436 nm is due to light scattered from the 436-nm line of the mercury arc, which is not completely blocked by the exitation filter. Small changes in the intensity of these two peaks, reflecting changes in the scattering properties of the platelets attendant upon shape changes and release, can be observed subsequent to stimulation. As expected, a more substantial change was noted when aggregation was allowed to occur by replacing EDTA in the reaction mixture with 1 mM calcium. In principle, these scattering differences can be used as independent estimators of platelet shape changes, general release, and aggregation, although we have not made use of this information in the present report. The OMA is in essence a "500-wavelength" kinetics spectrophotometer and is capable of acquiring a considerable amount of data in real time. There are limitations, however, and at the present state of development one is necessarily restricted in the amount and kind of data that can be effectively processed.

The reaction was further characterized by using platelets doubly labeled with acriflavine and [^{14}C]serotonin. These were stimulated with 0.5 unit/mL thrombin in the OMA, and the reaction was stopped with 0.6% formaldehyde at different times following addition of the stimulus. The fixed suspensions were then analyzed for released material as described under Experimental Procedures. A typical experiment is shown in Figure 3. In this and in the following figures only the

Table I: Comparison of the Maximum Increase in Acriflavine Fluorescence with [^{14}C]Serotonin Release in Acriflavine-Loaded and Control Platelets[a]

effector	fluorescence increase (%)	serotonin release (%)	serotonin release (%)
0.2 μM A23187	62.1	67.3	65.2
0.5 μM A23187	76.2	72.4	75.8
5.0 μM A23187	68.3	67.4	66.3
0.05 unit/mL thrombin	71.6	73.6	81.4
0.50 unit/mL thrombin	97.7	90.8	96.1
3.0 units/mL thrombin	(100.0)	95.0	96.1

[a] Release stopped with 0.6% formaldehyde after 20 s, except for 0.05 unit/mL thrombin, which was stopped after 50 s. The fluorescence increase induced by units/mL thrombin was taken as 100%. Each value represents the mean of two to four separate determinations.

acriflavine fluorescence at the apparent maximum of 509 nm is plotted, but in each case the entire spectrum has been obtained and checked for possible shifts in the maximum wavelength and for changes in the scattered light intensities. The results of a number of such experiments are summarized in Table I, which demonstrates that acriflavine fluorescence and [^{14}C]serotonin release are reasonably well correlated. In combination with the kinetic pattern of release discussed below, this strongly suggests that the fluorescence increase is due to liberation of acriflavine trapped in dense bodies. Analogous results were obtained with A23187-stimulated platelets (Table I). There was no significant difference in serotonin release whether the platelets were preloaded with acriflavine or not, so that acriflavine treatment does not interfere with the normal physiological process of serotonin secretion.

Experiments in which platelet dense bodies were loaded with mepacrine or acridine orange gave stimulus/response patterns that were similar to those obtained with acriflavine (i.e., an increase in fluorescence when release occurred). However, the poor quantum yield of mepacrine in aqueous solutions makes it less suitable as an indicator of release, and heavy

Table II: Effects of 2-min Preincubation of Platelets with Different Effectors on the Kinetic Parameters of Acriflavine Secretion Induced by 3 units/mL Thrombin and 0.5 μM A23187

	3 units/mL thrombin			0.5 μM A23187		
preincubation with	delay[a] (s)	RP[b] (s^{-1})	yield (counts)	delay[a] (s)	RP[b] (s^{-1})	yield (counts)
nothing (control)	1.5	0.41	12000	3.3	0.22	8300
10 μM ADP	1.5	0.30	11100	1.8	0.12	8200
2 μM arachidonate	1.5	0.28	10500	1.6	0.15	8100
0.04 μM A23187	1.5	0.22	10700	2.0	0.16	7100
0.02 units/mL thrombin	1.5	0.22	11600	1.3	0.11	7800

[a] +0.0–0.5 s. [b] Rate parameter. For a classical A → B → C reaction with rate constants k_1 and k_2, the rate parameter for the appearance of product C is $k_1k_2(k_1 - k_2)^{-1}$ [$\exp(-k_2t') - \exp(-k_1t')$] where the time to inflection $t' = (k_1 - k_2)^{-1} \ln (k_2/k_1)$ does not include any prior delays. A decrease in RP is synonymous with a slower rate of actual secretion (see the text).

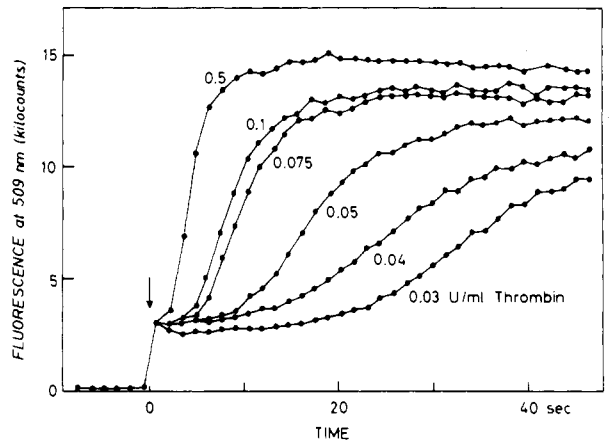

FIGURE 4: Stimulus/secretion coupling induced by varying concentrations of thrombin, as measured by the acriflavine fluorescence at 509 nm. The thrombin injection point concides with the opening of the excitation shutter (arrow + 0.0–0.5 s).

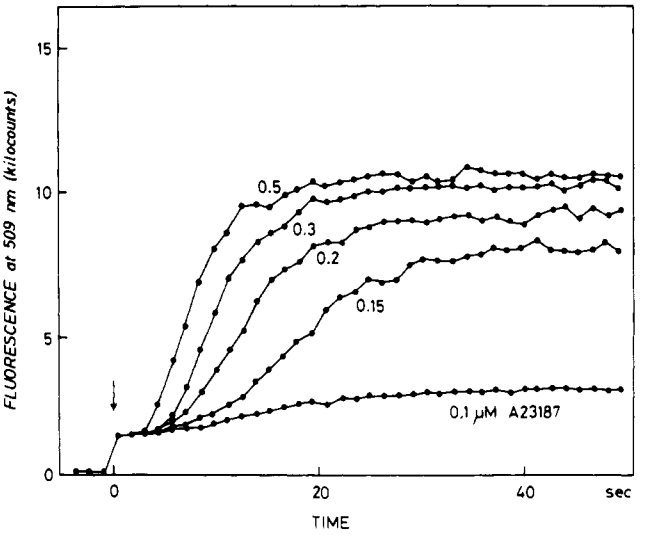

FIGURE 5: Stimulus/secretion coupling induced by varying concentrations of A23187, as measured by the acriflavine fluorescence at 509 nm. The A23187 injection point coincides with the opening of the excitation shutter (arrow + 0.0–0.5 s).

labeling with this substance (a known phospholipase inhibitor) markedly inhibited the A23187-induced release (50% with 50 μM mepacrine).

Kinetics of the Acriflavine Release. The kinetics of acriflavine release show three distinct phases, regardless of whether the reaction is induced with thrombin (receptor mediated) or with A23187 (diffusion controlled). An initial lag phase during which no changes in fluorescence are observed is followed by an apparently biexponential phase, resulting in a progress curve with a pronounced sigmoidal shape (Figures 1–5). This general picture is not new, and qualitatively in any case, the results are consistent with the classical model of Detwiler et al. for the room temperature release of ATP (Detwiler & Feinman 1973a,b; Martin et al., 1975). This model postulates an initial multistep process involving, for thrombin, binding and a catalytic step at the level of the thrombin–receptor interaction, followed by a sequence of irreversible, endogenous platelet reactions finally leading to secretion. Also according to this model, A23187 bypasses the receptor step, directly entering a pathway involving intracellular calcium fluxes that is common to both thrombin and ionophore activation (Friedman & Detwiler, 1975; Feinman & Detwiler, 1974). However, at similar thrombin concentrations the liberation of acriflavine appears to be as much as 10 times faster at 37 °C than ATP release measured by the firefly method at room temperature, an amount that cannot be accounted for on the basis of the temperature difference alone. The responses found here are in good agreement with more recent determinations of ATP/ADP secretion carried out at 37 °C by "stopping" methods (Holmsen et al., 1982) and with the room temperature experiments of Akkerman et al. (1982), who stopped the platelet reaction with formaldehyde as in the comparative experiments with acriflavine and [^{14}C]serotonin described above.

Because of data storage limitations, only 40 spectra were accumulated in the present experiments. There are thus not enough digitized points to curve fit the Detwiler model, and the individual progress curves were briefly characterized by means of the following empirical parameters: (a) the delay or lag time, i.e., the time from addition of the stimulator during which no change in fluorescence is observed, (b) the yield or total extent of release as determined from the difference in fluorescence counts before and after complete release, and (c) a rate parameter defined by the slope of the progress curve at the inflection point (maximum rate of fluorescence increase in counts per second) reduced by the yield. The rate parameter is one measure of how fast actual secretion takes place once it has started and, like the time to inflection used by Detwiler et al., is a complicated function of the rate constants (see footnote *a* of Table II). Finally, an apparent first-order rate constant was calculated from the slope of semilog plots of the final (single exponential) phase of the progress curve [time vs. the logarithm of the difference between the observed and final fluorescence, which was linear over most of the final fluorescence increase; see also Detwiler & Feinman (1973b)].

Progress curves for the release of acriflavine fluorescence are given in Figures 4 and 5 as a function of increasing thrombin and A23187 concentrations, and the results are summarized in Figure 6 in terms of the empirical reaction parameters. These values show the expected relationships—increasing stimulator levels increase the yield of the reaction

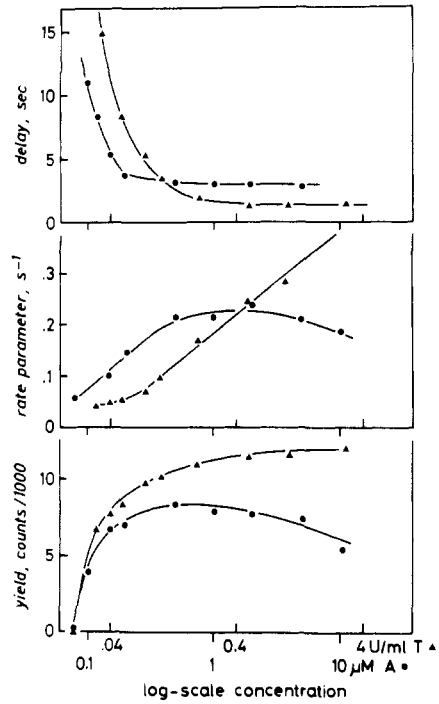

FIGURE 6: Effects of increasing concentrations (logarithmic scale) of thrombin (▲) and A23187 (●) on the delay (a), rate parameter (b), and yield (c) of acriflavine release. Each plotted point is the mean of from 3 to 25 separate determinations.

and decrease the delay time and the rate parameter for actual release. However, A23187 does not exhibit saturation kinetics, and excess reagent inhibits the responses. At saturating thrombin concentrations, release was 90% complete in 6–7 s and, with optimal concentrations of A23187 (0.5 μM), in around 11 s. The apparent rate constant for the final reaction phase at 37 °C was 0.62 ± 0.06 s^{-1} for 3 units/mL thrombin (11 experiments) and 0.30 ± 0.03 s^{-1} for 0.5 μM A23187 (7 experiments). The effect of temperature on the final phase apparent rate constants is shown in Figure 7. Arrhenius plots of these data were linear over the entire temperature range studied, and virtual activation energies calculated from the slopes of the plots were found to be 16.7 kcal/mol for 3 units/mL thrombin and 21.8 kcal/mol for 0.5 μM A23187.

Prestimulation to the Level of Shape Change. The effects of prestimulation of platelets with a number of different stimulators, each added 2 min before the secretion stimulus, are shown in Table II. The A23187 and thrombin levels used for prestimulation were sufficient to induce shape changes but not release, and ADP and arachidonic acid, which do not induce release under the present conditions, were used at levels that induce optimum shape change. In general, actual release is markedly slower with prestimulation (cf. the rate parameters), and while the delay prior to the onset of thrombin-induced stimulation is not affected by prestimulation, the delay of the A23187-induced response is markedly shortened. Calcium mobilization is believed to be an essential response to stimulation by ionophores (Rink et al., 1982a,b), and since prestimulation reduces the delay of the A23187 response, it seems likely that the membrane crossing rates of various A23187 complexes are the rate-determining features of ionophore activation. It is known (Kolber & Haynes, 1981) that the transport of the neutral complex CaA_2 (A = A23187) across synthetic membranes is slow (rate constant 0.1–0.3 s^{-1}) as compared with the crossing rate of the neutral protonated ionophore HA (rate constant ca. 30 s^{-1}) and that the charged 1:1 complex CaA^+ does not penetrate the membrane. While

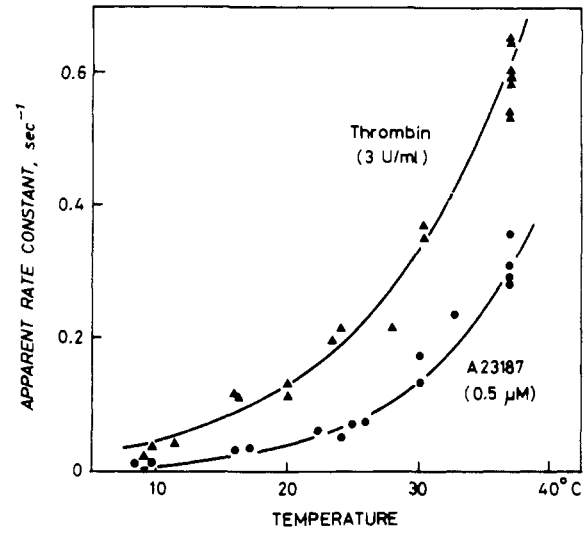

FIGURE 7: Effects of temperature on the apparent rate constant for acriflavine release induced by 3 units/mL thrombin (▲) or by 0.5 μM A23187 (●). Solid lines are the theoretical curves generated from the Arrhenius equation by using the activation energies given in the text.

not necessarily significant, it is at least interesting that the rate constant for the final phase of the A23187-induced release of acriflavine is similar to the rate constant for slow A23187 transport in the synthetic system.

According to Rink et al. (1982b), thrombin activation at the secretion level is different from ionophore activation in that much lower levels of free cytoplasmic calcium are associated with secretion. However, it is conceivable that prestimulation with thrombin leads to more efficient calcium utilization in the secretory process itself [e.g., tighter binding (Nishizuka, 1983)] and tighter binding would still require calcium mobilization in order to maintain even basal levels [see Rink et al. (1982b)]. Thus, prestimulation with either ionophores or thrombin could lead to partial depletion of mobilizable calcium storage pools and in turn to a decrease in the rate of generation of the extra calcium that may be needed in one way or another for secretion. While this is clearly speculation, it appears to be one way to explain why release induced by either thrombin or A23187 is slower following prestimulation (cf. the rate parameters in Table II).

Concluding Remarks. It is clear that the optical multichannel analyzer used in the present study has many advantages over other methods devised for kinetic studies of the release reaction. Further development of this method should allow curve fitting of the kinetic data; however, the system can store only a limited number of data points and these must be equitably divided between spectral and kinetic information. The present first attempt shows at least the feasibility of acquiring simultaneous spectral and kinetic information, and further efforts along these lines appear to be justified.

Acknowledgments

We thank Dorothy Brügger-Lichtenberg for technical assistance, Dr. H. Patscheke for discussion, and one of the referees for a critical assessment of an early version of the manuscript. We are grateful to the Emil Barell Foundation (Hoffmann-La Roche, Basel, Switzerland) and the Sandoz Foundation (Sandoz, Basel, Switzerland) for gifts of equipment.

Registry No. ADP, 58-64-0; A23187, 52665-69-7; Ca, 7440-70-2; acriflavine, 65589-70-0; serotonin, 50-67-9; arachidonic acid, 506-32-1; thrombin, 9002-04-4.

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7S Nerve Growth Factor α and γ Subunits Are Closely Related Proteins†

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ABSTRACT: The polypeptide composition and partial amino acid sequence of the 7S nerve growth factor (NGF) α subunit have been determined. Residues in 76 unique positions corresponding to 35% of the molecule were identified. The sequence shows that the NGF α subunit is closely related to the NGF γ subunit and thus a member of the same protein family as the serine proteases. This finding is unexpected since the NGF α subunit is devoid of detectable protease activity. However, the NGF α subunit differs in one important respect from the NGF γ subunit and related serine proteases. The highly conserved amino-terminal activation cleavage structure, common to most serine proteases, has been deleted, and an uncleaved activation peptide remains attached to the amino terminus of the mature NGF α subunit. It is suggested that this feature is causally related to the apparent lack of proteolytic activity.

The submaxillary gland of the adult male mouse is a rich source of nerve growth factor (NGF),[1] a polypeptide that promotes the differentiation and maintenance of certain nerve cells of neural crest origin (Server & Shooter, 1977). The growth factor is secreted into the saliva as a high molecular weight protein complex, 7S NGF (Varon et al., 1968; Burton et al., 1978). The core of the 7S NGF complex is a dimer of the biologically active polypeptide (the β subunit) and two molecules of a 28 000-dalton arginine esterase, the γ subunit (Stach et al., 1976). The 7S NGF complex also contains two molecules of the α subunit, which is a 27 000-dalton protein of unknown function (Stach et al., 1980). Like many other biologically active polypeptides, the NGF β subunit is synthesized as a precursor protein which is cleaved to yield the mature growth factor. The NGF γ subunit is responsible for this processing, and the enzyme remains bound to the growth factor after the cleavage has occurred (Berger & Shooter, 1977).

The NGF α and γ subunits have similar molecular weights and cross-react immunologically. When reduced and alkylated under denaturing conditions, the two proteins decompose into similar mixtures of 26 000-, 17 000-, 10 000-, and 6000-dalton chains (Anundi et al., 1978). These findings suggested that the two molecules may be related to one another. Sequence information on the NGF γ subunit (Thomas et al., 1981b) has demonstrated that it belongs to the trypsin branch of the serine proteases (DeHaën et al., 1975; Dayhoff, 1978). The aim of the present investigation was to obtain sequence information

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[1] Abbreviations: NGF, nerve growth factor; EGF-BP, epidermal growth factor binding protein; SDS, sodium dodecyl sulfate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PTH, phenylthiohydantoin.

0006-2960/84/0423-1229$01.50/0