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The internal calcium concentration of human platelets increases during chilling

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Abstract

Human platelets must be stored at 22°C in blood banks, because of the well-known phenomenon of cold-induced activation. When human platelets are chilled below room temperature, they undergo shape change and vesicle secretion that resembles physiological agonist-mediated activation. The trigger for the cascade of events leading to platelet activation at hypothermic temperatures is not known, although an increase in the internal calcium concentration ($[Ca]_i$) due to passage of the platelet membranes through their thermotropic phase transition has been proposed. We report here that the fluorescent calcium-sensitive probe, Indo-1, has been used to estimate the internal calcium concentration of human platelets during a reduction in temperature from 20°C to 5°C at a rate of 0.5°C/min. An increase on the order of 100 nM was recorded. Almost all of the increase in $[Ca^{2+}]_i$ occurs during the chilling process, as incubation of platelets for 1 h at low temperature did not lead to a continued calcium concentration increase. The increase in $[Ca^{2+}]_i$ during chilling is likely to be due to more than a single mechanism, but might include some release of the calcium stores from the dense tubule system. Loading platelets with the calcium chelator BAPTA (1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid) dramatically reduced the increase in $[Ca^{2+}]_i$ seen during chilling. Antifreeze glycoproteins (AFGPs) isolated from the blood serum of Antarctic fishes, which are known to protect platelets from cold-induced activation, did not eliminate the rise in $[Ca^{2+}]_i$ during chilling, suggesting that signaling mechanisms are likely to be involved in cold-induced activation.

Keywords: Platelet; Calcium; Membrane phase transition; Cold-induced activation; Indo-1

1. Introduction

The phenomenon of cold-induced activation of human platelets has greatly frustrated effective storage of these cell fragments. During hypothermic storage, platelets undergo shape change from discoid to spherical with multiple filopodia [1,2], and secretion of α-granule contents [3], in a process that mimics physiological platelet activation [4,5]. Such platelets are compromised in hemostatic capability as evaluated by platelet life-span and bleeding time analysis [6,7]. Because they must therefore be stored at room temperature (22°C), their storage in blood banks is limited by regulation to 5 days, due primarily to the high level of bacterial contamination associated with storage at such high temperature (for review, see [8]). Even with this restriction in shelf-life, however, septic shock due to transfusion of contaminated platelet

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fractions continues to represent a serious threat [8,9]. Understanding the process of cold-induced activation is clearly significant, therefore, and may lead to more favorable platelet storage conditions.

The mechanism causing cold-induced activation is not known, although it has recently been suggested to accompany passage of platelets through a membrane phase transition [10]. It has been proposed [10,11] that a possible trigger for cold-induced activation might involve an increase in the intracellular calcium concentration ($[Ca^{2+}]_i$), which could then initiate the platelet activation cascade, as has been shown with the calcium ionophore A23187 [12–14]. This would be consistent with the results of Winokur and Hartwig [11] who found that actin fragmentation and subsequent nucleation, key steps in the platelet shape-change process, occurred when platelets were chilled from 37°C to 4°C, but that they required a rise in $[Ca^{2+}]_i$ as they were blocked by the calcium chelators Fura-2 and Quin-2.

It is not a simple matter to measure the $[Ca^{2+}]_i$ of human platelets during chilling, however. Platelets are extremely small cell fragments, with diameters in the range of 1.0 μm [11], making impalement by a microelectrode unpractical. Further, the various spectroscopic methods used to measure the calcium concentration within cells all depend on the equilibrium balance of some reaction involving free calcium ions [15,16], and therefore all are affected by temperature. The fluorescent calcium-sensitive probes used most extensively to determine the $[Ca^{2+}]_i$ of various cell types are no exception.

A detailed report of the effect of temperature on the calcium-sensitive dyes is beyond the scope of the current article, and will be presented separately. Briefly, there are many aspects of the physical environment that can affect the fluorescence intensity of a given fluorophore, including but not limited to temperature, pH, viscosity, and level of oxygen quenching [17]. These variables can affect such properties as excited state lifetimes and rates of non-radiative decay, among others. In addition, if the fluorophore is a calcium chelator, as is the case for the calcium sensitive dyes, there are other considerations as well. The binding properties must be taken into account, because the dissociation constant is also affected by temperature, pH, and ionic strength [15,18,19]. Clearly, this is an extremely complicated issue. One approach to this problem would be to separate, quantify, and subtract the contribution of each of these effects to the report of calcium by Indo-1, the complexity of which would be virtually prohibitive. An alternative approach would be to demonstrate that the effects of temperature on the fluorophore itself cannot be responsible for any experimentally obtained result. This method, although not quantitative, can still give important information regarding trends, and we have used this approach to estimate the internal calcium concentration of human platelets during chilling.

The mechanism of cold-induced activation may be more complex than a simple increase in the internal calcium concentration, however. The possibility that calcium is the trigger and only requirement for cold-induced activation, can be tested using antifreeze glycoproteins (AFGPs) as a tool, because they have been shown, by morphological assessment and fluorescence activated cell scanning analysis, to inhibit activation during storage of platelets for up to three weeks at 5°C [10]. These glycoproteins, isolated from the blood serum of certain Antarctic fishes, consist of the repeating triplet alanine–alanine–threonine, with each threonine glycosylated by a galactose-*N*-acetylgalactosamine moiety [20]. In vivo, AFGPs lower the freezing temperature of the serum in a non-colligative manner [20–22], but they have also been shown in vitro to protect membranes and whole cells during chilling at temperatures well above freezing [10,23,24] (and for review, see [25,26]). For instance, AFGPs prevent lipid vesicles composed of dielaidoylphosphatidylcholine (DEPC) from leaking during chilling through their membrane phase transition (10°C) [24]. Because AFGPs effectively protect platelets from cold-induced activation [10], they emerge as a means by which to discriminate between different steps in the activation cascade. Evidence will be presented that although AFGPs are known to prevent cold-induced activation in platelets, they do not inhibit the rise in the internal calcium concentration. This suggests that the rise in calcium is probably not solely responsible for cold-induced activation and raises the possibility that signaling processes may also be involved.

2. Materials and methods

2.1. Isolation and washing of human platelets

Blood was drawn from healthy human volunteers into acid citrate dextrose buffer (ACD) (56.7 mM citric acid, 112 mM sodium citrate, 170 mM dextrose) using a large bore needle (#19) and without vacuum assistance to ensure a low shear force during the draw. Approximately 30 ml blood was drawn into 3 ml ACD, and prostaglandin E-1 (PGE-1) was added to this mixture (from a 1 mg/ml stock solution in 100% ethanol) to a final concentration of 10 μg/ml. Platelet rich plasma (PRP) was produced by centrifuging the whole blood in a pre-warmed Beckman GS-15R centrifuge using an S41-80 variable bucket rotor at $325\times g$ for 14 min with an acceleration setting of 1. At all times, platelets were maintained between 30°C and 37°C and handled only with polypropylene or siliconized glass (coated with Sigmacote, Sigma Chemical Co., St. Louis, MO) pipettes and polypropylene test tubes. Unless otherwise indicated, buffers, salts, and column chromatography materials were purchased from Sigma.

Washed platelets were produced by gel filtering the PRP (5–7 ml) over a Sepharose 2B column (7×2 cm) [27] rinsed thoroughly with buffer A (100 mM NaCl, 10 mM KCl, 10 mM ethylene glycol-bis(β-aminoethyl ether)*N,N,N′,N′*-tetraacetic acid (EGTA), and 10 mM imidazole; pH 6.8) (imidazole and EGTA from Research Organics, Cleveland, OH). Washed platelets (7–9 ml) were then collected in buffer A and again supplemented with PGE-1 to a final concentration of 10 μg/ml. The washed platelets were then counted on a T-890 Coulter cell counter (Miami, FL) and allowed to 'rest' at 37°C for 15–30 min before loading.

2.2. Loading platelets with Indo-1

The fluorescent calcium-sensitive probe Indo-1-acetoxymethyl ester (Indo-1-AM) (Molecular Probes, Eugene, OR) was purchased in 50-μg aliquots, and each aliquot was used for a single experiment. Dry dimethyl sulfoxide (DMSO) was purchased in sealed ampoules (Sigma), and each ampoule was also used for a single experiment. A 3 mM stock solution of Indo-1-AM was prepared in the dry DMSO. Indo-1-AM stock solution (~5 μl) was mixed well with 0.5 ml of warm buffer A with 10 μg/ml PGE-1, and this mixture was again warmed to 37°C for 10 min before combining with the washed platelets. The final loading solution consisted of 5–6 ml of approximately 2×10^8 platelets/ml with 3 μM Indo-1-AM and 10 μg/ml PGE-1 in buffer A. This solution was mixed by gentle pipetting and allowed to incubate at 37°C for 45 min. The AM form of the dye is membrane permeable, but once inside the AM-ester is cleaved by non-specific esterases, resulting in the calcium-sensitive form of the dye [19].

The Indo-1-loaded platelets were washed over a Sepharose 2B column rinsed thoroughly with phosphate-buffered saline (PBS) (9.4 mM Na_2HPO_4, 0.6 KH_2PO_4, 100 mM NaCl) supplemented with calcium and magnesium (10 μM $CaCl_2$, 10 μM $MgCl_2$). The loaded, washed platelets were again counted on a Coulter cell counter showing a concentration of approximately 1×10^8 platelets/ml. Calcium was then added gradually to a final external calcium concentration of 1 mM, in five aliquots of Tyrodes wash (148 mM NaCl, 2.5 mM KCl, 23 μM $MgCl_2$, 5 mM glucose with 10 mM $CaCl_2$) at 5-min intervals, in order to avoid shocking the platelets. The only exception to this procedure was the experiment done in the absence of extracellular calcium. In this case, the platelets were washed over a column using PBS without added calcium and magnesium.

In the experiments involving the calcium chelator 1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid (BAPTA), a trace of the non-toxic detergent pluronic was placed in a clean 1.5 ml microfuge tube to which the Indo-1-AM (6 μl of 3 mM) and BAPTA-AM (3 μl of 20 mM) stock solutions in dry DMSO were added. This sample was centrifuged at 10 000 rpm on a Microfuge-11 (Beckman, Palo Alto, CA) for 2 min to ensure the pluronic and DMSO were well mixed and subsequently warmed to 37°C for 10 min. A 0.5-ml aliquot of buffer A with 10 μg/ml PGE-1 was also warmed to 37°C for 10 min and subsequently mixed well with the DMSO stock solution mixture. Washed platelets (5.5 ml) were combined with the DMSO/buffer solution giving final concentrations of 3 μM Indo-1-AM and 10 μM BAPTA-AM with approximately 2×10^8 platelets/ml in buffer A with 10 μg/ml PGE-1. The

solution was mixed gently by pipetting, and allowed to incubate at 37°C for 45 min. Removing the excess Indo-1-AM and BAPTA-AM was accomplished by column chromatography as described above.

2.3. Measurement of internal calcium concentration of human platelets

The internal calcium concentration of the loaded platelets was quantified using a Hitachi F-2000 fluorometer controlled with a PC using the F-2000 IC cation-measurement software (Hitachi). Unless noted, platelets were measured at the final concentration of 1×10^8 platelets/ml in PBS with 1 mM $CaCl_2$. Samples of 770 μl were loaded into 1.0 ml methacrylate UV/vis cuvettes (Fisher Scientific, Pittsburgh, PA) and stirred using a small magnetic stirbar and built-in stir-plate. Samples were excited at 350 nm, and emission measured at 405 and 480 nm. The internal calcium concentration during a particular experiment was calculated after the addition of the detergent digitonin (5 mM in ethanol to a final concentration of 0.25 mM; Aldrich Chemical Co., Milwaukee, WI) to give a maximal calcium value and the calcium chelator EGTA (250 mM in 2.5 M Tris base (tris[hydroxy-methyl]aminomethane) to a final concentration of 12 mM) to give a minimal calcium value, using the K_d of 250 nM [19] except where otherwise noted. The equation used to determine $[Ca^{2+}]_i$ was as follows:

$$C = K_d \times \frac{R-R_{min}}{R_{max}-R} \times \frac{F_{min}(\lambda 2)}{F_{max}(\lambda 2)}$$

At the beginning and end of each set of experiments, platelets were tested for their responsiveness to the agonist thrombin. After a baseline was collected, thrombin was added (to a final concentration of 1 U/ml) before the digitonin and EGTA additions. Thrombin, digitonin, and EGTA were added by injection with a Hamilton syringe directly to the cuvette through the injection port.

2.4. Chilling human platelets

Two circulating water baths were connected by insulated tubing to the jacketed cuvette holder and separated from each other by valves. The heated water bath was held at 42°C, and the chilled water bath was held at −5°C. After a baseline was collected at a sample temperature of 37°C (∼100 s), the heated bath valve was closed and the chilled bath valve was opened. The samples were cooled at 2°C/min from 37°C to 20°C, then at 0.5°C/min from 20°C to 5°C. When sample temperature reached 5°C, the process was reversed, and samples were heated to 37°C at approximately 2°C/min. Platelet samples were then treated sequentially with thrombin, digitonin, and EGTA, and calcium concentration was calculated as stated above.

2.5. Antifreeze glycoproteins from antarctic fishes

Antifreeze glycoproteins (AFGPs) isolated from the blood serum of *Trematomus bernachii* and *T. borchgrevinki* as previously described [21] were a generous gift of Dr. Robert E. Feeney. Pooled fractions 7 and 8 (*T. bernachii*) and fractions 3, 5′, 6 (*T. borchgrevinki*) with molecular mass in the range of 2.6–33.7 kDa were used at 1 mg/ml in chilling experiments with Indo-loaded human platelets at a concentration of 5×10^7 platelets/ml.

3. Results and discussion

3.1. Indo-1-loaded platelets are thrombin-responsive

It was important, as a first control, to show that the Indo-1-loaded platelets were healthy and physiologically active. Fig. 1 shows the response of the platelet internal calcium concentration ($[Ca^{2+}]_i$) to the physiological agonist thrombin. The top panel (Fig. 1A) displays the raw fluorescence data in arbitrary fluorescence units (AFU), while the bottom panel (Fig. 1B) shows the $[Ca^{2+}]_i$, calculated as described in Section 2. It is clear that the platelets exhibited the well-known increase in $[Ca^{2+}]_i$ that is consistent with normal platelet function [28–30] (for review, see [31,32]).

Also shown in Fig. 1 are the maximal and minimal calcium concentrations achieved by the additions of digitonin and EGTA, respectively. It is not unusual that the calcium concentration value seen after the addition of digitonin does not match exactly the known external calcium concentration (1 mM), because this concentration is so great in comparison to

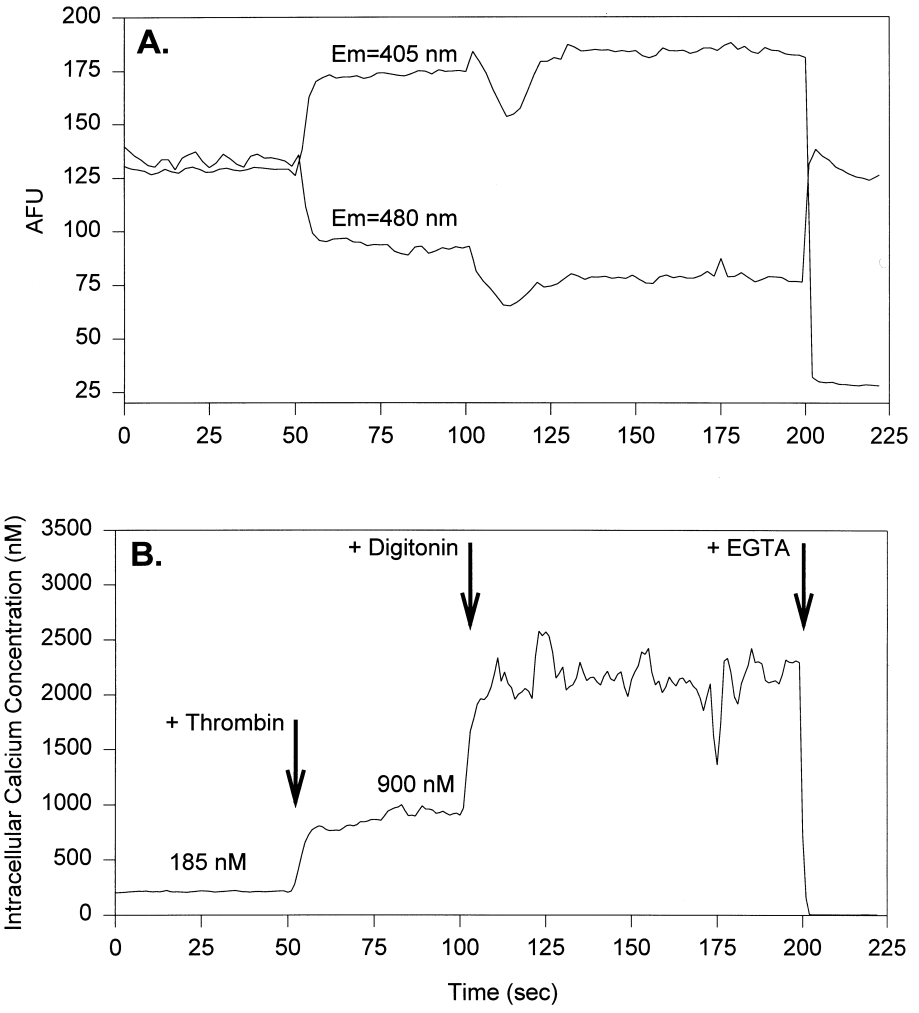

Fig. 1. Response of Indo-1-loaded platelets to 1 U/ml thrombin. (A) Fluorescence intensity traces for both emission wavelengths in arbitrary fluorescence units (AFU). (B) Platelet intracellular calcium concentration. Thrombin, digitonin, and EGTA additions are indicated by arrows. Tests for thrombin-responsiveness were performed at 35°C.

the K_d of Indo-1 (250 nM) that the calculation will not be accurate in this range. The digitonin and EGTA are added only to give maximal and minimal values for the two Indo-1 emission wavelengths, which are then used for calculation of the internal calcium concentration.

As mentioned in Section 1, most fluorescent probes are temperature-sensitive. Thus, another important control was to determine how temperature affected the report of calcium by Indo-1. Fig. 2 shows the results of such controls. The first type of condition studied was a saturating calcium concentration. Either platelets were chilled in the presence of digitonin (Indo-1 is exposed to 1 mM Ca^{2+}), or the pentapotassium salt of Indo-1 (1 μM in PBS) was chilled in the presence of 1 mM Ca^{2+}. Although the calculated values for the calcium concentration from measurements in the presence of 1 mM Ca^{2+} are not accurate, it is still important to observe the effect of temperature on the reported calcium value. As the dye will be continuously saturated at such a high calcium concentration, the effects of temperature on K_d will be negligible, and the effect of temperature on fluorescence intensity will predominate. In both cases, although there was a significant effect of temperature between 38°C and 20°C, there was very little change in the reported calcium value between 20°C and 5°C. The small change that was apparent between 20°C and 12°C actually showed a decrease in the reported calcium value during chilling. This ef-

fect, as it is a change in the opposite direction from what is observed in human platelets, could lead to an underestimation in the elevation of $[Ca^{2+}]_i$ during chilling.

The second type of condition studied was a low, more physiologically relevant calcium concentration, in the range of 0–500 nM. The simplest possible system was constructed in order to focus specifically on the effects of temperature on Indo-1, thus Indo-1 itself served as the only calcium buffer. Using 10 mM TES to buffer the pH to 7.2, 0.1 M KCl, and 10 μM Indo salt, free calcium was adjusted to ~300 or ~500 nM by the addition of $CaCl_2$. The approximate K_d of Indo-1 under these conditions was estimated using the program MaxChelator [33] to be 165 nM at 37°C. Under these circumstances, the effect of temperature on K_d of the dye becomes apparent. As temperature decreases, the dye reports a small but consistent drop in free calcium between 38°C and 5°C, if the Indo-1 K_d is not adjusted for temperature (see Fig. 2). This trend is important, because it would be extremely difficult to know the exact conditions of pH, ionic strength, and viscosity inside the platelets at each temperature in the series, all of which affect indicator K_d [15,18,19,34]. It would, therefore, be next to impossible to have an accurate K_d for Indo-1 in the internal environment of the platelets at each temperature during chilling and warming. This is an admittedly imperfect system, and an exact measurement of the free calcium concentration inside human platelets during chilling is probably an unreasonable goal. Nevertheless, cold-induced activation of platelets is such a critical issue in the blood banking community, that evidence for its mechanism, even if only an approximation and not a quantitative measurement, would still be important information. Since the effect of decreased temperature on Indo-1 is a *decrease* in the reported free calcium, any *increase* in free calcium seen in the platelets during chilling would be a valid increase and also an underestimate.

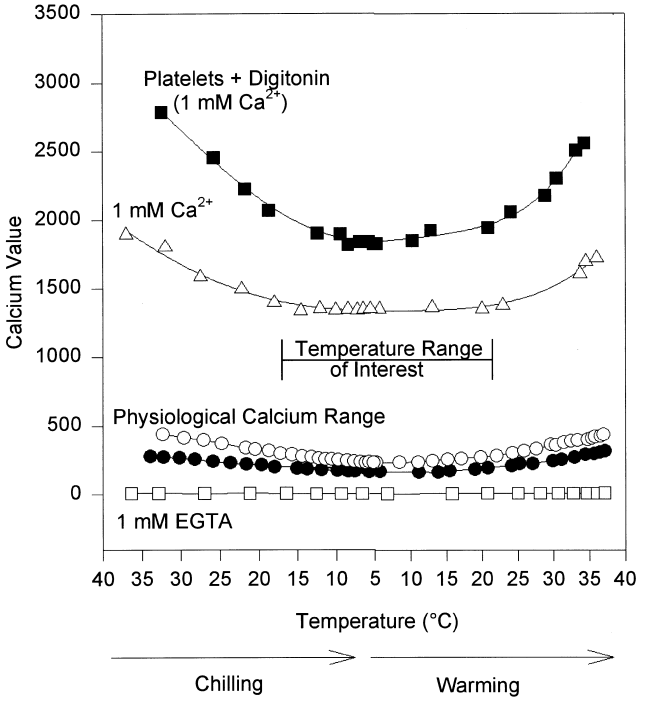

Fig. 2. Effect of temperature on Indo-1 fluorescence under conditions of different calcium concentrations. For a saturating calcium concentration, platelets were either disrupted with digitonin, exposing the Indo-1 to 1 mM $CaCl_2$ (■), or the pentapotassium salt of Indo-1 (1 μM in PBS) was studied in the presence of 1 mM $CaCl_2$ (△). For non-saturating conditions, a simplified system using Indo-1 as the only calcium buffer was used (10 μM Indo-1 salt, 0.1 M KCl, pH 7.2). The free Ca^{2+} concentration was adjusted to ~300 (●) or ~500 (○) nM by the addition of $CaCl_2$ as described in the text, or free Ca^{2+} was eliminated (1 mM EGTA) (□).

A third condition was also examined. EGTA (1 mM) was included in the absence of added calcium. Indo-1 showed the same trend in this case, but exhibited a smaller change in the reported calcium value.

One additional set of controls was conducted to ascertain if Indo-1 leaked out of the platelets during the course of these experiments. Since there was 1 mM $CaCl_2$ in the extracellular buffer, if Indo-1 leaked out of the cells, it would report an apparent increase in $[Ca^{2+}]_i$, even if $[Ca^{2+}]_i$ had not changed. Platelets maintained at constant temperature (25°C) showed no change in the $[Ca^{2+}]_i$ during a 50-min incubation (data not shown).

3.2. Intracellular calcium concentration increases in human platelets during chilling

Chilling human platelets from 20°C to 5°C resulted in an increase in $[Ca^{2+}]_i$, as shown by a representative experiment (Fig. 3). The raw fluorescence data appear in Fig. 3A, and the calculated $[Ca^{2+}]_i$ appears in Fig. 3B. It is clear that the $[Ca^{2+}]_i$ in-

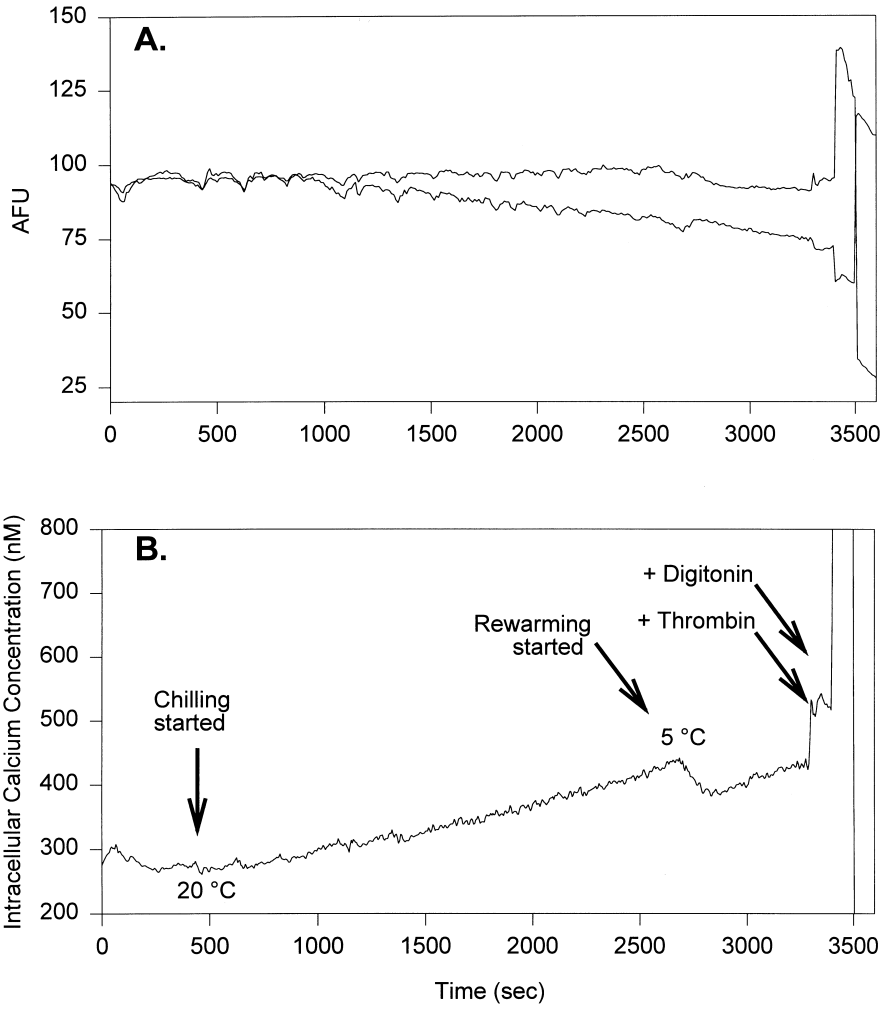

Fig. 3. Temperature dependence of intracellular calcium concentration during chilling of human platelets from 20°C to 5°C at a rate of 0.5°C/min. (A) Fluorescence intensity traces for both emission wavelengths in AFU. (B) Platelet intracellular calcium concentration. The initiation of chilling below 20°C and rewarming, as well as thrombin, digitonin, and EGTA additions are indicated by arrows.

creased by more than 100 nM during chilling. Again, the magnitude of this increase is underestimated and varied somewhat, depending presumably on differences in donor platelet physiology as well as other factors.

The $[Ca^{2+}]_i$ then decreased during rewarming to 37°C, which we suggest may be due to a variety of factors. We will return to a discussion of the possible mechanisms of the calcium changes below. However, if this phenomenon were due to a simple effect of temperature on the dye, one would expect the opposite pattern. That is, the Ca^{2+} value would be expected to *decrease* during chilling and *increase* during rewarming as can be seen with the Indo-1 salt in solution (cf. Fig. 2).

3.3. Increase in $[Ca^{2+}]_i$ occurs primarily during chilling

In order to determine if the $[Ca^{2+}]_i$ continues to increase if platelets are stored for short periods at low temperature, Indo-loaded platelet samples were chilled as usual to 5°C, followed by a 1-h incubation at this temperature. The results indicate that most of the elevation in the $[Ca^{2+}]_i$ occurred during the chilling process, as the $[Ca^{2+}]_i$ changed little during the hypothermic incubation (Fig. 4). The platelets remained viable after the 1-h period at 5°C, however, as the $[Ca^{2+}]_i$ decreased as usual during rewarming, and the platelets were still thrombin-responsive (Fig. 4). The possibility remains, of course, that the $[Ca^{2+}]_i$

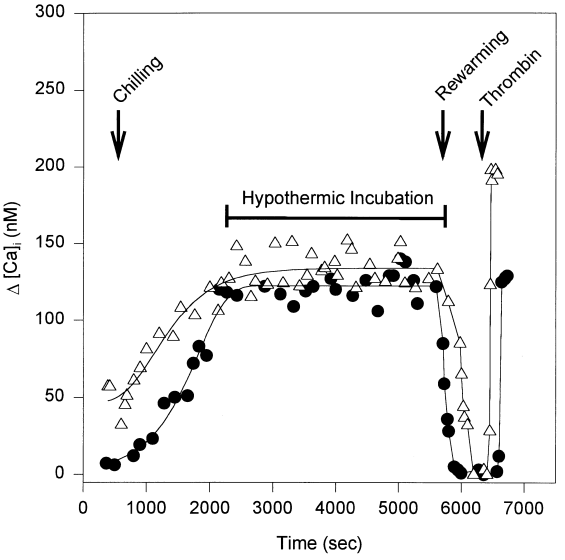

Fig. 4. The change in intracellular calcium concentration of human platelets chilled from 37°C to 20°C at a rate of 2°C/min, then to 5°C at 0.5°C/min. The platelets were then incubated for 1 h at 5°C and subsequently rewarmed to 37°C at 2°C/min. Time points corresponding to the initiation of chilling and rewarming, as well as the addition of thrombin, are indicated by arrows. Two independent data sets are shown.

might continue to increase during long-term hypothermic storage. This scenario would be problematic to test, however, as the fluorescent dye leaks out over time and can be somewhat toxic to the cells [35], making the differentiation between the long-term effect of low temperature and the long-term effect of Indo-1 extremely difficult.

3.4. Calcium increases in human platelets chilled under various conditions

In order to show the progression of the $[Ca^{2+}]_i$ increase during chilling, the change in $[Ca^{2+}]_i$ was plotted as a function of temperature for human platelet samples chilled under various circumstances (Fig. 5A). A representative trace is shown for each of three conditions, and the mean and standard deviation of the $[Ca^{2+}]_i$ increases for each condition are

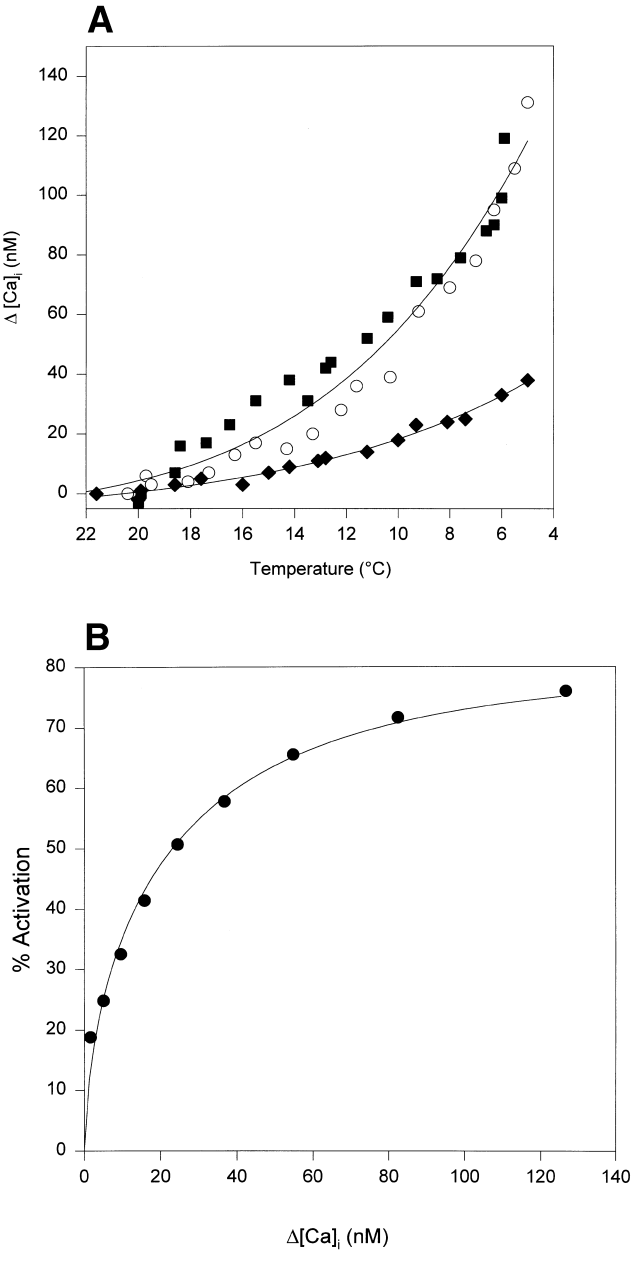

Fig. 5. A. The change in $[Ca^{2+}]_i$ of human platelets chilled to 5°C at a rate of 0.5°C/min under various conditions: control (PBS, 1 mM Ca^{2+}) (○), no external Ca^{2+} (■), Indo-1/BAPTA-loaded platelets (◆). All data points except those taken in the presence of BAPTA were fit with a single curve ($r^2 = 0.96$) with TableCurve software (Jandel Scientific, San Rafael, CA). Data points taken in the presence of BAPTA were curve fit separately ($r^2 = 0.99$). (B) The relationship between the increase in $[Ca^{2+}]_i$ during chilling and cold-induced activation in human platelets. Temperature-specific activation data were taken from [10] and are based on morphological assessment. Briefly, cells were examined microscopically, and classified as resting based on discoid morphology. Cells exhibiting spherical shape or having one or more filopodia were classified as activated. Data points were taken from the respective curve fits, in order to obtain activation and calcium data for identical temperatures.

Table 1
Increases in the $[Ca^{2+}]_i$ in human platelets chilled from 20 to 5°C under various conditions

Sample	*n*	Mean $\Delta[Ca^{2+}]_i$ (nM)	S.D.	*t*-Test
Control	8	113	34	
No extracellular Ca^{2+}	5	92	23	NS
BAPTA (intracellular)	5	45	5	$P = 0.001$
+AFGP 7&8	4	108	38	NS
+AFGP 3,5′,6	5	91	16	NS

NS, no significant difference from controls.

given in Table 1. The striking result from this series of experiments was that, there was very little difference between the $[Ca^{2+}]_i$ increase seen in control platelet samples (external buffer solution = PBS+1 mM Ca^{2+}) as compared to samples chilled in the absence of extracellular Ca^{2+} (external buffer solution = PBS, tested for and showing no contamination with calcium). Platelets in this test group were never exposed to high extracellular calcium. Their starting calcium concentration and change over time at constant temperature were similar to control samples. Statistical (*t*-test) analysis revealed no significant difference between this group and the control. This finding suggests that the $[Ca^{2+}]_i$ increase seen during the chilling of platelets cannot be due to leakage across the plasma membrane, and must be due to release from some internal source. These results correlate well with previous data regarding cold-induced activation of platelets, which is known to proceed when washed platelets are stored in the absence of extracellular calcium (10 mM EGTA) [10].

If the elevation of the $[Ca^{2+}]_i$ seen in Indo-1 loaded platelets chilled to 5°C were a genuine effect, co-loading the cells with the non-fluorescent calcium chelator BAPTA should be able to reduce it. Indeed, as shown in Fig. 5A, when platelets co-loaded with both Indo-1 and BAPTA were chilled to 5°C, they exhibited a dramatically reduced elevation in $[Ca^{2+}]_i$ (*t*-test revealed significant difference from controls at $P = 0.001$). This result suggests that the increase in $[Ca^{2+}]_i$ measured continuously during the chilling of human platelets is a real effect, as it can be markedly diminished by introducing a calcium chelator into the intracellular space. BAPTA itself does not fluoresce at these wavelengths, as control experiments showed no difference between the fluorescence intensities of a BAPTA salt solution in buffer and buffer alone.

The correlation between cold-induced activation in platelets and the intracellular calcium increase can be evidenced by plotting degree of platelet activation against $\Delta[Ca^{2+}]_i$ (Fig. 5B). Cold-induced activation was assessed morphologically (data from [10]) on the basis of discoid (classified as resting) versus spherical shape with one or more filopodia (classified as activated). This correlation between $\Delta[Ca^{2+}]_i$ and activation agrees well with a report showing that platelets exposed to 4°C underwent calcium-dependent actin reorganization resulting in platelet shape change [11].

3.5. Possible mechanisms

There are several mechanisms that may contribute to the elevation in calcium seen in chilled platelets, which the data suggest is released from some internal store. The dense tubule system (DTS), which is the calcium storage organelle in platelets [36–38], is one logical source from which calcium could be released by either active or passive leak processes. Four alternative, but not mutually exclusive mechanisms might account for the temperature-dependent increase in intracellular calcium.

3.5.1. Lipid phase transitions

A well-known property of membranes passing through this transition is that they become transiently leaky, which is thought to be due to the mismatch of molecules at the interfaces between regions of lipid in the gel and those in the liquid crystalline state [24,39–43]. Therefore, passage of the DTS membranes through their phase transition could result in a significant calcium leak and an increase in the $[Ca^{2+}]_i$. The membrane phase transition of intact human platelets, as determined by Fourier transform infrared spectroscopy, occurs between 20°C and

10°C [10], roughly the same temperature range in which we see the elevation in $[Ca^{2+}]_i$. However, $[Ca^{2+}]_i$ continues to rise, as does platelet activation [10] after the phase transition appears to be complete, suggesting that other mechanisms are likely to be involved.

3.5.2. Pump-leak imbalance

It is likely that the DTS permits passive leakage of calcium at all temperatures, but that under physiological conditions, this leak is compensated by active calcium pumps [44–47], thus leading to a steady state low concentration of calcium in the cytosol [30,31,48]. It would not be unusual if the platelet Ca^{2+}-pumps could not operate normally while the samples were subjected to hypothermic conditions, because membrane fluidity is reduced at low temperature [17,49–51]. The physical state of the membrane is an important determinant of activity for many integral membrane enzymes. In fact, a more rigid membrane environment has been shown to decrease the activity of Ca^{2+}-ATPases from skeletal muscle sarcoplasmic reticulum [52], and erythrocyte plasma membrane [53]. A similar dependence on membrane fluidity has been shown for the Na,K-ATPase [54–57]. At decreased temperatures, therefore, it would be reasonable to expect that activity of the calcium pumps would be depressed, leading to accumulation of calcium in the cytosol.

3.5.3. Signaling mechanisms

It is well known that phospholipase A_2 activity depends heavily on the physical state of the membrane and exhibits increased activity near the phase transition, due to disruption of the membrane bilayer [58,59]. There is some evidence that phospholipase C (PLC) activity is also affected by the physical state of the bilayer [60,61]. If phospholipase C were activated at the membrane phase transition in platelets, it could initiate its signaling pathway, causing release of calcium from internal stores by the production of inositol 1,4,5-trisphosphate [62,63] (or for review, see [64,65]).

3.5.4. Release of calcium from chelation sites

A fourth possibility is that various calcium-binding molecules within the platelet could be affected by temperature in the same manner as are EGTA and BAPTA, such that decreasing temperatures cause a decrease in their calcium affinity [18,33,66]. This would result in a release of free calcium into the internal milieu. Calcium released by this mechanism would be likely to continue as temperatures dropped. In any case, an increase in the free calcium concentration, regardless of its origin, could affect the activation state of human platelets [12–14].

The mechanisms causing the decrease in calcium seen in the platelets during warming (e.g., Figs. 3 and 4) are likely to be complex as well. One possibility is that any effects that the decreased temperatures had on the binding properties of internal calcium-binding molecules would be reversed as the samples were warmed. Free calcium released by this mechanism would be removed by rebinding. Another possible mechanism for the decrease in internal calcium during warming would be active pumping. It is possible that when the platelets are re-warmed, and normal membrane fluidity restored, the Ca^{2+}-ATPase might be again capable of pumping some of the calcium back into the DTS or out of the cell through the plasma membrane.

3.6. AFGPs do not prevent calcium increase in human platelets during chilling

Since the physiological activation cascade in platelets involves several different signaling pathways [48,67–69], it is reasonable to hypothesize that signaling mechanisms are also important in the process of cold-induced activation. The AFGPs from polar fish provide a unique tool with which to investigate this possibility, because they are known to inhibit cold-induced shape change and secretion of α-granules in platelets stored at 5°C for up to 3 weeks [10]. The AFGPs do not, however, inhibit the elevation of intracellular calcium of platelets chilled to 5°C (Fig. 6). Platelets chilled in the presence of 1 mg/ml AFGP, a concentration sufficient to protect platelets from cold-induced activation during hypothermic storage, showed the same magnitude of calcium increase as control platelet samples. Statistical (*t*-test) analysis revealed no significant difference between the AFGP and control samples. Representative traces are shown in Fig. 6, and the mean and standard deviation of the $[Ca^{2+}]_i$ increases for each condition are given in Table 1. AFGPs must, therefore, inhibit

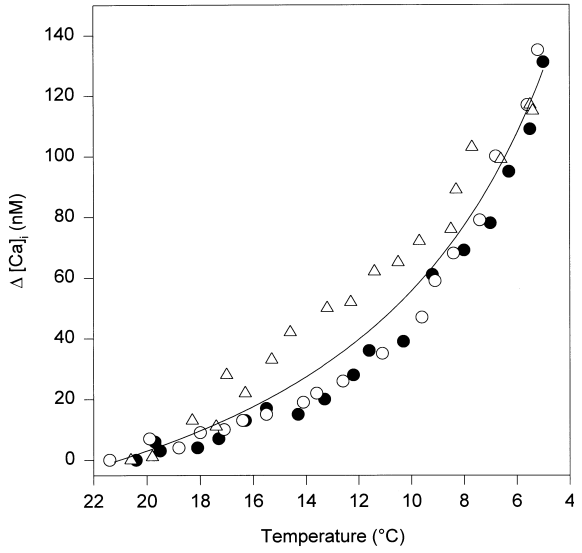

Fig. 6. The change in intracellular calcium concentration of human platelets chilled to 5°C at a rate of 0.5°C/min. Control (PBS, 1 mM Ca^{2+}) (●), +1 mg/ml AFGP 7&8 (○), +1 mg/ml AFGP 3,5′,6 (△). All data were fit with TableCurve to a single curve ($r^2 = 0.95$).

some event in the platelet activation cascade either downstream of the calcium increase, or along a separate pathway from calcium. It is possible that more than one signal transduction pathway may be operating during chilling. Studies regarding the specific contribution of signal transduction to cold-induced activation in platelets are currently underway, and will be reported separately.

In summary, we demonstrate here that chilling human platelets to 5°C results in an elevation of the $[Ca^{2+}]_i$ that is likely to be released from some internal source. This study represents, to the authors' knowledge, the first continuous measurement of the $[Ca^{2+}]_i$ during the chilling of human platelets. The elevation in the $[Ca^{2+}]_i$ probably contributes to cold-induced activation of human platelets by initiating downstream events in the activation cascade.

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