DIRECT EVIDENCE FOR INTRACELLULAR DIVALENT CATION REDISTRIBUTION ASSOCIATED WITH PLATELET SHAPE CHANGE

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Summary: Chlortetracycline was used as a fluorescent probe to monitor shifts in divalent cation distribution when blood platelets were induced to change shape. Human platelets in their native plasma were incubated at 25°C with 50 μM chlortetracycline. It was found that when platelet shape change was stimulated by ADP or the divalent cation ionophore A 23187, a significant decrease in platelet-chlortetracycline fluorescence occurred. This fluorescence shift was consistent with the time course for the change in platelet shape. ATP, which inhibits ADP-induced shape change, also inhibited the decrease in platelet-chlortetracycline fluorescence.

In response to various stimuli, blood platelets undergo shape change followed by aggregation and release of their granules. Platelets, like muscle, contain Ca^{2+}-sensitive contractile proteins. Since platelet shape change can be induced in the absence of external Ca^{2+}, e.g., by exogenous ADP (1), it has been suggested that shape change may be stimulated by a redistribution of intraplatelet Ca^{2+} (2,3). Indirect observations which support this contention include: 1. ionophores, which are thought to redistribute intraplatelet divalent cation, induce shape change (4); 2. D_2O, which blocks muscle contraction by interfering with Ca^{2+} release from the sarcoplasmic reticulum (5), inhibits ADP-induced shape change (6); and 3. a high concentration of the antibiotic chlortetracycline (500 μM), which chelates intracellular divalent cation (7), also inhibits ADP-induced shape change (6). Although these findings suggest that platelet shape change is associated with a mobilization of intraplatelet Ca^{2+}, they do not provide direct evidence for such intracellular redistribution.

Using the metallochromic indicator murexide, shifts in the level of intraplatelet Ca^{2+} were directly measured (8). It was found that induction of shape change by ADP

resulted in the formation of intraplatelet Ca^{2+}-murexide. However, since dimethylsulfoxide was required as a murexide vehicle, normal platelet Ca^{2+} ion distribution may have been altered. On the other hand, the divalent cation indicator CTC* is known to freely permeate biological membranes (9), and, thus can be incorporated into platelets without the use of dimethylsulfoxide. CTC forms a highly fluorescent pH-insensitive adduct (pH 6.0-8.5) (10) when chelated with divalent cations bound to biological membranes (7, 9, 10, 11), and does not interfere with platelet function in low concentrations (5-50 μM). Furthermore, the fluorescence intensity of the CTC-divalent cation membrane adduct markedly decreases when the divalent cation is released from the membrane to a more polar environment (9). Although CTC chelates either Ca^{2+} or Mg^{2+}, it has been shown, using red blood cell membranes, that in the presence of Ca^{2+} and Mg^{2+}, CTC fluorescence stems predominantly from the Ca-CTC adduct (12).

Using the fluorescent probe CTC, we found that platelet shape change induced by ADP or A 23187 is, indeed, associated with a redistribution of intraplatelet divalent cation.

Materials and Methods

Human blood, anticoagulated with acid-citrate-dextrose solution, was obtained from commercial blood banks within 4 hours of bleeding. Platelet-rich plasma was prepared by centrifuging the blood at 164 x g for 15 minutes.

CTC was incorporated into the platelet by incubating platelet-rich plasma at 25°C with the antibiotic (obtained from Sigma Chemical Co., St. Louis, Mo.) dissolved in normal saline. One ml of plasma was layered over 0.075 ml silicone oil (17 parts Dow 220; 4 parts Dow 550). The platelets were pelleted by centrifugation at 7000 x g for 1 minute (Fisher Model 59) (13). The platelet pellet was transferred to a depression milled in a Plexiglass slide, and the fluorometric spectra was determined with a photon counting microspectrofluorometer developed by Dinerstein, Ferber and Roth (14). Platelet shape change was separately determined in CTC-treated platelet-rich plasma by monitoring the intensity of scattered light at 90° (15).

*CTC = chlortetracycline

Results and Discussion

Platelet uptake of CTC: Platelet-rich plasma was incubated at 25°C with concentrations of CTC (5 or 50 μM) which did not significantly inhibit platelet shape change. When the platelets were isolated and analyzed for fluorescence, a progressive enhancement of fluorescence intensity with time of incubation was observed (Fig. 1). In approximately 25 minutes the levels of the fluorescence activity for both CTC concentrations reached a plateau, suggesting an equilibration of the antibiotic with the platelets. Although the rate of increase in fluorescence intensity was the same for 5 μM or 50 μM CTC, the absolute fluorescence of the platelet pellet was directly proportional to the concentration of CTC contained in the incubation medium. Thus, platelets treated with 50 μM CTC exhibited 10 x-greater fluorescence than platelets incubated with 5 μM CTC. Furthermore, the intensity of platelet fluorescence was 50-fold that observed in an equal volume of platelet-free plasma containing CTC, indicating that the antibiotic was substantially bound to platelet membrane components.

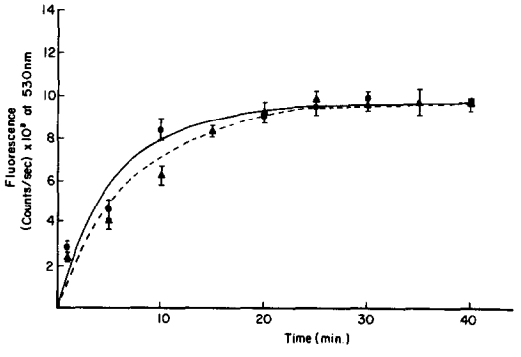

Fig. 1. Platelet fluorescence of chlortetracycline as a function of time. Human platelets in their native plasma were incubated at 25°C with 5 μM CTC (dashed line) or 50 μM CTC (solid line). The platelets were pelleted through silicone oil at 5 minute intervals and the pellet fluorescence determined in a microspectrofluorometer (400 nm excitation; 530 nm emission). The number of counts observed for the platelets incubated with 50 μM CTC has been divided by a factor of 10. The above values represent data obtained from four separate blood donors; error bars indicate standard error of the mean. At 15 minutes and 35 minutes the values for the two concentrations are superimposed.

Caswell (9) and others (12) demonstrated that significant fluorescence enhancement occurs when CTC binds to biological membranes only if divalent cations are present. Thus, the increase in platelet-CTC fluorescence observed with time must reflect binding of the drug to platelet membranes which are associated with a divalent cation, e.g., Ca^{2+} and/or Mg^{2+}.

In membrane systems, the wavelengths of peak emission for the Ca-CTC and Mg-CTC adducts have been described as 530 nm and 520 nm, respectively (9, 12, 16). We found (Fig. 2) that the emission spectrum of CTC-treated platelets coincides with that of the Ca-CTC adduct. Therefore, the platelet-CTC fluorescence must be predominantly associated with the formation of Ca-CTC. This contention is consistent with the relative

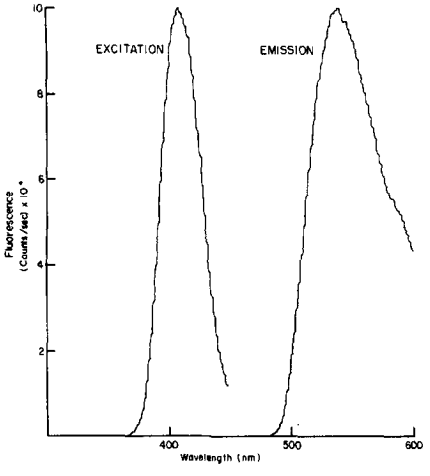

Fig. 2. Excitation and emission spectra of chlortetracycline-treated platelets. Human platelets in their native plasma were incubated at 25°C with 50 μM CTC for 40 minutes. The platelets were pelleted, and the excitation and emission spectrum of the pellet was determined in a microspectrofluorometer; for the excitation spectrum the 1/2 bandwidths were: 2.25 nm excitation and 6 nm emission; for the emission spectrum the 1/2 bandwidths were: 6 nm excitation and 2.25 nm emission. Note: Since a glass objective was employed, the spectrum below 350 nm is not accurate.

distribution of platelet Ca^{2+} and Mg^{2+} as determined by Steiner (17) who found that approximately 21% of the total platelet Ca^{2+} was lipid bound (i.e., about 2 mM).

Since the platelet Mg^{2+} concentration is 1.2 mM (18), the ratio Ca^{2+}/Mg^{2+} is approximately 1.67. In a lipid environment, Ca^{2+} has a 3-fold-greater affinity for CTC than does Mg^{2+} (16), and in membranes, Ca-CTC has a 1.5-fold-greater fluorescence at 530 nm than does Mg-CTC (12). The ratio of Ca-CTC, relative to Mg-CTC fluorescence at 530 nm in the intact platelet can therefore be estimated as: [(1.67) x (3) x (1.5)]/1, or 7.5/1. Thus, about 88% of the total fluorescence would be contributed by Ca-CTC.

Change in fluorescence intensity with platelet shape change: Shape change was induced in CTC-treated platelets with ADP or the divalent cation ionophore A 23187. Specifically, platelet-rich plasma was equilibrated with 50 μM CTC. The plasma was supplemented with EGTA (3 mM) to inhibit platelet aggregation. Subsequent to the addition of ADP or A 23187, samples of the platelet-rich plasma were taken at 60, 130 and 220 seconds, and the platelet pellets analyzed for fluorescence intensity.

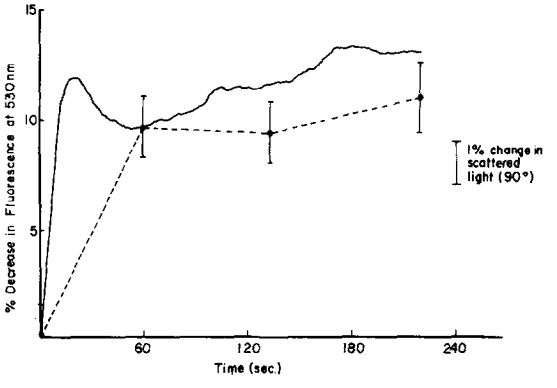

Fig. 3. Relationship between platelet shape change induced by 10^{-5}M ADP and fluorescence of platelets containing chlortetracycline. Human platelets were incubated at 25°C with 50 μM CTC for 40 minutes. The plasma was supplemented with 3 mM EGTA to prevent aggregation. Platelets from a portion of the plasma were then pelleted through silicone oil and the control pellet fluorescence determined in a microspectrofluorometer (400 nm excitation; 530 nm emission). ADP (10^{-5}M) was added to the remaining plasma and samples were pelleted and analyzed for fluorescence at 60, 130 and 220 seconds (dashed line). The fluorescence values at each time point represent the average of 16 samples from three different blood donors; error bars indicate standard error of the mean. Platelet shape change in response to 10^{-5}M ADP was separately determined by measuring the intensity of scattered light at 90°. The change in platelet shape (solid line) represents a typical platelet response in the presence of 50 μM CTC.

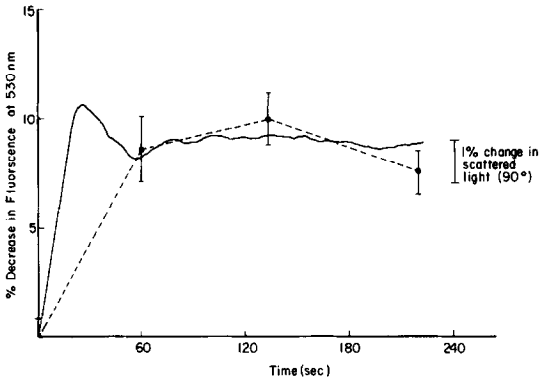

Fig. 4. Relationship between platelet shape change induced by 10^{-6}M ADP and fluorescence of platelets containing chlortetracycline. Conditions were as stated in Fig. 3 with the exception that the fluorescence values at each time point represent the average of 25 samples from three different blood donors; dashed line: fluorescence, solid line: shape change.

Figures 3 and 4 illustrate, that when platelet shape change is induced by 10^{-5}M ADP or by 10^{-6}M ADP, a significant decrease in the intensity of platelet fluorescence at 530 nm is observed. On the other hand, when shape change in response to 10^{-6}M ADP is inhibited by pretreatment with 10^{-5}M ATP, no significant decrease in fluorescence in-

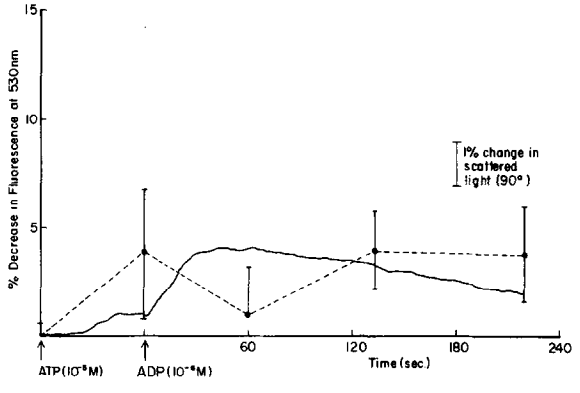

Fig. 5. Inhibition of ADP induced shape change and platelet fluorescence change by 10^{-5}M ATP. Conditions were as stated in Fig. 3 with the exception that the platelets were pretreated with 10^{-5}M ATP. The fluorescence values at each time point represent the average of 16 samples from two different blood donors; dashed line: fluorescence, solid line: shape change.

tensity occurs (Fig. 5). Furthermore, when platelet shape change is induced by 10^{-6} M A 23187, the time course for the change in platelet shape is consistent with the time course for the change in platelet CTC fluorescence (Fig. 6). In this case, however, the decrease in fluorescence relative to shape change is larger than might be expected on the basis of the ADP results. This may be due to the nonspecificity of the ionophore in causing Ca^{2+} redistribution unrelated to shape change.

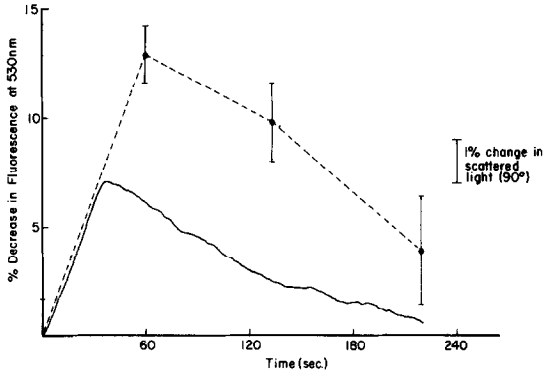

Fig. 6. Relationship between platelet shape change induced by 10^{-6}M A 23187 and fluorescence of platelets containing chlortetracycline. Conditions were as stated in Fig. 3 with the exception that the fluorescence values at each time point represent the average of 8 samples from two different blood donors; dashed line: fluorescence, solid line: shape change.

In order to establish that the changes in fluorescence intensity upon ADP or A 23187 addition were not merely associated with an alteration in light scattering properties of the platelet pellet, shape change was inhibited with 500 μM CTC (5) and the platelet fluorescence in response to 10^{-5}M ADP was measured. It was found that a 12% decrease in fluorescence intensity still occurred even when shape change was substantially blocked. Thus, the physical change in platelet shape is not responsible for the decrease in fluorescence observed upon the addition of ADP.

Since platelet fluorescence is almost exclusively due to CTC associated with mem-

brane-bound divalent cation (native platelet fluorescence constitutes 1% of the total), a change in fluorescence intensity must represent modification in the environment of the divalent cation CTC complex. This change in environment could represent a shift in polarity of the membrane itself or liberation of the membrane-bound CTC cation complex to the more polar cytosol. Horne et al. (19) have used the fluorescent probe 8-aniline-1 naphthalene sulfonate (ANS) to examine platelet membrane properties. This probe has no direct specificity for membrane-bound divalent cation, but rather alters its fluorescence efficiency as a function of membrane conformation. They reported that ANS fluorescence is not altered when platelets undergo shape change and aggregation. The present results which demonstrate a decrease in CTC-platelet fluorescence with ADP or A 23187, therefore, suggest that platelet shape change is associated with a redistribution of divalent cation from an apolar to a more polar environment.

Although the platelet fluorescence spectrum indicates that Ca-CTC is the primary component, it need not follow that the observed decrease in fluorescence intensity induced by ADP is wholly due to the redistribution of Ca^{2+}. In order to approach this question we determined the ratio of fluorescence at the wavelength of peak emission for the Ca-CTC adduct (530 nm) relative to the fluorescence at the wavelength of peak emission for the Mg-CTC adduct (520 nm) before ADP addition and at intervals thereafter. This was done on the basis that a spectrum composed of two different species, e.g., Ca and Mg-CTC adducts which exhibit different emission maxima, will have a characteristic shape. A change in fluorescence intensity of one of the species in the absence of a corresponding change in the other species will alter the ratio of fluorescence activity at peak emission for the two species. We found no substantial change in the ratio (i.e., 1.176 ± 0.004 before ADP addition and 1.175 ± 0.003, 1.171 ± 0.003 and 1.178 ± 0.004 at 60, 130 and 220 seconds after ADP). A change in ratio would not be detected if the platelet fluorescence (530 nm) were almost entirely due to Ca-CTC and the decrease in

fluorescence with ADP were also due to Ca-CTC. On the other hand, a change in ratio would not occur if Ca-CTC fluorescence and Mg-CTC fluorescence decreased in the same proportion. In either case, the fluorescence decrease induced by ADP would be predominately associated with a shift in Ca-CTC fluorescence.

Our results using CTC, therefore, demonstrate that platelet shape change is associated with a redistribution of intracellular divalent cation, i.e., presumably calcium.

Experiments are now in progress to further investigate intraplatelet divalent cation movements associated with platelet function.

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