CYCLIC AMP REGULATES PHOSPHORYLATION OF THREE PROTEIN COMPONENTS OF RAT CEREBRAL CORTEX MEMBRANES FOR THIRTY MINUTES

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1. Introduction

Adenosine 3′, 5′-monophosphate (cyclic AMP) has been implicated as the second messenger following neurotransmitter release from presynaptic terminals. As would be predicted by this hypothesis, a rise in cyclic AMP levels following synaptic transmission has been demonstrated [1]. The specific role which cyclic AMP plays in synaptic transmission has not been defined, although a recent clue to its function may be that this cyclic nucleotide regulates phosphorylation of specific proteins in synaptic membrane fractions of rat cerebrum [2–4].

The phosphorylation state of synaptic membrane proteins is maintained by membrane-bound protein kinases and phosphoprotein phosphatases, and this state was shown to be regulated by cyclic AMP [2, 5–7]. It has been suggested that this system plays a role in modifying the response of neurons for several minutes to subsequent inputs over long durations [8,9]. This implies that cyclic AMP, generated consequent to synaptic transmitter release, should also induce changes which last long enough to account for such modifiability. Supporting evidence for the extent of cyclic AMP involvement in such processes may be obtained if the regulated endogenous phosphorylation system is studied for periods longer than the two minutes previously reported [3,4]. In the present study, therefore, the time course of this enzymatic endogenous system was followed for 30 min. We have found that cyclic AMP-dependent phosphorylation affects each of several brain proteins in different ways throughout the 30 min period. These results contrast with previously reported [3,4] findings with regard to the number of major phosphoprotein bands, the time course of phosphorylation and its stimulation by cyclic AMP.

2. Methods

Membrane fractions were prepared by differential centrifugation from cerebral cortex of male adult Holtzman albino rats, weighing 200–250 g, dissected from the dorsal cortical surface in the cold room and placed on dry ice. The P2 fraction of Whittaker [10] was osmotically shocked in the presence of 50 μM calcium chloride [11] under slightly basic conditions, a procedure which reduces the attachment of synaptic vesicles to the membrane [12]. After centrifugation at 20 000 g for 20 min, the pellet was resuspended in a neutral medium containing 1 mM magnesium acetate, and washed three times. The final pellet is referred to as the crude synaptosomal preparation in this study. An enriched synaptic plasma membrane (SPM) fraction prepared as described by Franklin and Cox [13], and five membrane subfractions of rat cerebrum prepared by Dr Gary Davis on a sucrose density gradient by the recently developed method of Davis and Bloom [14] were also used. The various membrane preparations used were frozen and stored at −20°C for periods up to a month, and no differences in activity were noticed following storage during this period.

The phosphorylation assay procedure began with a 5 min preincubation at 30°C of a mixture containing 100 mM sodium acetate buffer pH 6.5,

20 mM magnesium acetate and 4 mg/ml protein of the cerebral cortex membrane fraction. The reaction was initiated by addition of an equal volume of commercially available [γ-^{32}P]ATP (I.C.N.), with or without cyclic AMP (Sigma), dissolved in 50 μM Tris–HCl, pH 7.4. Radioactive ATP was diluted with non-radioactive Tris–ATP (Sigma). The final concentrations in the reaction mixture of ATP (7.5 μM), cyclic AMP (5 μM) and salts were as described by Johnson et al. [3]. Determination of protein was done according to the Lowry method [15] with bovine serum albumin (Sigma, Type V) as standard, and that of protein-bound [^{32}P]phosphate according to Dods and Burdowski [16]. The reaction was terminated by 3% SDS (with 2% β-mercaptoethanol and 9% sucrose) which solubilized the membrane fragments. After denaturation (80°C for 30 sec) bromphenol blue, a dye marker, was added and samples each containing 130 μg protein, were subjected to a discontinuous gradient (7%, 9%, 12%) SDS-polyacrylamide gel electrophoresis in the Ortec 4207 verticle slab-gel cells, using the discontinuous buffer system of Grossfeld and Shooter [17], with 0.1% SDS added to the cathodic chamber. Electrophoresis was carried out at room temperature for 27 hr, at 100 V, 150 pulses per sec/gel and 0.1 mF, using the Ortec 4100 pulsed constant power power supply. The gel was stained for protein with Coomassie Brilliant Blue, and then autoradiographed by placing Kodak Royal blue X-ray film in close contact with Saran Wrap (DOW) covered gel for 4–7 days. For quantitation of the results, 2 mm gel slices were solubilized with NCS (Amersham/Searle) and radioactivity was measured by scintillation counting. Densitometric scanning of the autoradiograms was performed using a Joyce–Loebel Microdensitometer. For estimation of molecular weight, electrophoreses of fibrinogen, alpha and beta globulin, bovine serum albumin, bovine catalase, alpha amylase, ovalbumin and bovine chymotrypsinogen-A (Sigma) were performed. The relative mobility was found to be linearly proportional to the logarithm of molecular weight within each layer of the gel.

Since our assay does not measure the activities of protein kinase and phosphoprotein phosphatase separately, but rather the net result of the activity of both enzymes, the terms phosphorylation and dephosphorylation will be used to describe increments and decrements, respectively, in phosphate content of each phosphorylated component, but such terms do not refer to the capacity of a specific enzyme.

3. Results

Before describing the major results, it should be noted that we have found the overall incorporation of phosphate into the SPM prepared as described previously [13], to be 30–40% lower than into proteins of osmotically shocked, crude synaptic membrane preparations. Furthermore, the specific activity of the Davis and Bloom [14] preparation was similarly reduced relative to the crude synaptosome. Thus, partial inactivation of the system occurs during ultracentrifugation in hyperosmotic sucrose solutions. In the present study, then, the time course of endogenous phosphorylation and the regulatory effects of cyclic AMP were measured using the crude synaptosome preparation, as described in Methods. Figs. 1 and 2 illustrate a representative analysis of one of six experiments. The qualitative pattern of results did not vary for these six separate membrane preparations.

As shown in fig. 1 three protein components, designated D, E and F, demonstrated higher levels of phosphate incorporation than any other proteins. The time course of phosphorylation of these three protein components in the absence of cyclic AMP is shown in fig. 2a. It can be seen that for all components peak phosphorylation is reached at 20 sec and then dephosphorylation occurs, at a higher rate for band D in comparison to bands E and F. The time course of phosphorylation in the presence of cyclic AMP is shown in fig. 2b. Band E shows a peak incorporation at 20 sec, with rapid dephosphorylation in the next 40 sec. Band F, in contrast, rises slightly in incorporation from 20 sec to 1 min. Band D shows a negligible decline in phosphorylation state from 20 sec to 1 min.

Perusal of fig. 2c reveals that each protein component follows a separate time course of percent stimulation by cyclic AMP. Protein component D has its peak stimulation at 1 min. In contrast, protein component E has a peak stimulation at the 20 sec time point, and then a gradual rise in percent stimulation after 2 min. This contrasts with band D and band F which show a gradual decline in cyclic AMP stimula-

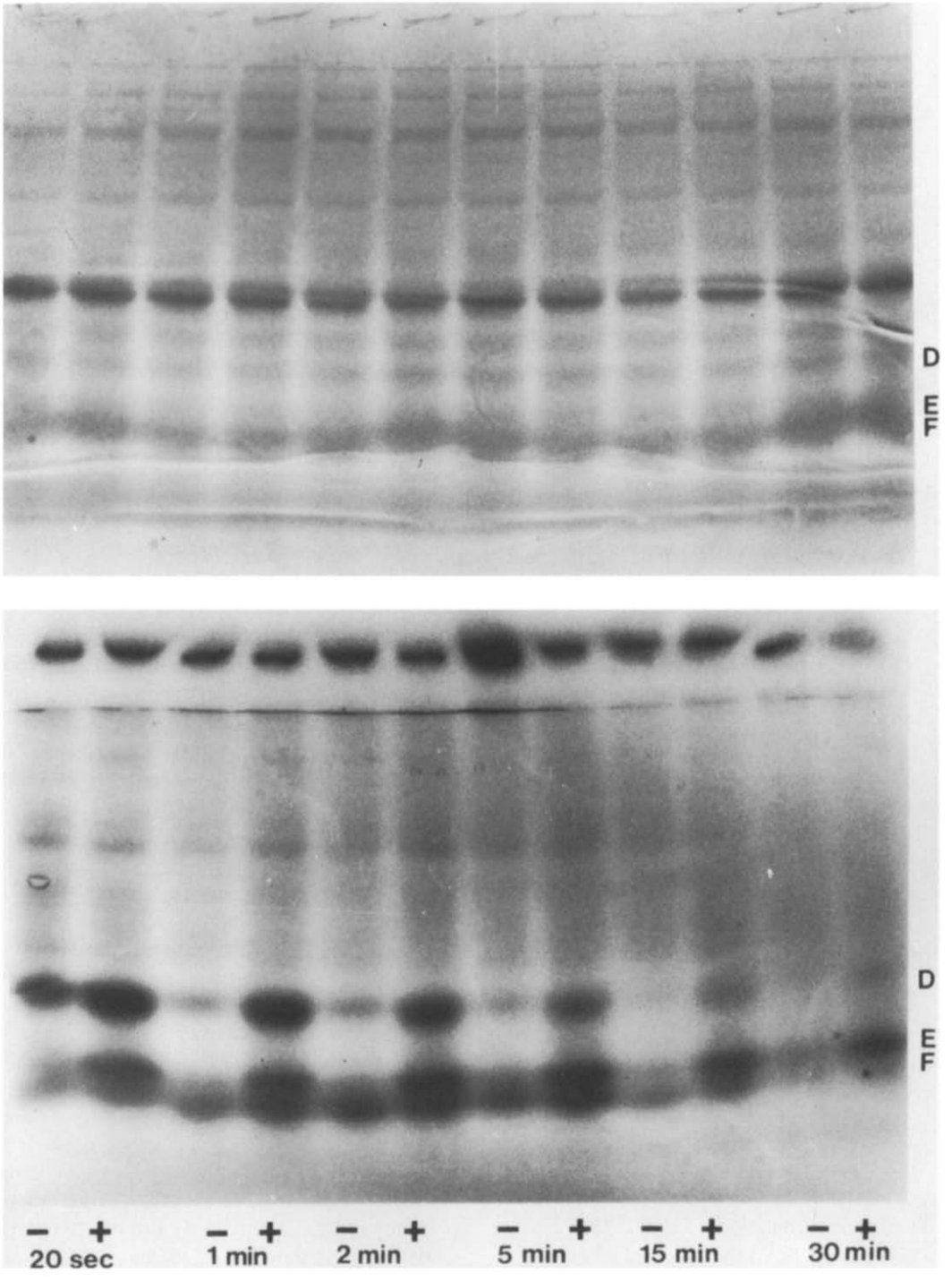

Fig. 1. Samples obtained during the in vitro assay of cerebral cortex membranes in the absence (–) or presence (+) of 5 μM cyclic AMP were solubilized in SDS, electrophoresed in polyacrylamide gel, stained for protein (top) and autoradiographed (bottom) as described in Methods. The indicated times were measured from the initiation of the reaction by [γ-^{32}P]ATP (7.6×10^6 cpm per nmole), following preincubation without cyclic AMP.

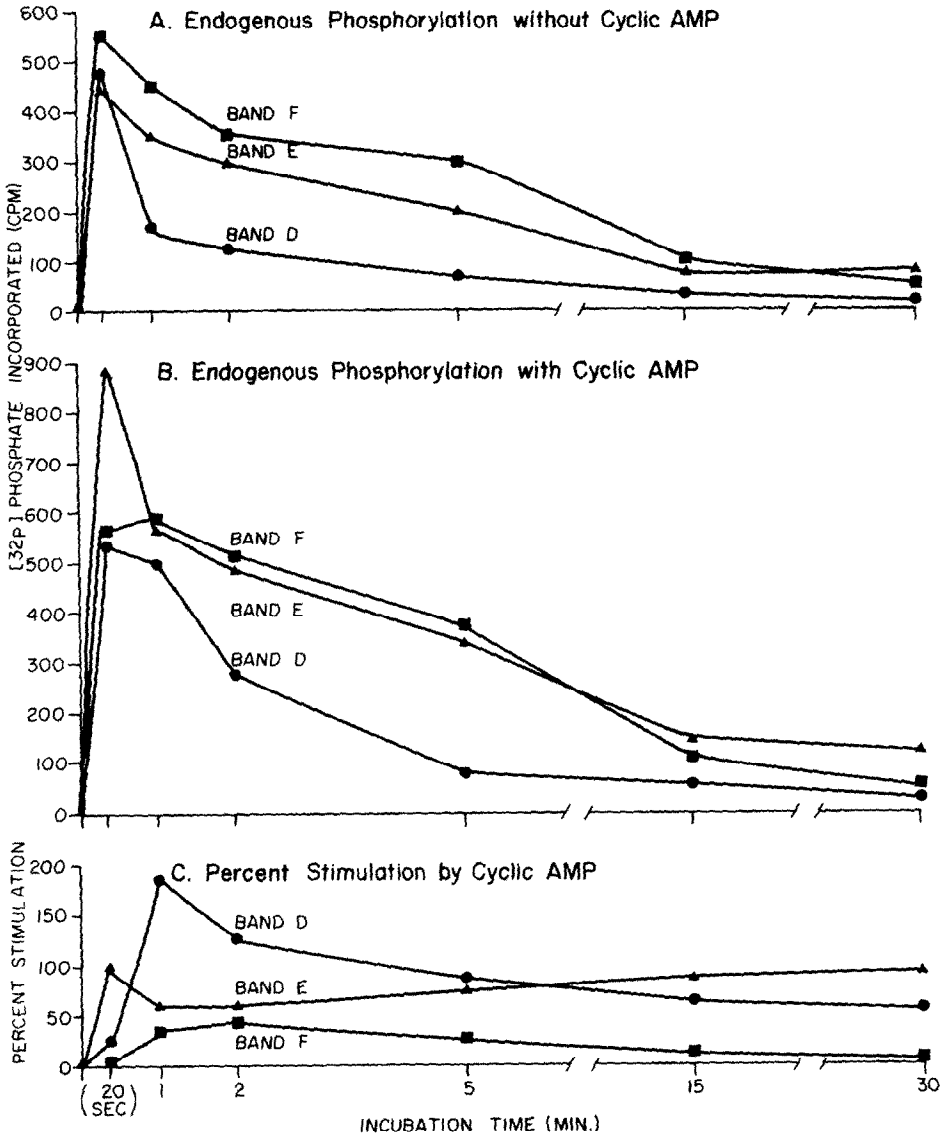

Fig. 2. Endogenous phosphorylation of proteins D, E and F without (A) and with (B) 5 μM cyclic AMP. Results are expressed, as cpm. Note break in abscissa between 5 and 15 min, and 15 and 30 min. (C) Percent stimulation by cyclic AMP of band D, E and F.

tion after 2 min. Band F differs from D and E on two counts: 1) percent stimulation by cyclic AMP is lowest in component F; 2) the maximal stimulatory effects of cyclic AMP on F occur at 2 min in contrast with the 1 min peak of band D and the 20 sec peak of band E.

We compared the five fractions obtained from the Davis and Bloom [14] subcellular fractionation procedure using the same assay conditions described in Methods. As shown in fig. 3, and confirmed by microdensitometry, the highest level of phosphate incorporation in the absence of cyclic AMP is in the 1.2 fraction which is the SPM-enriched fraction. In the presence of cyclic AMP, this fraction also showed the

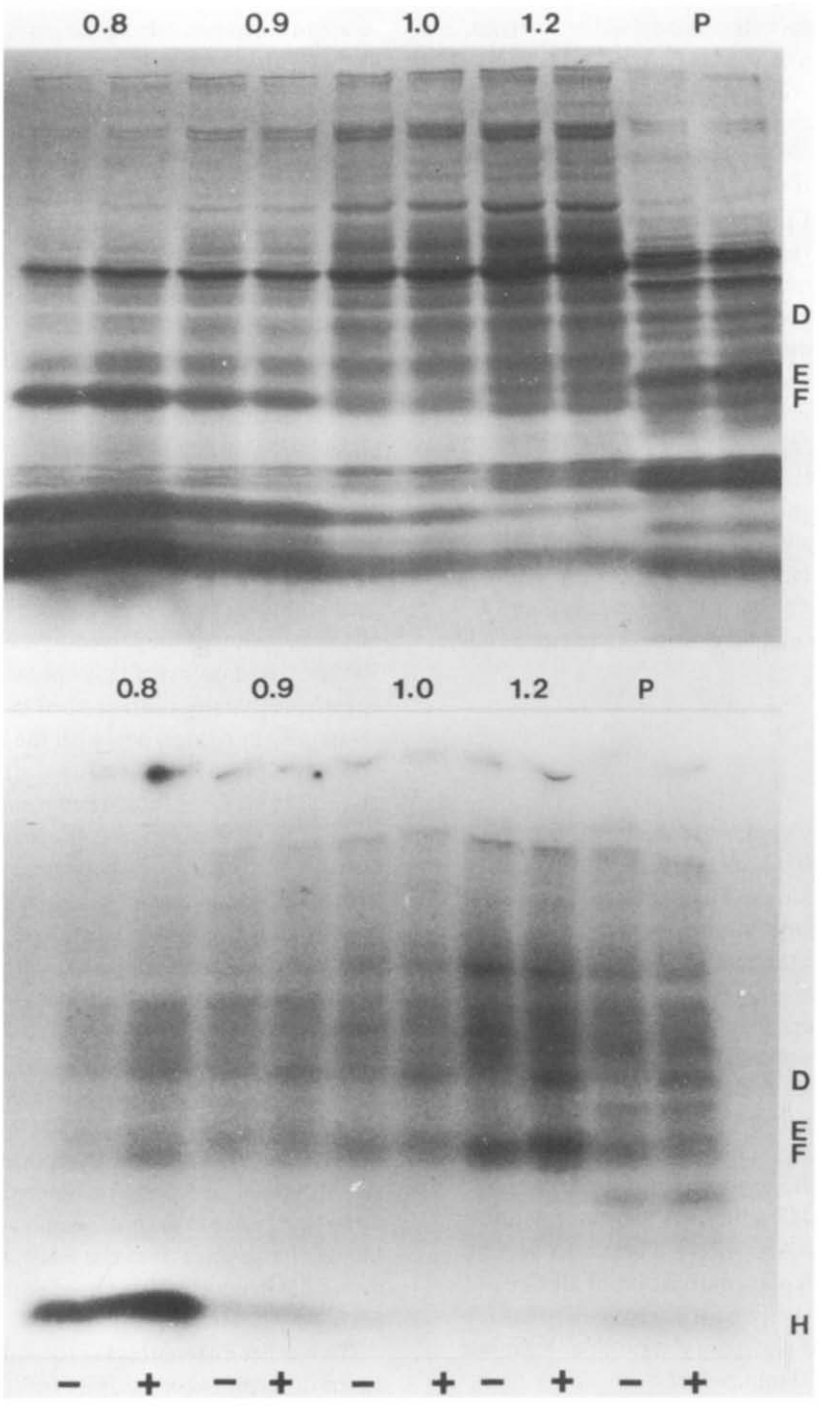

Fig. 3. Separation of phosphorylated membrane subfractions of rat cerebrum. Five subfractions, prepared by Dr Gary A. Davis of the University of Wisconsin, according to his recently published method [14] were assayed under the conditions described in fig. 1. Samples were withdrawn for electrophoresis 2 min after the initiation of the reaction with (+) or without (−) 5 μM cyclic AMP. Pairs from left to right are from gradient fractions of 0.8, 0.9, 1.0 and 1.2 M sucrose and the mitochondrial pellet. Top, figure-protein staining. Bottom, figure-autoradiograph.

highest level of incorporation. Quantitative analysis of the microdensitometric data revealed that, except for the 0.8 fraction which showed non-specific stimulation throughout the gel, cyclic AMP stimulation of band D was greatest in the 1.2 fraction.

According to their relative mobility, the estimated molecular weights of proteins D, E and F are 80 000, 52 000 and 47 000, respectively. Quantitative analysis of the phosphate turnover in each band was performed both by scintillation counting of gel slices and by microdensitometric analysis of the autoradiograms. The correlation between the two measures of phosphate incorporation into band D, E and F was highly positive ($r = +0.879, t = 7.41, N = 16, p < 0.01$) indicating that the less expensive and less time consuming microdensitometric analysis represents a reliable measure of phosphate incorporation. Agreement between microdensitometry and scintillation counting was better for band D than bands E and F because of the close mobility of the latter two bands (see fig. 1).

4. Discussion

The present study demonstrates several features of phosphorylation of brain membranes that have not been described previously. First, at least three protein components are major phosphoproteins of brain membranes. Second, the rate of phosphorylation and dephosphorylation in the absence and presence of cyclic AMP is different for each component. Third, the time course of cyclic AMP stimulation of phosphorylation differs for each component and, in particular, the time of peak stimulation is different. Fourth, the autoradiographic demonstration of phosphorylation of specific protein components in the presence of cyclic AMP with little phosphorylation in the absence of cyclic AMP [3,4] is best seen beyond 2 min, at a time of dephosphorylation of all three components. Fifth, the present study shows that cyclic AMP regulates phosphorylation of at least one brain protein for a 30 min period.

Because this study was initiated by the Johnson et al. [3] report and completed before the Ueda et al. [4] paper, it is necessary to compare their results and the procedures which they used to understand both the consistent and conflicting findings. On the basis of approximate molecular weight determinations, it is likely that their protein I is protein D, and, considering the time course of phosphorylation (fig. 2b), their protein II is probably protein E of the present report. Their failure to observe protein F may be due to the single concentration gel system which they used. The disparity cannot be accounted for by the cruder preparation used here since Ueda et al. [4] state that a similar crude preparation gave 'qualitatively similar results' (p. 8302).

The difference in subcellular fractionation procedure may, however, account for differences in time course. In the Ueda et al. study [4] the rate of phosphorylation and dephosphorylation in the absence of cyclic AMP was low during the 2 min period studied. In the present experiment such was not the case. It is unlikely that this difference is due to contamination of vesicular protein since we have used a procedure for releasing vesicles which is more efficient than that employed by Ueda et al. [4]. Rather, based on overall phosphate incorporation, enzymatic activity is diminished in our SPM preparations [13] in comparison with the activity of our crude synaptosome. Maeno et al. [5] and DeRobertis et al. [18] have reported results consistent with this finding. Such findings raise the issue of the generality of the time course of phosphorylation reported by Ueda et al. [4] since the endogenous enzymatic system studied appears to be altered in some way (e.g., shearing forces in high osmotic sucrose solutions). This interpretation may explain why we observed in the absence of cyclic AMP a rapid dephosphorylation of protein D, while Ueda et al. [4] observed no such decline over the 2 min period studied. It is interesting that in the presence of cyclic AMP band D and band E follow similar time course reported for protein I and protein II, respectively. Since there are significant differences over time in the endogenous phosphorylation in the absence of cyclic AMP, the stimulation by cyclic AMP reported here is, of course, different than previously reported [4].

Two other differences in procedure may be relevant to the differing observations. First, the entire cerebrum served as the source of membrane in the study of Ueda et al. [4] while we prepared our membrane fractions from a somewhat more defined brain region, the cerebral cortex. Most importantly, as two regions of the cerebrum, the cerebral cortex and the caudate

nucleus, were reported to differ in the pattern of regulated phosphorylation of specific proteins in their membranes [4], the time course measured for membrane phosphoproteins of cerebrum could indeed differ from that of corresponding components of cerebral cortex membranes. Second, in our assay procedure preincubation without ATP and cyclic AMP were conducted. Such a treatment, which was shown to alter the initial phosphorylation state of membrane-bound proteins [9], could partially explain differences in observations made at the initial phase of the reaction.

The fact that the cyclic AMP effects can be observed throughout a 30 min period after its addition to the reaction reinforces the hypothesis that the role of cyclic AMP is to modify proteins, providing a residual following presynaptic activation. Whether such a residual plays a role in regulating membrane permeability [2–4] and/or in regulating other cellular processes cannot be stated. The different time course of phosphorylation of several protein components described here could, however, permit the occurrence of a sequence of postsynaptic membrane events following presynaptic transmitter release.

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