On the Mechanism of Activation of Phosphorylase b Kinase by Calcium*

GEORGE I. DRUMMOND AND LOVERNE DUNCAN

From the Department of Pharmacology, University of British Columbia School of Medicine, Vancouver 8, Canada

SUMMARY

Kinase-activating factor, a protein required for the activation of phosphorylase b kinase by Ca++, was purified from brain tissue and from skeletal muscle and myocardium. All fractions contained calcium-activated proteinase activity as measured by the formation of acid-soluble, tyrosine-positive material with casein as substrate. The ratio of kinase-activating factor activity to proteinase activity remained constant throughout the purification, and both activities coincided on Sephadex G-100 filtration. Kinase-inhibitory factor, which prevents activation of kinase by Ca++, inhibited proteinase activity. Kinase-inhibitory factor also prevented the activation of kinase by trypsin. Acid-soluble, ninhydrin-positive material was formed during the activation of phosphorylase kinase by kinase-activating factor in the presence of Ca++. It is concluded that kinase-activating factor is a calcium-activated proteolytic enzyme, that kinase-inhibitory factor is a proteolytic inhibitor, and that activation of phosphorylase b kinase by Ca++ involves proteolysis.

Phosphorylase b kinase present in extracts of skeletal muscle (1, 2) and cardiac muscle (3, 4) is markedly activated in vitro when incubated with Ca++ before assay. Activation is particularly marked if the enzyme is assayed at neutral pH or below, where the nonactivated enzyme (as isolated from tissue) has very low activity. At pH values above neutrality, the activity of the nonactivated enzyme rises sharply, with a maximum at pH 8.5. When the calcium-activated enzyme is assayed at alkaline pH, the effect of Ca++ is much less marked, so that activation is characterized by increase in the ratio of activity at pH 6.8 to that at pH 8.2 (1, 2). Meyer, Fischer, and Krebs (2) recently reported that it caused the release of 32P-peptides from 32P-labeled phosphorylase b kinase and of ninhydrin-positive material from nonactivated kinase. From such studies they concluded that kinase-activating factor is a Ca++-requiring proteolytic enzyme. 1

While examining the enzymes of glycogenolysis in nerve tissue, we observed that brain phosphorylase kinase is also activated by Ca++ and that brain is an excellent source of both the activating and inhibitory factors. During these studies, it became apparent that kinase-activating factor had several properties in common with a calcium-activated proteinase isolated from brain by Guroff (6). The present report provides data indicating that kinase-activating factor and calcium-activated proteinase are identical, and that the mechanism of calcium activation of kinase is proteolytic.

EXPERIMENTAL PROCEDURE

Materials

Rabbit muscle phosphorylase b, glycogen, and glucose 1-phosphate were prepared or purified by methods previously used (3). Nonactivated phosphorylase b kinase was purified from rabbit muscle by the procedure of Krebs et al. (7). The 40-precipitate fraction was reoxidized three times at 100,000 × g in order to reduce contamination from kinase-activating factor. Enzyme preparations were dissolved in 50 mM β-glycerophosphate and 2 mM EDTA, pH 7.0, at a protein concentration of about 20 mg per ml, and stored at -18°C. Inhibitory factor was prepared from bovine heart (4).

1 We wish to thank Professor E. G. Krebs for making available his manuscript prior to publication.

2 G. I. Drummond and G. Bellward, to be published.
Measurement of Kinase Activation

The activation reaction was conducted by a slight modification of that previously described (4). Nonactivated kinase was appropriately diluted in 50 mM β-glycerophosphate containing 2 mM EDTA, pH 7.0, immediately before use. Usually 500 pM 8.2 units were added, in a volume of 0.05 ml. Kinase-activating factor and inhibitory factor preparations were appropriately diluted in 15 mM neutral cysteine immediately before addition. These components were mixed, and the volume was adjusted to 0.2 ml with neutral cysteine solution. Following a 1-mm equilibration at 30°C, 0.05 ml of 50 mM calcium acetate was added to start the reaction. To ensure linearity of response to activating factor protein added, amounts were chosen so that activation was not more than 30% of maximum. After 4 min at 30°C, 5 ml of ice-cold 15 mM neutral cysteine were added; the tubes were placed in ice, and a 0.2-ml aliquot was immediately used for the phosphorylase kinase assay, which was conducted at pH 6.8 (4) and, when necessary, at pH 8.2. Since with skeletal muscle kinase preparations there is negligible formation of 5'-AMP during the assay, it was not necessary to incubate the diluted kinase assay mixtures with adenylic deaminase before measurement. Units of kinase, activating factor, and inhibitory factor were those previously defined (4). Nonactivated kinase and appropriate amounts of soybean trypsin inhibitor or kinase-inhibitory factor were equilibrated at 30°C for 2 min in a volume of 0.2 ml. The reaction was started by the addition of 0.05 ml of trypsin solution (0.02 µg of protein). After 10 min, 10 µg of soybean trypsin inhibitor (in 0.05 ml) were added to each tube, followed immediately by 5 ml of cold 15 mM neutral cysteine. Kinase assays were then conducted as above.

Activation of Kinase by Trypsin

Nonactivated kinase and appropriate amounts of soybean trypsin inhibitor or kinase-inhibitory factor were equilibrated at 30°C for 2 min in a volume of 0.2 ml. The reaction was started by the addition of 0.05 ml of trypsin solution (0.02 µg of protein). After 10 min, 10 µg of soybean trypsin inhibitor (in 0.05 ml) were added to each tube, followed immediately by 5 ml of cold 15 mM neutral cysteine. Kinase assays were then conducted as above.

Homogenization of Brain Tissue

Cortex from freshly killed animals was packed in ice, transported to the laboratory, and thoroughly cleaned of blood and vascular and membranous tissue. Four 125-g portions were homogenized for 2 min at 4°C in a Waring Blender with 4 volumes of buffer consisting of 50 mM β-glycerophosphate, 2 mM EDTA, and 10 mM cysteine, pH 6.8. The homogenate was centrifuged at 35,000 x g for 30 min. The volume of supernatant fluid was 2,020 ml.

pH 5.1 Precipitation—The supernatant fluid was adjusted to pH 5.1 in 700-ml portions with 1 N acetic acid, and after 10 min the suspension was centrifuged at 38,000 x g for 30 min at 4°C in 40-ml tubes (Sorvall centrifuge). The second and third portions were added on top of the precipitates from the preceding centrifugation. The supernatant fluid was discarded or was neutralized and stored at −18°C as a source of inhibitory factor. The walls of the centrifuge tubes were carefully washed with 50 mM sodium acetate containing 2 mM EDTA and 5 mM mercaptoethanol, pH 6.0. The pellets were transferred to a Waring Blender chamber and briefly homogenized in fresh acetate buffer. The heavily turbid suspension was adjusted to pH 6.4 (final volume, 300 ml) and stored at −18°C.

Ammonium Sulfate Fractionation and Ultracentrifugation—The above preparation was thawed and refrozen once each day for 4 days. Extensive coagulation resulted; freezing and thawing at shorter intervals was not satisfactory. After the final thawing, the preparation was centrifuged at 30,000 rpm for 1 hour in the No. 30 rotor of a Spinco model L ultracentrifuge. The clear supernatant fluid was removed; the pellets were suspended in 100 ml of buffer and recenterfuged, and this wash fluid was added to the main supernatant (final volume, 360 ml). The solution was made 10 mM with respect to EDTA, and 104 g of ammonium sulfate were added slowly at 4°C to yield a final concentration of 40% saturation. After 20 min, the preparation was centrifuged at 38,000 x g at −6°C. The tightly packed pellets were dissolved in the above acetate buffer and dialyzed for 8 hours against two 1-liter changes of this buffer. The resulting solution was centrifuged at 100,000 x g for 1 hour. The volume of supernatant solution was 34 ml, and the protein concentration was 10.2 mg per ml.

DEAE-cellulose Chromatography—A portion (25 ml) of the above solution was diluted with 25 ml of 50 mM sodium acetate containing 2 mM EDTA and 5 mM mercaptoethanol, pH 6.0, and applied to a DEAE-cellulose column (2 x 20 cm) that had been equilibrated with this buffer at 30°C and packed under maximum hand bulb pressure. Elution was effected at 1°C with a stepwise gradient, beginning with starting buffer and proceeding with increasing concentrations of NaCl: 0.1, 0.15, 0.2, and 0.23 M in buffer. The flow rate was 1 ml per min; fraction volumes were approximately 12 ml, and elution of protein was followed optically at 280 nm. Activating factor was eluted at 0.2 M NaCl (Peak IV, Fig. 1). Tubes representing this peak were combined; 1.4 volumes of saturated ammonium sulfate containing 2 mM EDTA were added at 4°C; the fraction was centrifuged at 38,000 x g at −5°C, and the precipitated protein was dissolved in about 4 ml of acetate buffer. The resulting solution was dialyzed (final volume, 5 ml; protein, 4.5 mg per ml) and stored at −18°C. Activating factor was isolated from bovine heart in a similar manner. The activating factor from rabbit skeletal muscle was prepared by the method of Meyer, Fischer, and Krebs (2), except that DEAE-cellulose chromatography was conducted as for brain preparations.

Sephadex G-100 Fractionation—A portion (20 ml) of the
DEAE-cellulose column eluate was applied to a column of Sephadex G-100 (2.5 x 100 cm) equilibrated with 50 mM sodium acetate, 2 mM EDTA, and 5 mM mercaptoethanol, pH 6.0, by means of upward flow at 1°. Two milliliters of 10% sucrose solution were applied immediately after the sample, and development was continued with acetate buffer at a flow rate of 25 ml per hour. Shortly before the void volume had passed through, the outflow was delivered to a fraction collector, and approximately 4.5-ml fractions were collected. Each fraction was assayed for proteinase and activating factor activity within 24 hours.

Unless otherwise indicated, protein was determined by the method of Lowry et al. (10).

Electrophoresis of Large Scale Activation Reaction

For the attempted isolation of peptide material produced during activation of kinase, the reaction had to be considerably scaled up. Thus 0.5-ml aliquots of skeletal kinase, 10.5 mg of protein (300,000 pH 8.2 units), were placed in each of four tubes. To two tubes (Nos. 1 and 2) were added 0.5 ml of brain activating factor solution, 1.25 mg of protein (DEAE-cellulose eluate; specific activity, 134,000); to the remaining tubes (Nos. 3 and 4) were added 0.5 ml of skeletal muscle activating factor, 0.65 mg of protein (specific activity, 226,000). The volume of each mixture was brought to 2.0 ml with 15 mM mercaptoethanol (instead of cysteine), and the tubes were equilibrated at 30° for 2 min. The activation reaction was started by the addition of 0.5 ml of 50 mM calcium acetate to tubes 1 and 3 (tubes 2 and 4 served as controls). After a 5 min incubation period, a 0.02 ml aliquot of each tube was removed, diluted, and assayed for kinase at pH 6.8 to ensure that full activation had taken place. To the main reactions, 0.5 ml of 12% trichloracetic acid was added, and the tubes were placed in ice; 0.5 ml of 50 mM calcium acetate was added to tubes 2 and 4. Denatured protein was removed by centrifugation at 3000 rpm, each precipitate was washed by stirring with 3 ml of 3% trichloracetic acid, and, following centrifugation, the wash solution was added to the main acid supernatant. Each acid solution was extracted four times with 5-ml portions of ether. Excess ether was removed from the aqueous phase by aspiration, and the solutions were lyophilized. The residues were dissolved in 0.2 ml of water; each solution was neutralized, spotted on Whatman No. 3 MM paper, and subjected to high voltage electrophoresis (3000 volts) for 40 min in pyridine-acetate, pH 6.5 (pyridine, 50 ml; acetic acid, 2 ml; water to 500 ml). Toluene was the coolant. The paper was thoroughly dried at 65°, sprayed with ninhydrin, heated at 65° for 30 min, and then sprayed with 0.25 x nickel sulfate (11).

RESULTS

Preliminary

The yield of phosphorylase kinase is considerably greater from skeletal muscle than from myocardium (3) or brain. Unlike heart preparations, assay of the skeletal muscle enzyme is not complicated by the degradation of ATP by contaminating enzymes to 5'-AMP, which interferes with the phosphorylase assay (3, 8). The skeletal muscle enzyme has therefore been used in this study as a substrate for the activation process. Activating factor in unfractionated extracts of both heart and brain cannot be reliably estimated because of the presence of inhibitory factor. However, activating factor is precipitated in high yield at pH 5.1, while the inhibitory factor remains in solution. Such isoelectric precipitates were used to estimate the yield of activating factor activity in various tissues. Thus, three different preparations from bovine brain averaged 13,000 units per g; fresh weight; rabbit heart preparations averaged 17,000 units per g; and rabbit skeletal muscle was more active, at 50,000 units per g.

Purification of Brain Kinase-activating Factor

It was apparent from early studies that brain activating factor might be identical with a calcium-activated proteolytic enzyme isolated from rat brain by Guroff (6). In particular, brain proteinase purified by Guroff's method (6) was an excellent source of kinase-activating factor. Conversely, early fractions obtained in efforts to purify kinase-activating factor from brain all contained calcium-activated proteinase activity when casein was used as substrate. More conclusive evidence regarding the identity of the two activities came from extensive purification of the kinase-activating factor. The purification procedure as described above is essentially a combination of the method used for heart activating factor (4) and for brain proteinase (6). In a typical purification (Table I), an 85-fold purification was achieved based on the pH 5.1 precipitate fraction. Each fraction was found to contain proteinase activity, and the ratio of the two activities remained remarkably constant throughout the purification (Table I).

When the DEAE-cellulose column eluate (Fraction I, Table I) was passed through Sephadex G-100, activating factor was sharply separated from the main protein peak (Fig. 2). The recovery of activity was 90%. Protein in the peak tube (tube 13, Fig. 2) was estimated to be 23 μg per ml, representing a specific activity of 1.37 x 10⁶ at least a 1300-fold increase over the...
the pH 5.1 precipitate fraction. Calcium-activated proteinase was recovered in the same tubes as activating factor (Fig. 2); the ratio of the two activities remained essentially constant in each fraction (Table II) and were very similar to those obtained in the earlier purification steps.

### Proteinase Activity in Skeletal Muscle and Cardiac Activating Factor Preparations

The above experiments provided evidence that brain kinase-activating factor and calcium-activated proteinase were either identical or remarkably similar. It was of interest to determine whether such similarities existed between the activating factor from skeletal muscle and heart and calcium-activated proteinase. Activating factor from skeletal muscle was conveniently prepared by procedures similar to those for brain (see "Experimental Procedure"), and all fractions were found to contain proteinase activity. Throughout a purification which represented a 20-fold increase in specific activity with respect to kinase-activating factor, the ratios of this activity to proteinase remained quite constant in each fraction (Table III). Although the ratios are somewhat higher than those obtained for brain preparations, we consider these variations to be well within the accuracy of the two assays. Similar ratios were obtained with two heart preparations (Table III), one representing a pH 5.1 precipitate fraction of rabbit myocardium, and the other, a DEAE-cellulose eluate prepared from a bovine heart ammonium sulfate fraction. These data indicate that kinase-activating factor from skeletal muscle and heart may also be equated with calcium-activated proteinase.

### Calcium Lability of Activating Factor

Guroff (6) observed that brain proteinase was destroyed when incubated with Ca++ in the absence of protein substrate. If kinase-activating factor and proteinase were identical, the former activity should also be calcium-labile. When kinase-activating factor from both brain and skeletal muscle was incubated at 30° for 30 min without Ca++ before starting the activation reaction, some loss of activating factor was apparent (Fig. 3, horizontally

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### Table I

**Purification of activating factor from bovine brain**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Activating factor</th>
<th>Proteinase</th>
<th>Ratio of activating factor to proteinase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total units</td>
<td>Specific activity</td>
<td></td>
</tr>
<tr>
<td>pH 5.1 precipitate...</td>
<td>27.5</td>
<td>8.25</td>
<td>1.10</td>
</tr>
<tr>
<td>100,000 × g supernatant.</td>
<td>116</td>
<td>3.94</td>
<td>11.4</td>
</tr>
<tr>
<td>DEAE-cellulose Fraction IV (0.2 M NaCl)...</td>
<td>380</td>
<td>2.74</td>
<td>84.5</td>
</tr>
</tbody>
</table>

* Values have been extrapolated to represent the total volume of the 100,000 × g supernatant fraction.

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### Table II

**Kinase-activating factor and proteinase activity in Sephadex G-100 fractions**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Activating factor</th>
<th>Proteinase</th>
<th>Ratio of activating factor to proteinase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>units/ml × 10⁻²</td>
<td>units/ml</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>3.0</td>
<td>33.2</td>
<td>118</td>
</tr>
<tr>
<td>10</td>
<td>11.1</td>
<td>93.3</td>
<td>120</td>
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<td>11</td>
<td>24.3</td>
<td>146.0</td>
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<td>12</td>
<td>28.1</td>
<td>160.0</td>
<td>172</td>
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<td>13</td>
<td>31.5</td>
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<td>184</td>
</tr>
<tr>
<td>14</td>
<td>28.3</td>
<td>138.0</td>
<td>160</td>
</tr>
<tr>
<td>15</td>
<td>13.1</td>
<td>93.0</td>
<td>140</td>
</tr>
<tr>
<td>16</td>
<td>6.9</td>
<td>53.4</td>
<td>130</td>
</tr>
<tr>
<td>18</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

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### Table III

**Calcium-activated proteinase in activating factor preparations from skeletal muscle and heart**

<table>
<thead>
<tr>
<th>Heart</th>
<th>Skeletal muscle</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

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**Guroff (6) observed that brain proteinase was destroyed when incubated with Ca++ in the absence of protein substrate. If kinase-activating factor and proteinase were identical, the former activity should also be calcium-labile. When kinase-activating factor from both brain and skeletal muscle was incubated at 30° for 30 min without Ca++ before starting the activation reaction, some loss of activating factor was apparent (Fig. 3, horizontally...**
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FIG. 3. Calcium lability of kinase-activating factor. Brain pH 5.1 precipitate fraction (14 µg of protein) and rabbit skeletal activating factor (6.7 µg of protein) were used. The standard activation reaction was modified as follows: open bars, kinase + Ca++, no activating factor; solid bars, standard assay conditions; horizontally hatched bars, activating factor incubated in neutral 15 mM cysteine for 30 min at 30°, then kinase added, followed by CaCl₂; diagonally hatched bars, activating factor incubated for 30 min at 30° in the presence of Ca++, then kinase added. After the usual 4-min activation interval, 5 ml of cold 15 mM neutral cysteine were added, and 0.2-ml aliquots were assayed for kinase in the usual manner at pH 6.8.

TABLE IV

Action of inhibitory factor on proteinase activity

The standard proteinase assay was used, except that brain inhibitory factor was present where indicated. The factor was prepared by fractionating a pH 5.1 supernatant ("Experimental Procedure") with ammonium sulfate between 35 and 60% saturation. The specific activity of inhibitory factor was 3,000 and 4.3 mg of protein were used. In each experiment, 3 mg of activating factor protein were added.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Activating factor added</th>
<th>Proteinase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>units x 10⁻²</td>
<td>units/ml</td>
</tr>
<tr>
<td>Experiment 1: 0-40% ammonium sulfate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bovine brain</td>
<td>13.2</td>
<td>448</td>
</tr>
<tr>
<td>+Inhibitory factor</td>
<td>13.2</td>
<td>44</td>
</tr>
<tr>
<td>Experiment 2</td>
<td>100,000 x g supernatnat</td>
<td>7.6</td>
</tr>
<tr>
<td>+Inhibitory factor</td>
<td>7.6</td>
<td>0.04</td>
</tr>
</tbody>
</table>

hatched bars). When incubated in the presence of Ca⁺⁺, the activating factor underwent a complete loss of activity (Fig. 3, diagonally hatched bars). This loss of activity is quite analogous to that reported by Guroff for the calcium-activated proteinase (6).

Effect of Inhibitory Factor on Proteinase Activity

The foregoing experiments provide evidence that kinase activating factor activity and calcium-activated proteinase reside in the same protein, and that activation of kinase by Ca⁺⁺ is therefore likely proteolytic. Previous experiments (4) have shown that the inhibitory factor antagonized activating factor activity. Inhibitory factor might therefore be expected to inhibit the release of tyrosine-containing material in the protein-assay. To examine this, two preparations of activating factor were assayed for proteolytic activity with casein as the substrate in the presence and absence of rabbit brain inhibitory factor. In each case, proteinase activity was markedly reduced (Table IV), providing evidence that kinase-inhibitory factor may be a proteolytic inhibitor.

Action of Inhibitory Factor on Activation of Kinase by Trypsin

Krebs et al. (7) have shown that trypsin is a powerful activator of phosphorylase kinase. The pattern of trypsin activation was analogous to that of activating factor in that pH 6.8:8.2 activity ratio increased. In view of the foregoing results, it was of interest to determine whether inhibitory factor preparations would prevent activation of kinase by trypsin and, conversely, whether trypsin inhibitor could prevent the action of kinase-activating factor. When nonactivated kinase was incubated with small amounts of trypsin (0.02 µg) in the standard assay, marked activation took place (Fig. 4, Experiment I, Bar B).

FIG. 4. Effect of inhibitory factor on activation of kinase by trypsin, and effect of trypsin inhibitor on activating factor. In Experiment I, the standard activating factor assay was used (20 µg of kinase, 700 pH 8.2 units), except that Ca⁺⁺ was omitted, activation was effected with 0.02 µg of trypsine, and the activation time was 10 min. Bar A, kinase incubated alone; B, plus 0.02 µg of trypsin. For Bars C, D, E, and F, the conditions were the same as for B, except that increasing amounts of heart inhibitory factor were added before trypsin. The following amounts of inhibitory factor protein were used: C, 3.5; D, 8.75; E, 35; F, 350 µg of protein. For Bars G, H, and I, soybean trypsin inhibitor, 0.1, 1.0, and 5.0 µg, respectively, was added instead of inhibitory factor. In Experiment II, brain activating factor (DEAE-cellulose Fraction IV; specific activity, 2.5 x 10⁴) was used (0.6 µg of protein). The following amounts of soybean trypsin inhibitor were added: Bar A, none; B, 5; C, 25; D, 50; E, 200 µg. In Bars F and G, heart inhibitory factor, 8.75 and 17.5 µg, respectively, was used instead of trypsin inhibitor. In both experiments, activation in the absence of inhibitory material was not complete; i.e. activating factor in Experiment II and trypsin in Experiment I were limiting.
Inhibitory factor markedly inhibited the action of trypsin (Bars C to F), 65% inhibition being achieved with 8.75 μg of inhibitor protein. These data provide further evidence that the inhibitory factor is a proteolytic inhibitor. For comparative purposes, the inhibitory action of soybean trypsin inhibitor is also shown (Bars G, H, and I). Soybean trypsin inhibitor was not an effective antagonist of kinase-activating factor (Fig. 4, Experiment II).

Amounts added to the standard assay, ranging from 5 to 50 μg, caused only slight inhibition, and 200 μg produced only 30% reduction in activating factor activity. This is in keeping with the fact that soybean trypsin inhibitor is highly specific for trypsin.

To provide further information on the nature of the action of the inhibitory factor, its effect was examined on trypsin activity when benzoyl-L-arginine ethyl ester was used as substrate, a system in which the hydrolytic action of trypsin could be followed directly. It is apparent from Fig. 5 that inhibitory factor was capable of preventing the action of trypsin on this substrate.

Evidence for Peptide Formation during Activation of Kinase

If indeed the activation of kinase by kinase-activating factor was proteolytic, more conclusive proof would be available if a peptide or peptides could be isolated from the activation reaction mixture. In order to examine this, the activation reaction had to be greatly scaled up. It was found that the amount of kinase and kinase-activating factor could be increased 50-fold in the activation reaction while the calcium acetate concentration was kept constant. Mercaptoethanol (a volatile thiol) could be used in place of cysteine. In the method outlined in "Experimental Procedure," the activation reaction was scaled up 500-fold beyond the standard assay (10-fold increase in volume). After activation, protein was removed by precipitation with trichloroacetic acid, and the presence of ninhydrin-positive material was sought in the acid-soluble fraction following high voltage electrophoresis. At this pH, all ninhydrin-positive material migrated to the positive electrode. All four reaction mixtures (both controls and experimental) contained a spot 3 cm from the origin; Mixtures 2 and 4 contained no other ninhydrin-positive material. Mixtures 1 and 3 (complete systems for both brain and skeletal muscle activating factor) each contained a second, clearly defined spot 10.5 cm from the origin, and faint spots 12.5 and 17.5 cm from the origin. Some streaking occurred between each spot. It was clear that in those tubes containing the complete system, at least three ninhydrin-positive spots were present which were not discernible in the controls in which Ca++ had been added after protein precipitation with trichloroacetic acid. No attempt was made to characterize these products further.

**DISCUSSION**

The results of these experiments are consistent with the identity of kinase-activating factor with a calcium-activated proteinase described by Guroff (6). Kinase-activating factor was purified from both brain and skeletal muscle. Each fraction contained calcium-activated proteinase activity, as determined by the formation of acid-soluble, tyrosine-containing material with casein as substrate. Moreover, the two activities coincided during fractionation on Sephadex G-100. Both activities were destroyed when incubated with Ca++ in the absence of substrate. Acid-soluble, ninhydrin-positive material was formed during the activation of kinase by Ca++ and activating factor preparations purified from both brain and skeletal muscle. It seems reasonable to conclude that the activation of phosphorylase kinase by kinase-activating factor involves proteolysis. Kinase-inhibitory factor prevented proteinase activity and markedly inhibited the activation of phosphorylase kinase by trypsin. It also inhibited the action of trypsin on the synthetic substrate benzoyl-L-arginine ethyl ester. Kinase-inhibitory factor thus appears to be simply a proteolytic inhibitor.

Very little is known about the specificity of this calcium-activated proteinase. In addition to casein, Guroff (6) reported that it attacked the oxidized β chain of insulin. However, a large number of peptides failed to react. We have incubated a variety of purified enzymes with activating factor in the presence and absence of Ca++ under conditions of either the proteinase assay or the kinase activation assay in an attempt to determine whether enzyme activity would be altered. One might expect that if protoclysin were to occur, the action of a particular enzyme might be decreased, rather than increased, as in the case of kinase. The enzymes tested were phosphorylase b, intestinal mucosa adenosine deaminase, ribonuclease, acetylcholinesterase, hexokinase, isocitrate dehydrogenase, alcohol dehydrogenase, glyceraldehyde 3-phosphate dehydrogenase, aldolase, and glucose 6-phosphate dehydrogenase. None of the activities was altered. Very little can be said regarding the specificity of the inhibitory factor except that it antagonizes both the activating factor and trypsin. Whether it is identical with trypsin inhibitor activity, which has been identified in many tissues (12), is not known.
The possibility has been considered that activation of phosphorylase kinase by Ca++ might be a physiologically important process in regulating glycogenolysis during muscle contraction. Thus, Ca++ released from the sarcoplasmic reticulum during excitation could, in addition to initiation of the contractile event, serve to couple metabolic events by activation of phosphorylase kinase with subsequent increased glycogenolysis through the conversion of phosphorylase b to the a form. It seems unlikely now that activation of kinase by the mechanism described here could serve such a physiological role. The present mechanism would mean that the enzyme would be activated by cleavage of peptide bonds and deactivated by resynthesis of such bonds. The activation reaction has not been found to be reversible, however. Such a mechanism would hardly seem adequate to account for the rapid changes in glycogenolytic rate which are known to occur during muscle contraction.

REFERENCES
On the Mechanism of Activation of Phosphorylase \( b \) Kinase by Calcium
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