Regulation of Endogenous Phosphorylation of Specific Proteins in Synaptic Membrane Fractions from Rat Brain by Adenosine 3':5'-Monophosphate*

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SUMMARY

The phosphorylation of specific membrane proteins by endogenous protein kinase in synaptic membrane fractions from rat cerebrum has been studied using the technique of acrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. The endogenous phosphorylation of two specific membrane proteins was found to be regulated by adenosine 3':5'-monophosphate (cyclic AMP). The minimal molecular weights of these proteins were determined, by gel electrophoresis in the presence of sodium dodecyl sulfate, to be 86,000 (Protein I) and 48,000 (Protein II). The endogenous phosphorylation of Protein I and Protein II was stimulated by cyclic AMP in the presence of magnesium ions. Approximately 80% and 75% of the 32P incorporated into Protein I and Protein II, respectively, was found in phosphoserine. The concentration of cyclic AMP required for maximal stimulation of the phosphorylation of both Protein I and Protein II was approximately $5 \times 10^{-4}$ M. Guanosine 3':5'-monophosphate had no significant effect, at concentrations up to $10^{-4}$ M, on the phosphorylation of either of these proteins. The endogenous phosphorylation occurred rapidly, the phosphorylation of both Protein I and Protein II reaching maximal levels within 5 sec. After reaching a maximal level of phosphorylation, Protein II underwent rapid dephosphorylation. The cyclic AMP-stimulated endogenous phosphorylation of a protein corresponding in position on acrylamide gel electrophoresis to Protein I was also observed in membrane fractions prepared from other neural tissues known to contain synapses, but membrane fractions prepared from several neural and non-neural tissues devoid of synapses, including lingual nerve, lung, liver, spleen, and kidney, all failed to reveal cyclic AMP-dependent phosphorylation of a protein band corresponding in position to Protein I. In contrast, cyclic AMP-dependent phosphorylation of a protein band corresponding in position to Protein II was observed in several neural and non-neural tissues. The results are compatible with the hypothesis that the mediation of certain types of synaptic transmission may involve regulation by cyclic AMP of the level of phosphorylation of specific protein constituents of the synaptic membranes.

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Evidence has recently accumulated which suggests that adenosine 3':5'-monophosphate may mediate the actions of neurotransmitters at certain types of neuronal synapses (1-5). However, little information is available to indicate how cyclic AMP might achieve such effects. Following the discovery of cyclic AMP-dependent protein kinases, initially in muscle (6), and then in liver (7) and brain (8), it was proposed (9, 10) that the diverse effects of cyclic AMP in various tissues may be mediated through regulation of the activity of cyclic AMP-dependent protein kinases. The discovery of cyclic AMP-dependent protein kinase activity in mammalian brain (8, 11), and particularly in those subcellular fractions enriched in synaptic membranes (12), suggested the possibility of a role for this enzyme in neuronal function. Subsequently, synaptic membrane fractions were also found to be highly effective as substrate for exogenous cyclic AMP-dependent protein kinase (13) and to be enriched in phosphoprotein phosphatase activity (14). More recently, the endogenous phosphorylation of a specific protein component of synaptic membrane fractions was shown to be markedly stimulated by cyclic AMP (15). These results are compatible with the hypothesis (9) that the phosphorylation of a particular protein in the postsynaptic membrane, catalyzed by a membrane-bound cyclic AMP-dependent protein kinase, might result in an alteration of the permeability of the postsynaptic membrane to inorganic ions. Such an effect could account for the known physiological responses of neurons to neurotransmitters and to cyclic AMP, and could provide a mechanism by which cyclic AMP mediates the actions of neurotransmitters at synaptic junctions. In view of the possible involvement, in the process of synaptic transmission, of cyclic AMP-dependent phosphorylation of a specific protein in synaptic membranes, it seemed of value to characterize the cyclic AMP-dependent endogenous phosphorylation of synaptic membrane proteins. In addition to the protein (Protein I) shown, in our earlier studies, to exhibit cyclic AMP-dependent phosphorylation, we have been able, by varying the experimental conditions, to demonstrate the existence of an additional protein (Protein II), in synaptic membrane fractions prepared from whole cerebrum, the endogenous phosphorylation of which is markedly affected by cyclic AMP.

*1The abbreviations used are: cyclic AMP, adenosine 3':5'-monophosphate; cyclic IMP, inosine 3':5'-monophosphate; cyclic GMP, guanosine 3':5'-monophosphate; cyclic CMP, cytidine 3':5'-monophosphate; cyclic dTMP, 2'-deoxythymidine 3':5'-monophosphate; FMN, flavin mononucleotide.
In the present study, some of the characteristics of the phosphorylation of these two endogenous membrane proteins catalyzed by endogenous membrane-bound protein kinase have been investigated.

**Experimental Procedure**

**Materials**—The cerebra of male Sprague-Dawley rats (140 to 160 g body weight) were used as the source of synaptic membranes. Synaptic membrane fractions, designated M-1 (0.9) and M-1 (1.0) were prepared as described previously (12, 13). In initial experiments, results obtained using the M-1 (0.9) subfraction and those obtained using the M-1 (1.0) subfraction were found to be almost identical. Therefore, unless stated otherwise, the two fractions were combined, centrifuged, the pellet suspended in 0.32 M sucrose, and this suspension used as the synaptic membrane preparation in all of the experiments to be described. This membrane preparation was stored at −20° and used within 1 week. Under these conditions no significant loss of activity was observed for cyclic AMP-dependent endogenous phosphorylation of membrane proteins, nor was any significant effect of freezing and storage observed on the pattern of phosphorylation. The M-1 (0.9) fraction was also prepared from cerebral cortex, cerebellum and caudate nucleus of rats, using procedures similar to those used for whole rat cerebra. Subcellular fractionation of bovine lingual nerves, and of various non-nervous rat tissues was carried out using the same procedures as were used for rat cerebra (12, 13); membrane fractions corresponding to the M-1, M-2, and microsomal fractions of rat cerebra were prepared from each of these various tissues.

Cyclic AMP was purchased from Sigma BioResearch. Cyclic IMP, cyclic GMP, cyclic UMP, cyclic CMP, and cyclic dTMP were purchased from Boehringer-Mannheim. [γ-32P]-ATP was prepared by the method of Post and Sen (16). Other substances obtained from the following sources: ATP, ADP, and 5'-AMP from Schwarz BioResearch; adenosine, adenosine, FMN, p-chloromercuribenzoic acid, dithiothreitol, morpholineethane sulfonic acid, and phosphorylase a (two times crystallized) from Sigma; p-ribose from Nutritional Biochemicals; cytochrome c, myoglobin, chymotrypsinogen, ovalbumin, and bovine serum albumin from Mann Research Laboratories. Protein kinase modulator was prepared from lobster tail muscle by a procedure described by Donnelly et al. (17).

**Standard Assay**—Synaptic membrane fractions (0.75 mg of protein) were preincubated for 1 min at 20° in a mixture containing 10 μmoles of sodium acetate, pH 6.0, and 2 μmoles of magnesium chloride, in the absence or presence of 1 n mole of cyclic AMP. One nanomole of [γ-32P]ATP (1 to 3 × 10^6 cpm per nanomole) was then added to initiate the reaction. The final volume was 0.2 ml. Although the concentration of ATP (5 μM) used in the standard assay was not saturating, it was chosen both to achieve a maximal stimulation by cyclic AMP, as well as to use the highest specific activity of ATP possible, in order to minimize the time required for autoradiography. The mixture was incubated for 15 sec at 20°, and the reaction was terminated by the addition of 0.1 ml of 9% sodium dodecyl sulfate in 0.03 M Tris-Cl buffer, pH 8.0, containing 6% mercaptoethanol, 3 mM EDTA, and 15% glycerol. The final sodium dodecyl sulfate concentration of 3% abolished protein kinase activity and dissolved membrane proteins. A small amount of bromphenol blue was added as tracking dye for electrophoresis. Aliquots of 0.2 ml of each incubation mixture were then subjected to acrylamide slab gel electrophoresis followed by autoradiography.

**Electrophoresis and Autoradiography**—Electrophoresis was carried out for 7 to 8 hours at 60 ma on a vertical plate gel of 5.6% polyacrylamide in the presence of 1% sodium dodecyl sulfate with a running buffer of 0.04 M Tris-acetate, pH 7.4, containing 1% sodium dodecyl sulfate and 2 mM EDTA as described by Fairbanks et al. (18). The dimensions of the gel were 11 cm × 16 cm × 4 mm, and the apparatus used was similar to that described by Reid and Bieleski (19). The gel was stained for protein with 0.025% Coomassie blue in 25% isopropyl alcohol and 10% acetic acid, and was destained, first with 10% isopropyl alcohol and 10% acetic acid containing 0.0025% Coomassie blue, and then with 10% acetic acid with several changes. The gel was dried on Whatman No. 50 filter paper under vacuum, placed in close contact with Kodak Royal X-Omat film, generally for 2 to 8 days, and the film was developed. The resulting autoradiograph revealed those proteins into which radioactive phosphate had been incorporated during incubation. Intensity of the darkness of bands on the film was measured quantitatively by scanning with a Joyce-Loebl microdensitometer, and the optical density was expressed in arbitrary units. In order to correct for the background darkness of the films, dotted lines as shown in Fig. 2 were taken as base-lines for the optical density of Protein I and Protein II. Protein concentration was determined by the method of Lowry et al. (20) with bovine serum albumin as protein standard.

**Comment on Terminology**—The phosphorylation studies described in this paper were carried out using membrane fragments, which contained endogenous protein kinase, endogenous substrate protein for the protein kinase, and endogenous phosphoprotein phosphatase capable of removing the phosphate from the substrate protein. The observed level of phosphorylation of the membrane proteins represents a balance between protein kinase and protein phosphatase activity. In order to describe the results in a reasonably concise manner, the term "phosphorylation," as used in the present study, is intended to indicate the level of [32P]phosphate present in the substrate protein, and not the activity of the protein kinase. Similarly, "an increase in phosphorylation" is meant to imply simply an increase in the amount of [32P]phosphate present in the substrate protein without regard to the mechanism involved, i.e. it does not necessarily imply an increase in protein kinase activity.

**Results**

**Endogenous Phosphorylation of Synaptic Membrane Preparation from Rat Cerebrum**—When the cerebral synaptic membrane preparation was incubated under the standard assay conditions with [γ-32P]ATP, in the presence or absence of cyclic AMP, and then subjected to gel electrophoresis in the presence of 1% sodium dodecyl sulfate, it was found that the phosphorylation of two specific protein bands was stimulated significantly by cyclic AMP (Fig. 1). (Slight (10 to 20%) stimulation by cyclic AMP of the phosphorylation of several other protein bands was observed, but the effect was too small to permit a detailed quantitative study.) One of these two bands, which was described earlier (15), will hereafter be referred to as Protein I; the other band will be referred to as Protein II. The per cent stimulation by cyclic AMP of the endogenous phosphorylation of Protein I was considerably greater than that of Protein II. Protein I and Protein II were eluted from the gel by the techniques described previously (15), subjected to acid hydrolysis, and the hydrolysate was analyzed for radioactive phosphoserine and phosphothreonine as described elsewhere (13). After correction for hydrolysis of authentic phosphoserine and phosphothreonine, 80% of the total incorporated phosphate in Protein I was found...
to be associated with phosphoserine, 5% with phosphothreonine, and 10% with other amino acids or polypeptides. Using the same corrections, 75% of the total incorporated phosphate in Protein II was found to be associated with phosphoserine, 10% with phosphothreonine, and 10% with other amino acids or polypeptides. With both proteins, the remaining phosphate was recovered as inorganic phosphate.

Quantitative Estimation of Extent of Phosphorylation of Protein Bands—In order to carry out quantitative studies on the endogenous phosphorylation of the specific proteins, Protein I and Protein II, two different procedures were examined. The autoradiographs shown in Fig. 1 were scanned with a microdensitometer, and the density of phosphorylated bands was recorded as shown in Fig. 2. For comparison the same dried gel that produced the autoradiographs in Fig. 1 was cut into small pieces and each piece was counted in a liquid scintillation spectrometer. The electrophoretograms thus obtained are shown in Fig. 3. The electrophoretogram obtained by densitometry and that obtained by liquid scintillation counting were similar. In either case, a relatively high background was noted around Protein I and Protein II, as well as around other bands, which was not readily observed on the autoradiographs (Fig. 1). For the quantitative estimation of the amount of radioactive phosphate incorporated into a specific protein band, base-lines were constructed, as indicated for Protein I and Protein II by the broken lines in Figs. 2 and 3. With the electrophoretogram obtained using either procedure, the per cent stimulation of phosphorylation by cyclic AMP, calculated from the area of the peak above the base-line, was not significantly different from that calculated from the height of the peak above the base-line. However, the calculated per cent stimulation of phosphorylation by cyclic AMP, determined by scintillation counting (Fig. 3) was, consistently, somewhat greater than that determined by densitometric measurement (Fig. 2), especially when the per cent stimulation was large. For instance, in the experiment illustrated in Figs. 1 to 3, cyclic AMP caused a 9.1-fold stimulation of Protein I when analyzed by liquid scintillation counting, and a 6.2-fold stimulation when analyzed by densitometric measurement. In
FIG. 2. Densitogram obtained from the autoradiograph shown in Fig. 1. The autoradiograph of proteins from the synaptic membrane preparation incubated in the presence (left) or absence (right) of cyclic AMP was scanned with a Joyce-Loebl microdensitometer, and the density of bands was recorded.

FIG. 3. Scintillation counting of slices of the gel which had given the autoradiographic pattern shown in Fig. 1. After the destained, dried gel had been subjected to autoradiography, it was cut into pieces of 2-mm thickness, and the radioactivity in each piece of gel was determined in a liquid scintillation spectrometer, and plotted as a function of distance from the origin of the gel. The area of Peak II above the broken line, in the presence of cyclic AMP, corresponds to 0.096 pmole of radioactive phosphate. The scintillation mixture contained 8 g of 2,5-diphenyloxazole and 160 mg of bis-(O-methylstyryl)-benzene dissolved in 2 liters of toluene and ethylene glycol monoethyl ether (1:1), and the counting efficiency of $^{32}$P was greater than 95%.

the same experiment, cyclic AMP caused a 3.0-fold stimulation of Protein II when analyzed by liquid scintillation counting, and a 2.6-fold stimulation when analyzed by densitometric measurement. The reason for this discrepancy is not known at the present time. However, since the densitometric measurement was felt to be more accurate because of continuous recording, and, in addition, was less time-consuming to perform, the extent of phosphorylation of Protein I and Protein II was determined, except where otherwise stated, from a densitogram with base-lines similar to those indicated in Fig. 2.

Time Course of Endogenous Phosphorylation of Protein I and Protein II—Under the standard assay conditions, the endogenous phosphorylation of Protein I reached a maximal level rapidly in the presence of cyclic AMP as shown in Fig. 4. The maximal level of phosphorylation occurred within 5 s, the briefest incubation time which could be accurately studied, and this level of phosphorylation decreased slowly afterwards, an observation similar to that made earlier (15); in both studies, the level of phosphorylation had decreased by 10 to 20% at 2 min as compared to the maximal level. The stimulatory effect of cyclic AMP was observed at all incubation times tested. The phosphorylation of Protein II also occurred very rapidly, reaching a maximal level within 5 s (Fig. 5). However, in contrast to the case with Protein I, at times later than 5 s there was a rapid dephosphorylation in the presence of cyclic AMP. As a result, the effect of cyclic AMP on the phosphorylation of Protein II was significant only when incubation was carried out for a short time; little or no stimulation by cyclic AMP was observed 2 min after the reaction was initiated. The percent stimulation by cyclic AMP of Protein I phosphorylation was invariably greater than that of Protein II phosphorylation, even at 5 s of incubation, at which time the stimulatory effect on Protein II was maximal.

Effect of Varying the Amount of Membrane, the pH, and the Concentration of ATP—The endogenous phosphorylation of Protein I and Protein II was nearly proportional to the amount of membrane protein added up to about 0.75 mg (Fig. 6). The pH optimum, for the phosphorylation of both proteins, was about 6.0 (data not shown). The phosphorylation of Protein II was markedly dependent upon pH, whereas that of Protein I was relatively insensitive to pH.

The effect of cyclic AMP on the endogenous phosphorylation
Fig. 4. Endogenous phosphorylation of Protein I in synaptic membrane preparation from rat cerebrum as a function of incubation time, in the absence or presence of $5 \times 10^{-6}$ M cyclic AMP. Incubation conditions were as described under "Experimental Procedure," except for the variation in incubation time. Amount of $[^{32}\text{P}]}$phosphate incorporated from $[^{32}\text{P}]$ATP into Protein I was calculated from the intensity of darkness of the band corresponding to Protein I on the x-ray film, and is expressed in arbitrary units.

Fig. 5. Endogenous phosphorylation of Protein II in synaptic membrane preparation from rat cerebrum as a function of incubation time, in the absence or presence of $5 \times 10^{-6}$ M cyclic AMP. Incubation conditions were as described under "Experimental Procedure," except for the variation in incubation time. Amount of $[^{32}\text{P}]}$phosphate incorporated from $[^{32}\text{P}]$ATP into Protein II was calculated from the intensity of darkness of the band corresponding to Protein II on the x-ray film and is expressed in arbitrary units.

of Protein I and Protein II as a function of the concentration of ATP is shown in Fig. 7. It is clear that cyclic AMP caused a decrease in the concentration of ATP required for half-maximal phosphorylation of both proteins. From these data, it was estimated that the concentrations of ATP required for half-maximal phosphorylation of Protein I were $2.3 \times 10^{-5}$ M and $5.5 \times 10^{-6}$ M, in the absence and presence of $5 \times 10^{-6}$ M cyclic AMP, respectively. Similarly, values of $2.4 \times 10^{-5}$ M and $7.9 \times 10^{-6}$ M were estimated for the concentration of ATP required for half-maximal phosphorylation of Protein II, in the absence and presence of cyclic AMP, respectively. It should be emphasized that these values for ATP, and the corresponding values for cyclic AMP and for Mg$^{2+}$, given below, should not be equated with apparent $K_m$ values, since these latter could not be measured under our standard assay conditions owing to the rapid rate of phosphorylation of Protein I and Protein II. Moreover, the presence in the membrane preparations of interfering enzymes, such as ATPase, cyclic AMP phosphodiesterase, and protein phosphatase, further complicates any quantitative interpretation of the data.

Effect of Varying Concentration of Cyclic AMP and Cyclic GMP—The effect of varying the concentration of cyclic AMP and cyclic GMP on the endogenous phosphorylation of Protein I and Protein II is shown in Fig. 8. From these data, the concentrations of cyclic AMP required to give half-maximal phosphorylation of Protein I and Protein II were estimated to be about $5 \times 10^{-6}$ M and $9 \times 10^{-7}$ M, respectively. The maximal activation was observed in the presence of approximately $5 \times 10^{-6}$ M cyclic AMP in both cases. Increasing the concentration of cyclic AMP above $5 \times 10^{-6}$ M caused a progressively lower stimulation of phosphorylation, possibly by acting as a competitive inhibitor of ATP in the phosphorylation reaction (21). Under the standard conditions of 1 min preincubation plus 15 s incubation, at 20°C, only about 10% of the added (5 µM) cyclic AMP was hydrolyzed. Moreover, the presence of 4 mM papaverine, a known phosphodiesterase inhibitor, did not alter significantly the concentration of cyclic AMP required for half-maximal activation. In contrast to the marked effect of cyclic AMP, cyclic GMP exhibited little effect on the endogenous phosphorylation of Protein I and Protein II.

Effect of Analogs of Cyclic AMP—The effect of several cyclic 3':5'-mononucleotides on the endogenous phosphorylation of
TABLE I

<table>
<thead>
<tr>
<th>Cyclic nucleotide</th>
<th>Protein I</th>
<th>Protein II</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 X 10^-4 M</td>
<td>5 X 10^-4 M</td>
</tr>
<tr>
<td>Cyclic AMP</td>
<td>23.0</td>
<td>14.5</td>
</tr>
<tr>
<td>Cyclic IMP</td>
<td>5.5</td>
<td>10.0</td>
</tr>
<tr>
<td>Cyclic GMP</td>
<td>2.5</td>
<td>6.5</td>
</tr>
<tr>
<td>Cyclic UMP</td>
<td>2.0</td>
<td>5.4</td>
</tr>
<tr>
<td>Cyclic CMP</td>
<td>0.3</td>
<td>3.0</td>
</tr>
<tr>
<td>Cyclic dTMP</td>
<td>-0.2</td>
<td>-0.2</td>
</tr>
</tbody>
</table>

Protein I and Protein II is shown in Table I. At the lowest concentration tested, cyclic AMP was far more effective than its analogs in its ability to stimulate the endogenous phosphorylation of each of the proteins. Cyclic IMP was capable, at a sufficiently high concentration, of causing a stimulation of the phosphorylation of both proteins as great as that produced by optimal concentrations of cyclic AMP. Cyclic GMP, cyclic UMP, and cyclic CMP caused a slight stimulation of the phosphorylation of Protein I, but only at high concentrations. Cyclic dTMP was ineffective, at all concentrations examined, in stimulating protein phosphorylation.

Effect of Divalent Cations—The effect of varying the concentration of Mg^{2+} on the endogenous phosphorylation of Protein I and Protein II is shown in Fig. 9. In the presence of cyclic AMP, the concentrations of Mg^{2+} required for half-maximal phosphorylation of Protein I and Protein II were about 4 mM and 2 mM, respectively. The optimal concentration of Mg^{2+} was in the range of 10 to 20 mM for the phosphorylation of both proteins. There was no significant effect of cyclic AMP on the concentration of Mg^{2+} required for half-maximal phosphorylation of either protein.

The effect of replacing Mg^{2+} by a variety of other divalent cations, on the endogenous phosphorylation of Protein I, is shown in the upper part of Table II. Co^{2+} and Mn^{2+} were more effective metal ions than Mg^{2+} in supporting the phosphorylation reaction, both in the absence and in the presence of cyclic AMP; however, the per cent stimulation by cyclic AMP seen in the presence of these cations was no greater than that seen in the presence of Mg^{2+}. Zn^{2+} and Ni^{2+} were able to substitute partially for Mg^{2+} in supporting the phosphorylation of Protein I, but the stimulatory effect of cyclic AMP was less in the presence of these cations than of Mg^{2+}. When Mg^{2+} was replaced by either Ca^{2+}, Fe^{2+}, Cu^{2+}, or Ba^{2+}, little or no phosphorylation of Protein I occurred either in the absence or in the presence of cyclic AMP.

The effects of various divalent cations on the endogenous phosphorylation of Protein II, seen in the lower part of Table II, showed some similarities to, and some differences from, their effects on the phosphorylation of Protein I. When Mg^{2+} was replaced by either Co^{2+} or Mn^{2+}, the phosphorylation of Protein II was increased, but the per cent stimulation by cyclic AMP was about the same as that observed in the presence of Mg^{2+}. A particularly interesting result was obtained when Mg^{2+} was replaced by Zn^{2+}. Thus, Zn^{2+} inhibited the phosphorylation observed in the absence of cyclic AMP, but inhibited the phosphorylation observed in the presence of cyclic AMP. Cyclic AMP strongly inhibited the phosphorylation of Protein II.

Fig. 8. Effect of varying the concentration of cyclic AMP and cyclic GMP on the endogenous phosphorylation of Protein I (left) and Protein II (right). Incubation conditions were as described under "Experimental Procedure," except for the variation in the concentration of the cyclic nucleotide. Incorporation of radioactive phosphate was measured by the densitometric method.

Fig. 9. Effect of Mg^{2+} concentration on the endogenous phosphorylation of Protein I (left) and Protein II (right), in the absence (O--O; D---D) or presence (●---●; □---□) of 5 X 10^-5 M cyclic AMP. Incubation conditions were as described under "Experimental Procedure," except for the variation in the concentration of Mg^{2+}. Incorporation of radioactive phosphate was measured by the densitometric method.
Effect of various divalent cations on endogenous phosphorylation of Protein I and Protein II in synaptic membrane preparation from rat cerebrum

Incubation conditions were as described under "Experimental Procedure," except for the variation in the kind and amount of each cation as indicated. Values are given for the phosphorylation of Protein I and Protein II in the absence of cyclic AMP, in the presence of 5 × 10⁻⁴ M cyclic AMP, and for the ratio of these two phosphorylations.

<table>
<thead>
<tr>
<th>Divalent cation</th>
<th>Phosphorylation of Protein I and Protein II at following concentration of divalent cation:</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Minus cyclic AMP</td>
<td>Plus cyclic AMP</td>
</tr>
<tr>
<td>Protein I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>0.5</td>
<td>1.3</td>
</tr>
<tr>
<td>Mg⁺⁺</td>
<td>1.4</td>
<td>5.8</td>
</tr>
<tr>
<td>Ca⁺⁺</td>
<td>4.0</td>
<td>16.0</td>
</tr>
<tr>
<td>Mn⁺⁺</td>
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<td>14.0</td>
</tr>
<tr>
<td>Zn⁺⁺</td>
<td>2.8</td>
<td>3.0</td>
</tr>
<tr>
<td>Ni⁺⁺</td>
<td>1.0</td>
<td>3.0</td>
</tr>
<tr>
<td>Ca⁺⁺</td>
<td>1.6</td>
<td>0.6</td>
</tr>
<tr>
<td>Fe⁺⁺</td>
<td>0.0</td>
<td>1.5</td>
</tr>
<tr>
<td>Cu⁺⁺</td>
<td>0.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Ba⁺⁺</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Protein II</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>7.0</td>
<td>13.2</td>
</tr>
<tr>
<td>Mg⁺⁺</td>
<td>12.0</td>
<td>24.3</td>
</tr>
<tr>
<td>Ca⁺⁺</td>
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<td>41.0</td>
</tr>
<tr>
<td>Mn⁺⁺</td>
<td>25.6</td>
<td>49.0</td>
</tr>
<tr>
<td>Zn⁺⁺</td>
<td>87.8</td>
<td>10.5</td>
</tr>
<tr>
<td>Ni⁺⁺</td>
<td>14.2</td>
<td>11.5</td>
</tr>
<tr>
<td>Ca⁺⁺</td>
<td>10.3</td>
<td>10.1</td>
</tr>
<tr>
<td>Fe⁺⁺</td>
<td>21.2</td>
<td>41.4</td>
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<tr>
<td>Cu⁺⁺</td>
<td>17.0</td>
<td>13.0</td>
</tr>
<tr>
<td>Ba⁺⁺</td>
<td>8.5</td>
<td>8.0</td>
</tr>
</tbody>
</table>

AMP also caused a marked inhibition of the phosphorylation of Protein II in the presence of 10 mM Ni⁺⁺, Ca⁺⁺, or Ba⁺⁺. In other experiments, it was observed that the inhibitory effect of cyclic AMP on the phosphorylation of Protein II, seen in the presence of 10 mM Ca⁺⁺, was diminished by the addition of 10 mM Mg⁺⁺. In contrast to the inhibition of phosphorylation of Protein II observed in the presence of several metal ions, inhibition by cyclic AMP of the phosphorylation of Protein I was observed only in the presence of 2.5 mM Ca⁺⁺. The phosphorylation of either protein, even in the absence of added divalent cations, was not significantly affected by the addition of 1 mM EDTA, either in the absence or presence of cyclic AMP.

Effect of Inhibitors—The various substances listed in Table III, which had been studied earlier for their effects on partially purified cyclic AMP-dependent protein kinases from brain (11) and other tissues (22), were tested for their ability to affect the endogenous phosphorylation of Protein I and Protein II. These compounds were found to affect the phosphorylation of the two proteins similarly. Adenosine strongly inhibited the phosphorylation of both Protein I and Protein II, causing approximately 70% inhibition at a concentration of 10⁻⁴ M, and almost abolishing phosphorylation at a concentration of 10⁻³ M. 5'-AMP and adenosine were also effective inhibitors, whereas ADP caused a substantially weaker inhibition, and ribose was virtually ineffective as an inhibitor. FMN and protein kinase modulator, each of which had previously been shown (17, 22) to inhibit the phosphorylation of histone catalyzed by soluble protein kinases prepared from bovine brain, did not exhibit any significant effect on the endogenous phosphorylation of Protein I or Protein II in synaptic membrane fractions.

Sodium fluoride, at a concentration of 10⁻³ M, enhanced the net phosphorylation of Protein I and Protein II, both in the absence and in the presence of cyclic AMP. In other experiments, it was found that the net increase in the level of phosphorylation of Protein I and Protein II, due to the presence of sodium fluoride, was observed at all incubation times from 5 to 120 s, both when the standard ATP concentration (5 × 10⁻⁴ M) was used and when a higher ATP concentration (10⁻⁴ M) was present in the incubation mixture. The decrease in the level of radioactive phosphate in Protein II, which occurred after 5 to 10 s in the presence of cyclic AMP under standard incubation conditions (Fig. 5), was also observed in the presence of the higher concentration of ATP (Fig. 10). The decrease in the level of Protein II phosphate was abolished when sodium fluoride was present in the incubation medium (Fig. 10); the results obtained with sodium fluoride can be explained by the well known ability of sodium fluoride to inhibit ATPase activity and by its demonstrated ability (14) to inhibit protein phosphatase activity.

Tissue Specificity—Membrane fractions from various tissues were examined for the endogenous phosphorylation of protein bands. As shown in Fig. 11, the cyclic AMP-stimulated phosphorylation of a protein band corresponding in position to Protein I occurred in the membrane fraction, M-1 (0.9), prepared from either cerebral cortex or cerebellum of the rat; the effect of

Table II

Effect of various possible inhibitors on endogenous phosphorylation of Protein I and Protein II in synaptic membrane preparation from rat cerebrum

Incubation conditions were as described under "Experimental Procedure," except for the addition of various test substances. The test substances were present, in the final concentrations indicated, either in the absence or presence of 5 × 10⁻⁴ M cyclic AMP. Protein kinase modulator used (0.26 mg per 0.2 ml of incubation volume) was a hydroxylapatite eluate prepared as described previously (17).

<table>
<thead>
<tr>
<th>Test substance</th>
<th>Concentration (M)</th>
<th>Phosphorylation of Protein I</th>
<th>Phosphorylation of Protein II</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Minus cyclic AMP</td>
<td>Plus cyclic AMP</td>
</tr>
<tr>
<td>None</td>
<td>0.3</td>
<td>9.2</td>
<td>33.0</td>
</tr>
<tr>
<td>Adenosine</td>
<td>10⁻⁴</td>
<td>3.4</td>
<td>10.0</td>
</tr>
<tr>
<td>5'-AMP</td>
<td>10⁻⁴</td>
<td>1.5</td>
<td>12.2</td>
</tr>
<tr>
<td>Adenine</td>
<td>10⁻⁴</td>
<td>4.0</td>
<td>17.0</td>
</tr>
<tr>
<td>ADP</td>
<td>10⁻⁴</td>
<td>5.6</td>
<td>23.3</td>
</tr>
<tr>
<td>Ribose</td>
<td>10⁻⁴</td>
<td>9.3</td>
<td>33.8</td>
</tr>
<tr>
<td>FMN</td>
<td>10⁻⁴</td>
<td>9.2</td>
<td>27.0</td>
</tr>
<tr>
<td>Sodium fluoride</td>
<td>10⁻⁴</td>
<td>7.0</td>
<td>29.5</td>
</tr>
<tr>
<td>Dithiobutyllysine</td>
<td>10⁻⁴</td>
<td>9.3</td>
<td>30.9</td>
</tr>
<tr>
<td>Modulator</td>
<td>10⁻⁴</td>
<td>13.5</td>
<td>43.0</td>
</tr>
<tr>
<td>Modulator</td>
<td>10⁻³</td>
<td>7.0</td>
<td>25.5</td>
</tr>
</tbody>
</table>

Table III

Effect of various divalent cations on endogenous phosphorylation of Protein I and Protein II in synaptic membrane preparation from rat cerebrum

Incubation conditions were as described under "Experimental Procedure," except for the variation in the kind and amount of each cation as indicated. Values are given for the phosphorylation of Protein I and Protein II in the absence of cyclic AMP, in the presence of 5 × 10⁻⁴ M cyclic AMP, and for the ratio of these two phosphorylations.
From cerebellum. Thus, the results obtained using the mem-
brane fractions prepared from the cerebral cortex (Fig. 11) were
similar to those observed with the synaptic membrane prepara-
tion from the whole cerebrum (Fig. 1).

In other experiments, subcellular membrane fractions were
prepared from the rat caudate nucleus, and examined for the
presence of cyclic AMP-regulated endogenous phosphorylation
of proteins, using the same procedures as were used to study the
cerebrum. Preparations of the membrane fraction, M-1 (0.9),
from the caudate nucleus, showed much weaker phosphate incor-
poration, per mg of protein, than did membranes from the
cerebral cortex or cerebellum, but the caudate preparation did
not show cyclic AMP-dependent phosphorylation of any protein band. The various membrane fractions from various neural and non-neural tissues, and examined for regulation by cyclic AMP of endogenous phosphorylation of membrane pro-
tein. The M-1 mitochondrial subfraction of whole rat cerebra
gave results qualitatively similar to those obtained with the more
highly purified synaptic membrane fractions, M-1 (0.9) and M-1
(1.0). The M-2 and microsomal subfractions from whole rat
cerebra did not show a cyclic AMP-dependent phosphorylation of any protein band. The various membrane fractions from various neural and non-neural tissues, and examined for regulation by cyclic AMP of endogenous phosphorylation of membrane protein.

The properties of the enzyme systems controlling the level of
phosphorylation of Protein I and Protein II differed in some

**DISCUSSION**

In the present experiments, we have studied two distinct protein
components in synaptic membrane fractions from wholeebra, designated Protein I and Protein II, the endogenous
phosphorylation of which is regulated by cyclic AMP. It seems
possible that cyclic AMP, a compound whose concentration in
nerve tissue is altered by physiological activity (28), as well as
by a variety of chemical and physical stimuli (29-35), may play
an important role in the physiology of synaptic transmission
(1-5), possibly through regulation of the endogenous phos-
phorylation of these or similar specific membrane proteins. In-
terestingly, the cyclic AMP-dependent endogenous phosphoryla-
tion of these proteins occurred at an extremely rapid rate, an
observation compatible with the possible involvement of cyclic
AMP in the rapid events associated with synaptic transmission.

The properties of the enzyme systems controlling the level of
phosphorylation of Protein I and Protein II differed in some

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3 A. M. Malkinson, J. E. Casnellie, and P. Greengard, unpublished observations.
FIG. 11. Effect of cyclic AMP on the endogenous phosphorylation of synaptic membrane preparations from rat cerebral cortex and cerebellum. Incubation conditions were as described under "Experimental Procedure," except that the synaptic membrane preparation used was the M-1 (0.9) fraction (0.4 mg of protein) prepared from cerebral cortex or from cerebellum. The arrows indicate the locations of the bands corresponding in position to Protein I and Protein II. The - and + signs at the bottom of the gel indicate the absence and presence, respectively, of 5 × 10⁻⁴ M cyclic AMP in the incubation medium.

Fig. 12. Determination of minimal molecular weight of Protein I and Protein II by polyacrylamide gel electrophoresis in presence of sodium dodecyl sulfate. Details of the electrophoretic procedure were as described under "Experimental Procedure," except that the gels were not dried. The molecular weights of proteins used as standards were 11,700 (cytochrome c), 17,200 (myoglobin), 25,700 (chymotrypsinogen), 43,000 (ovalbumin), 68,000 (bovine serum albumin), and 94,000 (phosphorylase a).

respects for the two membrane proteins. Phosphorylated Protein II, unlike phosphorylated Protein I, underwent a rapid net dephosphorylation, presumably by a specific phosphoprotein phosphatase present in the membrane. A clear difference was also noted between the intrinsic phosphorylation of Protein I and Protein II with respect to Zn²⁺. Zn²⁺ caused an increase in the level of phosphorylation of Protein II in the absence of cyclic AMP and a decrease in the level of phosphorylation of Protein II in the presence of cyclic AMP. The magnitude of these effects was such that, in the presence of Zn²⁺, cyclic AMP caused a decrease in the level of phosphorylation of Protein II. Interestingly, Zn²⁺ did not cause such a qualitative alteration in the effect of cyclic AMP on the phosphorylation of Protein I. Apparent inhibition by cyclic AMP of cyclic AMP-dependent protein kinase activity has been observed in other systems (11, 13, 21, 24, 36). For instance, the net phosphorylation of histone by a protein kinase from bovine brain was shown to be decreased by cyclic AMP, when Ca²⁺ or Zn²⁺ was used as the divalent cation rather than Mg²⁺ (11); this effect of cyclic AMP has recently been shown, using a highly purified preparation of the brain enzyme, to be due to an inhibition of the protein kinase activity of the preparation, and not due to stimulation of a phosphoprotein phosphatase contaminant. On the other hand, it has recently been shown (37), in elucidating the mechanism by which cyclic AMP causes a decrease in the net endogenous phosphorylation of a protein in toad bladder membrane, that cyclic AMP causes an increase in the activity of a protein phosphatase present in the membrane. It will be of value to determine whether the apparent inhibition of phosphorylation of Protein II by cyclic AMP, in the presence of Zn²⁺, is due to a decrease in protein kinase activity, or to an increase in protein phosphatase activity or both.

In our preliminary studies (15), Protein I was the only protein whose endogenous phosphorylation was markedly stimulated by cyclic AMP. In that study, cyclic AMP was observed to cause a small stimulation of several other proteins, including

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what we now refer to as Protein II; the per cent stimulation of phosphorylation of Protein II was only 10 to 30% in those experiments. In the present study, we observed a more pronounced dependence on cyclic AMP of the endogenous phosphorylation of Protein II. Since there were a number of experimental differences between the previous (15) and the present studies, we have attempted to clarify the factor(s) responsible for the ability to demonstrate a marked effect of cyclic AMP on the endogenous phosphorylation of Protein II, observed in the present study.

In our preliminary studies, the size of the rats used was about 200 g to 250 g, the synaptic membrane fractions were used immediately upon preparation, assay tubes were preincubated for about 15 min at 0° and the incubation was carried out at 30°. In the present studies, the size of the rats used was 140 g to 160 g, the synaptic membrane fractions were stored at −20° between preparation and use, assay tubes were preincubated for about 5 min at 0°, followed by 1 min at 20°, and the incubation was carried out at 20°. The factor found to be primarily responsible for the difference in results was the preincubation time during which cyclic AMP was exposed to the synaptic membrane preparation at 0°. Thus, in experiments carried out to study the effect of varying the time of preincubation at 0° on the phosphorylation of Protein II, it was found that when the preincubation lasted for 15 min or longer, the cyclic AMP-stimulated incorporation of 32P into Protein II was much smaller than that observed after a shorter (1 to 7 min) preincubation period. (With the longer period of preincubation, the cyclic AMP-stimulated incorporation of 32P into Protein I was also reduced.) Freezing and thawing, change in incubation temperature from 30° to 20°, and change in the size of the rats did not cause a significant change in the per cent stimulation of Protein II phosphorylation by cyclic AMP. The effect of prolonged preincubation at 0° in reducing the cyclic AMP-stimulated endogenous phosphorylation of Protein I and Protein II was shown to be due to depletion of cyclic AMP by phosphodiesterase activity in the synaptic membrane fraction. It has been reported that cyclic AMP phosphodiesterase purified from bovine heart is also active at 0° (38). Preincubation of synaptic membranes at 20° for 2 min in the absence or presence of cyclic AMP, in the hope of removing endogenous phosphoprotein phosphatase, did not lead to increased incorporation of radioactive phosphate into membrane proteins during the incubation period. It should be emphasized that under any assay conditions tested thus far, cyclic AMP always caused a greater per cent stimulation of the endogenous phosphorylation of Protein I than of Protein II.

The endogenous phosphorylation of Protein I and Protein II appeared to be quite similar with respect to a number of properties, including optimal pH, optimal concentrations of Mg2+ and cyclic AMP, and the concentration of ATP required for half-maximal activity, and responses to several inhibitors. On the other hand, the endogenous phosphorylation of these membrane proteins seemed to differ from the phosphorylation of histone catalyzed by a partially purified brain protein kinase (11), with respect to the effects of certain inhibitors. For instance, adenosine was the most potent inhibitor for the endogenous membrane phosphorylation, whereas ADP, which showed only a weak inhibitory effect on the endogenous membrane phosphorylation, was the most effective antagonist for the partially purified protein kinase system. FMN and protein kinase modulator were ineffective as inhibitors of endogenous membrane phosphorylation, in contrast to their strong inhibitory effect (22, 17) on the phosphorylation of histone catalyzed by the partially purified brain protein kinase. The lack of effect of the modulator on the membrane-bound protein phosphorylation system may be due to an inability of the modulator, itself a protein, to reach the appropriate site in the phosphorylation system.

It has recently been shown (24), with a particular fraction of toad bladder, which was enriched in fragments of plasma membrane, that cyclic AMP altered the level of phosphorylation of a specific protein, designated Protein D. This effect of cyclic AMP on the phosphorylation of Protein D in isolated toad bladder membranes was mimicked in intact toad bladders by either antidiuretic hormone or monobutyrly cyclic AMP. There is good evidence (39) that antidiuretic hormone, through controlling the level of cyclic AMP, regulates the transport of sodium and of water in toad bladder. It was therefore postulated (24) that the cyclic AMP-induced change in the level of phosphorylation of Protein D in the toad bladder membrane might be responsible for the action of antidiuretic hormone, in that tissue, on sodium transport or water transport or both. There are a number of similarities between the properties of the membrane system responsible for the cyclic AMP-dependent phosphorylation of that protein in toad bladder membranes and Protein II in membranes from rat cerebra. For instance, cyclic AMP causes a decrease in the net phosphorylation of Protein D in toad bladder membranes (24) and, in the presence of Zn2+, cyclic AMP causes a decrease in the net phosphorylation of Protein II (Table II). Moreover, the Protein D phosphatase activity of the toad bladder membrane (37) and the Protein II phosphatase activity of the synaptic membrane are each increased in the presence of cyclic AMP. In addition, the minimal molecular weights of the two proteins are similar. Therefore, it is tempting to speculate that the level of phosphorylation of Protein D or other ions across the postsynaptic neuronal membrane. The functional significance of the cyclic AMP-regulated endogenous phosphorylation of the specific proteins observed in the synaptic membrane fractions is an important subject for further investigation.

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Tetsufumi Ueda, Hiroo Maeno and Paul Greengard


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