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RELEASE OF INTRACELLULAR MEMBRANE-BOUND CALCIUM PRECEDES THE ONSET OF STIMULUS-INDUCED EXOCYTOSIS IN PLATELETS

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Summary: The time courses of the intracellular release of membrane-bound Ca^{2+} measured by chlortetracycline fluorescence, and exocytosis measured with an extracellular calcium electrode have been determined simultaneously in platelets at 21°C. Thrombin, trypsin and the thiol reagent, thimerosal produced concentration dependent release of intracellular membrane calcium which always significantly preceded the onset of secretion. Thrombin and trypsin initiated Ca^{2+} mobilization in 0.75 to 1.8 sec. whereas secretion commenced 4-8 sec. later. Ca^{2+} release was 30-50% completed before exocytosis started. This is the first direct evidence that the release of intracellular Ca^{2+} in platelets occurs with a time course consistent with its proposed role in activation-secretion coupling.

Stimulation of platelets by a variety of agents (i.e. collagen, thrombin and certain other proteases, and ionophore A23187) causes the release of the contents of dense secretory granules, notably adenine nucleotides, serotonin and Ca^{2+}, as well as some secretion of lysosomal enzymes. It has been proposed, by analogy with endocrine and neurosecretory cells, that Ca^{2+} plays an essential role in exocytosis in platelets (1-3). However, it has been difficult to prove this hypothesis because secretion evoked by proteases and A23187 can occur in the absence of extracellular Ca^{2+}. If Ca^{2+} triggers secretion in platelets, it must therefore be mobilized from intracellular sources. An increase in free cytoplasmic Ca^{2+} brought about by stimulation may also serve to activate; (a) lipases involved in the mobilization of arachidonic acid from phospholipids (4) and (b) the myosin-light chain kinase (5) necessary for actomyosin ATPase activity. The onset of protease-induced secretion of Ca^{2+} or ATP from platelets (6,7) and the O_2 consumption burst due to oxidation of arachidonic acid (8) characteristically occurs after a lag period following addition of the

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stimulating agent. The duration of the lag period is inversely related to the concentration of the stimulant protease (i.e., thrombin, trypsin or papain) but it reaches a lower limit of about 4-5 sec. at 22°C (6). It is likely that during this lag period the requisite processes required for exocytosis must be taking place. If Ca^{2+} is actually a second messenger, or "coupling factor", linking stimulation to response, bound or sequestered Ca^{2+} must be released into the cytoplasm prior to the initiation of platelet responses. To date it has not been possible for various reasons to apply either photoproteins (i.e., aequorin) or Ca^{2+}-sensitive dyes intracellularly to successfully measure free cytoplasmic Ca^{2+} levels in platelets. Another approach to the problem is to employ the fluorescent probe chlortetracycline (CTC) to measure membrane-bound Ca^{2+}, as has been successfully accomplished in mitochondria (9), sarcoplasmic reticulum (10), pancreatic exocrine cells (11), and leukocytes (12). In the pioneering experiments of Le Breton et al. (13) CTC fluorescence was measured in platelets after their rapid centrifugation through silicone oil. A fall in fluorescence, indicating redistribution of Ca^{2+}, was associated with platelet shape change in response to ADP and A23187. However, the earliest time point measured was 60 sec. after stimulation, a time at which many of the platelet responses (i.e., secretion, shape change, O_2 burst) are largely already completed.

In this paper it is shown, through the use of simultaneous recording of CTC fluorescence and Ca^{2+} secretion, that a rapid release of intracellular Ca^{2+} does in fact occur in platelets during the lag period preceding secretion and arachidonic acid oxidation.

METHODS: CTC fluorescence in platelets was measured in a Perkin Elmer MPF2 fluorometer whose sample compartment was modified to contain a cuvette in which a Ca^{2+} electrode (Radiometer F2112Ca) and a Ag/AgCl reference electrode were placed. The electrodes were connected to a Radiometer 25 pH meter. Rapid mixing in the cuvette was provided by a powerful magnetic stirrer. The electrode response was calibrated by additions of known amounts of standard Ca^{2+} solutions to platelet suspensions. The stability, rapid response and calibration of this Ca^{2+} electrode has been repeatedly

verified (7,14). The overall response time of the instrument for both Ca^{2+} activity measurements and chlortetracycline fluorescence was such that for both signals at least 90% of maximal response was attained within 1.0 second (fig. 1A). The fluorescence method can detect changes in binding of Ca^{2+} to membranes when the free Ca^{2+} in the medium is in the micromolar range (10,15,22). Human platelet concentrates provided by the Connecticut Red Cross Blood Center were sedimented by centrifugation and resuspended in their own plasma to a concentration of 20-30 mg platelet protein per ml and incubated with 10-50μM chlortetracycline for about 30 min. at 21-22°C. Aliquots were centrifuged for 15 sec in an Eppendorf microcentrifuge to remove plasma by aspiration, and the platelets were resuspended in a CTC-free buffered saline solution to a concentration of about 120-380μg platelet protein per ml. The medium initially contained 50-100μM Ca^{2+} to ensure rapid response of the Ca^{2+} electrode (7,14).

RESULTS AND DISCUSSION: Two different types of platelet stimulating agents were employed: (a) proteases, e.g., trypsin, thrombin and papain and (b) certain thiol reagents; i.e., ethylmercurythiosalicylate (thimerosal) and N-ethylmaleimide (NEM). The addition of either proteases or the thiol reagents produced a concentration dependent fall in platelet chlortetracycline fluorescence, indicating that the release of membrane-bound divalent cation had occurred (figs. 1,2). LeBreton et al. (13) estimated that about 88% of CTC fluorescence in unstimulated platelets was due to Ca-CTC. Stimulation by proteases shifted the peak of the excitation spectrum from 395 nm to 382 nm whereas thiol reagents induced a greater shift from 395 nm to 375 nm. These spectral shifts indicate that the fall in fluorescence is due to a release of membrane Ca^{2+} rather than Mg^{2+} (11,13,15). Furthermore in both cases a blue-shift of about 5 nm occurred in the peak of the emission spectrum which is characteristic for a loss of the Ca-CTC chelate (12,15). The addition of EGTA or EDTA to the medium did not affect the initial fluorescence readings nor the fall in fluorescence due to stimulation, and thus the fluorescence changes must be attributed to effects on intracellular calcium. The time course of the fluorescence change produced by proteases was more rapid than that induced by the thiol reagents, but the magnitude of the decrease was about 4 to 5-fold greater in the case of the thiol reagents. NEM blocks platelet aggregation and secretion by unknown mechanisms, yet it causes release of membrane Ca^{2+} (fig. 1B) (CTC fluorescence) and stimulates release of arachidonic acid from phospholipids

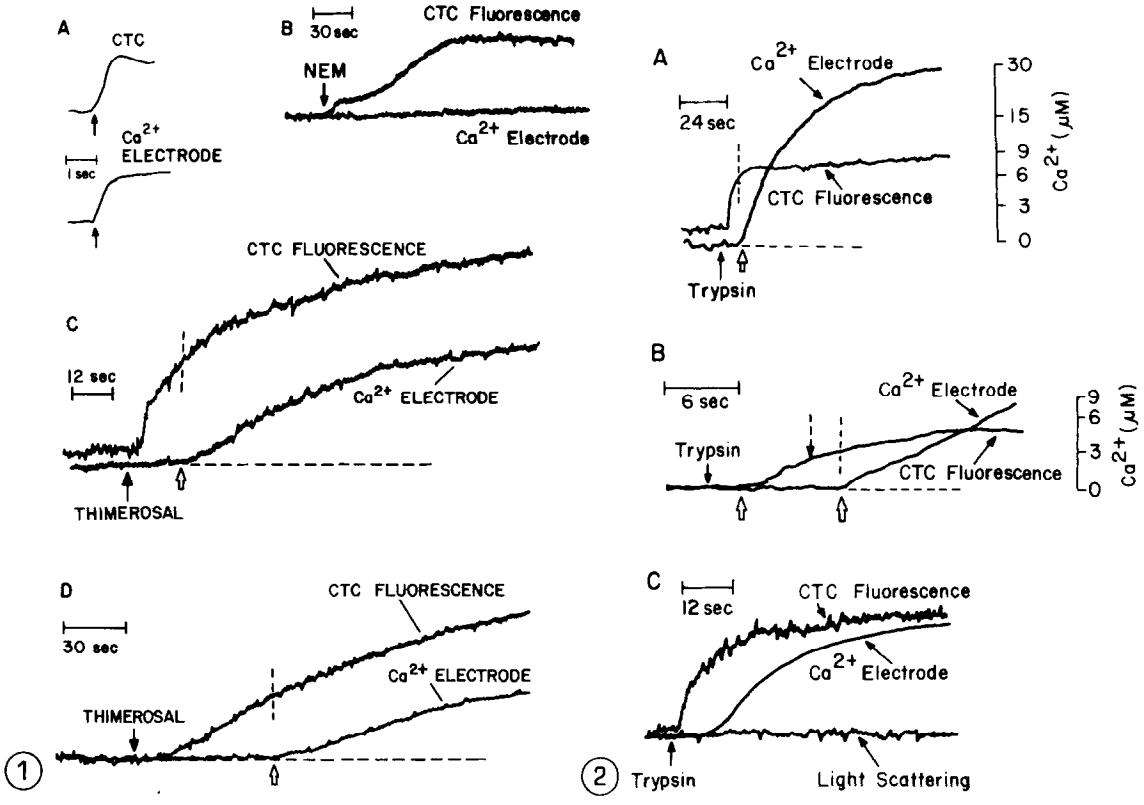

Figure 1. (A) Response time of fluorometer and calcium electrode to $CaCl_2$ (6μM) added into cuvette containing 60μM CTC in buffer solution. (B) Addition of NEM (1.0mM) to platelet suspension preincubated with 50μM CTC. (C) Addition of thimerosal (0.90mM) and (D) 0.38 mM to platelets preincubated with 50μM CTC. Open arrows mark onset of secretion (Ca^{2+} electrode). Platelets (0.2mg protein/ml) suspended in solution containing 137mM NaCl, 5.4mM KCl, 0.2% (w/v) dextrose, 25mM Tris-HCl, adjusted to pH 7.5. Fluorescence excitation at 390nm, emission measured at 530nm with 430nm cut off filter. A decrease in fluorescence is recorded as an upward signal for ease of comparison with Ca^{2+} electrode signal.

Figure 2. Trypsin induced changes in CTC fluorescence and Ca^{2+} secretion. Platelet protein concentration 0.38mg/ml.
(A) Initial responses occur 1.5 sec. (CTC) and 8 sec. (Ca^{2+} secretion) respectively after trypsin (4μM) addition. CTC signal attains 90% of maximum before 3μM Ca^{2+} has been secreted. Note the logarithmic relationship between electrode response and Ca^{2+} concentration.
(B) Responses recorded at faster chart speed. Open arrows: onsets of CTC fluorescence signal and Ca^{2+} secretion. Dashed arrow marks 50% of maximum fluorescence response.
(C) Effect of trypsin on CTC fluorescence and light scattering. Platelets were incubated with 50μM CTC or in absence of CTC and then stimulated with trypsin (4μM). Excitation at 390nm. Light emission measured at 530nm with 430nm cut off filter in front of phototube. Cells without CTC show no changes in emission due to light scattering or absorbance after exposure to trypsin. Ca^{2+} secretion was the same in both cases.

and its subsequent oxidative metabolism to prostaglandins (16). Thimerosal also stimulates the arachidonate cascade but unlike NEM, this mercurial induces secretion. Intracellular release of membrane-bound Ca^{2+} measured by CTC fluorescence preceded the onset of secretion due to addition of thimerosal (fig. 1C,D). The lag time between the CTC fluorescence signal and onset of secretion decreased, as a function of thimerosal concentration, to a minimum of 10-12 sec. The release of intracellular Ca^{2+} produced by the thiol reagents also commenced prior to the production of malonyldialdehyde (a metabolite of arachidonic acid) and the O_2 consumption burst (data not shown) and was not blocked by eicosatetraynoic acid, an inhibitor of both pathways of arachidonate metabolism. The initial Ca^{2+} release measured by CTC therefore was probably not due to products of arachidonic acid metabolism. Since the enzymes responsible for the mobilization of arachidonate require Ca^{2+} (4), the release of intracellular Ca^{2+} by thiol reagents detected by CTC fluorescence may be responsible for initiating their effects on arachidonate metabolism. NEM and mercurials are known to induce release of Ca^{2+} from liver mitochondria (17). Yet, other agents which also produce release of mitochondrial Ca^{2+} detectable with CTC, such as antimycin A (14) and p-trifluoromethoxy(carbonylcyanide)phenylhydrazone (FCCP) (10), had little or no effect on CTC fluorescence of platelet suspensions, nor did they induce production of malonyldialdehyde or the O_2 burst due to the oxidation of endogenous arachidonate. Thus, the platelet mitochondria are probably not an important source of the releasable intracellular Ca^{2+}. NEM and thimerosal may possibly release Ca^{2+} from the dense tubules since they are both potent inhibitors of Ca^{2+}-transport ATPase, and suppress the cytochemical reaction for Ca^{2+}-ATPase activity in the platelet dense tubules (18).

Protease-induced secretion occurred with a minimum lag period, measured by the Ca^{2+} electrode, of about 6 seconds for 4μM trypsin and 4.8 seconds for 10 U/ml thrombin at 21^{o}C. The subsequent time course of Ca^{2+}

secretion was essentially the same as that previously described using murexide dye (19,20) or Ca^{2+} electrode (7) as detector. These protease concentrations, although supramaximal for inducing secretion were chosen to obtain the shortest lag period (6). During the lag period trypsin or thrombin induced a striking decrease in CTC fluorescence (fig. 2, 3), whose time course was quite distinct from that of the secretory response. In 25 experiments the decrease in fluorescence commenced abruptly within 0.75 to 1.8 seconds after the addition of trypsin or thrombin, usually 4-8 seconds before the first sign of Ca^{2+} secretion. The fluorescence signal usually reached 30-50% of its maximum change prior to the onset of secretion, and was typically at least 90% completed at 6-12 sec. after addition of the protease, a time when secretion of Ca^{2+} had only risen to about 30% or less of its maximum value (fig. 2). As the concentrations of secretogogues (proteases) was increased, the fall of CTC fluorescence commenced earlier and both the rate and magnitude of the fluorescence change increased to a maximum. Concurrently the onset of secretion occurred with a shorter lag period and the initial rate and final magnitude of Ca^{2+} secretion also increased up to

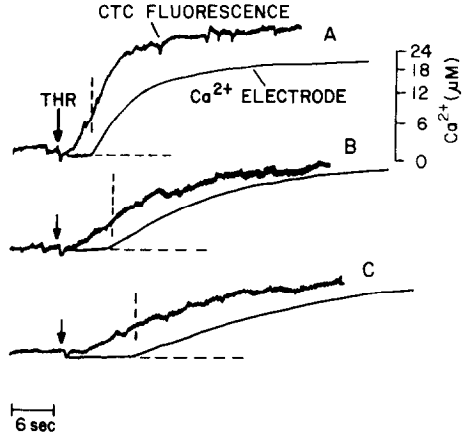

Figure 3. Thrombin-induced changes in CTC fluorescence and Ca^{2+} secretion. Platelet protein concentration 0.25mg/ml. Thrombin concentrations: (a) 5 U/ml, (b) 0.5 U/ml, (c) 0.25 U/ml. Lag times for onset of CTC signal are 1.8 (a) 1.8 (b) and 3.2 (c) secs; for secretion 4 (a), 7.2 (b) and 10.8 (c) secs. (solid arrows).

a maximum (fig. 3). The fluorescence signal observed during the lag period was not due to light scattering or absorbance artifacts (fig. 2C).

The fluorescence signal represents a response distinct from, and not dependent on, the secretory response not only because the time courses of each are so different, but also because the fluorescence signals occur unimpaired even when secretion is completely blocked (as with NEM) or strongly inhibited by (a) preincubation of platelets with high concentrations of CTC (150-200 μM) which probably chelate cytoplasmic Ca^{2+} (21), or (b) by low concentrations of trifluoroperazine (experiments to be published elsewhere). However, a causal relationship between the intracellular release of membrane Ca^{2+} and secretion is suggested, since in 35 experiments secretion never occurred without detection of a CTC-fluorescence signal which preceded it in onset. Furthermore, whenever the CTC fluorescence signal was depressed (e.g. by chlorpromazine), secretion was also always inhibited.

These experiments demonstrate for the first time that intracellular Ca^{2+} release from platelet membranes occurs almost immediately after stimulation, and precedes the onset of exocytosis. The intracellular Ca^{2+} which is released may initiate reactions which lead to exocytosis, phosphorylation of myosin and the activation of lipases involved in the mobilization of arachidonic acid. The sites from which Ca^{2+} is mobilized, and the mechanisms responsible for its release by stimuli remain to be elucidated.

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