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

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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 Mr, 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 Mr) 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_{2}O) 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^{2+} 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 Mr proteins and/or their kinases obtains during shape change.
**MATERIALS AND METHODS**

**Solutions for Platelet Suspension**

Medium A consisted of 0.12 M NaCl, 4.3 x 10^{-3} M KCl, 8.5 x 10^{-4} M MgCl₂, 3 x 10^{-4} M glucose and 1 x 10^{-3} M HEPES (pH 7.4). Medium B was the same as medium A, but included creatine phosphate and creatine phosphokinase, 10 mM and 40 mg/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 ½ vol of medium A. The blood was then spun for 15 min at 200 gmax in a microfuge (Beckman Instruments, Inc., Spincio 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 gmax for 5 min, then at 600 gmax for 3 min. Platelets were collected from above the BSA-plasma interface, pelleted at 600 gmax, and resuspended in medium B at a cellular concentration of 5-8 x 10⁸ 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 [³²P]orthophosphate at 1 ml/ml for 1 h at 25°C. The cells were separated from unincorporated label by centrifugation at 600 gmax for 3 min, and resuspended in medium B to ~3 x 10⁸ platelets/ml. 25-μl aliquots of these labeled cells were mixed with an equal volume of 2 x 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% 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% polyacylamide gradient slab gels, subjected to electrophoresis, and prepared for autoradiography.

**In Vitro Protein Phosphorylation**

Platelets in medium B were pelleted by centrifugation at 600 gmax for 3 min and the supernatant fluid was removed. The pellets (~10⁹ 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 Vironsonic cell disruptor (VirTis Co., Inc., Gardiner, N.Y.) at maximum microprobe energy. These lysates were then incubated with γ-³²P]ATP (2 mCi/ml; 50 μM) for the indicated times and with the indicated amounts of Ca²⁺. The samples were processed and analysed as described in the previous section. Calcium concentrations were determined using a Radiometer (Copenhagen, Denmark) Ca²⁺ ionspecific electrode coupled with a Radiometer pH meter (13).

**[³H]5-HT Release Assay**

Platelets in medium B were incubated with [³H]serotonin creatinine sulfate for 30 min at 25°C (3 x 10⁻³ M, 8.75 μCi/ml). Labeled cells were washed and resuspended in medium B at ~3 x 10⁵ 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 gmax for 15 s. The supernatant fluids were then assayed for released [³H]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 rewarving in the presence and absence of Ca²⁺, 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 freez 113. Critical-point drying in halocarbon 13 was carried out on a Bomer SAP-900/EX critical-point dryer (The Bomer Co., Tacoma, Wash.). The samples were then gold coated for 3 min at 10 mA using a Hummer II sputterer (Techsinc Inc., Alexandria, Va.), and were examined under Hitachi S-500 scanning electron microscope.

**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 rewarving (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 [³²P]orthophosphate for 1 h at 25°C, then diluting the cell suspensions with ice-cold medium B with or without Ca²⁺, 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, (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 LYSES 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 Ca²⁺) 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, proteins.
FIGURE 1 Scanning electron microscopy of chilled and rewarmed platelets. Platelets in medium B were treated as follows: (a) 0.5 mM CaCl₂, 25°C for 30 min, (b) 0.5 mM EGTA, 0°C for 5 min; (c) 0.5 mM CaCl₂, 0°C for 5 min; (d) 0.5 mM CaCl₂, 0°C for 5 min, followed by 25°C for 30 min. Glutaraldehyde (0.4 mM in H₂O) 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-[³²P]ATP at 0°C with either no added Ca²⁺ (≈0.1 μM free Ca²⁺), a Ca²⁺/EGTA ratio of 0.25:0.5 mM (≈1 μM free Ca²⁺), or 100 μM excess Ca²⁺. 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 [³²P]orthophosphate. As expected, phosphorylation of the protein bands at 20,000 and 40,000 M₉ displays a strong Ca²⁺ requirement, but interestingly, phosphorylation of several other proteins exhibits similar Ca²⁺ dependency. The protein band at 76,000 M₉, for example, appears to have a very strong Ca²⁺ requirement for in vitro phosphorylation, despite the fact that in situ labeling conditions produce no Ca²⁺ 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. 32P-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 CaCl2; (-) 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 32P 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-32P]ATP in media containing (a) 0.5 mM EGTA (b) 0.5 mM EGTA + 0.25 mM CaCl2, and (c) 0.5 mM EGTA + 0.6 mM CaCl2. 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 μl/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 Mr, 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 32P 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 Mr 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 Mr proteins. It follows then that phosphorylation is
cytoplasmic actin can be detected on the platelet surface only for the initial few minutes of the platelet shape change reaction (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 and 40,000 Mr 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 Mr 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 Mr protein resembles the α-subunit of pyruvate dehydrogenase: both phosphoproteins are reported to have isoelectric points of 6.5–6.8 and Mr of ~40,000 (11, 15). However, comparison using limited proteolysis shows dissimilarity between the 40,000 Mr 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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