Preparation and Properties of 5'-Nucleotidase from Smooth Muscle of Small Intestine*

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SUMMARY

5'-Nucleotidase prepared from smooth muscle is strongly inhibited by ADP. This inhibition is much stronger than the previously described inhibition of 5'-nucleotidase by ATP. The α,β-methylene phosphonate analogue of ADP is an exceptionally powerful inhibitor of the enzyme. The enzyme possesses three pH optima. The lowest of these optima is observed only when the enzyme is not inhibited by buffer anions; the highest is observed only in the presence of magnesium ions.

METHODS

Small intestines of pig were obtained from Trelegan Wholesale Meat Company, Cambridge, Massachusetts. Purified enzymes were obtained from Boehringer-Mannheim, except for hexokinase which was purchased from Sigma. Lactate dehydrogenase was a gift from F. Stolzenbach. Crystalline bovine serum albumin was obtained from Armour Pharmaceutical. Nucleosides and nucleotides were purchased from P-L Biochemicals except as noted. They were dissolved in water, neutralized with NaOH or Tris base, and standardized spectrophotometrically. The extinction coefficients used were those recommended in Reference 14. Stock solutions were stored frozen at -20°C. DPNH solutions were prepared on the day of use and kept on ice. The mixed isomers of 2'- and 3'-AMP and glucose 1-phosphate were purchased from Sigma. 5'-AMP methyl ester was a gift from Dr. F. Cramer of the Institut für Organische Chemie der Technische Hochschule, Darmstadt, Germany. 3-β-D-Ribofuranosyladenine 5'-monophosphate (isoAMP) and the analogous isoATP were the gift of Dr. N. L. Leonard of the University of Illinois. Adenosine-5'-α,β-methylene triphosphonate and other phosphonate nucleotide analogues were purchased from Miles Laboratories Inc., Eckhart, Indiana. 32P-AMP was obtained from Schwarz BioResearch, and 3H-AMP, from International Chemical and Nuclear Corporation, Burbank, California, and purified as described previously (1). All other substances were of reagent grade or were recrystallized twice before use.

5'-Nucleotidase Assays

Three assays were used for 5'-nucleotidase. In Assay 1 the adenosine formed in the reaction was converted to inosine with the aid of adenosine deaminase. In Assay 2 the orthophosphate formed in the reaction was determined by the method of Morszky, Pettinati, and Kolman (15). Assay 3 involved the use of [3P]labeled AMP and the extraction of the orthophosphate liberated in the reaction by the method of Berenblum and Chain (16), followed by counting of the extract (17).

Assay 1—Reaction mixtures contained buffer consisting of 45 mM 2-(N-morpholino)ethane sulfonic acid neutralized to pH 6.5 with Tris base (hereafter referred to as MES-Tris buffer),

1 The abbreviations used are: isoATP and isoAMP, 3-β-D-ribofuranosyladenine 5'-triphosphate and 5'-monophosphate, respectively; MES, 2-(N-morpholino)ethane sulfonate; AOPCP, α,β-methylene adenosine 5'-diphosphates; AOPCP, α,β-methylene adenosine 5'-triphosphate; AOPCP, α,β-methylene guanosine 5'-triphosphate.
100 &mu;M AMP, 3.3 &mu;g of adenosine deaminase, and 5'-nucleotidase, in a final volume of 3 ml. The reaction, which was started by addition of AMP, was run at 24° in a water-jacketed cuvette. The change in absorbance at 262.5 nm was followed in a Zeiss PMQ II spectrophotometer linked to a Beckman model 1005 strip chart recorder (AE_{262.5} = 0.2 \times 10^6). A set of 17 identical reactions varied &pm; 4% in velocity. The reaction proceeded at a nearly constant rate, which diminished slightly as substrate was consumed. When inhibition of adenosine deaminase by constituents of the reaction mixture was suspected (for example by divalent cations), the activity of adenosine deaminase was assayed by adding adenosine after the 5'-nucleotidase reaction had reached completion.

Determination of the K_m for AMP of 5'-nucleotidase, and other kinetic work, necessitated following changes in AMP concentration in the range of 0 to 20 &mu;M. In these experiments the reaction volume was increased to 15 ml and the reaction was followed in cuvettes with a light path of 5 cm in a Cary model 14 recording spectrophotometer. In a modification of Assay 1, which will be referred to as Assay 1a, the reaction was allowed to proceed until the AMP was exhausted. The concentration of AMP was determined at suitable intervals from the absorbance shown on the chart recording, and the corresponding reaction velocity was determined from the slope of the curve at the same point. The final absorbance of the completed reaction appeared on the same trace and served as an internal reference. The standard error of 10 reaction velocity versus substrate concentration points obtained in this manner was considerably less than that obtained from 10 separate incubations started at different AMP concentrations.

Assay 2—Reaction mixtures contained 45 mM MES-Tris buffer, pH 6.5, substrate as indicated, and enzyme, less than 2 &mu;g of protein per ml, in a final volume of 2 ml. The reaction was started by addition of substrate and was run at 24°. It was stopped by addition of 1 ml of 2.5 N NaOH. This was followed by 3 ml of isobutyl alcohol-benzene (thiophene-free) (1:1, by volume), and 0.6 ml of 25 mM ammonium molybdate. The mixture was shaken vigorously on a Vortex mixer for 15 sec and was then centrifuged in a clinical centrifuge for 10 min. Two milliliters of the organic phase were added to 1 ml of dry isobutyl alcohol-benzene (1:1, by volume), and the absorbance at 313 nm was measured immediately. A constant molar absorbance of 1.9 \times 10^4 was obtained over the range 0.01 to 0.1 &mu;mol of phosphate. Thus the method of Moresky et al. (15) is about 5 times as sensitive as the method of Fiske and SubbaRow (18), as modified by Jones and Spector (19). Blanks were run without substrate. In addition, “zero time” blanks were run for each substrate, to correct for phosphate contamination of reagents. Since enzyme preparations of high specific activity were used, deproteinization was found to be unnecessary.

Assay 3—This was similar to Assay 2, except that ^32P-labeled AMP was used, and that the organic phase was assayed for radioactivity.

Myokinase Assay

Formation of ADP from ATP and AMP was measured by the method of Lamprecht and Trautschold (20).

p-Nitrophenylphosphatase Assay

Reaction mixtures contained 45 mM Tris-HCl buffer, pH 8.0, enzyme, and 100 &mu;M p-nitrophenylphosphate, in a final volume of 3 ml. Reactions were started by addition of p-nitrophenylphosphate, and run at 24°. The reaction was followed continuously at 410 nm with a 1-cm light path (E_{410} = 1.8 \times 10^4) (21).

Reaction mixtures in the pH range of 5.2 to 6.5 contained 45 mM MES-Tris buffer, enzyme, and 138 &mu;M p-nitrophenylphosphate, in a volume of 2.5 ml. Reactions were started by addition of p-nitrophenylphosphate, and were run at 24° without recording the absorbance. After 30 min the mixture was adjusted quickly to pH 8.7 with 0.5 ml of 2 M Tris base, and the absorbance at 410 nm was recorded continuously from a time about 15 sec after addition of base. The alkaline hydrolysis curve was extrapolated to the time of adding Tris base and the amount of enzymatic hydrolysis under acidic conditions was obtained by difference. The change in rate of hydrolysis was such that an error of 4 sec in estimating the time of adding Tris base would have resulted in an assay error of 10%. A blank lacking enzyme was used as a control for contamination of the substrate by nitrophenol.

Miscellaneous

Protein was assayed by the method of Lowry et al. (22) with bovine serum albumin as standard.

Preparation of 5'-Nucleotidase from Pig Intestine

Small intestines were removed from a hog and placed in crushed ice immediately after the animal was killed. Hereafter the material was kept on ice when it was not being manipulated. The intestinal tube was freed manually of its surrounding mesenteries and fat, and lengths of 4 feet were rinsed in cold tap water. Chyme and water were then expressed manually. The intestine was everted over a glass tube which was 50 cm long and 2.5 cm in diameter by passing a piece of gut down the tube, everting it over the far end, and drawing it back over the outside of the glass tube, which thus provided a firm backing. Mucoa and other material overlying the muscle were removed by alternately rubbing with paper towels and rinsing with tap water. The muscle was then separated from the tough membrane which encased it with the aid of a loop of thin, strong string. The loop was passed around the tube and was tightened against it with one hand while the glass tube was held in the other. The loop was now drawn along the length of the tube with a rotary motion. In this way the string cut the muscle away from the underlying membrane. When all of the exposed portion of the muscle had been dissected in this manner, more was brought into position by drawing the gut back over the outside of the glass tube. One intestine yielded 500 g of muscle at the expense of 2 to 3 hours of labor.

The purification procedure to be described is summarized in Table I. The muscle was rinsed in a colander, drained, and suspended in 1 volume by weight of ice-cold 0.9% KCl, pH 6.8. It was disintegrated in a Waring blender for 1 min, and was then further subjected to sonic disruption for 5 min, care being taken to keep the temperature below 25° and to keep the suspension stirred. The resulting material was centrifuged at 16,000 \times g for 30 min. The low speed supernatant obtained in this way from 500 g of muscle contained 12 g of protein. If the sonic oscillation step was omitted the recovery of enzyme activity in this supernant was halved.

The supernatant obtained by the above procedure was treated with \&frac13; volume of 5% ammonium deoxycholate, pH 8.4, with
Preparation of 5'-nucleotidase from pig intestine

Activities are quoted in micromoles per min; specific activities, in micromoles per mg of protein per min. Assay 1 was used to measure activity.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total protein</th>
<th>Total activity</th>
<th>Specific activity</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Low speed supernatant 1</td>
<td>12.1</td>
<td>602</td>
<td>0.05</td>
<td>73</td>
</tr>
<tr>
<td>2. Deoxycholate-solubilized low speed supernatant</td>
<td>11.7</td>
<td>459</td>
<td>0.04</td>
<td>60</td>
</tr>
<tr>
<td>3. First high speed supernatant above cloudy zone</td>
<td>10.1</td>
<td>163</td>
<td>0.16</td>
<td>63</td>
</tr>
<tr>
<td>4. Cloudy zone washed three times</td>
<td>0.024</td>
<td>128</td>
<td>5.62</td>
<td>60</td>
</tr>
<tr>
<td>5. Combined washings from Fraction 4</td>
<td>0.80</td>
<td>40</td>
<td>0.05</td>
<td>65</td>
</tr>
</tbody>
</table>

Substrate specificity of 5'-nucleotidase in presence and absence of ADP

Assay 2 was used to estimate orthophosphate release. Incubation mixtures contained 0.56 μg of protein per ml, and 60 μm substrate. The incubations were run at 24°C for 5 min. Each substrate was tested in the absence and presence of 5 PM ADP. A zero time blank was run with each substrate.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Orthophosphate liberated</th>
<th>Activity remaining</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μmoles per mg protein per min</td>
<td>%</td>
</tr>
<tr>
<td>AMP</td>
<td>6.7</td>
<td>1.1</td>
</tr>
<tr>
<td>IMP</td>
<td>2.8</td>
<td>0.5</td>
</tr>
<tr>
<td>GMP</td>
<td>2.8</td>
<td>0.5</td>
</tr>
<tr>
<td>CMP</td>
<td>3.9</td>
<td>2.1</td>
</tr>
<tr>
<td>UMP</td>
<td>1.9</td>
<td>0.5</td>
</tr>
<tr>
<td>dAMP</td>
<td>0.9</td>
<td>0.16</td>
</tr>
<tr>
<td>dGMP</td>
<td>0.45</td>
<td>0.05</td>
</tr>
<tr>
<td>dCMP</td>
<td>0.3</td>
<td>~0</td>
</tr>
<tr>
<td>TMP</td>
<td>1.1</td>
<td>0.3</td>
</tr>
</tbody>
</table>

When a mixture of 2'- and 3'-AMP was tested as substrate in Assay 1 at a total concentration of 100 μM, adenosine was liberated at 0.5% of the rate observed with 100 μM 5'-AMP. Addition of 3.3 μM ADP diminished the rate of 5'-AMP hydrolysis by 67%, but slowed that of the 2'- and 3'-isomers by only 11%. Under similar circumstances, 1 mM glucose 1-phosphate did not alter the rate of 5'-AMP hydrolysis, but halted that of 2'- and 3'-AMP. Finally, addition of 2'- and 3'-AMP did not inhibit the hydrolysis of 5'-AMP. Assay 1 was used in these experiments, the protein concentration was 24.7 μg per ml for the assay of 2'- and 3'-AMP hydrolysis, and 1.23 μg per ml for the assay of 5'-AMP hydrolysis.

No adenosine formation was detected when the following compounds were tested instead of AMP in Assay 1: ADP, ATP, cyclic 3',5'-AMP, AMP methyl ester, and adenosine monosulfate. No orthophosphate was released from ADP, ATP, pyrophosphate, or tripolyphosphate in Assay 2. At a pH of 7.2, the analogue isoAMP was hydrolyzed at 16% the rate of AMP.
from pig gut, in the absence (-) and presence (---) of 17 μM ADP. Assay 1 was used with the following buffers (45 mM): Δ, acetate-Tris; O, glutarate-Tris; X, MES-Tris; C, β,β-diethylglutarate Tris; △, Tris HCl; O, diethanolamine HCl; ■, lysine-HCl. The pH values were checked at the end of the reaction with a Radiometer pH meter, model PHM 25, using the expanded scale.

Lack of Contamination with Myokinase—The presence of myokinase would seriously prejudice studies of the inhibition of 5′-nucleotidase by ADP and ATP. When the 5′-nucleotidase preparation from pig gut was assayed for myokinase, as described under “Methods,” none was found. A myokinase activity of as little as 0.002 amole per mg of protein per min could have been detected in the assay. The same preparation showed a 5′-nucleotidase activity of about 10 amoles per mg of protein per min. An additional factor which limits both myokinase and nucleoside monophosphate kinase activities is the absence of magnesium ions from the reaction mixture. Moreover, the levels at which ADP and ATP were generally used as inhibitors fell well below the Km of myokinase for these substrates.

p-Nitrophenylphosphatase Activity—In the pH region 5.2 to 6.5, the p-nitrophenylphosphatase activity of the 5′-nucleotidase preparation was about 0.3% of the AMP-phosphatase activity. Moreover, in this pH region ADP strongly inhibited the 5′-nucleotidase activity while hardly affecting the p-nitrophenylphosphatase activity (Table III). At pH 8.6 the p-nitrophenylphosphatase activity was 7.5% of the AMP-phosphatase activity. It is shown below that the ADP inhibition of 5′-nucleotidase is greatly reduced at alkaline pH values; nevertheless it remains greater than the inhibition of p-nitrophenylphosphatase activity.

The AMP-phosphatase and p-nitrophenylphosphatase activities of the 5′-nucleotidase preparation obtained from pig gut showed different rates of heat inactivation. After incubating 0.67 mg of enzyme per ml in 50 mM Tris-HCl buffer, pH 7.2, at 60° for 3 min the activities remaining were 50 and 13%, respectively. These values are selected from a denaturation curve which extended from 1 to 10 min. In contrast the heat denaturation of AMP and isoAMP-phosphatase activities proceeded at the same rate.

Inhibitors—Nucleoside diphosphates are powerful inhibitors of 5′-nucleotidase from pig gut. The order of effectiveness of the inhibitors is TDP > ADP > UDP > CDP > GDP > IDP. The hydrolysis of 80 μM AMP is inhibited 53 and 59% by 1 μM ADP and 1 μM TDP, respectively (Table IV). A comparison of the inhibitory effectiveness of the nucleoside di- and triphosphates showed the triphosphates to be considerably weaker in each case.

Although the 5′-nucleotidase preparation used by us contained negligible amounts of myokinase (see above), it seemed prudent to test ADP and ATP analogues which cannot be converted to ADP or ATP in the myokinase reaction. This led to the discovery that the analogue AOPCPOP is an even more powerful inhibitor than ADP (Table V). The effects of this analogue are described in greater detail in a later paper. In addition it was found that one of the ATP analogues, AOPCPOP, is a more powerful inhibitor than ATP, whereas the analogue AOPOPCP has about the same effect as ATP.

Table V shows that GTP and its β,γ-methylene analogue are also inhibitors of 5′-nucleotidase from pig gut, although they are not as effective as ATP. The α,β-methylene analogue of GTP was not available at the time of testing. IPATA (6) stated that sheep brain 5′-nucleotidase is not inhibited by GTP.

Effect of pH on Activity—The activity of 5′-nucleotidase as a function of pH is shown in Figs. 1 and 2. The enzyme shows optimum activity at a pH of about 7 in β,β-diethylglutarate-Tris buffer, and Tris-HCl buffer. However, a somewhat higher activity is obtained in MES-Tris buffer in this pH region. An interesting feature of the pH activity profile is the occurrence of a second optimum at pH 5.5, when the activity is determined in MES-Tris buffer. The optimum in the region of pH 5.5 is not observed in acetate-Tris, or glutarate-Tris buffers. As is shown below, with the exception of MES, all buffer anions which were tested inhibit 5′-nucleotidase at pH 5.5 in the presence of 100 μM AMP.

In the presence of ADP the pH optimum at pH 7 is displaced to higher values. The degree of this shift depends on the concentrations of AMP and ADP. At 100 μM AMP and 17 μM ADP the pH optimum is about 8.5. The pH optimum which is observed at pH 5.5, in the presence of MES-Tris buffer is not affected by the presence of ADP (Fig. 1).

In the presence of ATP the pH optimum is displaced from pH 7 to higher values in a way similar to that observed with ADP. The inhibition by ATP is relatively greater at pH 5.5 than it is at pH 7.0, in contrast to the inhibition of the enzyme by ADP (Fig. 2).

The dual pH optimum might reflect the presence of two enzymes. As has already been shown, the enzyme preparation contains very little (<0.5%) p-nitrophenylphosphatase activity in the pH range 5.2 to 6.5. The possible presence of two differ-
TABLE V

Inhibition of 5'-nucleotidase by some nucleoside di- and triphosphates and their phosphonate analogues

Assay 1 was used with 100 μM AMP and 1.23 μg of 5'-nucleotidase protein per ml. The uninhibited reaction released adenosine at 6.9 μmoles per mg of 5'-nucleotidase per min.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Activity remaining 17 μM inhibitor</th>
<th>3.3 μM inhibitor</th>
<th>0.1 μM inhibitor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>AOPCP</td>
<td>0</td>
<td>2</td>
<td>21</td>
</tr>
<tr>
<td>ADP</td>
<td>5</td>
<td>10</td>
<td>69</td>
</tr>
<tr>
<td>AOPCP</td>
<td>4</td>
<td>12</td>
<td>86</td>
</tr>
<tr>
<td>ATP</td>
<td>43</td>
<td>43</td>
<td>10</td>
</tr>
<tr>
<td>GTP</td>
<td>54</td>
<td>12</td>
<td>86</td>
</tr>
<tr>
<td>GTP</td>
<td>66</td>
<td>12</td>
<td>86</td>
</tr>
<tr>
<td>GOPOCP</td>
<td>74</td>
<td>74</td>
<td>74</td>
</tr>
</tbody>
</table>

Fig. 2. Effect of pH on activity of 5'-nucleotidase in the absence (---) and presence (---) of 33 mM ATP. The conditions were as described in Fig. 1.

Fig. 3. Effect of pH on activity of 5'-nucleotidase in the absence ( ) and presence ( ) of 10 mM MgCl₂. Assay 1 was used at pH values below 7. Assay 2 was used in the presence of 0.5 mM AMP at pH values above 7. This was necessary because adenosine deaminase, which is used in Assay 1, is inhibited by magnesium ions at pH values greater than 7. The buffers were as described in Fig. 1.

Fig. 4. Effect of magnesium ions on inhibition of 5'-nucleotidase by ADP and ATP. Assay 1 was used in conjunction with 1.0 mM AMP, 36 mM Tris-HCl buffer, pH 7.2, and MgCl₂ as indicated. Additions were as follows: X, none; Y, 0.25 mM ADP; Δ, 0.75 mM AMP; O, 0.75 mM ATP; and □, 1.47 mM ATP. Reactions were run at 37° with a 1-mm light path; they were started by addition of AMP.

Effect of Magnesium Ions on Activity—Magnesium ions markedly stimulate 5'-nucleotidase from pig gut above pH 8. For example, when the AMP concentration is 0.5 mM, addition of 10 mM MgCl₂ results in a 25, 100, and 290% increase in activity at pH 8.5, 9.0, and 9.8, respectively (Fig. 3). Magnesium ions thus reveal a third pH optimum of 5'-nucleotidase. This finding serves to relate the enzyme studied by us with the 5'-nucleotidase prepared from bull seminal plasma. Levin and Bodansky (4), Song and Bodansky (5), and Bodansky and Schwartz (23) showed that the latter is stimulated by magnesium ions, and that magnesium ions produce a new pH optimum at pH 9.2. However, with AMP as substrate the pH activity curves of the enzymes from pig gut and bull seminal plasma show
Fig. 5. Inhibition of 5'-nucleotidase by ADP and ATP as a function of the free nucleotide concentration. The results shown in Fig. 4 have been replotted.

Fig. 6. Inhibition of 5'-nucleotidase by various buffers at pH 5.6. Each complete reaction mixture, less AMP, was made up to \( \frac{1}{2} \) of the final volume and was titrated to pH 5.63 with Tris base. The reaction was then started by addition \( \frac{1}{2} \) of the final volume of 1 mM AMP-Tris, pH 5.63. The final protein concentration was 2.5 \( \mu \)g per ml. Immediately after each assay the pH was measured again. It was found to be within ±0.05 unit of the starting pH. The buffers were prepared by partial neutralization of the free acids and of hydroxylamine hydrochloride with Tris base.

considerable differences both in the presence and absence of magnesium ions.

Magnesium ions partially reverse the inhibition of pig gut 5'-nucleotidase by ADP and ATP (Fig. 4). A simple explanation to account for this observation would be that only the unchelated nucleotides inhibit the enzyme, and that magnesium ions serve to remove the inhibitory species by chelation. The results shown in Fig. 4 were replotted to show the inhibition of 5'-nucleotidase as a function of the free nucleotide concentration. Fig. 5 shows that the simple explanation is incorrect. Even at high magnesium ion concentrations, ADP, and to a lesser extent ATP, still exert a relatively strong inhibition on the enzyme.

Effects of Various Buffer Ions—It has already been mentioned that the enzyme possesses an additional pH optimum at pH 5.5 (Fig. 1). The additional optimum was observed in the presence of MES but not of other buffers tested by us. Apparently this is not the result of an activation of the enzyme by MES in the pH region of 5.5, but to the inhibition of the enzyme by other buffers. The simplest way to show this is to determine the activity of the enzyme as a function of the concentration of different buffers. In the experiment shown in Fig. 6 the pH of each reaction mixture was adjusted to 5.63 just before starting the reaction. The pH was measured again at the end of the run and was found to have remained between pH 5.6 and 5.7. The pH measurements were considered necessary to insure that a large change in pH had not occurred at the low buffer concentrations used in about half of the reaction mixtures. Citrate is the strongest inhibitor of the anions tested, but even acetate causes an appreciable inhibition.

The \( K_m \) for AMP in glutarate-Tris buffer, pH 5.67, is 4 \( \mu \)M. This value is not changed when the pH is raised from 5.67 to 6.18 in the presence of glutarate-Tris buffer. On the other hand, the \( K_m \) for AMP in MES-Tris buffer, pH 5.67 and 6.18 is 15 and 4 \( \mu \)M, respectively (Fig. 7). The \( K_m \) in MES-Tris buffer, pH 5.30, is 5 \( \mu \)M (not shown in Fig. 7).

DISCUSSION

Adenylate and adenosine are vasodilators. It is not certain whether the vasodilatory action of adenylate is caused by its conversion to adenosine, or whether both adenylate and aden-
sine possess this property. Adenosine is deaminated to inosine, which possesses no vasodilatory properties (24, 20). The present paper shows that 5'-nucleotidase is inhibited strongly by ADP and, to a lesser degree, by ATP. Adenosine deaminase apparently possesses no regulatory properties. The regulation of the activity of 5'-nucleotidase may therefore be a key control mechanism in determining AMP and adenosine levels, and hence in the control of blood flow.

5'-Nucleotidase activity is particularly high in smooth muscle, while being relatively low in organs such as heart. At first sight this may appear to rule out any important function for 5'-nucleotidase in the regulation of blood flow of the heart. However, 5'-nucleotidase is found largely in the walls of the coronary blood vessels (8). It is now recognized that most of the regulation of blood flow is exerted in the precapillary resistance vessels (31). The diameter of these vessels is controlled by smooth muscle, which accounts for only a small fraction of the total protein of the heart. The heart may require relatively low levels of 5'-nucleotidase provided that the enzyme is present in strategic places, namely in or near the precapillary resistance vessels. Similar considerations apply to the regulation of blood flow through other organs.

Studies at the level of ultrastructure indicate that 5'-nucleotidase may be located on the outside of the cell surface (5, 8, 12, 23-35). This is supported by the observation that when AMP is perfused through hearts of rat or guinea pig it is dephosphorylated (36, 37); indeed it is largely converted to adenosine and inosine during a single passage through the heart (12). Control experiments showed that the hydrolysis of AMP was catalyzed by the perfused hearts and not by enzymes leached out of the hearts into the perfusion fluid. Moreover, the enzyme involved was 5'-nucleotidase and not a nonspecific phosphatase, since a mixture of 2'- and 3'-AMP remained virtually unaffected (12).

It has been suggested by Baer et al. (2) and by Baer and Drummond (12) that during oxygen sufficiency 5'-nucleotidase is inhibited by virtue of high ATP concentrations. During anoxia ATP levels decline, and 5'-nucleotidase becomes deinhibited and causes the formation of adenosine. This results in an increase in the coronary blood flow and in the correction of the anoxia. Our finding that ADP is an even more powerful inhibitor of 5'-nucleotidase than ATP throws doubt on this otherwise attractive proposal. At present there is a lack of information concerning the accessibility of intracellular ADP and ATP to 5'-nucleotidase. Because of this it is probably not profitable to attempt to integrate the inhibition of the enzyme by adenine nucleotides into physiological control mechanisms.

The discovery that AOPCP is a very powerful inhibitor of 5'-nucleotidase (K = 2 nM), makes available an inhibitor of adenosine production. This makes it possible to test, among other things, whether AMP possesses vasodilator properties, or whether it must first be converted into adenosine. A kinetic study of 5'-nucleotidase and of its inhibition by AOPCP will be presented in a future paper.
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