Low-temperature Induction of Calcium-dependent Protein Phosphorylation in Blood Platelets

WILLIAM F. BENNETT and GARY LYNCH
School of Biological Sciences, University of California, Irvine, California 92717

ABSTRACT Exposure to low temperature causes platelets to change shape in a manner similar to the shape change that precedes secretagogue-induced serotonin release. Previous studies have shown that two proteins, of ~20,000 and ~40,000 M_r, become phosphorylated before secretion. We have investigated whether low temperature can induce phosphorylation of these proteins and/or serotonin secretion. The data indicate that low-temperature-induced shape change has no requirement for extracellular calcium, whereas phosphorylation of the two proteins and subsequent serotonin release both have strong calcium requirements. Because cold treatment is thought to influence platelet shape through an effect on microtubules, the events in the shape change–release sequence would seem to be ordered as follows: microtubule disassembly→shape change→protein phosphorylation→secretion.

Protein phosphorylation has been established as one of the more widely used posttranslational mechanisms for regulation of enzyme activity (see reference 14 for review). As an outgrowth of this, numerous studies have been performed in an attempt to link specific *in situ* protein phosphorylations to various physiological events (e.g., see reference 8), with the aim of determining whether phosphorylation regulates the observed physiology. A notable example of this approach is the blood platelet system, in which very selective phosphorylation of two proteins of indeterminate function (40,000 and 20,000 M_r) occurs under conditions that stimulate platelets to secrete serotonin (5-hydroxytryptamine, 5-HT) (2, 9, 17). Because of the simplicity of these anucleate cells, the ease with which homogeneous populations can be prepared and manipulated, and the magnitude of the phosphorylation response, platelets are an excellent system in which to investigate the possible contribution of phosphorylation to secretion. A necessary step in the analysis will be to identify the stage in the stimulus-release sequence at which phosphorylation occurs. Upon exposure to an activator such as thrombin, blood platelets change shape from smooth discoid to convoluted spherical, release the contents of their dense granules (including 5-HT), and aggregate. These processes do not necessarily occur in the order given, although shape change is generally thought to be a prerequisite to granule release (see reference 12). Previous studies have eliminated the possibilities that phosphorylation of the proteins is a consequence of either aggregation or release (2) or that it is required for aggregation (9). Therefore we have begun to concentrate on the relationships between shape change and protein phosphorylation and on the contributions of these processes to serotonin release. Studies using the lectin concanavalin A (Con A) as secretagogue have shown that shape change and protein phosphorylation take place in the same time frame and to similar extents; moreover, several treatments that influence shape change (e.g., colchicine and D_2O) evoke concomitant changes in the phosphorylation and secretion responses (see reference 2). There are three possible explanations for this pattern of results: (*a*) the secretagogue triggers two independent processes, phosphorylation and shape change, and one or both of these are steps in release; (*b*) the secretagogue induces phosphorylation, which then initiates or facilitates shape change; or (*c*) the secretagogue causes shape change, which then stimulates selective protein phosphorylation. In an effort to distinguish among these possibilities, we have eliminated the secretagogue and instead have produced shape change by lowering the temperature of the platelet suspension (22). Such treatment induces the apparent collapse of peripheral microtubules and a rounding of the cells that morphologically resembles the shape change seen after secretagogue treatment (19–21). The present communication demonstrates that low-temperature-induced shape change can occur without concomitant phosphorylation of the two proteins, although the presence of Ca^{++} during cold treatment does trigger such phosphorylation. Furthermore, experiments using platelet extracts at 0°C indicate that phosphorylation of a number of platelet proteins is calcium sensitive, a result which suggests that compartmentalization of the 20,000 and 40,000 M_r proteins and/or their kinases obtains during shape change.

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MATERIALS AND METHODS

Solutions for Platelet Suspension

Medium A consisted of 0.12 M NaCl, 4.3×10^{-3} M KCl, 8.5×10^{-4} M $MgCl_2$, 3×10^{-3} M glucose and 1×10^{-2} M HEPES (pH 7.4). Medium B was the same as medium A, but included creatine phosphate and creatine phosphokinase, 10 mM and 40 μg/ml, respectively. This medium is prepared from medium A immediately before use.

Preparation of Platelets

Washed rat platelets were prepared essentially as described previously (1). Briefly, whole blood was collected into acid-citrate-dextrose and diluted with ⅓ vol of medium A. The blood was then spun for 15 min at 200 g_{max} in a microfuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) equipped with a variable speed control and a stroboscopic tachometer. Platelet-rich plasma was collected, layered over 30% bovine serum albumin (BSA), centrifuged at 225 g_{max} for 5 min, then at 600 g_{max} for 3 min. Platelets were collected from above the BSA-plasma interface, pelleted at 600 g_{max} and resuspended in medium B at a cellular concentration of $5–8 \times 10^8$ platelets/ml.

In Situ Protein Phosphorylation Assay

In situ protein phosphorylation was assessed essentially as previously described (2). Platelet suspensions in medium B were incubated with [^{32}P]orthophosphate at 1 mCi/ml for 1 h at 25°C. The cells were separated from unincorporated label by centrifugation at 600 g_{max} for 3 min, and resuspended in medium B to $\sim 3 \times 10^9$ platelets/ml. 25-μl aliquots of these labeled cells were diluted into 75 μl of medium B at 0°C, and at designated times 25 μl of these suspensions were mixed with an equal volume of 2 × concentrated SDS sample buffer at 95°C and incubated for 2 min. After dilution, this sample buffer contained 2.3% sodium dodecyl sulfate (SDS), 5% β-mercaptoethanol, 10% glycerol, and 0.06 M Tris-HCl, pH 6.8. After 2 min at 95°C, the samples were loaded onto 7.5–20% polyacrylamide gradient slab gels, subjected to electrophoresis, and prepared for autoradiography.

In Vitro Protein Phosphorylation

Platelets in medium B were pelleted by centrifugation at 600 g_{max} for 3 min and the supernatant fluid was removed. The pellets ($\sim 10^9$ cells) were quickly resuspended in 1 ml ice-cold 20 mM HEPES, 5 mM/mg acetate, 0.5 mM EGTA, pH 7.0, and maintained at 0°C. The suspensions were then sonicated for 5 s in 1-s bursts at 30-s intervals using a Virsonic cell disruptor (VirTis Co., Inc., Gardiner, N. Y.) at maximum microprobe energy. These lysates were then incubated with γ-[^{32}P]ATP (2 mCi/ml; 50 μM) for the indicated times and with the indicated amounts of $CaCl_2$. The samples were processed and analysed as described in the previous section. Calcium concentrations were determined using a Radiometer (Copenhagen, Denmark) Ca^{++} ion specific electrode coupled with a Radiometer pH meter (13).

[^{3}H]5-HT Release Assay

Platelets in medium B were incubated with [^{3}H]serotonin creatinine sulfate for 30 min at 25°C (3×10^{-7} M, 8.75 μCi/ml). Labeled cells were washed and resuspended in medium B at 3×10^9 cells/ml. After dilution into ice-cold medium B, aliquots were removed at designated times and the cells were pelleted by centrifugation at 11,000 g_{max} for 15 s. The supernatant fluids were then assayed for released [^{3}H]5-HT using a Packard liquid scintillation counter (Packard Instrument Co., Inc., Downers Grove, Ill.)

Preparation for Scanning Electron Microscopy

Platelets in medium B were subjected to chilling and rewarming in the presence and absence of Ca^{++}, as described in the legend to Fig. 1. Fixation was carried out by addition of 0.4 M glutaraldehyde to yield a final concentration of 0.1 M, followed by incubation at 4°C for 1 h. The cells were allowed to settle onto poly-L-lysine–treated glass coverslips for ~90 min; the coverslips were then washed three times with 0.1 M potassium phosphate, pH 7.2. The solvent was exchanged for increasing concentrations of ethanol, then freon 113. Critical-point drying in halocarbon 13 was carried out on a Bomar SPC-900/EX critical-point dryer (The Bomar Co., Tacoma, Wash.). The samples were then gold coated for 3 min at 10 mA using a Hummer II sputterer (Technics Inc., Alexandria, Va.), and were examined under Hitachi S-500 scanning electron microscope.

Reagents

[^{32}P]orthophosphoric acid and γ-[^{32}P]ATP were purchased from ICN Pharmaceuticals, Irvine, Calif., and [^{3}H]5-hydroxytryptamine creatinine sulfate, from New England Nuclear, Boston, Mass. Con A, BSA (30% sterile solution), and HEPES were obtained from Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif. EGTA, phosphocreatine, creatine phosphokinase, poly-L-lysine, and the molecular weight standards for SDS-gel electrophoresis (excepting myosin) were from Sigma Chemical Co., St. Louis, Mo.

RESULTS

Low-temperature-induced In Situ Phosphorylation

At physiological temperatures, resting platelets have a characteristic disk shape (shown in Fig. 1 *a*) that is thought to be maintained to some extent by a peripheral ring of microtubules (see references 19, 20). Low temperature, which tends to disaggregate microtubular structures (3, 7, and Tilney as cited in reference 18), causes platelets to lose their discoid appearance and collapse to a more spherical cell with an irregular surface (Fig. 1 *b* and *c*; also see references 21 and 22). This collapse is similar in many respects to the shape change induced by numerous platelet-activating agents. Low-temperature-induced shape change can be gradually reversed by rewarming (21, 22), as shown in Fig. 1 *d*, which indicates that rewarmed cells bear a strong resemblance to resting disk-shaped platelets. The cold-induced transformation shown in Fig. 1 *b* and *c* was observed in >90% of the cells, and, like secretagogue-induced shape change (1), it occurs in either the absence or presence of extracellular calcium (Figure 1 *b* and *c*, respectively).

Protein phosphorylation was assessed under similar conditions by preincubating the cells with [^{32}P]orthophosphate for 1 h at 25°C, then diluting the cell suspensions with ice-cold medium B with or without Ca^{++}, under conditions such that the temperature of the diluted platelet suspension reaches 2°C within 15 s. Aliquots of the platelets were removed at various intervals and prepared for SDS–polyacrylamide gel electrophoresis. Fig. 2 is an autoradiograph from such an experiment. In the presence of extracellular calcium, the protein bands at 20,000 and 40,000 M_r (and only these) can be seen to become phosphorylated with time. If calcium is omitted, there is no induced phosphorylation of either protein. This is in contrast to secretagogue-induced phosphorylation, which does not display the dramatic dependency on extracellular calcium shown in Fig. 2 (2, 16).

PHOSPHORYLATION BY CELL LYSATES AT 0°C: Phosphorylation of the two proteins takes place over a period of minutes after the cells have been diluted into ice-cold medium. In view of the fact that the temperature of the medium reaches 2°C within 15 s under these conditions, it is difficult to escape the conclusion that not only is phosphorylation induced by exposure to low temperature (in the presence of Ca^{++}) but phosphate transfer is taking place at a temperature well below the physiological range. Because this biochemical reaction occurs under rather unusual circumstances, experiments using lysed platelets were carried out to determine whether, and to what extent, protein phosphorylation in platelet lysates takes place in the cold. In addition, we hoped in these experiments to gain information about the mechanisms responsible for the extreme selectivity of the phosphorylation observed *in situ*. This specificity suggests either possession by the platelet of a limited number of calcium-sensitive kinases (and/or phosphatases) or a compartmentalization of the 20,000 and 40,000 M_r proteins

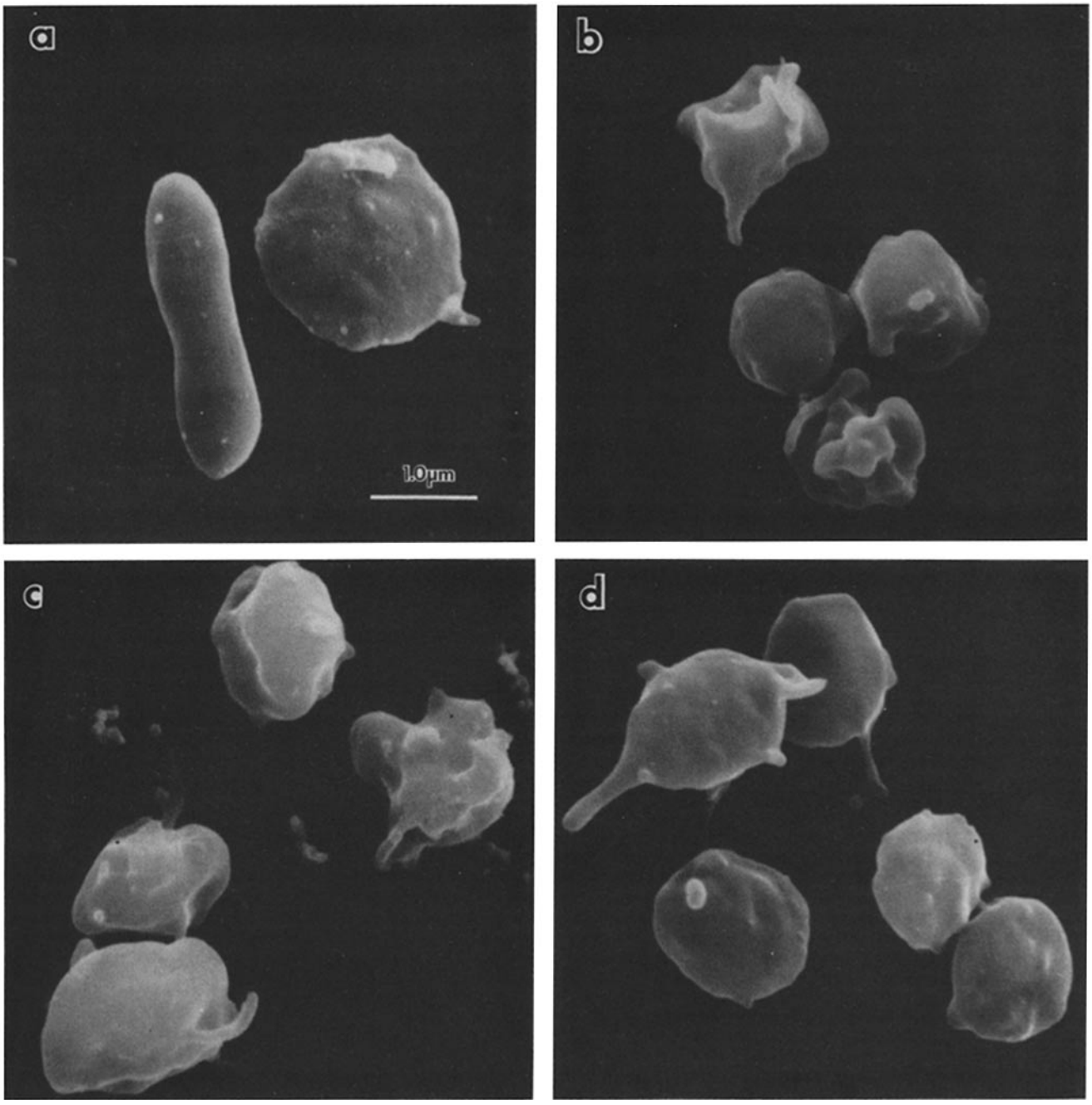

FIGURE 1 Scanning electron microscopy of chilled and rewarmed platelets. Platelets in medium B were treated as follows: (*a*) 0.5 mM $CaCl_2$, 25°C for 30 min, (*b*) 0.5 mM EGTA, 0°C for 5 min; (*c*) 0.5 mM $CaCl_2$, 0°C for 5 min; (*d*) 0.5 mM $CaCl_2$, 0°C for 5 min, followed by 25°C for 30 min. Glutaraldehyde (0.4 mM in H_2O) was then added to give a final concentration of 0.1 M, and the samples were processed for scanning EM as described in Materials and Methods.

or their kinases, such that only these peptides are exposed to calcium during shape change. Comparison of calcium-dependent phosphorylation in lysates with that seen *in situ* should help to distinguish between these possibilities.

Platelet lysates were prepared in an EGTA-containing pH 7.0 buffer, then incubated with γ-[^{32}P]ATP at 0°C with either no added Ca^{++} (~0.1 μM free Ca^{++}), a Ca^{++}/EGTA ratio of 0.25:0.5 mM (~1 μM free Ca^{++}), or 100 μM excess Ca^{++}. Aliquots were removed at various times after ATP addition and analysed by gel electrophoresis and autoradiography as shown in Fig. 3. The autoradiographic patterns indicate that considerable overall protein phosphorylation takes place at 0°C and that the proteins phosphorylated under these in vitro conditions show a pattern similar though not identical to that of those labeled *in situ*, using [^{32}P]orthophosphate. As expected, phosphorylation of the protein bands at 20,000 and 40,000 M_r displays a strong Ca^{++} requirement, but interestingly, phosphorylation of several other proteins exhibits similar Ca^{++} dependency. The protein band at 76,000 M_r, for example, appears to have a very strong Ca^{++} requirement for in vitro phosphorylation, despite the fact that *in situ* labeling conditions produce no Ca^{++} effect on this protein band. This is also the case for several other proteins. In contrast, there are some proteins whose phosphorylation is apparently inhibited by the

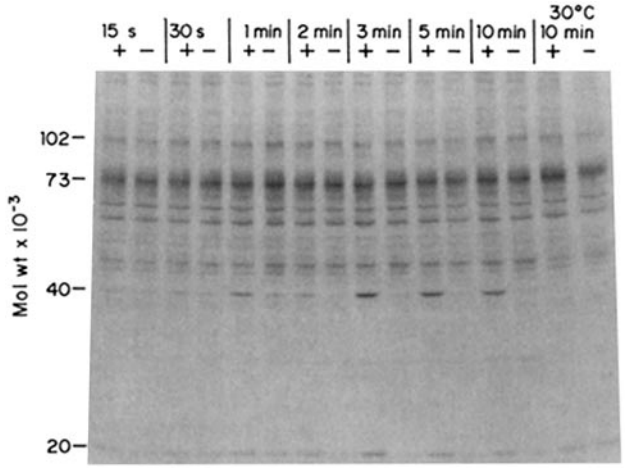

FIGURE 2 Time-course of low-temperature-induced protein phosphorylation by *in situ* labeled platelets. ^{32}P-labeled cells were diluted into 3 vol of medium B at 0°C. At the indicated times, samples were removed and analysed by SDS-gel electrophoresis and autoradiography; (+) indicates the presence of 0.5 mM $CaCl_2$; (−) represents no calcium addition. Molecular weight standards are: platelet myosin, 200,000; rabbit muscle phosphorylase *b*, 96,000; bovine serum albumin; 67,000; rabbit muscle glyceraldehyde-3-phosphate dehydrogenase, 36,000; horse heart cytochrome *c*, 13,000. Liquid scintillation analysis revealed the ^{32}P content of the 40 kdalton band at 3 min after chilling to be: +Ca, 800 dpm; $-Ca^{++}$, 75 dpm.

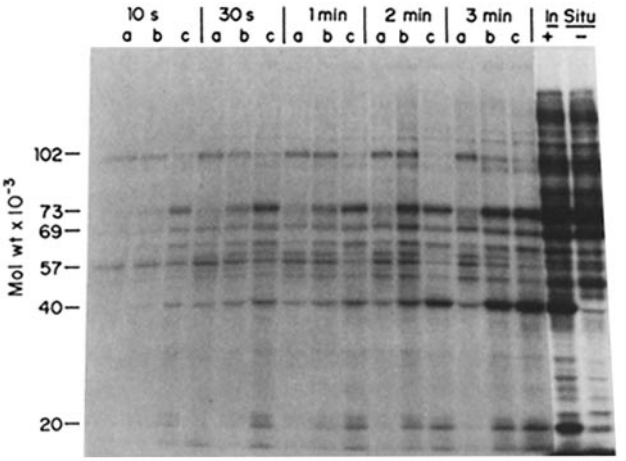

FIGURE 3 Platelet lysate phosphorylation at 0°C. Sonicated lysates were incubated with γ-[^{32}P]ATP in media containing (*a*) 0.5 mM EGTA (*b*) 0.5 mM EGTA + 0.25 mM $CaCl_2$, and (*c*) 0.5 mM EGTA + 0.6 mM $CaCl_2$. Aliquots were removed at the indicated times and processed for SDS-gel electrophoresis and autoradiography. *In situ* labeled platelet samples have been included on the gel for reference. (+) is a 1.0 μ/ml thrombin-stimulated sample, (−) is unstimulated. The molecular weight standards are the same as in Fig. 2.

presence of Ca^{++}. The most interesting examples are two proteins at 57,000 and 102,000 M_r, which are phosphorylated very quickly at 0°C at low Ca^{++} levels. In fact, these proteins are almost maximally phosphorylated at the earliest time-point tested (10 s), and at later times both phosphoproteins undergo apparent Ca^{++}-dependent dephosphorylation. In contrast, neither of these bands displays any stimulation-induced changes in ^{32}P content with *in situ* labeling in either the presence or absence of calcium. Taken together, these results suggest that if phosphorylation is mediated by an influx of extracellular calcium, then that influx is confined to the intracellular compartment containing the 20,000 and 40,000 M_r proteins and/or their attendant kinase(s).

LOW-TEMPERATURE INDUCTION OF 5-HT RELEASE: We have previously raised the suggestion that only two conditions need be satisfied to allow physiological serotonin release from platelets: (*a*) that the two proteins be phosphorylated to a certain level and (*b*) that a sufficient level of external calcium be present. (There are situations where extracellular calcium need not be present for release, e.g., where thrombin or the Ca^{++}-ionophore A23187 is used as a secretagogue; but these treatments also produce aggregation, a complicating variable in release studies [1].) The present situation with low-temperature induction provided a test for this model. If Ca^{++} and phosphorylation together produce release, then 5-HT should be secreted in a Ca^{++}-dependent manner when the temperature of the cells is lowered. This is in fact the case, as shown in Fig. 4. The level of release is only ~25% of that obtained at 25°C, and the rate is somewhat slower than that observed for phosphorylation; nevertheless the Ca^{++} dependency is striking (approximately fivefold over background). This result not only supports the notion that release might be a synergistic function of Ca^{++} and protein phosphorylation, but indicates that few enzymatic steps are likely to be involved in release following the Ca^{++}-dependent step, because the entire process takes place at 0°C.

DISCUSSION

The present experiments somewhat clarify the possible roles of protein phosphorylation in platelet behavior. The data show that in the absence of calcium, low-temperature-induced shape change occurs without detectable phosphorylation of the 20,000 and 40,000 M_r proteins. It follows then that phosphorylation is

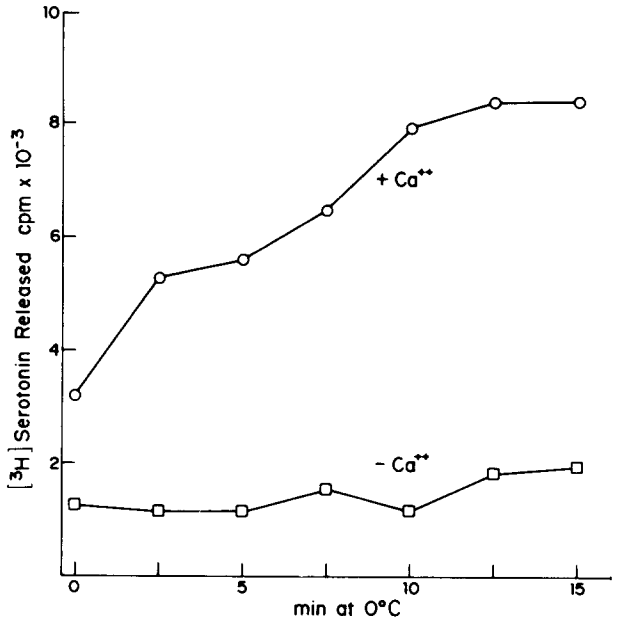

FIGURE 4 Low-temperature-induced 5-HT release. [^{3}H]Serotonin-labeled platelets were diluted with 3 vol of ice-cold medium B, in either the absence or presence of 0.5 mM $CaCl_2$, and were maintained at 0°C. Aliquots were removed at the indicated times and assayed for released 5-HT. No backgrounds have been subtracted; under these conditions, centrifugation itself produces very low levels of release. The maximal release in this experiment represents 20% of the cells' [^{3}H]5-HT content, which is ~25% of the thrombin-releasable [^{3}H]5-HT at 25°C.

neither necessary for induction of the platelet shape change reaction nor mandated by shape change. This conclusion is also supported by recent experiments using the phosphodiesterase inhibitor papaverine. This drug completely and selectively blocks phosphorylation of the 20,000 and 40,000 M_r peptides, while only slightly influencing the shape change reaction (2). In the same study it was shown that robust phosphorylation of the 20,000 and 40,000 M_r proteins can be obtained in the absence of detectable serotonin release. These results, combined with the present findings, suggest that the events after platelet stimulation (in the absence of aggregation) can be placed in the following order: stimulus → peripheral microtubule disassembly → shape change → protein phosphorylation → serotonin release. Low temperature initiates microtubule depolymerization in vitro (3, 7), and the *in situ* effects of low temperature on platelets have been ascribed to a similar effect on the equatorial microtubular ring (20, 21). It seems possible, therefore, that all subsequent steps (including protein phosphorylation) are triggered by microtubule disassembly.

Our data also demonstrate for the first time that phosphorylation can be obtained through an intrinsically activated system, without added secretagogues. It now appears likely that stimulus-induced shape change is the factor that elicits phosphorylation, rather than some direct action of these agents on the biochemical machinery of the platelet. It should be noted that previous experiments have shown that phosphorylation of the 20,000 and 40,000 M_r proteins had components dependent on and independent of external divalent cation levels (2, 16). In the present study, only the calcium-dependent aspect of the reaction appeared to be present. The existence of two components may indicate that phosphorylation of the same proteins can be achieved by two different cellular processes, only one of which requires external calcium. Alternatively, it is possible that phosphorylation is always dependent upon calcium but that only under some experimental circumstances are internal stores of the cation accessible and/or sufficient.

Assuming that the temperature inside the platelets drops to near 0°C within 15–30 s of dilution with cold buffer, and given that most phosphorylation occurs 1–5 min later, it can be concluded that phosphorylation of the 20,000 and 40,000 M_r proteins takes place at a temperature at which many enzymatic reactions are strongly inhibited. However, phosphorylation of platelet proteins takes place at 0°C not only in *in situ* experiments but also in those using platelet lysates; it therefore appears that protein phosphorylation in platelets does not have a prohibitive free-energy requirement.

The experiments using platelet lysates also provide clues to the mechanisms by which the extremely selective phosphorylation seen after shape change is achieved. It is clear that several platelet phosphorylation and dephosphorylation reactions are calcium sensitive but, in contrast to the 20,000/40,000 M_r system, these other reactions are apparently unaffected by shape change in the presence of calcium. This result would be consistent with segregation of the 20,000/40,000 M_r system in a cellular compartment distinct from the other Ca^{++}-sensitive proteins. Such compartmentalization might be into intracellular organelles such as mitochondria or any of the various types of platelet granules. Alternatively, shape change might promote exposure of the 20,000 and 40,000 M_r proteins (or their kinases) to the external environment. There is some precedent for this type of activity by the stimulated platelet. For example, immunological studies with actin-specific antibody indicate that cytoplasmic actin can be detected on the platelet surface only following shape change–producing stimulation (4). At present the data available are insufficient to distinguish among the above possibilities.

Because the two phosphoproteins appear to be important in platelet physiology, identification of their functions would be the next obvious point of interest. This task would be much easier if they could be shown to be identical to already characterized proteins. This is not yet the case, although there is some experimental support for the idea that the 20,000 M_r protein is the light chain of myosin (5), a reasonable hypothesis in view of the preponderance of contractile proteins in platelets as well as of the recent demonstration (10) that calmodulin activates the platelet myosin light-chain kinase. About all that is known about the 40,000 M_r protein is that it is soluble (6) and that it is neither actin, tubulin, nor the regulatory subunit of cAMP-dependent protein kinase (15). On the basis of molecular weight and isoelectric point, the 40,000 M_r protein resembles the α-subunit of pyruvate dehydrogenase: both phosphoproteins are reported to have isoelectric points of 6.5–6.8 and M_r of ~40,000 (11, 15). However, comparison using limited proteolysis shows dissimilarity between the 40,000 M_r band and the rat liver pyruvate dehydrogenase α-subunit band (W. F. Bennett, and M. D. Browning, unpublished observations). Further analyses of this sort are in progress, as identification of the phosphoprotein would help suggest possible functions in the secretion process.

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