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PROTEIN-BOUND HISTIDINE, AS WELL AS PROTEIN-BOUND SERINE, RESIDUES ARE SITES OF PHOSPHORYLATION IN THE SYNAPTIC PLASMA MEMBRANE

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Summary

When synaptic plasma membrane fragments are incubated with ATP in the presence of Mg^{2+}, phosphate is transferred, not only to protein-bound serine, but also to protein-bound histidine. The phosphorylation of protein-bound serine is stimulated by cyclic AMP and has a K_m for ATP of about 0.12 mM, both in the presence and absence of cyclic AMP. By contrast, the phosphorylation of protein-bound histidine is unaffected by cyclic AMP and does not follow Michaelis-Menton kinetics since a non-linear double reciprocal plot is given when activity is measured at various ATP concentrations.

Introduction

It is well known that synaptic plasma membranes (in common with subcellular membranes from a number of other tissues [1]) contain a tightly bound protein kinase (or kinases) which catalyses the phosphorylation of endogenous proteins in a reaction which is stimulated by cyclic AMP [2—4]. The enzyme and its substrate(s) are concentrated in the region of the synaptic junction [5] and are mainly presynaptic [6]. The phosphorylation reaction is rather slow [3,7] and phosphorylation of synaptic membrane proteins reduces the permeability of the membrane to Ca^{2+} [8]. It has been proposed that the reaction may have a role in modulating synaptic transmission at the presynaptic level [8] although the reaction is unlikely to be involved in such fast neuronal processes as the generation of the postsynaptic potential [1,7].

In earlier studies protein-bound serine was identified as a major site of phosphorylation in the synaptic membrane, but several points suggested that it might not be the only site. In the first case, cyclic AMP causes a greater percentage stimulation when the phosphorylation of serine is specifically measured than it does when total protein phosphorylation is examined (Weller,

M., unpublished observations). In the second case a half maximal rate of phosphorylation of serine residues is observed with an ATP concentration of, very approximately, 0.25 mM [9] while, if total protein phosphorylation is examined, the concentration is nearer to 15 μM [10].

For this reason experiments were carried out to determine the possible presence of sites of protein phosphorylation apart from serine in the synaptic plasma membrane.

Materials and Methods

Synaptic plasma membranes were prepared as described by Morgan et al. [11]. [γ^{32}P]ATP was prepared by the method of Glynn and Chappell [12] and purified by the method of Rodnight et al. [13].

Preparation of phosphorylated amino acids

Phosphorylated lysine, histidine and argenine were prepared by the method of Zetterqvist and Engstrom [14]. Samples (10 mmol) of the appropriate amino acids were dissolved in 35 ml of 7 M NaOH and cooled in an ethanol-ice bath. Phosphorus oxychloride (3.8 ml) was added dropwise over 1 h with constant stirring, taking care that the temperature stayed between 0 and 10°C. The mixture was left for 30 min then filtered at 4°C through a scintered glass funnel. The filter was washed with 10 ml of ice-cold water and the combined filtrates adjusted to pH 7.6 with 2 M NaOH and freeze dried. The solid was taken up in 5 ml of water and filtered again. Magnesium acetate (21 ml of a 1 M solution) was added to the filtrate and the pH adjusted to 9.0 with 1 M NaOH. The mixture was then centrifuged at low speed and the pellet washed once by resuspension in, and centrifugation, from 10 ml of ice-cold water. Ethanol (4 vols.) was then added to the combined supernatants and the precipitated phosphorylated amino acids filtered off and dried.

Phosphorylation of synaptic plasma membrane proteins

The intrinsic protein kinase activity of synaptic plasma membrane was determined by incubating samples (about 50 μg of protein) with 50 mM Tris · HCl (pH 7.4), 1 mM $MgCl_2$ and the indicated concentration of [γ^{32}P]ATP (specific radioactivity about $2 \cdot 10^7$ cpm/μmol). After 10 s (or the stated time) reactions were stopped by the addition of 1.5 ml of ice-cold 15% trichloroacetic acid. If total protein phosphorylation was to be determined the protein precipitates were washed twice by suspension in, and centrifugation from 2-ml lots of 10% trichloroacetic acid containing 1 M orthophosphoric acid. The pellets were then suspended in 0.5 ml of 0.1 M NaOH and warmed at 37°C for 10 min. Aliquots (2 ml) of 10% trichloroacetic acid were then added and the mixtures centrifuged. In some cases (indicated in the text) the pellets were then suspended in 2 ml of 5% trichloroacetic acid, heated in a boiling-water bath for 10 min and then centrifuged. Whether or not this step was included the pellets were finally suspended in 0.5 ml of ethanol/diethyl ether (1 : 1, v/v) and centrifuged again. The pellets were then dissolved in 0.1 M NaOH by heating in a boiling-water bath and the Cerenkov radiation counted.

When protein-bound phosphorylated amino acids were to be identified the

washing procedure was slightly modified. The initial protein precipitates were washed twice with 2-ml lots of 10% trichloroacetic acid containing M orthophosphoric acid, once with 10% trichloroacetic acid and twice with ethanol/diethyl ether (1 : 1, v/v). The pellets were then dried in an oven at 50°C, suspended in 75 μl of 1 mM Tris · HCl, pH 8.0, containing 40 μg of trypsin, and incubated for 1 h at 37°C. Aliquots (10 μl) of a solution containing 8 mg of pronase/ml were then added to each tube and incubation continued at 37°C for 14 h.

Separation of phosphorylated amino acids

Aliquots of the digested phosphoproteins were applied to silica gel thin layer chromatography plates (Merck, Darmstadt, G.F.R.) and the plates developed with a solvent mixture consisting of water/2-methoxyethanol/aqueous ammonia (specific gravity 0.910)/methanol (20 : 10 : 10 : 60, by vol.). Various phosphorylated amino acids and inorganic phosphate were chromatographed at the same time as markers. The chromatography plates were dried at 50°C and stained with ninhydrin to detect the amino acids. Inorganic phosphate was detected by the method of Wade and Morgan [15].

ATP breakdown

This was determined by measuring the amount of $^{32}P_i$ in the first supernatant following the addition of trichloroacetic acid to the phosphorylation reaction described above. Aliquots (1 ml) of the supernatant were taken and 0.5 ml of 5% ammonium molybdate/10 M perchloric acid (4 : 1, v/v) and 2 ml of isobutanol/benzene (1 : 1, v/v) added. The ssmples were well mixed then briefly centrifuged. Aliquots (1 ml) of the organic layer were taken and ^{32}P counted by measuring the Cerenkov radiation.

Results

Identification of the phosphorylated amino acids in syanptic plasma membrane proteins

Protein-bound phosphorylated amino acids can be divided into three groups: (1) those which are labile to alkali but stable to acid (phosphoserine and phosphothreonine), (2) those which are labile to acid but stable to alkali (phosphohistidine and phospholysine) and (3) acyl phosphates (phosphoglutamate and phosphoaspartate) which are very labile to alkali and not very stable to acid.

Preliminary results indicated that if synaptic plasma membranes are phosphorylated by incubation with [γ^{32}P]ATP and washed free of non-protein-bound phosphate, as described above, a portion of the protein phosphate could be removed by heating in a boiling-water bath for 10 min in the presence of 10% trichloroacetic acid. The washing procedure which was designed to remove non-protein-bound phosphate involved a treatment with 0.1 M NaOH which would remove protein-bound acyl phosphate. Hence the loss of phosphate on treatment with hot acid suggests the presence of acid-labile, protein-bound, phosphorylated amino acids such as phosphohistidine or phospholysine.

For this reason experiments were carried out to determine which (if any) of

these phosphorylated amino acids occur in phosphorylated synaptic plasma membrane proteins.

Samples of synaptic plasma membranes (200 μg of protein) were incubated with 0.2 mM [γ^{32}P]ATP (specific radioactivity approx. $2 \cdot 10^8$ cpm/μmol) and the reaction stopped after 30 s by addition of trichloracetic acid. The precipitated protein was then washed and digested and the phosphorylated amino acids separated by silica gel thin layer chromatography as described in Materials and Methods.

Strips (1 cm wide) of the chromatograms were scraped into 10-ml lots of scintillation fluid (Insta-gel, Packard Instrument Company Inc., U.S.A.) and counted to determine the distribution of radioactivity. It may be seen from Fig. 1 that there was an excellent separation of inorganic phosphate, phosphoargenine, phospholysine, phosphoserine and phosphohistidine. Phosphoserine was not separated from phosphothreonine but previous experiments had demonstrated the lack of protein-bound phosphothreonine in acid hydrolysates of phosphorylated synaptic plasma membranes [9]. The digested synaptic plasma membrane proteins may be seen to contain, in addition to phosphoserine, a radioactive compound which co-chromatographed with phosphohistidine. Identification of this compound as phosphohistidine is confirmed by the fact that if the phosphorylated synaptic plasma membranes are treated with 5% trichloroacetic acid for 10 min in a boiling-water bath before enzymic digestion phosphoserine is the only radioactive compound (apart from inorganic phosphate) which can be detected.

It may thus be concluded that protein-bound histidine, as well as protein-bound serine, residues are sites of phosphorylation in the synaptic plasma membrane.

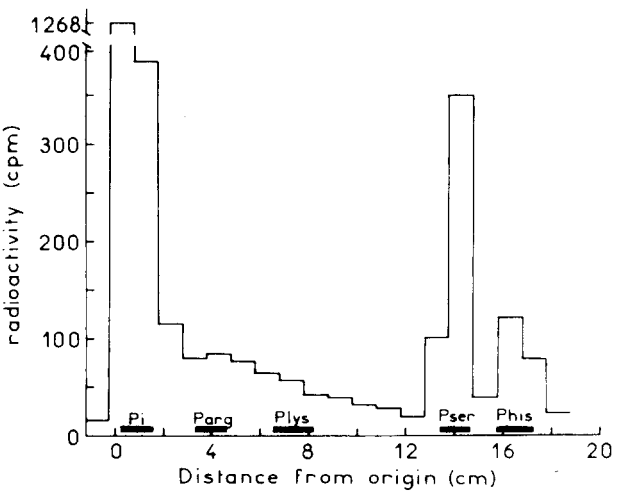

Fig. 1. Separation of phosphorylated amino acids by thin layer chromatography on silica gel plates. The method is described in the text.

The characteristics of the phosphorylation of protein-bound histidine and serine residues in the synaptic plasma membrane

The two major discrepancies between the results obtained by examining total protein phosphorylation and determining the phosphorylation of protein-bound serine in synaptic plasma membranes concern the percentage stimulation of phosphorylation caused by cyclic AMP and the affinity of the system for ATP. Experiments were thus carried out in which synaptic plasma membrane fragments (50 μg of protein) were incubated for 10 s with various concentrations of [γ^{32}P]ATP in the presence or absence of 10 μM cyclic AMP and the amount of ^{32}P transferred to protein-bound serine or histidine determined as described above. Preparations of synaptic plasma membrane fragments contain substantial amounts of ATPase activity, but using these amounts of protein and short times of incubation less than 10% of the ATP used was broken down at any of the concentrations used. The results of the experiment are shown in Fig. 2A from which it may be seen that in contrast to the phosphorylation of serine the phosphorylation of protein-bound histidine was insensitive to cyclic AMP. A double reciprocal plot of the results (Fig. 2B) revealed that the phosphorylation of protein-bound serine followed normal Michaelis-Menton kinetics and had a K_m for ATP of approx. 0.12 mM both in the presence and absence of cyclic AMP. By contrast, the phosphorylation of protein-bound histidine gave a non-linear double reciprocal plot. There are several possible explanations for this behaviour but, in view of the fact that when examining protein phosphorylation in membrane fragments one is forced to work with a very heterogenous system, the situation appears too complex to warrant detailed analysis.

The technique used to obtain the results described above has a severe limitation in that the enzymic digestion results in a massive breakdown of phosphoserine. The yield of phosphoserine found after enzymic digestion of phos-

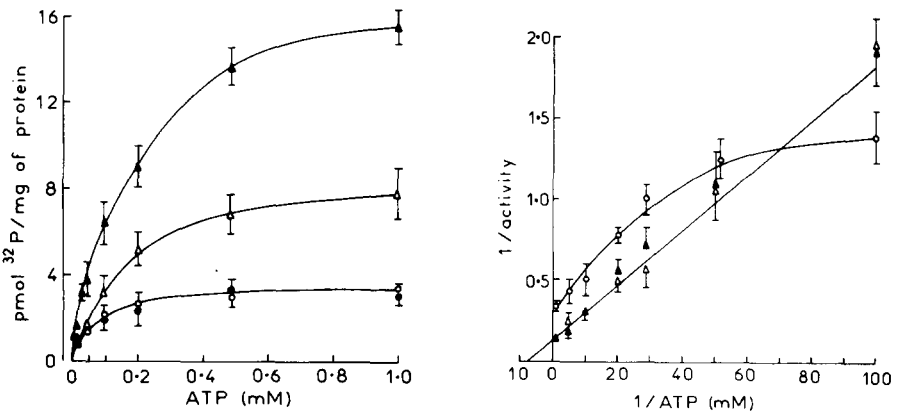

Fig. 2. A. The effect of cyclic AMP at different concentration of ATP on the phosphorylation of protein-bound histidine (●,○) and serine (▲,△) residues. Phosphorylation was measured as described in the text in the presence (●,▲) or absence (○,△) of 10 μM cyclic AMP. Results are shown as means ± S.D. and are taken from 6 observations. B. The results of A are shown as a double reciprocal plot, but in this figure filled triangles represent the cyclic AMP-stimulated phosphorylation of protein-bound serine (i.e., the difference between the activities observed in the presence and absence of cyclic AMP).

phorylated synaptic plasma membrane fragments was only 20% of that obtained after digestion for 6 h at 100°C with 2 M HCl (results not shown), a procedure which, in itself, results in some breakdown of phosphoserine [9]. It also seems probable that the enzymic digestion would result in a similar breakdown of phosphohistidine. It thus seemed advisable to examine the effect of ATP concentration on the phosphorylation of intact proteins in the synaptic plasma membrane, i.e., without attempting to release and separate the phosphorylated amino acids.

The effect of ATP concentration on the phosphorylation of proteins in the synaptic plasma membrane

The results described above demonstrate that phosphoserine and phosphohistidine are the only phosphorylated amino acids which occur in the synaptic plasma membrane. Phosphohistidine differs from phosphoserine in that it is unstable in hot acid and it was found that if phosphorylated preparations of synaptic plasma membranes are treated with 5% trichloroacetic acid in boiling-water bath for 10 min (as described in Materials and Methods) only phosphoserine could be detected. It was, however, observed that the treatment with hot acid caused a loss of protein-bound phosphoserine of 30 ± 7% (mean ± S.D. of 5 observations). This is somewhat surprising as protein-bound phosphoserine is generally somewhat more stable to acid [16]. Preliminary results indicate that the treatment with hot trichloroacetic acid may cause an initial rapid loss of protein-bound serine phosphate over the first few minutes followed by a relatively very slow loss of a few percent per hour. This suggests that a portion of the protein-bound phosphoserine may be less stable (it may, perhaps, be of higher energy) than the rest.

In view of these results the effect of treating the acid-denatured protein precipitates with hot trichloroacetic acid after completion of the phosphorylation reactions at different ATP concentrations was determined. It may be seen from Fig. 3 that treatment with hot acid causes a loss of protein-bound phosphate which is relatively greater at low than at high ATP concentrations. This loss is partially due to breakdown of phosphoserine but when allowance is made for a 30% loss of this amino acid it may be seen that there is still a difference between the results obtained with and without hot trichloroacetic acid treatment. This is presumably due to loss of protein-bound phosphohistidine. This conclusion is supported by the fact that if the cyclic AMP-stimulated phosphorylation (that is the difference between the activities observed in the presence and absence of cyclic AMP) is examined, although treatment with hot trichloroacetic acid does cause some loss of protein-bound phosphorus, this can all be accounted for by breakdown of phosphoserine (Fig. 4). This is in complete accord with the observation noted above that the phosphorylation of serine is stimulated by cyclic AMP while the phosphorylation of histidine is not.

If the results of Fig. 3, obtained after treatment with hot acid, are drawn as a double reciprocal plot a linear result is given showing that the transfer of phosphate to an acid-stable, protein-bound form follows normal Michaelis-Menton kinetics with a K_m for ATP of about 0.12 mM which is in agreement with the results obtained when specifically examining the phosphorylation of protein-

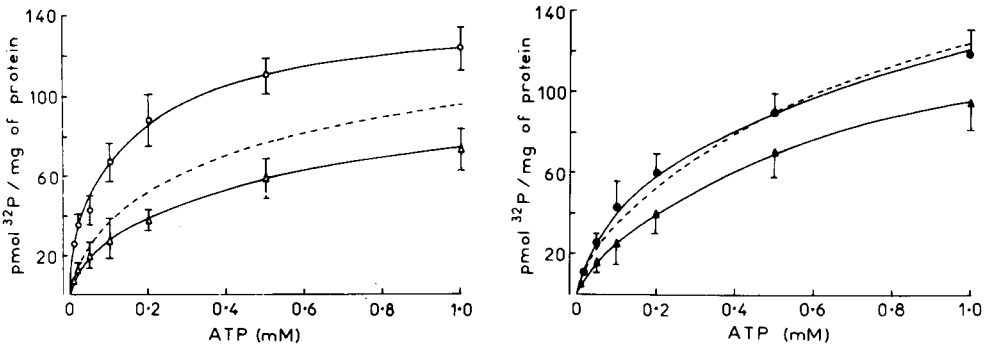

Fig. 3. The influence of ATP concentration on the phosphorylation of synaptic plasma membrane proteins and the effect, after the completion of the phosphorylation reactions, of treating the acid-denatured protein with hot trichloroacetic acid. Synaptic plasma membrane proteins were phosphorylated by incubation with various concentrations of ATP and the reaction stopped by addition of trichloroacetic acid. The acid-denatured protein precipitates were washed free of non-protein-bound phosphorus and some of the samples (△) but not others (○) were treated with hot trichloroacetic acid as described in the text. Results are shown as mean ± S.D. from 6 observations. The dotted line represents the amount of protein-bound phosphate expected to be present after treatment with hot trichloroacetic acid if allowance is made for a 30% loss of protein-bound phosphoserine.

Fig. 4. The influence of ATP concentrations on the cyclic AMP-stimulated phosphorylation of synaptic plasma membrane proteins and the effect of treating the acid-denatured protein with hot trichloroacetic acid after the completion of the phosphorylation reactions. Synaptic plasma membrane proteins were phosphorylated by incubation with various concentrations of ATP in the presence or absence of 10 μM cyclic AMP and the reaction stopped by addition of trichloroacetic acid. The acid-denatured proteins were washed free of non-protein-bound phosphorus and some of the samples (▲) but not others (●) were treated with hot trichloroacetic acid as described in the text. Results are shown as the difference between the activities measured in the presence and absence of cyclic AMP and are shown as mean ± S.D. from 6 observations. The dotted line represents the amount of protein-bound phosphate expected to be present after treatment with hot trichloroacetic acid if allowance is made for a 30% loss of protein-bound phosphoserin.

bound serine. If treatment with hot acid is not employed, the double reciprocal plot is non-linear due to the peculiar kinetics of the phosphorylation of protein-bound histidine described above. If a double reciprocal plot is made of the effect of ATP concentration on the cyclic AMP-stimulated activity (Fig. 4) a linear plot is given whether or not a treatment with hot trichloroacetic acid is employed. This indicates that the phosphorylation reaction follows normal Michaelis-Menton kinetics and has a K_m for ATP of approx. 0.12 mM. This agrees with the conclusion noted above that phosphorylation of serine residues accounts for all the cyclic AMP-stimulated phosphorylation of synaptic plasma membrane proteins.

Discussion

The results described in this paper indicate that there are two different types of sites of protein phosphorylation in synaptic plasma membrane preparations. The first consists of series residues which are phosphorylated in a reaction which is stimulated by cyclic AMP and which has a K_m for ATP of approx. 0.12 mM. The second consists of histidine residues which are phosphorylated in a reaction which is unaffected by cyclic AMP and which does not follow

Michaelis-Menton kinetics since a non-linear double reciprocal plot is given when activity is measured at various ATP concentrations.

Although the membrane preparations of Morgan et al. [11] used in this study are the purest available even these are slightly (less than 10%) contaminated by fragments from other subcellular fractions. It might thus be suggested that the protein-bound histidine which can be phosphorylated in the preparations may have derived from a subcellular fraction other than the synaptic plasma membrane. In view of the low level of contamination of the membrane preparations and the relatively high amount of histidine phosphorylation this seems rather unlikely.

Protein-bound phosphohistidine may be readily removed by treatment with hot trichloroacetic acid, thus allowing a simpler method of specifically measuring the phosphorylation of protein-bound serine in synaptic plasma membrane proteins than the alternative digestion and separation of phosphorylated amino acids. It must, however, be noted that the treatment with hot acid causes some loss of protein-bound phosphoserine (a criticism which can also be made of techniques which isolate phosphoserine from acid digests of phosphorylated synaptic plasma membranes) and it may be that the phosphorylation of the more labile compound has different characteristics to the phosphorylation of the less labile.

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