Cyclic AMP-stimulated Protein Kinases at Brain Synaptic Junctions*

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We have examined endogenous cyclic AMP-stimulated phosphorylation of subcellular fractions of rat brain enriched in synaptic plasma membranes (SPM), purified synaptic junctions (SJ), and postsynaptic densities (PSD). The analyses of these fractions are essential to provide direct evidence for cyclic AMP-dependent endogenous phosphorylation at discrete synaptic junctional loci. Protein kinase activity was measured in subcellular fractions using both endogenous and exogenous (histones) proteins as substrates. The SJ fraction possessed the highest kinase activity toward endogenous protein substrates, 5-fold greater than SPM and approximately 120-fold greater than PSD fractions. Although the kinase activity as measured with histones as substrates was only slightly higher in SJ than SPM fractions, there was a marked preference of kinase activity toward endogenous compared to exogenous substrates in SJ fractions but not in SPM fractions. Although overall phosphorylation in SJ fractions was increased only 36% by 5 mM cyclic AMP, there were discrete proteins of M, = 85,000, 82,000, 78,000, and 55,000 which incorporated 2- to 3-fold more radioactivity in the presence of cyclic AMP. Most, if not all, of the cyclic AMP-independent kinase activity is probably catalyzed by catalytic subunit derived from cyclic AMP-dependent kinase, since the phosphorylation of both exogenous and endogenous proteins was greatly decreased in the presence of a heat-stable inhibitor protein prepared from the soluble fraction of rat brain.

The specific retention of SJ protein kinase(s) activity during purification and their resistance to detergent solubilization was achieved by chemical treatments which produce interprotein cross-linking via disulfide bridges. Two SJ polypeptides of M, = 55,000 and 49,000 were photoaffinity-labeled with [32P]8-N-2-cyclic AMP and probably represent the regulatory subunits of the type I and II cyclic AMP-dependent protein kinases. The protein of M, = 55,000 was phosphorylated in a cyclic AMP-stimulated manner suggesting autophosphorylation as previously observed in other systems.

Ever since protein kinases and their companion "second messenger" molecules were discovered in high quantities in brain, many studies have sought to elucidate their function, particularly in synaptic transmission. The involvement of protein kinases in synaptic transmission is supported by recent observations that cyclic nucleotide-stimulated protein kinases are associated with particular fractions containing synaptic plasma membranes (1-14) and synaptic junctional structures (8). To date, however, direct evidence demonstrating the presence at high specific activities of cyclic nucleotide-dependent protein kinases at discrete pre- or postsynaptic junctional loci (or both) is missing. Evidence of this nature is of obvious importance in assigning roles of protein kinases in synaptic transmission.

This study has examined the distribution of endogenous and cyclic nucleotide-stimulated phosphorylation activity in subcellular fractions enriched in synaptic plasma membranes and in highly purified synaptic junction and postsynaptic density fractions (15-17). The synaptic junction fractions used in these studies represent a 20-fold enrichment in synaptic junctional structures over the SPM fraction from which SJs were obtained (17). Concomitant with this purification of synaptic junctions we observed a 6-fold increase in endogenous phosphorylation activity and a compartmentation in the SJ fraction of specific proteins whose phosphorylation was stimulated 2- to 3-fold by cyclic AMP. The localization of protein kinases and their apparent substrate phosphoproteins in such high quantities in SJ fractions strongly suggests a functional involvement for these macromolecules in synaptic function.

EXPERIMENTAL PROCEDURES

Subcellular Fractionation—Sprague-Dawley male rats (60 to 100 days old) were used. Forebrains were obtained following decapitation, and a crude mitochondrial and synaptosomal fraction (I°) was prepared according to the method of Cotman and Taylor (15). A purified SPM fraction was prepared by the treatment of the lysed P fraction with succinate and INT followed by buoyant density centrifugation of this fraction on a gradient containing 8 ml each of 1.2 M, 1.0 M, and 0.8 M sucrose. Following centrifugation (65,000 × g, 90 min), the SPM fraction was harvested from the 1.0 to 1.2 M sucrose interface as described previously (15). An SJ fraction was prepared from the SPM fraction following treatment of the latter with Triton X-100 (0.4%, v/v) and centrifugation through 1.0 M sucrose. The Triton X-100 treatment solubilized between 88 and 95% of the protein in SPM fractions. A purified PSD fraction was isolated as previously outlined (16) by treatment of the SPM fraction with 3.5% (w/v) N-lauryl sarcosinate.

Electron Microscopy—Synaptic fractions were examined occasionally by electron microscopy to monitor their particulate composition.

* The abbreviations used are: SPM, synaptic plasma membrane; SJ, synaptic junction; PSD, postsynaptic density; INT, iodonitrotetrazolium violet; EGTA, ethylene glycol bis[β-aminoethyl ether]N,N-tetraacetic acid.

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Samples were prepared for electron microscopic examination as previously described (16). All synaptic fractions prepared for these studies compared quite favorably with respect to purity to those obtained in earlier studies from this laboratory (15-19).

**Standard Phosphorylation Assay**—The different synaptic fractions (20 μg of protein) were suspended in 20 mM phosphate buffer (pH 7.2) in a final volume of 20 μl. To this was added 50 μl of standard incubation mixture which contained (final concentration) MgCl₂ (5 mM), sodium phosphate (20 mM; pH 7.2), theophylline (1 mM), dithiothreitol (2 mM), and [γ-³²P]ATP (20 μM; 3.5 Ci/mM/sample).

Some incubations also included cyclic AMP (5 μM). These steps were conducted at 0°C. Some fractions were preincubated under varying conditions (see Results), and this was performed in the initial steps of the assay. The reaction mixture was then incubated for 3 min unless otherwise stated. The phosphorylation reaction was terminated by shifting the temperature back to 0°C and sedimenting the particulate material for 2 min at 10,000 X g at 0°C. The supernatant was removed, and the resulting pellet (100% yield from starting particulate material) was immediately resuspended in 100 μl of sodium dodecyl sulfate sample buffer (20) and then heated for 2 min at 50°C. Part of the sample was then precipitated with trichloroacetic acid and collected on glass fiber filters (Whatman GF/C). Filters were then washed with cold trichloroacetic acid (5%, v/v), and incorporated radioactivity was measured as previously described.

In experiments measuring the phosphorylation of exogenous histones, the above supernatant fraction was likewise precipitated with trichloroacetic acid and incorporated radioactivity was measured by liquid scintillation counting.

**Assay for Cyclic AMP-dependent Protein Kinase Activity** Protein kinase activity was measured by the method described by Hofmann et al. (21) with minor modifications. Activities were measured at pH 7.2 in a final volume of 0.1 ml containing (final concentration) MgCl₂ (5 mM), EGTA (0.5 mM), sodium phosphate (20 mM), theophylline (1 mM), dithiothreitol (2 μM), [γ-³²P]ATP (20 μM, 1.0 Ci/mM/reaction), 10 μg of protein from subcellular fractions, 100 μg of histone f2b (Sigma Chemical Co.), and ± cyclic AMP (5 μM). Some reactions also contained 15 μg of cyclic AMP-dependent protein kinase inhibitor (obtained from rat brain cytosol) which was purified by the method of Walsh et al. (22). Protein kinase activity was measured in various subcellular fractions which were diluted appropriately so that reaction rates were linear and were proportional to the amount of enzyme added (see Hofmann et al. (21)). With the dilutions of the various subcellular fractions used in these assays, the transfer of ³²P to non-histone proteins was less than one-fifth of the total protein-associated radioactivity. Control activities from incubations without histones were subtracted from experimental values to give final estimates of kinase activity. In addition, all reactions were dialyzed overnight at 2°C in phosphate buffer (20 mM) to remove unbound ATP and cyclic AMP. Incubations were carried out for 1 min at 30°C. Reactions were terminated by the addition of 1 ml of ice-cold trichloroacetic acid (10%, v/v), and the precipitated material was collected on glass fiber filters (Whatman GF/C). Filters were washed with cold trichloroacetic acid (5%, v/v), and radioactivity was measured as described above. As in the studies of Hofmann et al. (21), 1 unit of activity is equal to the amount of enzyme catalyzing the transfer of 1 μmol of phosphate to histone f2b per min.

**Binding of [³²H]cyclic AMP to Synaptic Fractions and Photofinity Labeling of Cyclic AMP-binding Proteins with [³²P]8-Azido Cyclic AMP**—The binding of [³²H]cyclic AMP and [³²P]8-N-cyclic AMP to synaptic fractions was carried out by the filter method of Gilman (23). Fractions (20 to 50 μg of protein) were routinely incubated in the standard phosphorylation incubation mixture (100 μl) to which was added [³²H]cyclic AMP at various concentrations (0-20 nM) in a final volume of 20 μl. Non-specific binding was determined from incubations in which a 100-fold excess of unlabelled cyclic AMP was present. Binding reactions were incubated for 60 min at 0°C and then filtered onto 0.45 μm Millipore membranes. Bound radioactivity was determined by liquid scintillation counting.

Following incubation in the presence of [³²P]8-N-cyclic AMP some samples were photolyzed with short wavelength ultraviolet light (253.4 nm) from a UV-8-11 Mineralight lamp located approximately 2 cm away. Following photolysis samples were concentrated by centrifugation at 0°C and solubilized in sodium dodecyl sulfate sample buffer. Covalently bound radioactivity was measured by liquid scintillation counting of trichloroacetic acid-precipitated material as described above.

**Polyacrylamide Gel Electrophoresis**—Sodium dodecyl sulfate-gel electrophoresis and autoradiography were done as previously described (19). All slab gels were cast in an exponential-linear gradient fashion as described elsewhere (20) and varied in acrylamide concentration from 7.5 to 15% (w/v). In some cases the distribution of radioactivity in gels was determined by liquid scintillation counting of gel slices. Because of the high counting efficiency (95%) for ³²P, this was performed directly on slices of gels placed in 1 ml of scintillation mixture (19).

**Materials**—Reagents used for electron microscopy were obtained from Polysciences, Inc. Sodium dodecyl sulfate was obtained from BDH Chemicals Ltd. N-Lauryl sarcosinate was purchased from K & K Laboratories, [³²P]ATP and [³²P]H-N-s-cyclic AMP were purchased from ICN. Type f2 histone (Sigma), cyclic AMP-dependent protein kinase (predominantly type II from bovine heart), INT, N-ethylmaleimide, ATP, dithiothreitol, succinate, o-phenanthroline, 5.5-di-thiobis(2-nitrobenzoic acid), cyclic AMP, 8-bromo-cyclic GMP, and 8-azido-cyclic AMP were obtained from Sigma Chemical Co.

**RESULTS**

**Analysis of Synaptic Fractions**—Two types of synaptic fractions were used in this study; one was prepared with INT and succinate and the other without INT and succinate. In the INT/succinate preparation, the crude mitochondrial fraction was incubated with INT and succinate after osmotic lysis in order to weight the mitochondria so that they can be completely separated from the SPM fraction (15). Succinate serves as a substrate and proton donor for succinic dehydrogenase, a major mitochondrial enzyme, and INT acts as a general proton acceptor. In the above reaction of succinate with succinic dehydrogenase, the formazan (reduced INT) produced forms a dense precipitate in mitochondria, thus increasing their buoyant density, and results in relatively pure SPM fractions which are free of mitochondrial contamination. The SPM fraction is then treated with either Triton X-100 or N-lauryl sarcosinate and subjected to a second gradient centrifugation to obtain the SJ and PSD fractions, respectively. These fractions are sufficiently refined and pure to allow analysis of the macromolecular components present at synaptic junctions. Isolated SJ fractions are comprised of integral synaptic junctions (20%) and postsynaptic membrane specializations (50%) which have lost their attached presynaptic membranes (17). The PSD fraction is greater than 80% pure in postsynaptic densities (18).

**Kinase Activity in Subcellular Fractions**—The level of endogenous phosphorylation (i.e. the amount of ³²P incorporation) in the different subcellular fractions was measured in the presence and absence of cyclic AMP (data not shown). The highest level of cyclic AMP-stimulated ³²P incorporation (disintegrations per min per μg of protein per min) was in the postmitochondrial supernatant fraction (S₂). Total homogenate (H) and SJ fraction displayed the next highest levels of endogenous phosphorylation (i.e. the amount of ³²H incorpo-

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protein kinase activity (units/mg) was in the Sz fraction, soluble SPM, and Pa fractions. The PI fraction displayed fractions from brain. As shown in Table I, the highest specific S2 P? may be quite different in systems containing soluble versus particulate components. Hofmann et al. (21) have recently cellular fractions, especially membrane and particulate fractions, developed a method for determining the concentration of catalytic subunit in tissue fractions by activity measurements using exogenous histones as substrates in the phosphorylation reaction. We have also applied this method to subcellular fractions from brain. As shown in Table I, the highest specific protein kinase activity (units/mg) was in the Sz fraction, followed by decreasing levels in Sz, H, SPM, Triton X-100-soluble SPM, and P2 fractions. The P1 fraction displayed considerably less activity, and the PSD fraction's activity was not significantly different from background values. SPM fractions prepared without INT treatment was only slightly lower (~20%) than standard SPM fractions, the SJ fractions prepared without INT displayed nearly a 6-fold decrement in 32P incorporation compared to the standard SJ preparation. These findings suggest that the high level of phosphorylation in SJ fractions was not an artifact of mitochondrial contamination.

A true measure of protein kinase activity in various subcellular fractions, especially membrane and particulate fractions, is difficult because the nature of kinase-substrate interactions may be quite different in systems containing soluble versus particulate components. Hofmann et al. (21) have recently developed a method for determining the concentration of catalytic subunit in tissue fractions by activity measurements using exogenous histones as substrates in the phosphorylation reaction. We have also applied this method to subcellular fractions from brain. As shown in Table I, the highest specific protein kinase activity (units/mg) was in the Sz fraction, followed by decreasing levels in Sz, H, SPM, Triton X-100-soluble SPM, and P2 fractions. The P1 fraction displayed considerably less activity, and the PSD fraction's activity was not significantly different from background values. SPM fractions prepared without INT treatment (see below) displayed one-eighth of the kinase activity which was present in the standard SJ fraction. The results with Triton-soluble SPM fractions show that this detergent has little effect on kinase activity per se and that Triton solubilizes nearly all of the kinase activity from SPMs prepared without INT. All fractions, except for PSDs, displayed high increments in 32P incorporation in the presence of cyclic AMP. The addition of purified protein kinase inhibitor resulted in a large reduction in cyclic AMP-dependent phosphorylation with an average of 87% inhibition for all assays assayed. The total recovery of protein kinase activity as assayed with histones was approximately 85%.

A comparison of the two assays for kinase activity, i.e. the use of endogenous versus exogenous substrates, underscores the importance of a difference between SJ and SPM fractions. On a protein weight basis, the endogenous protein substrates of SJs incorporate more than 5 times the amount of radioactivity than did comparable incubations with SPM fractions. In contrast, when the phosphorylation of histones by the kinases in these two fractions are compared (Table I), SPMs and SJs display almost equal levels of catalytic activity. These results suggest that whereas the phosphorylation of endogenous substrates are largely preferred in SJ fractions, the kinases of SPMs show little preference for endogenous over exogenous substrates. We have not as yet determined whether this difference is a result of steric or kinetic factors (or both) for SJ kinase-substrate interactions.

Alkaline hydrolysis was performed on SPM and SJ fractions which had incorporated 32P in the standard incubation reaction. Of the total trichloroacetic acid-insoluble radioactivity in either SJ or SPM fractions, 95 ± 2% (n = 8) was hydrolyzed following incubation for 90 min at 90°C in 0.1 N NaOH. This suggests that the incorporated radioactivity in SPM and SJ fractions is present as phosphoserine and phosphothreonine.

**CAMP-stimulated Protein Kinases**

**Table I**

Specific protein kinase activities as assayed with histones

<table>
<thead>
<tr>
<th>Fraction</th>
<th>N*</th>
<th>Specific activity (units/mg)</th>
<th>Activity recovered</th>
<th>Stimulation by cyclic AMP</th>
<th>Inhibition by protein kinase inhibitor</th>
</tr>
</thead>
<tbody>
<tr>
<td>H</td>
<td>4</td>
<td>3.03 ± 0.39</td>
<td>100</td>
<td>506</td>
<td>91</td>
</tr>
<tr>
<td>P1</td>
<td>4</td>
<td>1.63 ± 0.13</td>
<td>20</td>
<td>298</td>
<td>82</td>
</tr>
<tr>
<td>P2</td>
<td>4</td>
<td>2.22 ± 0.45</td>
<td>10</td>
<td>408</td>
<td>85</td>
</tr>
<tr>
<td>Sz</td>
<td>6</td>
<td>3.77 ± 0.18</td>
<td>45</td>
<td>652</td>
<td>88</td>
</tr>
<tr>
<td>SPM</td>
<td>8</td>
<td>2.90 ± 0.21</td>
<td>3</td>
<td>423</td>
<td>83</td>
</tr>
<tr>
<td>Triton X-100-soluble SPM</td>
<td>4</td>
<td>2.48 ± 0.28</td>
<td>2.3</td>
<td>520</td>
<td>90</td>
</tr>
<tr>
<td>SJ</td>
<td>8</td>
<td>3.12 ± 0.31</td>
<td>0.3</td>
<td>583</td>
<td>92</td>
</tr>
<tr>
<td>SJ without INT</td>
<td>3</td>
<td>0.38 ± 0.07</td>
<td>0.01</td>
<td>220</td>
<td>78</td>
</tr>
<tr>
<td>PSD</td>
<td>3</td>
<td>0.03 (N.S.)*</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* N = the number of separated determinations (reactions were carried out in duplicate).

* N.S. = not significantly different from background values.

Protein kinase activity measurements (with histones f2b as substrates) and protein concentration determinations were made on the various fractions as described in the text. The percentage of increase in the presence of cyclic AMP and the percentage of inhibition by the protein kinase inhibitor of 32P incorporation were also measured. Background values of incorporation were obtained with reactions heated for 10 min at 95°C prior to assays.

The relative levels of phosphorylation were also examined in SPM and SJ fractions prepared without INT treatment (not shown). Electron microscopic examination of these fractions shows substantial increases in mitochondrial and undetectable particulate contamination (15, 24). While the level of phosphorylation in SPM fractions prepared without INT treatment was only slightly lower (~20%) than standard SPM fractions, the SJ fractions prepared without INT displayed nearly a 6-fold decrement in 32P incorporation compared to the standard SJ preparation. These findings suggest that the high level of phosphorylation in SJ fractions was not an artifact of mitochondrial contamination.

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contain only 1 nmol of ATP it is highly likely that the concentration of this substrate in the phosphorylating reactions becomes rate-limiting in a short period of time. Second, it is possible that the SPM fraction possesses more endogenous protein phosphatase activity than SJ fractions. These reasons would explain the shorter time to reach maximal levels of incorporation for SPM polypeptides and the much larger decrease in incorporated radioactivity with longer incubation times in the SPM compared to SJ polypeptides.

Electrophoretic and Autoradiographic Analysis of Phosphorylated Proteins in Synaptic Fractions—As seen in Fig. 2 many proteins incorporate $^{32}$P in the different synaptic fractions. Of primary interest to us were those proteins whose $^{32}$P incorporation was stimulated by cyclic AMP. The PSD fraction contained no observable proteins that exhibited cyclic AMP-stimulated phosphorylation (Fig. 2a). The major PSD protein of $M_r = 52,000$ (19) is not a major phosphorylated substrate in this fraction. The two major phosphorylated proteins have apparent molecular weights of 180,000 and 160,000. There is also significant incorporation into two polypeptides of $M_r = 54,000$ and 57,000. Following electrophoresis in the two-dimensional system developed by O'Farrell (25), we observed that these two PSD phosphoproteins co-migrated with the a and b subunits of purified cytoplasmic tubulin.

The SPM fraction contains many phosphorylated proteins having molecular weights between 12,000 and 300,000 (Fig. 2b). Some SPM polypeptides displayed modest cyclic AMP-stimulated phosphorylation. These proteins have molecular weights of 210,000, 160,000, 85,000, 82,000, and 55,000. The two proteins with $M_r = 85,000$ and 82,000 correspond in molecular weight to Proteins Ia and Ib, and the one of $M_r = 55,000$ is comparable to Protein II described by Ueda and Greengard (10). The levels of cyclic AMP-stimulated phosphorylation (~20%) appears considerably less in our SPM fractions than for SPM fractions prepared by Ueda and Greengard (10). Factors which possibly contribute to this difference in levels of cyclic AMP-stimulated phosphorylation are (a) the ATP concentration used by Ueda et al. (5) compared to 6 μM by Ueda et al. (5); and (b) the protein concentrations used by Ueda et al. (5) were 10 times higher than ours. Both of these factors contribute to a situation where the concentration of ATP in the phosphorylating reaction becomes severely rate-limiting, i.e. the lower initial ATP concentration used by Ueda et al. (5) and the higher protein concentration and thus a considerably greater endogenous Mg$^{2+}$-ATPase activity. Therefore, these differences in cyclic AMP-stimulated phosphorylation may result primarily from differences in optimal kinetic rates of cyclic AMP-stimulated $^{32}$P incorporation into SPM proteins under conditions of limited substrate concentration.

The SJ fraction contained the largest number of phosphorylated proteins (Fig. 2c), many of which displayed increased incorporation in the presence of cyclic AMP. The proteins showing maximal cyclic AMP stimulation have apparent molecular weights of 85,000, 82,000, 78,000, and 55,000. A number of SJ proteins displayed lower levels of stimulated incorporation in the presence of cyclic AMP than the bands noted above. This can be seen by comparing the radioactivity profiles from SJ fractions incubated with and without cyclic AMP and then subjected to gel electrophoresis and liquid scintillation counting of gel slices (Fig. 3d). The SJ fraction prepared without INT treatment, in addition to showing a different profile of phosphorylated proteins, also contained proteins that incorporated more $^{32}$P in the presence of cyclic AMP (Figs. 2c and 3b). Cyclic AMP stimulated incorporation into proteins with molecular weights of 85,000, 82,000, and 55,000. These appeared identical in molecular weights with proteins

![FIG. 1. Time course of cyclic AMP (5 μM)-stimulated endogenous phosphorylation of SPM and SJ polypeptides (apparent molecular weights of individual polypeptides designated in parentheses). Corresponding symbols represent measurements made from the same experiment and from the same gel. $^{32}$P incorporation was calculated as disintegrations per min per gel slice (band) following localization of radioactive bands by autoradiography. a, incorporation into SPM polypeptides; b, incorporation into SJ polypeptides; and c, ratio of incorporation into SPM and SJ polypeptides of similar molecular weights.](http://www.jbc.org/)
counterparts in standard SJ fractions although the levels of stimulation were much smaller (see Fig. 3 a and b, and Table II). The overall levels of cyclic AMP stimulation in SJ fractions were 36 ± 3% (n = 8), while that in fractions prepared without INT was approximately 15% (n = 3). The $M_r = 78,000$ component in SJs prepared with INT displayed the highest cyclic AMP stimulation (200%) followed in decreasing order by the $M_r = 82,000$, 55,000, and 85,000 species (Table II). Similar molecular weight proteins in SJ fractions prepared without INT and SPM fractions displayed an average of one-fifth to one-tenth as much cyclic AMP-stimulated phosphorylation (Table II). We have also surveyed the degree of cyclic AMP-stimulated phosphorylation of the $M_r = 85,000, 82,000$, and 55,000 proteins in SPM and SJ fractions following incubation periods of various duration (1/4 to 3 min). The degree of stimulated incorporation into these different SPM and SJ proteins following INT and SPM fractions displayed an average of one-fifth to one-tenth as much cyclic AMP-stimulated phosphorylation (Table II). We have also surveyed the degree of cyclic AMP-stimulated phosphorylation of any SPM or SJ polypeptide. Therefore, there appears to be no significant cyclic GMP-dependent protein kinase activity in these synaptic fractions.

The levels of endogenous and cyclic AMP-stimulated phosphorylation of the different synaptic fractions were quite stable. Little change in these parameters was observed as a consequence of freeze-thawing or storage at -60°C for periods of 1 to 3 months.

Studies on the Nature of Association of Protein Kinases at the Synaptic Junction—We have previously shown that substantial interprotein cross-linking via disulfide bonds exists in SJ fractions prepared via the INT procedure (18). Interprotein disulfide bonds are also present though to a lesser extent in SJ fractions prepared without INT treatment. Since INT is a proton acceptor in the reaction of succinate with succinic dehydrogenase it will also, under appropriate conditions, serve as a general oxidizing agent for other chemical groups such as free sulfhydryls, and in fact, INT will react with free cysteine (reduced form) to produce formazan. Therefore, it may have been that INT acted as a type of cross-linking reagent which caused the retention in SJ fractions of additional components supporting phosphorylation.

In an attempt to reproduce the oxidative effects of INT treatment on SJ polypeptides we used the o-phenanthroline·Cu²⁺ chelate complex which has been shown to greatly stimulate the oxidation of protein sulfhydryl groups, resulting in the formation of inter- and intraprotein disulfide bridges (26, 27). Following osmotic lysis of the crude synaptosomal fraction and centrifugation to remove soluble proteins, the INT treatment was omitted and instead the particulate fraction was treated with the o-phenanthroline (100 μM)·Cu²⁺ (20 μM) complex for 20 min at room temperature and processed by standard procedures to obtain an SJ fraction. These SJs were subjected to the standard incubation, and the phosphorylated proteins were examined by electrophoresis and autoradiography. Fig. 4 shows that the autoradiographic profile of this cross-linked fraction, with or without cyclic AMP, resembles closely the standard SJ preparation. In fact the cyclic AMP-stimulated incorporation into the $M_r = 85,000, 82,000$, 78,000, and 55,000 proteins is just as large in the o-phenanthroline·Cu²⁺ cross-linked SJs as in the SJs prepared with INT. To investigate the distribution of protein kinase protein substrates in SPM fractions, the partitioning of various proteins in Triton X-100 was examined. Following cyclic AMP-
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Fig. 3. Radioactivity profiles from polyacrylamide gels of SJ fractions incubated in the standard phosphorylation reaction. a, SJ fractions prepared with INT; b, SJ fractions prepared without INT. Closed symbols represent incubations in the absence of cyclic AMP and open symbols in its presence. Molecular weights ($\times 10^3$) are placed above appropriate peaks.

Table II
Cyclic AMP-stimulated endogenous phosphorylation of SPM and SJ polypeptides

<table>
<thead>
<tr>
<th>Molecular weights of proteins</th>
<th>Cyclic AMP-stimulated $^{32}$P incorporation</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>SJ with INT</td>
</tr>
<tr>
<td>85,000</td>
<td>95 ± 25</td>
</tr>
<tr>
<td>82,000</td>
<td>168 ± 74</td>
</tr>
<tr>
<td>78,000</td>
<td>195 ± 94</td>
</tr>
<tr>
<td>55,000</td>
<td>123 ± 60</td>
</tr>
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</table>

stimulated $^{32}$P incorporation into INT-prepared SPMs, Triton X-100-insoluble proteins of $M_r = 78,000$, 82,000 and 85,000 contained approximately 4 times more radioactivity than did Triton X-100-soluble proteins of similar molecular weight from the same preparation (data not shown). It appears, therefore, that protein kinases and their protein substrates are cross-linked into the synaptic junctional region by either treatment and thus resist solubilization by Triton X-100 and are isolated in enriched quantities in the SJ fraction.

To guard against the selective adsorption or cross-linking of soluble protein kinases in brain (6) to synaptic membranes during fractionation procedures, we have washed the crude synaptosomal fraction repeatedly following osmotic lysis and have then treated it with one-fifth the standard amount of INT in the absence of exogenous succinate. Under these conditions, the only proton donors in the reduction of INT will be endogenous substrates such as free sulfhydryl groups. The SJ fraction was then prepared and incubated in the standard phosphorylation reaction. As shown in Fig. 4 the autoradiographic patterns of phosphorylated proteins, with and without cyclic AMP, of these SJs were remarkably similar to those of the standard SJ preparation (compare Figs. 4 and 26). Thus, adventitious adsorption or cross-linking of soluble protein kinases to synaptic fractions does not occur.

Triton X-100 was examined for its ability to stimulate endogenous phosphorylation of the SPM fraction. In the presence of 0.4% (v/v) Triton X-100, the SPM fraction incorporated approximately 35% more radioactivity, and the addition of cyclic AMP increased these levels about 20%. It appears, therefore, that the high levels of endogenous phosphorylation in SJ fractions is not simply a result of detergent treatment.

Fig. 4. a, b, autoradiograms of SJ fractions prepared with Cu$^{2+}$/o-phenanthroline-stimulated cross-linking instead of INT treatment; c, d, SJ fractions were prepared from a lysed P3 fraction which was repeatedly washed in 0.32 M sucrose to remove soluble proteins and then treated with one-fifth the standard INT concentration in the absence of added succinate. The plus (+) and minus (−) symbols represent incubations in the presence and absence of cyclic AMP, respectively. The numbers to the left of the profiles indicate molecular weights ($\times 10^3$).
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SPM and SJ fractions isolated without INT treatment were also examined for endogenous phosphorylation following postisolation INT treatment. These results yielded no significant differences in total incorporated radioactivity or autoradiographic patterns (plus or minus cyclic AMP) from those obtained with respective fractions prepared without INT treatment. Thus, the differences in endogenous phosphorylation between SPM and SJ fractions prepared with and without INT treatment are not a result of the treatment alone but rather a consequence of the inclusion of INT in the preparation of synaptic fractions.

Further Characterization of SJ Endogenous Phosphorylation—The endogenous phosphorylation of SJ proteins was inhibited by Ca²⁺. Inclusion of Ca²⁺ (5 mM) in the standard incubation reactions or pretreatment of SJs with Ca²⁺ followed by its removal by repeated centrifugation and washing prior to the addition of Mg²⁺ inhibited ³²P incorporation greater than 95%.

No significant differences in labeling patterns, with or without cyclic AMP, were observed by the omission of dithiothreitol from the standard incubation. There is, however, a class of SJ proteins whose phosphorylation is more sensitive to free sulphydryl blocking agents. Treatment of SJs with N-ethylmaleimide or 5,5'-dithiobis(2-nitrobenzoic acid) prior to incubations reduced the amount of ³²P incorporation by 50% and 70%, respectively. Sulphydryl blocking agents did not, however, restrict the cyclic AMP-stimulated phosphorylation of specific SJ proteins (Fig. 5 a and b). In fact, it appears that these blocking agents have more pronounced inhibitory effects on cyclic AMP-independent phosphorylation. In SJs pretreated with either blocking agent the M_r = 85,000, 82,000, 78,000, and 55,000 proteins displayed a comparable and often greater level of stimulated incorporation with the addition of cyclic AMP (Fig. 5 a and b) compared to untreated SJs (see Figs. 2c and 3a).

To examine the nature and specificity of SJ protein kinases for endogenous protein substrates, the cyclic AMP-stimulated phosphorylation of SJ proteins was examined following the addition of 0.5 μg (1.0 pmol unit, as described by Sigma) of an exogenous cyclic AMP-dependent protein kinase (Sigma, type II prepared from bovine heart). In the presence of cyclic AMP (5 μM), the phosphorylation of standard SJ and SJ fractions prepared without INT treatment was stimulated 21% and 100%, respectively, by the addition of the commercially available cyclic AMP-dependent protein kinase. Following electrophoresis and autoradiography of these SJ preparations, the incorporation stimulated by exogenous kinase in the presence of cyclic AMP was determined to be nonspecific with respect to protein species. Phosphorylation endogenous to the SJ fraction appears, therefore, to be maximal with regard to the number of protein substrates since no new proteins were phosphorylated in the presence of added kinase.

To further characterize the cyclic AMP-stimulated phosphorylation of SJ fractions, the effect of a heat-stable cyclic

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**Fig. 5.** The effects of sulphydryl blocking agents on SJ phosphorylation. a, autoradiograms of SJ fractions pretreated with N-ethylmaleimide (N-EM) (10 mM) or 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) (5 mM) and then incubated in the standard phosphorylation reaction in the presence (+) or absence (−) of cyclic AMP. b, radioactivity profile of the polyacrylamide gel of the N-ethylmaleimide-pretreated SJ fraction above; closed and open symbols indicate incubations in the absence and presence of cyclic AMP, respectively.
AMP-dependent protein kinase inhibitor (isolated from a soluble rat brain fraction by the method of Walsh et al. (22)) on endogenous phosphorylation was examined. Fig. 6 shows the effect of adding the inhibitor (7 μg) to the standard incubation. Endogenous phosphorylation was decreased 66% by this concentration of inhibitor, either in the presence or absence of cyclic AMP. At higher inhibitor concentrations (14 to 20 μg) endogenous phosphorylation was inhibited approximately 90%. The SJ proteins normally showing maximal cyclic AMP-stimulated incorporation displayed no such stimulation in the presence of the inhibitor (Fig. 6). The inhibitor also abolished the cyclic AMP-stimulated phosphorylation of exogenous histones by SJ protein kinases. Thus, most if not all of the endogenous phosphorylation of SJ fractions, even that which is not strictly dependent on cyclic AMP, appears to be carried out by catalytic subunit derived from cyclic AMP-dependent protein kinases, a class of protein kinases which has been characterized in a number of other cellular systems (28).

Inoue et al. (29) recently characterized a cyclic AMP-independent protein kinase from brain soluble and particulate fractions whose activation was mediated via Ca⁡²⁺-dependent proteolysis. This protein kinase could only be purified in its inactive proenzyme form if media contained EDTA and EGTA. To determine whether or not a kinase of this type was present in SJ fractions, SJs were prepared following standard procedures except that all media contained EGTA (10 mM) and EDTA (5 mM). SJs were then subjected to the standard phosphorylation reaction and analyzed by gel electrophoresis and autoradiography. SJs prepared in the presence of chelators display phosphorylation profiles that are very similar to standard SJ preparations (data not shown). The SJ proteins whose phosphorylation is stimulated by cyclic AMP are also present in SJ/EDTA/EGTA preparations. Furthermore, treatment of SJ/EDTA/EGTA fractions with Ca⁡²⁺ (5 mM) prior to the standard phosphorylation reaction strongly inhibited (-95%) endogenous phosphorylation, while preincubations with Ca⁡²⁺ plus a brain-soluble enzyme fraction (a potential source of Ca⁡²⁺-activated proteases) had little if any effect on subsequent autoradiographic profiles (data not shown). Therefore, a cyclic AMP-independent protein kinase of the type described by Inoue et al. (29) does not appear to be present in isolated SJs.

Binding of [³²P]Cyclic AMP to Synaptic Fractions and Photoaffinity Labeling of Cyclic AMP-binding Proteins with [³²P]8-Azido Cyclic AMP—The concentration-dependent binding of [³²P]Cyclic AMP to SPM and SJ fractions is shown in the Scatchard plot in Fig. 7. The apparent dissociation
constants (Kd) for a high affinity [3H]cyclic AMP binding was 0.07 μM for the SJ fraction and 0.10 μM for the SPM fraction. As seen in Fig. 7, both fractions displayed saturable binding which was proportional to the amount of protein used up to 100 μg (data not shown). Both fractions displayed low affinity binding of [3H]cyclic AMP with a Kd of 2.0 μM. The Scatchard plot further shows that per mg of protein the SJ fraction contains about 75% more high affinity binding sites for cyclic AMP than does the SPM fraction. Additional [3H]cyclic AMP-binding studies were conducted with PSD fractions and SJ and SPM fractions prepared without INT. These binding experiments were conducted at final [3H]cyclic AMP concentrations which ranged from 150 nM to 300 nM. The SPM and SJ fractions prepared without INT bound 33% and 78% less cyclic AMP per mg of protein, respectively, than did their INT-prepared counterparts. The PSD fraction bound approximately 20-fold less [3H]cyclic AMP than did the SJ fraction.

To identify the specific cyclic AMP-binding sites (polypeptides) in SPM and SJ fractions, the incorporation of the photoaffinity analog [32P]8-N+yclic AMP was examined. Both SPM and SJ fractions bound similar amounts of [32P]8-N+yclic AMP as compared to respective values obtained with [3H]cyclic AMP. [32P]8-N+yclic AMP binding in incubations at an analog final concentration of 150 nM appeared quite specific since the amount bound was reduced 70% in the presence of unlabeled cyclic AMP (30 nM). The ability of 8-N+yclic AMP to mimic cyclic AMP-stimulated phosphorylation was tested. As seen in Fig. 8, the analog produces similar levels of stimulated phosphorylation of the M, = 49,000, 55,000, 78,000, and 55,000 SJ proteins. In this system, as in others (30, 31), 8-N+yclic AMP appears to be an appropriate cyclic AMP analog and a suitable candidate for the photoaffinity labeling of cyclic AMP-binding proteins. SJ and SPM fractions were incubated in the presence of [32P]8-N+yclic AMP (150 nM final concentration) and then subjected to photolysis (see “Experimental Procedures”). Fractions were subjected to electrophoresis, and covalently bound radioactivity was detected by autoradiography and measured by liquid scintillation counting. The amount of covalently bound radioactivity reached a maximum after 30 min of incubation at 0°C and photolysis for 10 min. As seen in Fig. 8, more than 95% of the bound radioactivity was present in two polypeptides with apparent molecular weights of 55,000 and 49,000. In SJ fractions approximately 36% more covalently bound radioactivity was present in the M, = 49,000 band than in the M, = 55,000 band (Table III). In contrast, the SPM fraction contained approximately 39% more radioactivity in the M, = 55,000 band than in the M, = 49,000 band. The total covalently bound radioactivity in SPM or SJ fractions represented about 8% of the total radioactivity reversibly bound to these fractions under the conditions used for [32P]8-N+yclic AMP for 3 min.

**Table III**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>M, = 55,000 (A)</th>
<th>M, = 49,000 (B)</th>
<th>Ratio, B/A</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPM</td>
<td>227 ± 29</td>
<td>0.115</td>
<td>0.083</td>
</tr>
<tr>
<td>SJ</td>
<td>281 ± 32</td>
<td>0.142</td>
<td>0.194</td>
</tr>
</tbody>
</table>

The M, of this protein was calculated on the basis of its electrophoretic migration compared to that of the larger M, subunit of purified tubulin which has a reported M, of 56,000 (36).
could be the result of endogenous cyclic AMP, the presence of cyclic AMP-dependent phosphorylation of any SJ protein has not been observed. The apparent absence of cyclic AMP-dependent phosphorylation of SJ proteins of mass 85,000 and 82,000 whose phosphorylation is stimulated by cyclic AMP have molecular weights considerably reduced. A major difference between the membrane preparations used in previous studies and our SJ fraction is that the SJ fraction is treated with detergent which may alter the ability of the inhibitor to interfere with the protein kinase-protein substrate interaction. Recently, Uno et al. (11) have in fact reported that in its purified form the activity of a cyclic AMP-dependent protein kinase from neuronal membranes is inhibited by a protein kinase modulator (inhibitor) from bovine brain.

Numerous reports have shown that cyclic AMP-dependent protein kinases are associated with plasma membrane fractions from brain (2, 3, 7, 8, 10, 12, 13) and other tissues (28). Weller and Morgan (8) have described a moderate increase (40%) in endogenous phosphorylation activity in a fraction enriched in synaptic junctional material compared to the activity in synaptic plasma membrane fractions. They also reported an approximate 2-fold increase in cyclic AMP-stimulated activity in these synaptic junction fractions compared to the same activity in SPM fractions. These results partially agree with those presented here. However, there are a number of significant differences. Their values for the enrichment of endogenous phosphorylation activity in SJ fractions are approximately one-fifteenth of our values. Furthermore, Weller and Morgan (8) reported, on the basis of electrophoretic separations, that differences in the amount and distribution of 32P-labeled proteins between SPM and SJ fractions both incubated with cyclic AMP were quite small except in a high molecular weight region (M, > 100,000) where SJ proteins incorporated about twice as much radioactivity. The fact that we used the INT method for preparing SJ fractions and Weller and Morgan did not most likely accounts for these differences.

DISCUSSION
Our studies show that cyclic AMP-stimulated4 protein kinases are enriched in SJ fractions of isolated SJIs. The level of endogenous phosphorylation in isolated SJIs is 6-fold higher than in the SPM fraction from which they are obtained. The SJ fractions used in these studies are greater than 60% pure by particulate volume in synaptic junctional structures (17) which represents a 20-fold enrichment over SPM fractions which contain 3% synaptic junctional structures as measured by particulate volume (17). It would appear, therefore, that a significant portion of SPM protein kinases are localized to the synaptic junction. This finding suggests an in vivo compartmentation of these enzymes at central nervous system synaptic junctions. The synaptic fractions used in earlier studies (7, 8, 12, 13) contain considerably smaller proportions of synaptic junctional structures and, therefore, are not ideal fractions in which to examine the functional roles of protein kinases at central nervous system synapses.

The endogenous protein substrates of the cyclic AMP-stimulated protein kinases also appear enriched in SJ fractions. Exogenous protein substrates can be phosphorylated in a cyclic AMP-stimulated fashion via SJ protein kinases; however, endogenous SJ proteins are preferred. In contrast, SPM kinase activity displays little substrate preference between endogenous and exogenous (histones f2b) proteins. The two SJ proteins of M, = 85,000 and 82,000 whose phosphorylation is greatly stimulated by cyclic AMP have molecular weights very similar to the phosphoproteins Ia and Ib described by Ueda et al. (10) in bovine SPM fractions. Moreover, Uno et al. (33) have shown that cyclic AMP-dependent protein kinase purified from neuronal membranes phosphorylates proteins Ia and Ib much more effectively than either lysine-rich or arginine-rich histones. Thus, our results from measurements of kinase activity using exogenous (histones f2b) versus endogenous protein substrates suggests two possible interpretations for the localization and possible enrichment of kinases in the SJ fraction: (a) although about the same relative amounts of specific kinase activity (assayed with histones) are present in SPM and SJ fractions, the high endogenous phosphorylation activity in SJIs results from an enrichment of intrinsic proteins which are much better substrates than histones; or (b) the specialized compartmentation of kinase-substrate components in SJ fractions sterically favors their interactions so that measurements of activity toward exogenous substrates results in abnormally low values for SJ fractions and more accurate values for SPMs.

Characteristics of Protein Kinases at Synaptic Junctions—The effect of a purified cyclic AMP-dependent protein kinase inhibitor on the phosphorylation of endogenous and exogenous substrates shows that the protein kinase activity in SJ fractions is predominantly, if not entirely, derived from catalytic subunit of cyclic AMP-dependent protein kinases. Previous studies with similar inhibitors but different membrane preparations have been unable to inhibit cyclic AMP-stimulated phosphorylation of endogenous substrates although the phosphorylation of exogenous substrates was considerably reduced (1, 14). A major difference between the membrane preparations used in previous studies and our SJ fraction is that the SJ fraction is treated with detergent which may alter the ability of the inhibitor to interfere with the protein kinase-protein substrate interaction. Recently, Uno et al. (11) have in fact reported that in its purified form the activity of a cyclic AMP-dependent protein kinase from neuronal membranes is inhibited by a protein kinase modulator (inhibitor) from bovine brain.

4 We use the term "stimulated" because a totally cyclic AMP-dependent phosphorylation of any SJ protein has not been observed. The apparent absence of cyclic AMP-dependent phosphorylation could be the result of endogenous cyclic AMP, the presence of catalytic subunit derived from cyclic AMP-dependent protein kinases, or the presence of cyclic AMP-independent protein kinase(s).

5 This band is often resolved as a narrowly spaced doublet.

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Organization of Protein Kinases at Synaptic Junctions—The nature of the association of cyclic AMP-stimulated protein kinases with SPM and SJ fractions is presently unknown. We would propose that a considerable portion of these kinases is associated with synaptic junctional plasma membranes, but they may not exist as integral membrane proteins. This idea is supported by a number of observations. The SJ fraction contains the highest endogenous protein kinase activity and also contains considerable amounts of synaptic junctional plasma membranes. Little protein kinase activity and [3H]cyclic AMP binding and no observable cyclic AMP-stimulated protein kinase activity is present in PSD fractions which are nearly devoid of identifiable plasma membranes. Finally, SJ fractions obtained from SPM fractions prepared without cross-linking agents lose a majority of their protein kinase activity following detergent treatment, and, therefore, protein kinases may not be integral membrane proteins.

The observation that SPM protein kinases are not integral
membrane proteins is supported by the studies of Uno et al. (11). They reported that approximately 80% of the cyclic AMP-dependent protein kinase activity of neuronal plasma membranes can be extracted with Triton X-100. This appears consistent with our findings using SPM fractions prepared without INT. In these SPM fractions approximately one-tenth of the total endogenous phosphorylation activity is retained in the insoluble fraction following Triton X-100 treatment of SPM fractions. The membrane fractions used by Uno et al. (11) were prepared from brain tissues homogenized by a Waring Blender and not by conventional homogenization methods which preserve a greater degree of in situ subcellular integrity. Furthermore, their SPM fractions were obtained from gradient interfaces of 0.9 M and 1.0 M sucrose compared to the 1.2 M sucrose interfaces used in this study. We and others have observed that SPM fractions which contain the most synaptic junctional structures are found at gradient interfaces below 1.0 M sucrose (15, 24). We have prepared SJ fractions from INT-treated SPM fractions obtained from the 1.0 M sucrose interface. These SJs contain similar levels of endogenous phosphorylation and autoradiographic patterns (± cyclic AMP) compared to SPM and SJ fractions prepared by the methods described above (see “Experimental Procedures”). The yield of SJ's on the basis of protein weight from 1.0 M sucrose interface SPMs, however, is 20-fold less than that from SPMs at 1.2 M sucrose interfaces. Although we have not attempted a systematic comparison between our SPM fractions and those prepared by Uno et al. (11), it is reassuring that our observations concerning neuronal membrane-associated protein kinases, such as their solubilization from uncross-linked SPMs, are in general agreement.

The precise distribution of synaptic junction protein kinases will undoubtedly await the electron microscopic immunochromatic localization of these enzymes. However, the observation that SJ fractions contain more than twice as many postsynaptic junctional membrane specializations (postsynaptic membrane plus PSD) than intact synaptic junctions (both pre- and postsynaptic membranes) suggests that the greatest proportion of cyclic AMP-dependent protein kinases are present in postsynaptic structures as previously suggested (9). This suggested postsynaptic localization of components responsible for cyclic AMP-stimulated protein phosphorylation is supported by the immunohistochemical localization of cyclic nucleotide phosphodiesterases, another component in this system, in predominantly postsynaptic structures (4).

The Importance of Protein Kinase Sulphydryl Groups—The cross-linking studies presented here show that cyclic AMP-stimulated protein kinases can be linked into the SJ, probably through reactive sulphydryl groups. The sulphydryl groups that mediate cross-linking are apparently not at important catalytic or cyclic AMP-binding sites since the protein kinase's response to cyclic AMP binding and subsequent induction of protein substrate phosphorylation is unrestricted. SJ fractions prepared without cross-linking agents contain considerably less endogenous cyclic AMP binding and subsequent phosphorylation activity than the standard SJ fraction. These differences are a result of the detergent solubilization of protein phosphorylation components from SJ fractions prepared without cross-linking.

The effects of sulphydryl blocking agents on endogenous phosphorylation in SJ fractions is interesting in view of the dramatic inhibitory action of these agents on purified catalytic subunit activity of cyclic AMP-dependent protein kinase reported by Bechtel et al. (34). N-Ethylmaleimide and 5,5'-dithiobis(2-nitrobenzoic acid) were found to inhibit the purified catalytic subunit activity 85% and 98%, respectively. Although these sulphydryl blocking agents inhibit endogenous phosphorylation activity in SJs approximately 50%, the phosphorylation of SJ proteins which normally display the largest cyclic AMP stimulation are affected little, if at all (Fig. 5).

This difference in sensitivity to sulphydryl blocking agents between SJ and purified catalytic subunit activities suggests that they may possibly be different enzymes or more likely that the SJ holoenzyme(s) and specific substrate proteins are organized in a way that resists these inhibitory phenomena.

The Nature of the Regulatory Subunit(s) of Synthetic Junction Protein Kinases—The measurement of cyclic AMP binding is generally accepted to be a quantitative measure of the regulatory subunit(s) of cyclic AMP-dependent protein kinases (21). Our results from [3H]cyclic AMP-binding experiments and the photoaffinity labeling with [32P]-N-cyclic AMP provide strong evidence that more than one type of cyclic AMP-dependent protein kinase is present in rat brain SPM and SJ fractions. [32P]-N-cyclic AMP-labeling patterns clearly show two major cyclic AMP-binding proteins. One of these, the M, = 55,000 protein, displays cyclic AMP-stimulated phosphorylation, a phenomenon analogous to the autophosphorylation observed for the regulatory subunit of the type II cyclic AMP-dependent protein kinase in heart (32) and brain soluble (6) particulate fractions (11). The M, = 55,000 protein we have observed appears slightly larger in molecular weight than the M, = 52,000 protein from bovine brain particulate fraction recently described by Uno et al. (11). At present, we are not certain whether the M, = 55,000 protein from SPMs is similar to the M, = 52,000 regulatory subunit described by Uno et al. (11) or the M, = 55,000 regulatory subunit from the type II cyclic AMP-dependent protein kinase (32). The second cyclic AMP-binding protein (M, = 49,000) observed in this study may represent the regulatory subunit of a different cyclic AMP-dependent protein kinase holoenzyme since its molecular weight is very similar to that of the regulatory subunit of the type I cyclic AMP-dependent protein kinase (32). Analogous to the type I regulatory subunit, the endogenous phosphorylation of the M, = 49,000 protein in SJ fractions is not stimulated by cyclic AMP. Our findings are different from those of Uno et al. (11) in that they found only one major cyclic AMP-dependent protein kinase regulatory subunit in Triton X-100 extracts of neuronal plasma membranes.

It is interesting to note that the ratio of M, = 55,000 to M, = 49,000 proteins determined by photoaffinity labeling is different between SPM and SJ fractions (Table III). The SPM fraction contains considerably more (≈38%) M, = 55,000 than M, = 49,000 protein while the ratio for SJ fractions is reversed so that SJs contain approximately 36% more M, = 49,000 than M, = 55,000 protein. Walter et al. (31) have recently used this same photoaffinity ligand in the identification and quantitative measurement of cyclic AMP receptor protein proteins from soluble and particulate extracts. The reported dissociation constants of 0.07 μM and 0.20 μM for cyclic AMP-binding proteins of M, = 47,000 and M, = 54,000, respectively. Although our studies on [3H]cyclic AMP binding (Fig. 7) did not employ purified cyclic AMP receptors, the apparent Kd values for SPM (0.10 μM) and SJ (0.07 μM) fractions are in general agreement with values reported by Walter et al. (31) if we assume some correspondence between soluble and particulate cyclic AMP receptor proteins. The difference in apparent Kd values for cyclic AMP-binding proteins between SPM and SJ fractions is in agreement with the photoaffinity labeling of two distinct components in these fractions and supports the notion that: 1) at least two types of cyclic AMP-dependent protein kinases are present in synaptic fractions; and 2) their distribution in SJ fractions appears distinct from that in SPMs.
Corbin et al. (35) have recently described a particulate fraction-associated cyclic AMP-dependent protein kinase in rabbit heart. This protein kinase appears to be almost exclusively the type II protein kinase holoenzyme. In addition, it appears that the catalytic subunit of the protein kinase holoenzyme is bound to membranes via the regulatory subunit and is dissociated from the membrane-holoenzyme complex following the binding of cyclic AMP to the regulatory subunit. In view of these findings, we have preincubated and washed SJ fractions in media containing cyclic AMP and were unable to remove any detectable amount of endogenous phosphorylation activity (±cyclic AMP) or change significantly the autoradiographic pattern of radiolabeled substrates. These results indicate a difference in the cyclic AMP-mediated dissociation of a presumptive membrane-holoenzyme complex between SJ fractions and the heart particulate fractions. However, the presumed protein kinase catalytic subunit in SJs may be sufficiently cross-linked to neighboring macromolecules and cannot be dissociated by cyclic AMP.

The high levels of endogenous phosphorylation in SJ fractions in the absence of cyclic AMP suggest an apparent excess of free catalytic subunit derived from cyclic AMP-dependent protein kinase. The high degree of inhibition (~90%) of endogenous phosphorylation by the purified inhibitor further supports the notion of excess free catalytic subunit in SJ fractions. Finally, the observation that in SJs there exist two levels of cyclic AMP-stimulated phosphorylation, i.e., the general 36% stimulation versus the 2- to 3-fold stimulation of the $M_c = 85,000, 82,000, 78,000$, and $55,000$ proteins, suggests that some degree of compartmentation exists between protein kinase holoenzymes and specific substrate proteins.

Evidence for the localization of a cyclic AMP kinase-substrate system at important synaptic loci comes from developmental and comparative studies. We have observed a dramatic appearance of both kinase activity and proteins whose phosphorylation is greatly stimulated by cyclic AMP in SJ fractions isolated from immature rat brains at a time when the major period of synapse formation begins (7 to 8 days post-natal). In addition, we have observed significant amounts of kinase activity as well as the major phosphoproteins whose phosphorylation is stimulated by cyclic AMP ($M_c = 82,000, 78,000$, and $55,000$) in SJ fractions isolated from human, steer, and chicken central nervous system tissues.

In summary, cyclic AMP-stimulated protein kinases are present in high concentrations at synaptic junctions, the locus of synaptic transmission in the central nervous system. Only a few proteins display large increments in phosphorylation in response to cyclic AMP, and they are not major proteins of the SJ fraction. The molecular weights of the putative protein kinase regulatory subunits in SJ fractions appear quite similar to those described in other tissues although the presumed membrane association of the type I regulatory subunit has not been previously reported. In situ, this class of protein kinases is presumably mediated by the action of transmitters on adenylyl cyclases present at catecholaminergic synapses (9).

The activity of these protein kinases is directly modulated by cyclic AMP and also perhaps by the status of intramolecular sulphydryl groups and disulfide cross-linking as demonstrated in these studies. Cyclical nucleotide-dependent protein kinases represent a reasonably well characterized molecular system which is discrete in operation and likely plays a key role in synaptic transmission.

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