Isolation and Characterization of a Protein from Rat Testis Which Inhibits Cyclic AMP-dependent Protein Kinase and Phosphodiesterase*

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A total of four chromatographically separable forms of cyclic AMP-dependent protein kinase (EC 2.7.1.37; ATP:protein phosphotransferase) inhibitor protein were found in mature rat testis. The most abundant form (50% of the total activity) in total testis was demonstrated to be the only form present in Sertoli cell enriched testis. Therefore, this form was isolated by conventional techniques with a 13% yield and judged to be homogeneous by polyacrylamide gel electrophoresis under both native and denaturing conditions. Several physical characteristics of the pure inhibitor were then determined. These included Stokes radius of 20.8 Å; a diffusion coefficient of $1.0 \times 10^{-6} \text{ cm}^2 \text{s}^{-1}$; a sedimentation coefficient of 1.2 S and a $f/f_0$ of 1.18. Molecular weight and partial specific volume of 0.72 cm$^3$/g was calculated from the isoelectric pH of 4.4. A molecular weight of 19,100 and a $f/f_0$ of 1.18. Molecular weight was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis was 26,100. Amino acid analysis of the inhibitor revealed 185 residues/mol with an acidic/basic amino acid ratio of 2.3. The protein was devoid of tyrosine but contained 13% proline. This acidic nature was reflected by an isolectric pH of 4.4. A molecular weight of 19,100 and a partial specific volume of 0.72 cm$^3$/g was calculated from the amino acid composition. The pure inhibitor had an $E_{1/2}$ nm of 7.6. Circular dichroism indicated a tertiary structure containing approximately 20% $\alpha$-helix, and 80% random coil, but the absence of $\beta$-pleated sheet. The estimated intracellular concentration of the protein kinase inhibitor was 0.3 μM and the $K_i$ was 11 μM. The homogenous inhibitor also specifically inhibits testicular cyclic AMP phosphodiesterase with an apparent $K_i$ of 20 μM, indicating that this protein may have multiple roles in the cell.

Walsh et al. (1) partially purified and characterized a heat-stable, low molecular weight protein from rabbit skeletal muscle which specifically inhibits cyclic AMP-dependent protein kinase. Since protein kinase mediates many of the effects of cyclic AMP the inhibitor is potentially an important regulator of the cyclic AMP-protein kinase system (2). In fact, evidence from several laboratories has suggested that this inhibitor has a regulatory function in heart (3), adipose tissue (4), pancreas (5), and perhaps other tissues (6). Recent evidence from our laboratory has suggested a regulatory role of protein kinase inhibitor in the rat tests; significant changes in the specific activity of the inhibitor were observed following hypophysectomy and subsequent treatment with follicle stimulating hormone (7). The inhibitor has not previously been purified to homogeneity and thus precise characterization of the inhibitor mechanism has not been possible. This report describes the purification to apparent homogeneity and the characterization of protein kinase inhibitor from rat testis. In addition, several physical properties of the purified protein have been determined.

**EXPERIMENTAL PROCEDURES**

**Materials**

ATP, cAMP, total histone (type II-A), bovine serum albumin (Fraction V), and 3-(N-morpholino)propanesulfonic acid, 1,4-piperazinediethanesulfonic acid, and 2-(N-morpholino)ethanesulfonic acid buffers were purchased from Sigma. Standard proteins for gel filtration were purchased from Schwarz/Mann. Sodium [32P]orthophosphate (carrier-free), [3H]cAMP (40 Ci/mmol), and [3H]water (1.9 × 10^6 dpm/ml) were purchased from New England Nuclear. DEAE-cellulose (DE52) and phosphocellulose (P-11) were from Whatman. Materials for polyacrylamide gel electrophoresis, AG 2-X8 anion exchange resin (200 to 400 mesh), and hydroxyapatite (Bio-Gel HTP) were obtained from Bio-Rad. Quaternary aminomethyl (QAE)-Sephadex (A-25) was obtained from Pharmacia. Crotalus atrox venom was purchased from Ross Allen's Reptile Institute, Inc. (Silver Springs, Fla.). All other chemicals were of analytical grade.

**Tissue**

Pregnant Sprague-Dawley rats were obtained from Holtzman Co. (Madison). The animals were maintained in our own animal care facilities and received water and Purina Lab Chow ad libitum. Male offspring with Sertoli cell enriched testes were prepared as described previously by fetal irradiation in utero (8). Animals were killed by cervical dislocation at 30 to 50 days of age and their testes removed. Testes were stored in a liquid nitrogen freezer. Frozen normal rat testes were purchased from Dr. A. Parlow, University of California at Los Angeles. Testes were stored in a liquid nitrogen freezer.

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Assay of Protein Kinase Inhibitor Assay

A cytosol (105,000 × g supernatant) was prepared from 2 g of rat testes using 6 volumes of a homogenization buffer (Buffer A) containing 10 mM imidazole, 1 mM disodium EDTA, pH 7.0. Homogenization was accomplished with a Polytron homogenizer equipped with a PT-10 generator. The tissue was disrupted for 30 s at a setting of 6.0 and centrifuged 1 h at 40,000 rpm in a Beckman type 65 rotor. The supernatant fluid was adjusted to 1 mM with EGTA and chromatographed on an Ultrogel ACA 44 column (2.5 × 90 cm). The column was eluted with a buffer containing 10 mM imidazole, 100 mM NaCl, and 1 mM EGTA, pH 7.0, at a flow rate of 16 ml/h. All operations were performed at 0–5°C. Fractions containing phosphodiesterase activity were pooled and stored at −5°C until used. This preparation maintained activity for at least 1 month under these conditions.

Purification of Protein Kinase Inhibitor

The initial steps of purification were carried out by modification of the procedures described by Walsh et al. (1).

Step 1: Homogenization—One kilogram of frozen whole rat testes was thawed and placed in 1 liter of ice-cold 1 mM EDTA, pH 7.0. Homogenization was performed in a 4-liter beaker using a Polytron homogenizer (Brinkmann) (setting = 6.0) equipped with a PT-65ST generator. The tissue was homogenized in three 1-min periods with 5-min cooling intervals to maintain temperature at 2–4°C.

Step 2: Heat Treatment and Acid Precipitation—The homogenate (15-m1 portions) in 30-ml corex test tubes was placed in a boiling water bath for 5 min until the homogenate temperature rose to 95°C. The homogenate was cooled to 4°C by immersion in an ice slush. Precipitated material was removed by centrifugation for 15 min at 12,000 rpm in a JA-14 rotor (Beckman). The pH of the heat-treated extract was adjusted to 4.0 by the addition of glacial acetic acid and this suspension was stirred 1 h at 4°C. The resulting precipitate was removed by centrifugation as described above. An insoluble "bead" of lyophilized material was removed by filtering the extract through glass wool. The pH was adjusted to 6.0 by the addition of 1 mM Tris base. Solid ammonium sulfate was added to a final saturation of 90%. After stirring 1 h in the cold, the precipitate was harvested by centrifugation as described above and the supernatant was discarded. The precipitate was dissolved in 10 mM imidazole, 1 mM EDTA, pH 6.2 (Buffer B) and dialyzed against 4 liters of the same buffer with two buffer changes at 12-h intervals.

Step 3: DEAE-cellulose Chromatography—The sample from Step 2 was applied to a DEAE-cellulose column (1.5 × 50 cm) equilibrated with Buffer B. The column was washed with 300 ml of the same buffer and eluted with 1 liter of a linear NaCl gradient (0 to 300 mM) in Buffer B. The flow rate was maintained at 35 ml/h. The 5-m1 fractions were collected and assayed for inhibitor activity. The fractions containing inhibitor activity (peak at 25 mM NaCl) were combined. The combined fractions were dried and redissolved in 18 ml of distilled water and lyophilized to dryness.

Step 4: Gel Filtration Chromatography—The lyophilized sample was dissolved in 10 ml of Buffer C containing 100 mM NaCl and 10% glycerol. This sample was chromatographed on a Ultrogel AcA 44 column (2.5 × 90 cm). The column was developed with Buffer C containing 100 mM NaCl at 16 ml/h. The peak fractions were dialyzed against 40 volumes of 5 mM potassium phosphate, pH 7.2 (Buffer C), for Step 5.

Step 5: Hydroxylapatite Chromatography—The Step 4 sample was applied to an hydroxylapatite column (1.5 × 20 cm). The column was developed with Buffer C containing 100 mM NaCl at 16 ml/h. The peak fractions were dialyzed against 40 volumes of 5 mM potassium phosphate, pH 7.2 (Buffer C), for Step 6.

Preparation of Testis cAMP Phosphodiesterase

Rats with Sertoli cell enriched testes were killed by cervical dislocation and their testes were excised. One gram of testes was homogenized in 1 ml of 10 mM Tris/HCl, 7 mM 2-mercaptoethanol, pH 7.8, with a Polytron homogenizer equipped with a PT-10 generator. The tissue was disrupted 30 s at a setting of 6.0 and centrifuged 1 h at 40,000 rpm in a Beckman type 65 rotor. The supernatant fluid was adjusted to 1 mM with EGTA and chromatographed on an Ultrogel ACA 44 column (2.5 × 90 cm). The column was eluted with a buffer containing 10 mM imidazole, 100 mM NaCl, and 1 mM EGTA, pH 7.0, at a flow rate of 16 ml/h. All operations were performed at 0–5°C. Fractions containing phosphodiesterase activity were pooled and stored at −5°C until used. This preparation maintained activity for at least 1 month under these conditions.

Preparation of cAMP dependent Protein Kinase Inhibitor

Protein kinase inhibitor was assayed by a modification of the method of Walsh et al. (1). The activity of a standard preparation of cyclic AMP-dependent protein kinase was determined in the presence and absence of test samples. One unit of protein kinase will catalyze the incorporation of 1 pmol of phosphate into histone/min. One unit of inhibitor activity is defined as that amount which would inhibit 1 unit of protein kinase.

The reaction mixture contained 50 mM 1,4-piperazine-dithanesulfonic acid, pH 7.0, 0.2 mM EDTA, 0.3 mM EGTA, 13 mM magnesium acetate, 1 mM dithiothreitol, 75 mM NaCl, 1 mCAMP, 0.3 mg/ml of mixed histone (Sigma Type II), 7 units of protein kinase, testis extract containing 1 to 10 units of protein kinase inhibitor, and 2 × 10^−6 M (γ-32P)ATP (20 to 200 cpm/pM). The total reaction volume was 75 µl. Reactions were initiated by the addition of (γ-32P)ATP in 5-µl volumes. The reaction mixtures were incubated at 30°C for 30 min and were terminated by pipetting 50 µl aliquots onto squares (0.5 × 2 cm) of filter paper (Whatman ET-311) which were immediately immersed in ice-cold 10% trichloroacetic acid. The filters were washed as described by Warna et al. (12), dried, and radioactivity determined with Liquifluor-toluene (New England Nuclear) scintillation mixture in a Beckman LS-280 scintillation spectrometer.

Assay of cAMP Phosphodiesterase

Phosphodiesterase activity was determined by the method of Thompson and Appleman (13) as modified by Dedman et al. (14). The standard reaction mixture contained 50 mM 3-(N-morpholino)propanesulfonic acid (pH 7.0), 5 mM magnesium acetate, 10 mM 2-mercaptoethanol, 0.1 mM CaCl₂, and 10^−5 M [32P]ATP (20 to 200 cpm/pM). Samples containing phosphodiesterase were added and the reactions were initiated by the addition of [32P]ATP in a volume of 20 µl which resulted in a final reaction volume of 200 µl. Following incubation at 30°C for 50 min, the reactions were terminated by the addition of 200 µl of a solution containing 10 mM cAMP and 20 mM EDTA, 25 µl of (0.5 mg/ml) snake venom (C. atrox), and 100 µl of 10 mM adenosine. This mixture was incubated for 20 min at 25°C. Unhydrolyzed cAMP was removed by the addition of 1.0 ml of settled anion exchange resin (Bio-Rad AG 2-X8, 200 to 400 mesh), equilibrated with 40 mM Tris/HCl, pH 7.0. Following vigorous vortexing (twice), this mixture was centrifuged at 3000 rpm for 5 min in a Sorvall General Laboratory Centrifuge. Radioactivity was assayed in 100-µl aliquots of the supernatant fluid using 10% BBS-3 (Beckman) in Liquifluor/toluene (New England Nuclear) as the scintillant. The recovery of reaction product ([32P]adenosine) was determined using [32P]adenosine as tracer. Results were corrected for adenosine recovery and expressed as picomoles of cAMP hydrolyzed per min. Phosphodiesterase concentrations in the assay were adjusted to give reaction rates that were linear for the duration of the incubation under the conditions utilized.

1 The abbreviations used are: EGTA, ethylene glycol bis(β-aminoethyl ether) N,N ′-tetraacetic acid; K_{app}, apparent inhibition constant.

Polyacrylamide Gel Electrophoresis

Polyacrylamide gels (0.5 × 5 cm) were prepared in glass columns as electrophoresed as described by O'Kane (15) and Davis (16). These non-denaturing gels were 12% acrylamide and 0.33% bisacryl-
amide. Sodium dodecyl sulfate gels were prepared as described by Laemmli (17) at a concentration of 15% acrylamide and 0.4% bisacrylamide. The dye front (bromphenol blue) was permanently marked with a piece of copper wire to compensate for gel shrinkage during soaking and destaining steps. Molecular weight standards used for sodium dodecyl sulfate gels were: ovalbumin (43,000), aldolase (40,000), lactate dehydrogenase (36,000), chymotrypsinogen (25,700), trypsin (25,000), soybean trypsin inhibitor (22,500), avidin (18,000), myoglobin (17,200), /-lactoglobulin (17,500), hemoglobin (15,900), ribonuclease A (13,700), and cytochrome c (12,400). A plot of log M, versus relative mobility was constructed and the "best fit" line was determined by the method of least squares.

**Gel Filtration Chromatography**

The Ultrogel AcA 44 column described in Step 4 of the purification procedure was calibrated with proteins of known molecular weight and Stokes radii. Samples were dissolved in 10 ml of Buffer B containing 10% glycerol. Elution conditions were identical with those described above. The Stokes radius of the inhibitor was determined as described by Siegel and Amos (18). Molecular weight was estimated by the method of Whitaker (19). The standard proteins and their respective Stokes radii were: (A) ovalbumin, 27.3 A; (B) bovine hemoglobin, 24.0 A; (C) chymotrypsinogen, 22.4 A; (D) myoglobin, 20.7 A; (E) ribonuclease A, 19.2 A; and (F) cytochrome c, 16.4 A. Hemoglobin and cytochrome c were located by measuring the absorbance at 410 nm. The remaining proteins were measured at 230 nm. Dextran blue and 3Hwater were used to determine void and included volumes, respectively.

**Sucrose Gradient Centrifugation**

The sedimentation coefficient was determined by sucrose gradient centrifugation as described by Martin and Ames (20). Protein kinase inhibitor was mixed with 400 /g each of cytochrome c (1.7 S) and hemoglobin (2.8 S) in 150 /l of Buffer B containing 100 mM NaCl. This sample was layered onto the surface of a linear gradient of 5 to 20% sucrose in the same buffer. The gradient was centrifuged 43 h at 48,000 rpm in an SW 50.1 rotor (Beckman) at 5° and was fractionated into 28 fractions using a Densflo-HC fractionator (Buchler Instruments). Standard proteins were located by measuring optical density at 410 nm. Inhibitor was assayed as described above.

**Amino Acid Analysis of Protein Kinase Inhibitor**

Amino acid analysis of the protein kinase inhibitor was performed on samples hydrolyzed for 22, 48, and 72 h in 6 N HCl at 110°. Analyses were obtained with a Beckman 117 amino acid analyzer. Tryptophan was determined by the ultraviolet absorbance method of Edelhoch (21). Inhibitor was dissolved in 0.1 mM EDTA, pH 7.0, and its amino acid composition revealed a partial specific activity peak, normal mature testes have three additional peaks (at 110 mM, 160 mM, and 230 mM NaCl) which together constitute 40 to 50% of the total inhibitor activity. In this communication we have restricted our purification and characterization to the major (Sertoli cell) inhibitor activity.

**Purification of Testis Protein Kinase Inhibitor**

The purified inhibitor was purified by the steps described in "Experimental Procedures." A summary of a typical purification procedure is shown in Table I. A total of 800 /g of protein was recovered at a yield of 13% from 1 kg of rat testis. Recovery was calculated for the major inhibitor peak only. The purity of the preparation was assessed by polyacrylamide gel electrophoresis under nondenaturing conditions and in the presence of sodium dodecyl sulfate. The stained polyacrylamide gels shown in Fig. 2 illustrate the size and charge homogeneity of the inhibitor preparation. No minor bands were detected on either type of gel, demonstrating that the protein kinase inhibitor is homogeneous by these criteria.

**Amino Acid Composition**

The amino acid composition of the protein kinase inhibitor was determined as described under "Experimental Procedures" and the results are shown in Table II. A total of 43 acidic residues but only 19 basic residues were present per mol of protein, indicating that the inhibitor is a highly acidic protein. It should also be noted that the inhibitor has 24 residues of proline but is devoid of tyrosine.

**Physical Properties**

Several physical properties of the testicular protein kinase inhibitor are shown in Table III. The experimental details are described under "Experimental Procedures." A Stokes radius of 20.8 A, diffusion coefficient of 1.0 × 10^5 cm^2/s, and M, = 21,400 were calculated from gel filtration data. The inhibitor sedimented at 1.2 S in sucrose gradients. The amino acid composition revealed a partial specific volume of 0.72 cm^3/g (23), and an anhydrous M, = 19,700. A frictional ratio of 1.17 was calculated from these latter two values and from the Stokes radius suggesting that the inhibi

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**RESULTS**

**Comparison of Protein Kinase Inhibitor in Normal and Sertoli Cell Enriched Testes**

Fig. 1 depicts the DEAE-cellulose column elution profiles of Sertoli cell enriched (A) and normal testes (B) extracts. The major inhibitor activity in both profiles is in a peak eluting at 20 to 40 mM NaCl. Whereas the Sertoli cell enriched extract appeared to contain a single activity peak, normal mature testes have three additional peaks (at 110 mM, 160 mM, and 230 mM NaCl) which together constitute 40 to 50% of the total inhibitor activity. In

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**Fig. 1.** DEAE-cellulose chromatography of Sertoli cell enriched (A) and normal (B) testes protein kinase inhibitor. A. Inhibitor was purified through Step 2 from 10 g of Sertoli cell enriched testis and applied to a DEAE-cellulose column (1.0 × 6.0 cm). The column was washed with 25 ml of Buffer B and eluted with a 100-mM linear NaCl gradient (0 to 400 mM) in Buffer B. The 2-ml fractions were collected and assayed for inhibitor activity. B, inhibitor was purified from 1 kg of normal rat testes and chromographed on DEAE-cellulose as described under "Experimental Procedures." The NaCl concentration was determined by conductivity measurements.
Inhibition of cAMP Phosphodiesterase by Testicular Protein Kinase Inhibitor—In other studies ongoing in our laboratory we noted that heat-treated cytosols from rat testes would inhibit cAMP phosphodiesterase from the same tissue. We subsequently found that such cytosol contains two major protein components: the calcium-dependent regulatory protein inhibitor (7). Therefore, we surmised that the phosphodiesterase inhibition might be the result of the protein kinase inhibitor. In order to test this possibility, cAMP phosphodiesterase from testis was partially purified by gel filtration as described under "Experimental Procedures." Activity measurements represent the major or 30 mM NaCl form of the enzyme.

Inhibition of cAMP phosphodiesterase by pure inhibitor—In other studies ongoing in our laboratory we noted that heat-treated cytosols from rat testes would inhibit cAMP phosphodiesterase from the same tissue. We subsequently found that such cytosol contained two major protein components: calcium-dependent regulatory protein (26) which activates phosphodiesterase and protein kinase inhibitor (7). Therefore, we surmised that the phosphodiesterase inhibition might be the result of the protein kinase inhibitor. In order to test this possibility, cAMP phosphodiesterase from testis was partially purified by gel filtration as described under "Experimental Procedures" to remove endogenous inhibitor. cAMP hydrolysis by this enzyme preparation was then measured in the presence of various concentrations of protein kinase inhibitor. The results presented in Fig. 4 demonstrate a concentration-dependent inhibition of enzyme activity. The \( K_{\text{m}} \) of this inhibition was \( 2 \times 10^{-8} \text{ M} \) estimated from the inhibitor concentration necessary for 50% inhibition of the phosphodiesterase. These data show that in addition to its well

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**Table I**

<table>
<thead>
<tr>
<th>Step</th>
<th>Procedure</th>
<th>Protein</th>
<th>Total protein kinase inhibitor</th>
<th>Specific activity</th>
<th>Yield</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Crude homogenate</td>
<td>90,700</td>
<td>( 8.5 \times 10^9 )</td>
<td>9.4</td>
<td>72</td>
<td>9.4</td>
</tr>
<tr>
<td>2</td>
<td>Heat-acid treatment</td>
<td>1,250</td>
<td>( 8.5 \times 10^9 )</td>
<td>680</td>
<td>100</td>
<td>72</td>
</tr>
<tr>
<td>3</td>
<td>DEAE-cellulose</td>
<td>110</td>
<td>( 3.9 \times 10^9 )</td>
<td>3,550</td>
<td>46</td>
<td>380</td>
</tr>
<tr>
<td>4</td>
<td>Ultrogel AcA 44</td>
<td>16</td>
<td>( 1.4 \times 10^8 )</td>
<td>8,760</td>
<td>16</td>
<td>920</td>
</tr>
<tr>
<td>5</td>
<td>Hydroxylapatite</td>
<td>0.8</td>
<td>( 1.1 \times 10^9 )</td>
<td>138,000</td>
<td>13</td>
<td>14,700</td>
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**Table II**

Amino acid composition of protein kinase inhibitor

<table>
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<tr>
<th>Residue</th>
<th>Molar composition</th>
<th>22 h</th>
<th>48 h</th>
<th>72 h</th>
<th>Residues/molecule*</th>
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<tr>
<td></td>
<td></td>
<td>%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asx</td>
<td>10.5</td>
<td>10.5</td>
<td>9.98</td>
<td>19</td>
<td></td>
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<tr>
<td>Glx</td>
<td>12.9</td>
<td>13.2</td>
<td>14.5</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>Threonine</td>
<td>4.47</td>
<td>4.10</td>
<td>4.30</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Serine</td>
<td>11.9</td>
<td>11.1</td>
<td>9.82</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>Proline</td>
<td>12.9</td>
<td>12.6</td>
<td>14.4</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>Glycine</td>
<td>8.72</td>
<td>9.17</td>
<td>8.70</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>Alanine</td>
<td>10.2</td>
<td>10.8</td>
<td>10.2</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>Valine</td>
<td>7.37</td>
<td>7.15</td>
<td>8.03</td>
<td>14</td>
<td></td>
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<tr>
<td>Methionine</td>
<td>0.52</td>
<td>0.57</td>
<td>0.57</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Isoleucine</td>
<td>1.33</td>
<td>1.49</td>
<td>1.43</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Leucine</td>
<td>5.61</td>
<td>5.11</td>
<td>4.78</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Tyrosine</td>
<td>12.2</td>
<td>11.9</td>
<td>12.2</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Tryptophan*</td>
<td>2.87</td>
<td>2.36</td>
<td>2.96</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>4.93</td>
<td>4.78</td>
<td>4.73</td>
<td>9</td>
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<tr>
<td>Lysine</td>
<td>1.49</td>
<td>1.43</td>
<td>1.43</td>
<td>3</td>
<td></td>
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<tr>
<td>Histidine</td>
<td>1.24</td>
<td>1.22</td>
<td>1.14</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Arginine</td>
<td>4.35</td>
<td>4.58</td>
<td>4.31</td>
<td>8</td>
<td></td>
</tr>
</tbody>
</table>

* Percentages of molar compositions were extrapolated to zero hydrolysis time. Protein kinase inhibitor was assumed to have 1 residue of methionine.

* Tryptophan was determined by the method of Edelhoch (21).
Kinase inhibitor protein from the rat testis and offer the first
documentation of protein kinase inhibitory activity, the inhibitor
can also inhibit cAMP phosphodiesterase in testis.

**DISCUSSION**

We report here the purification to homogeneity of a protein kinase inhibitor protein from the rat testis and offer the first suggestive evidence of multiple actions of this molecule. Physical characterization of the inhibitor revealed that it is an acidic protein with a molecular weight of approximately 20,000. Although it is slightly smaller (M₉ = 20,000 versus 26,000) and sediments somewhat slower (1.2 S versus 1.5 S) than the rabbit skeletal muscle protein described by Walsh et al. (1), it is clearly an analogous protein. The existence of several possible explanations for this apparent discrepancy may be suggested. First, the Kᵢ was measured at a protein kinase concentration which is approximately 250-fold lower than that present in the intact tissue. When the total rather than the free (which cannot as yet be measured) concentration of inhibitor is considered, the Kᵢ increases as a function of protein kinase concentration (27, 28). A second possible explanation might be that a substantial amount of the purified preparation may have lost activity during the course of its purification. Additional possible explanations include the existence of modulators which directly affect inhibitor activity (no evidence exists for this at the present time), intracellular compartmentalization of the inhibitor and the protein kinases (both are present in particulate fractions of the testis), and multiple forms of the inhibitor (Fig. 1) has not been described prior to this report. Evidence presented here is compatible with the idea that cell-specific isoforms are present in the testis. It will be interesting to determine if the multiple forms are covalent modifications of single protein or if they are completely different molecules and therefore might inhibit by separate mechanisms. Indeed, using partially purified preparations, Ashby and Walsh demonstrated that the inhibitor from skeletal muscle interacts specifically with the free catalytic subunit of cAMP-dependent protein kinase in a noncompetitive manner (27, 28). With homogeneous preparations of inhibitor it will be possible to extend these studies in order to gain a more complete understanding of the mechanism of inhibition and whether the multiple forms have different or similar mechanisms of action.

The studies reported herein suggest that the testicular protein is a very potent inhibitor of protein kinase having a Kᵢ of 11 nM (Fig. 5). Moreover, it can be calculated from the purification data (Table I) that the intracellular concentration of the inhibitor is approximately 0.3 μM. The cAMP-dependent protein kinase concentration of rat testis is approximately equivalent to rat heart which has been estimated at 0.2 μM by Beavo et al. (6). It would thus appear that testis contains sufficient inhibitor to inactivate all of the endogenous protein kinase, a condition which obviously does not occur in vivo. Several possible explanations for this apparent discrepancy may be suggested. First, the Kᵢ was measured at a protein kinase concentration which is approximately 250-fold lower than that present in the intact tissue. When the total rather than the free (which cannot as yet be measured) concentration of inhibitor is considered, the Kᵢ increases as a function of protein kinase concentration (27, 28). A second possible explanation might be that a substantial amount of the purified preparation may have lost activity during the course of its purification. Additional possible explanations include the existence of modulators which directly affect inhibitor activity (no evidence exists for this at the present time), intracellular compartmentalization of the inhibitor and the protein kinases (both are present in particulate fractions of the testis), and

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**Table III**

**Properties of testis protein kinase inhibitor**

<table>
<thead>
<tr>
<th>Property</th>
<th>Procedure</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stokes radius (Rₛ) (cm)</td>
<td>Gel filtration</td>
<td>20.8 x 10⁻⁶</td>
</tr>
<tr>
<td>Diffusion coefficient (D) (cm²/s)</td>
<td>Gel filtration</td>
<td>1.0 x 10⁻⁶</td>
</tr>
<tr>
<td>Sedimentation coefficient (s)</td>
<td>Sucrose gradient</td>
<td>1.2 x 10⁻¹³</td>
</tr>
<tr>
<td>Partial specific volume (Ω) (cm³/g)</td>
<td>Amino acid composition</td>
<td>0.72</td>
</tr>
<tr>
<td>Molecular weight (Mᵦ)</td>
<td>Sodium dodecyl sulfate-gel electrophoresis</td>
<td>26,100</td>
</tr>
<tr>
<td>Frictional ratio (f₀/fᵢ)</td>
<td>Calculated from Rₛ, Mᵦ, Ω</td>
<td>1.17</td>
</tr>
<tr>
<td>Isoelectric point (pI)</td>
<td>Isoelectric focusing</td>
<td>pH 4.4</td>
</tr>
<tr>
<td>Eₘₜₜₜ (mg⁻¹·cm⁻³)</td>
<td>Absorbance at 280 nm</td>
<td>0.76</td>
</tr>
<tr>
<td>Secondary structure</td>
<td>Circular dichroism</td>
<td>-20%</td>
</tr>
<tr>
<td>α Helix</td>
<td>None</td>
<td>-80%</td>
</tr>
<tr>
<td>β-pleated sheet</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>Random coil</td>
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<td></td>
</tr>
</tbody>
</table>

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**FIG. 4.** Inhibition of testis cAMP phosphodiesterase by protein kinase inhibitor. cAMP phosphodiesterase from testis was prepared and the hydrolysis of cAMP was measured as described under "Experimental Procedures" in the presence of increasing concentrations of inhibitor. The results are plotted on a log-logit scale as in the inset of Fig. 3. The initial Ca²⁺-dependent cAMP phosphodiesterase activity in the absence of inhibitor was 0.29 pmol of cAMP hydrolyzed/min.

Fig. 5. Inhibition of testis CAMP phosphodiesterase by protein kinase inhibitor (PKI). The activity of a standard preparation of protein kinase (normally used for inhibitor assay) was measured in the presence of increasing concentrations of inhibitor. The results are plotted on a log-logit scale as in the inset of Fig. 3. The initial Ca²⁺-dependent CAMP phosphodiesterase activity in the absence of inhibitor was 0.29 pmol of CAMP hydrolyzed/min.
finally, the inhibitor might have multiple functions in the testis as is discussed below. Thus, binding to molecules other than protein kinase might effectively lower the concentration of free inhibitor increasing $K_{inw}$.

During the course of the present investigation we discovered that the inhibitor also would inhibit a cAMP phosphodiesterase in rat testis (Fig. 4). The enzyme is insensitive to inhibition in an unfractionated state (cytosol). However, following gel filtration in the presence of EGTA its sensitivity to inhibitor is greatly enhanced. This inhibition is restricted to a Ca$_2^+$ dependent form of testis phosphodiesterase. The $K_{iapp}$ of phosphodiesterase inhibition was $2 \times 10^{-8}$ M. It is impossible to directly relate this to the $K_{iapp}$ in vivo since the intracellular concentration of the phosphodiesterase is unknown. Protein kinase inhibitor could serve to regulate the R-CAMP/catalytic subunit ratio through protein kinase catalytic subunit and phosphodiesterase inhibition. Whether the inhibition of phosphodiesterase occurs in vivo remains to be determined. Indeed, we must assess the physiological significance and mechanism of the "sensitization" by EGTA. It may be that physiological stimuli such as hormones might decrease the intracellular concentration of free calcium (29). In that sense, EGTA might mimic such stimuli. The inhibitor could then block the reactivation by calcium after the stimulus is removed. Nonetheless, this is an intriguing observation and suggests for the first time that inhibitor may have multiple functions.

The physiological role of protein kinase inhibitor has not as yet been firmly established. Current evidence suggests that it may serve a regulatory role associated with hormone action (3-5, 7). It is our hope that through the use of pure inhibitor the present study can be extended to delineate its function in the testis.

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REFERENCES
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