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ENDOGENOUS PHOSPHORYLATION OF RAT BRAIN SYNAPTOSOMAL PLASMA MEMBRANES IN VITRO: SOME METHODOLOGICAL ASPECTS

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The time course of endogenous phosphorylation in vitro of total or separated synaptic plasma membrane proteins (SPM) has been correlated with that of hydrolysis of the phosphate donor (ATP) in the incubation medium. The ATP/SPM ratio in the medium was varied. In a low-ratio medium (7.5 μM ATP; 2.2 μg SPM/μl) a complete hydrolysis of ATP occurred almost instantaneously as was measured by the release of free phosphate in and the disappearance of ATP from the medium. As a consequence, only a very short peak of phosphorylation, followed by dephosphorylation was observed. However, when higher ATP/SPM ratios were used (200 μM ATP; 0.4 μg SPM/μl and 500 μM ATP; 0.4 μg SPM/μl), the incorporation of phosphate into SPM proteins was linear for 20 sec, and the maximum level of phosphate incorporation was increased. Similar results were obtained after separation of ^{32}P-labeled phosphoproteins by slab gel electrophoresis. However, analysis of the autoradiographs obtained from *one* SPM preparation under different ATP/SPM ratios revealed dependence of phosphorylation of individual protein bands on the conditions used.

INTRODUCTION

Evidence exists that phosphorylation and dephosphorylation of brain cell membrane proteins are involved in the modulation of membrane perme-

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ability, thus influencing certain types of synaptic transmission (1, 2). Therefore, in the study of factors affecting neurotransmission, the measurement of endogenous phosphorylation of membrane proteins can provide important information. In experiments on in vitro phosphorylation of SPM proteins, [γ-^{32}P]-ATP is often used as a phosphate donor (see reference 3). Apart from protein-kinase activity, however, synaptic plasma membranes usually contain high levels of other ATP-consuming enzymes, causing rapid hydrolysis of ATP in the incubation medium. Consequently, depending on the ATP/protein ratio used, the ATP concentration might become limiting. When polyacrylamide gel electrophoresis is applied to study phosphorylation of separate SPM protein bands, usually low ATP concentrations are used (μM) in order to keep the specific activity of the [γ-^{32}P]ATP and, as a consequence, that of phosphorylated proteins as high as possible (4–6). In the present communication it is shown that an incubation system based on high specific activity of [γ-^{32}P]ATP (and consequently a low ATP concentration) in fact provides an ATP concentration that is limiting for the phosphorylation reaction. Therefore it seemed worthwhile to gain more insight in the effect of the ATP and SPM concentration on the endogenous phosphorylation of synaptic plasma membrane proteins in vitro.

EXPERIMENTAL PROCEDURE

Animals, Tissue Fractionation, and Protein Determination

Male albino rats of an inbred Wistar strain (TNO, Zeist, the Netherlands) were used, weighing 160–200 g. After decapitation of the animal the cerebral cortex was excised and used for the isolation of synaptic plasma membranes (SPM) according to Terenius (7). The enriched SPM pellet was suspended in a medium containing 50 mM Na acetate and 10 mM Mg acetate (pH 6.5). Protein determinations were carried out according to the method of Lowry et al. (8).

In Vitro Phosphorylation Assay

The phosphorylation assays were carried out at 30°C in the same medium under various conditions of SPM and ATP concentrations, and precursor specific activities.

Condition A. 7.5 μM ATP (2–4 μCi [γ-^{32}P]ATP); 2.2 μg SPM-protein /μl; final volume 50 μl. 110 μg SPM protein was preincubated for 5 min at 30°C. The reaction was initiated by the addition of the ATP, resulting in a final concentration of 7.5 μM and a final volume of 50 μl. The reaction was terminated by the addition either of a solution of ice cold TCA to a final concentration of 5%, or of 25 μl SDS solution, resulting in final concentrations of 62.5 mM Tris-HCl, pH 6.5; 2% SDS; 10% glycerol; 0.001% bromophenol blue; 5% 2-mercaptoethanol.

Condition B. 200 μM ATP (4–15 μCi [γ-^{32}P]ATP); 0.4 SPM protein/μl; final volume

25 μl. The incubation procedure was essentially the same as described for condition A and was terminated by addition of 12.5 μl SDS solution, resulting in the same final concentrations as described for condition A.

Condition C. 500 μM ATP (4–15 μCi[γ-^{32}P]ATP); 0.4 μg SPM protein/μl; final volume 25 μl. The incubation procedure was identical to that described for condition A. The reaction was terminated by addition of 12.5 μl SDS solution, resulting in the same final concentrations as described for condition A.

Measurement of ATP Hydrolysis

Hydrolysis of ATP during the incubation with SPM was studied under all three conditions mentioned above. The reaction was started by addition of [^{14}C]ATP (approx. 20.000 dpm) and terminated by adding TCA to a final concentration of 5%. After centrifugation the supernatant was neutralized with 1 N NaOH and loaded on a small Dowex 1 × 2 column (height 3 cm; diameter 0.4 cm). Subsequently, the ATP was eluted according to Glynn and Chappell (9) and the eluate counted for ^{14}C radioactivity.

Determinations of Free Phosphate

Production of free phosphate during the incubation was studied under the different conditions of incubation. The reaction was started by the addition of ATP and terminated by adding TCA, resulting in a final concentration of 5%. After centrifugation, free phosphate was measured in the supernatant using the method of Hurst (10).

Measurement of Phosphate Incorporation in Total Protein

The phosphorylation reaction was stopped with TCA. In the case of conditions B and C, 100 μg TCA-treated SPM protein was added to the sample as a carrier. Subsequently, phosphorylation of total SPM protein was determined after removal of phospholipids and unbound phosphates according to Rodnight et al. (3).

Separation of Individual Phosphoprotein Bands

The phosphorylation reaction was stopped with SDS solution; 35-μl aliquots of each incubation mixture were subjected to SDS–polyacrylamide gel electrophoresis on slab gels as described by Lugtenberg et al. (11). Staining, destaining, and autoradiography were carried out as described previously (6). Autoradiographs of the gels were scanned densitometrically using a linear gel scanner (slide width 0.1 mm) and a Zeiss PMQ II spectrophotometer. With this improved scanning method, more phosphoprotein bands could be identified than under the conditions previously used by us (6). Therefore, the labeling profile was divided into several areas (A–D) and the protein bands were renumbered. In one experiment, after the autoradiography was performed, the protein band B50 was excised from the gel. Subsequently, the incorporation of ^{32}P in this band was determined by liquid scintillation counting, and the total incorporation of phosphate was computed from the specific activity of the [γ-^{32}P]ATP in the incubation medium.

Estimation of molecular weight was done by comparing the electrophoretic mobilities of the various membrane proteins with those of the following marker proteins: phosphorylase *b* (92, 500), bovine serum albumin (67,000), catalase (60,000), ovalbumin (45,000), lactate dehydrogenase (36,000), chymotrypsinogen (25,000) and hen egg lysozyme (14,000).

Chemicals

All chemicals used, were of analytical grade. Acrylamide and methylene bisacrylamide were obtained from Serva, Heidelberg, GFR. Fast Green FCF from Sigma Chemical Company, St. Louis, Missouri, USA. Sodium dodecylsulfate from BDH, Poole, UK. Glycine from Merck, Darmstadt, GFR. Hen egg lysozyme from Fluka, Buch, Switzerland. Other standard proteins were obtained from Boehringer Mannheim, [γ-^{32}P]ATP (50 Ci/mmol) and [1-^{14}C]ATP (38 mCi/mmol) from the Radiochemical Centre, Amersham, UK.

RESULTS

Figure 1 illustrates that hydrolysis of ATP by synaptosomal plasma membranes (SPM) in vitro is a very rapid process. When the ATP/SPM ratio is kept low (condition A), essentially all the ATP is hydrolyzed within the first 5 sec of the incubation (Figure 1A). The amount of free phosphate released into the medium during the 120 sec of the incubation corresponds to three times the molar amount of ATP present at the beginning of the reaction.

When higher ATP/SPM ratios were used for the incubation (conditions B and C), a lower rate of depletion of ATP was observed. This resulted in much higher ATP levels during the incubation (Figure 1B and C). Under these conditions the amount of free phosphate released equals the amount of ATP that is hydrolyzed.

In Figure 2 the effect of the various ATP/SPM ratios on the time course of phosphorylation of total SPM protein is shown. The net incorporation

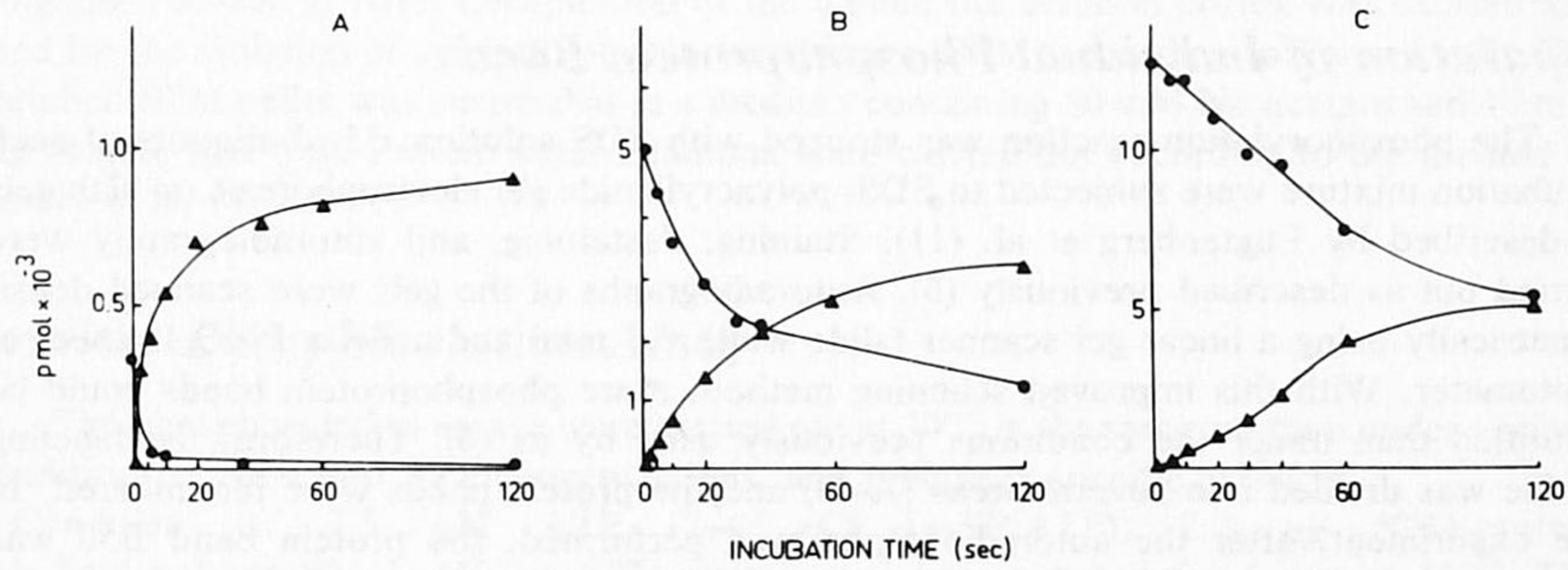

FIG. 1. Time course of the disappearance of ATP from (●——●) and accumulation of free phosphate in (▲——▲) the incubation medium. The incubations were performed under different conditions: (A) 7.5 μM ATP, 100 μg SPM protein, final volume 50 μl; (B) 200 μM ATP, 10 μg SPM protein, final volume 25 μl; (C) 500 μM ATP, 10 μg SPM protein, final volume 25 μl. The assays were carried out at 30°C in duplicate and the determinations were performed as described in the text.

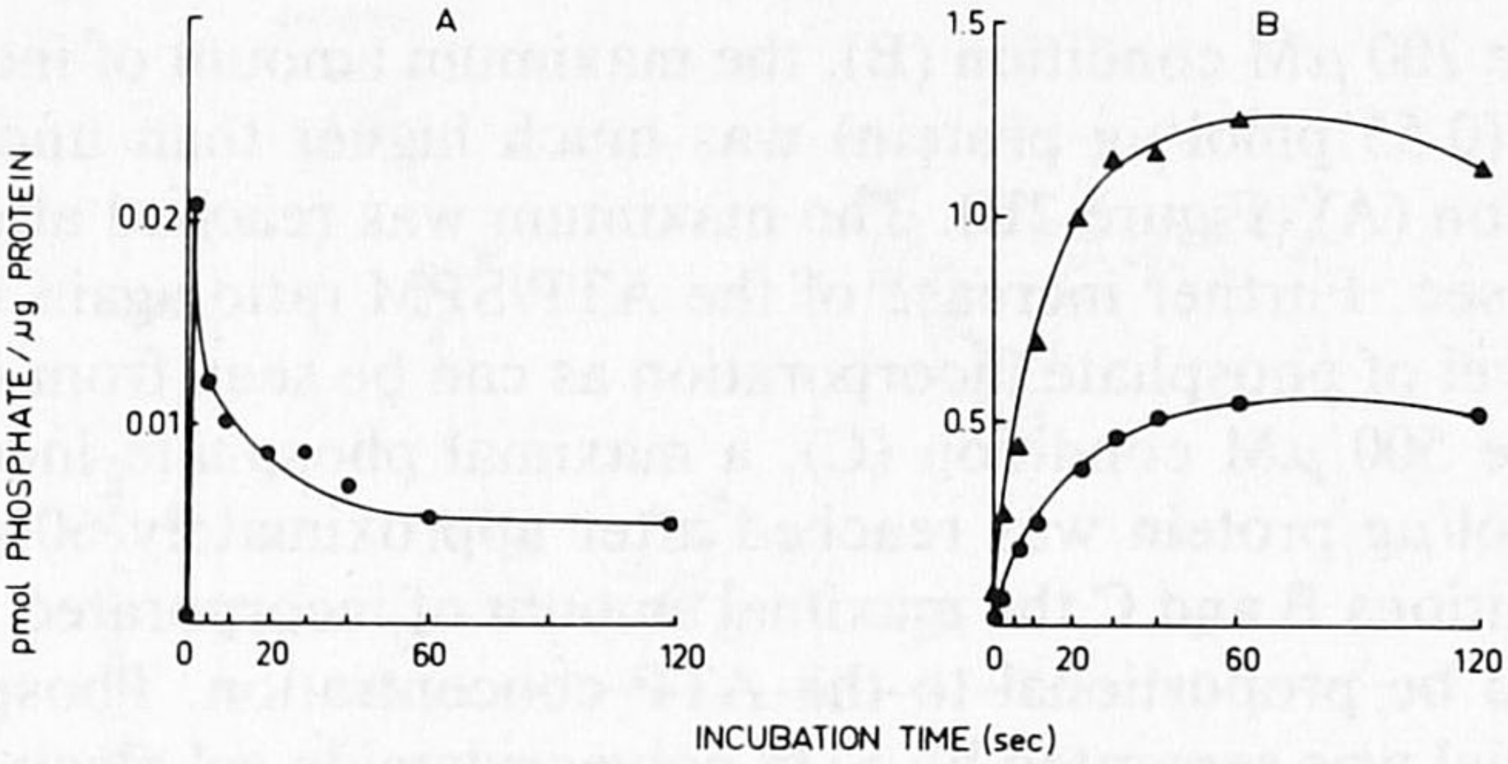

FIG. 2. Time course of incorporation of phosphate in total SPM protein. The incubations were done at 30°C under various conditions: (A) 7.5 μM ATP, 2.2 μg SPM/μl; (B) 200 μM ATP, 0.4 μg SPM/μl (●——●), and 500 μM ATP, 0.4 μg SPM/μl (▲——▲). The incubations were carried out in duplicate.

of phosphate clearly depends on both the incubation time and the ATP level used. Incubation under the low ATP condition (A) results in a maximal incorporation of 0.02 pmol phosphate per μg SPM protein (Figure 2A). This maximum was reached within 2 sec, being the shortest incubation time used. Incubation for longer than 2 sec resulted in a rapid net dephosphorylation.

If an additional aliquot of [γ-^{32}P]ATP was given at t = 90 sec to increase the actual ATP concentration by 7.5 μM, then again an increased phosphorylation instead of a net dephosphorylation could be observed (Figure 3). The pattern of phosphorylation matches that seen at the start of the incubation.

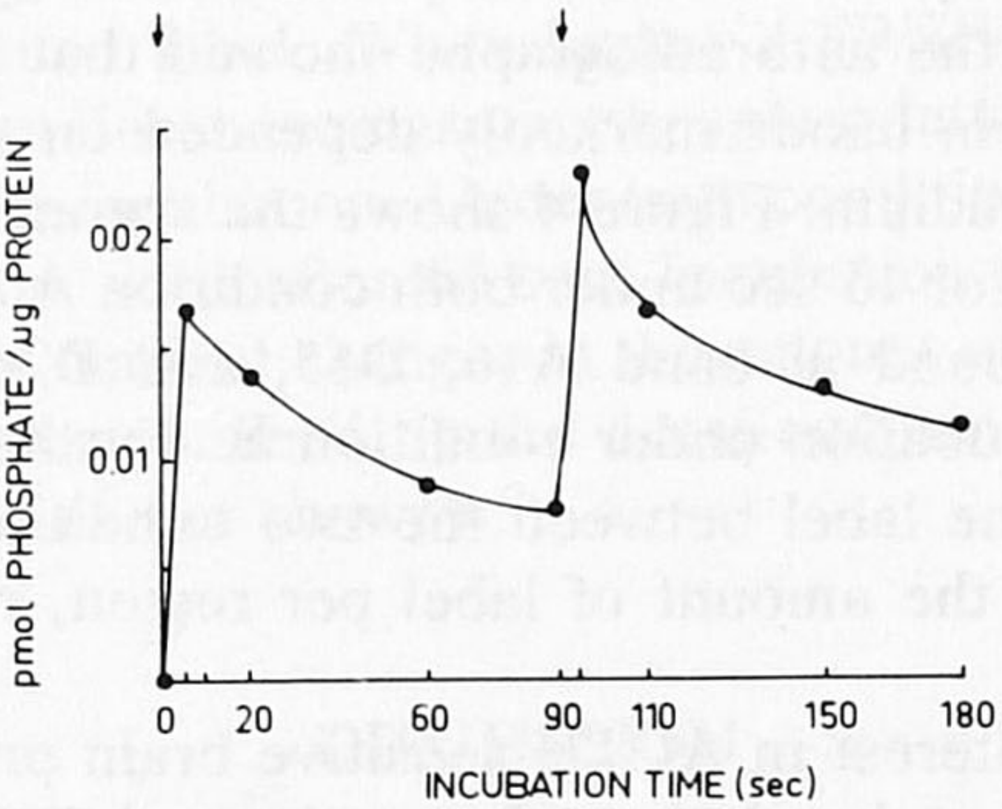

FIG. 3. Effect of renewed addition of [γ-^{32}P]ATP to the incubation medium on the incorporation of phosphate in total SPM protein. The assays were performed at 30°C in duplicate. [γ^{32}P]ATP was added to the medium at 0 sec resulting in a concentration of 7.5 μM, and at 90 sec resulting in an increase of the ATP concentration by 7.5 μM (arrows).

Using the 200 μM condition (B), the maximum amount of incorporated phosphate (0.55 pmol/μg protein) was much higher than under the 7.5 μM condition (A) (Figure 2B). The maximum was reached after approximately 60 sec. Further increase of the ATP/SPM ratio again resulted in a higher level of phosphate incorporation as can be seen from Figure 2C.

Using the 500 μM condition (C), a maximal phosphate incorporation of 1.25 pmol/μg protein was reached after approximately 60 sec. Thus, under conditions B and C the maximal amount of incorporated phosphate appeared to be proportional to the ATP concentration. Phosphorylated SPM material was separated by SDS polyacrylamide gel electrophoresis. Possible adhering free ^{32}Pi or [γ-^{32}P]ATP was removed during the electrophoretic separation, as was evidenced by the absence of radioactivity in gels having aliquots from incubations started and stopped at 0 sec (data not shown).

Protein staining of the gels revealed numerous protein bands, but no differences were found in this respect between patterns of SPM material phosphorylated under conditions A, B, and C.

Densitometric scanning of the autoradiographs was performed with higher resolution than described previously (6). As a result more phosphoprotein peaks could be identified and therefore the peaks were renumbered. In Figure 4A and B scanning patterns obtained after incubation of SPM for 15 sec under conditions A and B are shown. The scans were divided in 4 areas (A–D). Main peaks visible under all conditions were then numbered in tens, allowing numbering of bands visible only under special circumstances (like cAMP-sensitive bands, numbers A55, B35) in units. As a consequence, the peaks previously designated 1–10 (6) are now numbered A50, A55, B30, B35, B40, B50, D10, D20, D25, and D30, respectively. In contrast to protein staining patterns, densitometric scanning of the autoradiographs showed that labeling kinetics of the separated protein bands markedly depended on the ATP/SPM ratio in the incubation medium. Figure 4 shows the scanning patterns of SPM material incubated for 15 sec under both condition A and B. Pronounced differences were found in band A46, D15, and D26 which were only detectable after incubation under condition B. Furthermore, differences in distribution of the label between the two conditions were observed, i.e., differences in the amount of label per region, relative to total gel radioactivity.

In view of our interest in ACTH-sensitive brain protein phosphorylation (6, 12), the phosphorylation of one single ACTH-sensitive protein band (B50, MW 48,000 Dalton) was studied at different ATP/SPM conditions (Figure 5), as a function of the incubation time. The shortest incubation time used in this experiment was 5 sec. At 7.5 μM ATP, the

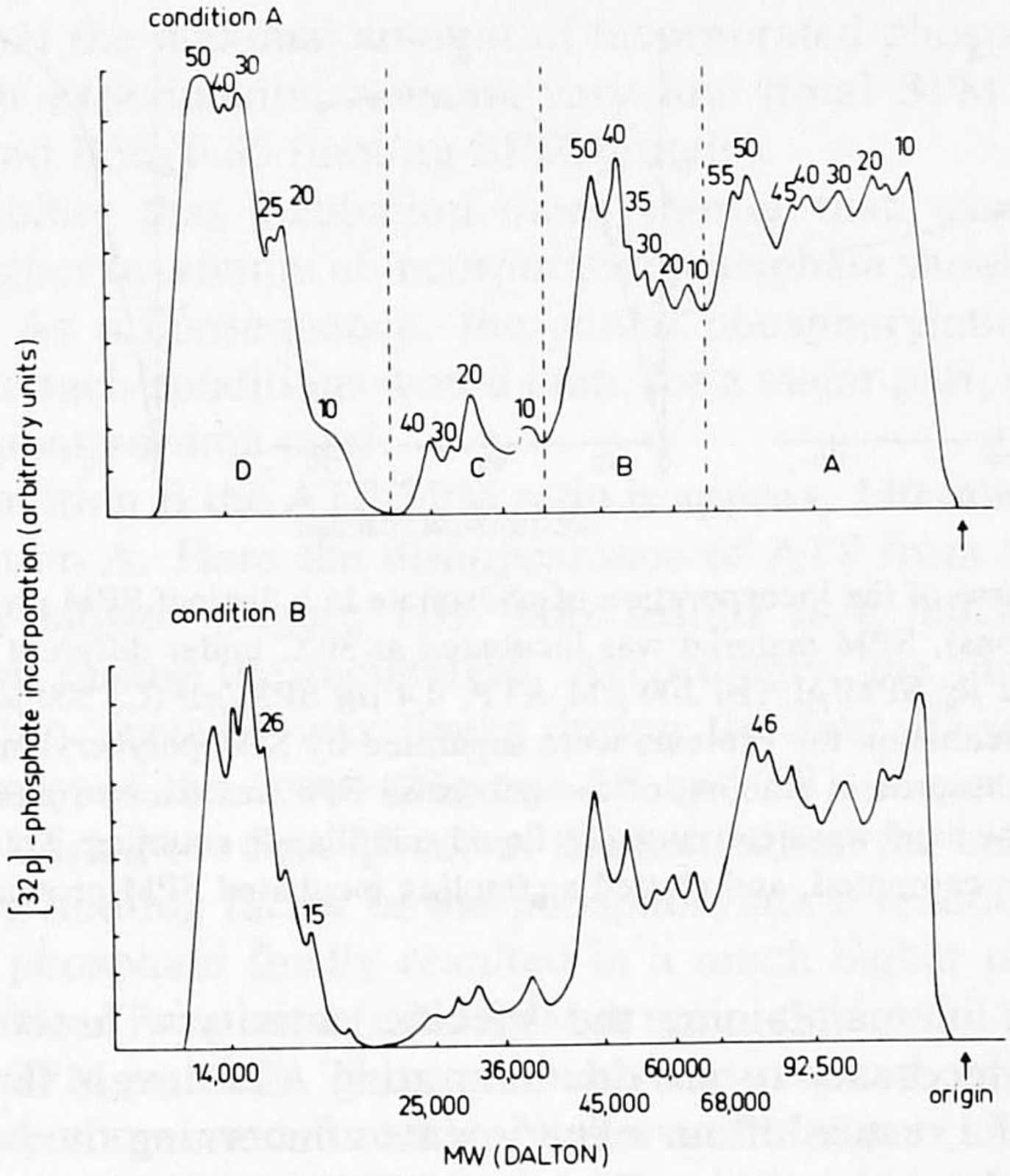

FIG. 4. Densitograms obtained from autoradiographs of the same SPM preparation incubated for 15 sec at 30°C under two different conditions: (A) 7.5 μM ATP, 2.2 μg SPM/μl; (B) 200 μM ATP, 0.4 μg SPM/μl. The electrophoretic mobility of 7 molecular weight protein standards (see Experimental Procedure) is shown at the bottom.

phosphorylation of the protein band reached its maximum instantaneously (Figure 5A; cf. Figure 2A). Longer incubation times resulted in a loss of incorporated label. When higher ATP/SPM ratios were used (Figure 5B and C), a linear increase in phosphorylation was found during the first 20 sec of the incubation. Under both conditions B and C maximal phosphorylation was seen after 60 sec. Incubation for an additional 60 sec did not result in marked changes in the amount of radioactivity in the protein band. Results obtained by the densitometric method were qualitatively identical (data not shown).

DISCUSSION

SPM preparations contain high ATP-hydrolyzing activity. Since [γ-^{32}P]ATP is used as the phosphate donor in studies on the endogenous phosphorylation of SPM material in vitro, an ATP-regenerating system

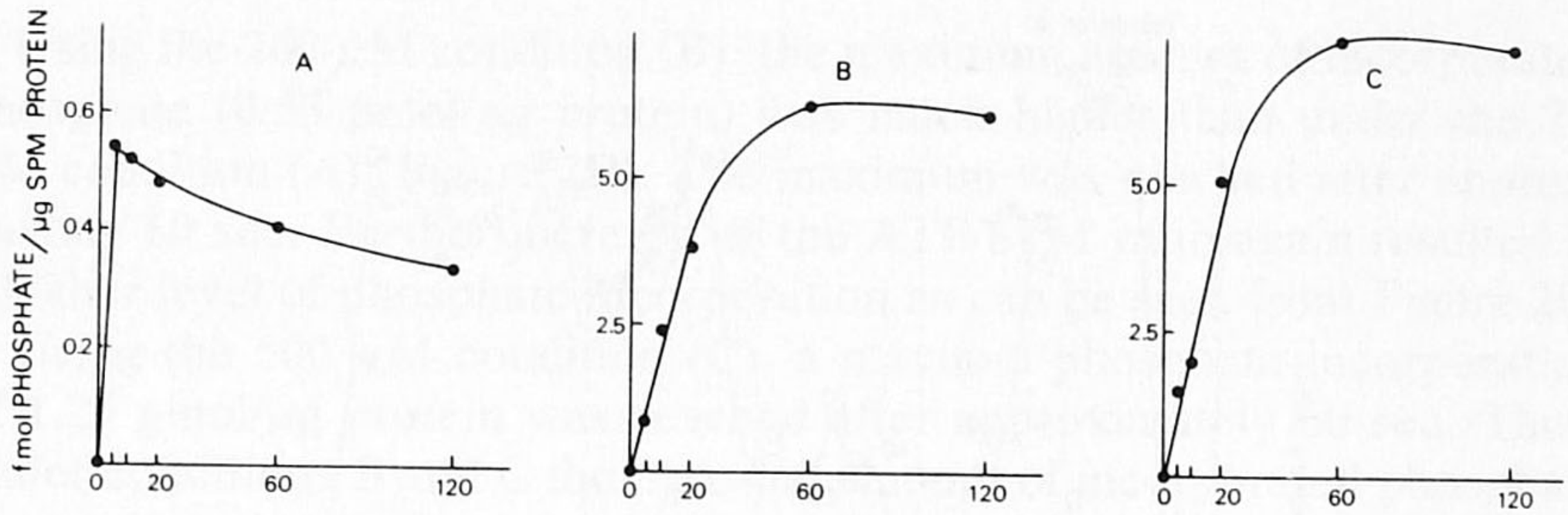

FIG. 5. Time course of the incorporation of phosphate in a distinct SPM protein band (B50; MW 48,000 daltons). SPM material was incubated at 30°C under different conditions: (A) 7.5 μM ATP, 2.2 μg SPM/μl; (B) 200 μM ATP, 0.4 μg SPM/μl; (C) 500 μM ATP, 0.4 μg SPM/μl. After incubation the proteins were separated by SDS-polyacrylamide gel electrophoresis. After the protein staining of the gel, band B50 was excised and the amount of radioactivity in the band was determined by liquid scintillation counting. Total incorporation of phosphate was computed, and plotted as fmol/μg incubated SPM protein.

is insufficient in maintaining the specific activity. Therefore, in such studies, it is necessary to provide saturating ATP levels throughout the incubation. The results of our experiments concerning the breakdown of ATP clearly show that when the low ATP/SPM ratio is used (condition A), essentially all the ATP is hydrolyzed within the first 5 sec of the incubation. This is confirmed by the rapid increase in free phosphate in the medium (Figure 1A). Obviously the high level of membrane-bound ATPases in SPM is responsible for this loss of ATP (rather than the phosphorylation reaction), because the total amount of ATP used for phosphorylation of total SPM protein (110 μg) under condition A is negligible (2.3 pmol = approx. 0.6%). The amount of free phosphate present after 120 sec corresponds to three times the molar amount of ATP at the beginning of the incubation and in fact this suggests hydrolytic cleavage of all three phosphate bonds of ATP. The hydrolysis of the α-phosphate bond can be accounted for by the high level of 5′-nucleotidase activity as this enzyme was taken as a membrane marker in the enrichment of the SPM fraction (7). The rapid hydrolysis of ATP results, under this condition, in a lack of phosphate donor in the medium, and this might explain why phosphorylation of total (Figure 2A) as well as separated (Figure 5A) SPM proteins stops instantaneously. Thus, increasing the ATP-concentration with 7.5 μM at t = 90 sec results, as expected, in a similar peak in phosphorylation as seen at the beginning of the incubation (Figure 3). Taken together, these observations indicate that under low ATP/high SPM conditions the ATP concentration becomes limiting immediately after the start of the reaction. Therefore it is not

surprising that the maximal amount of incorporated phosphate as measured in such experiments, remains very low (total SPM protein: 0.02 pmol/μg; band B50: 0.55 fmol/μg SPM protein).

The possibility that incubation times shorter than used here would result in a higher maximum of incorporated phosphate should be seriously considered. As a consequence, the actual phosphorylation profile observed under such conditions would then, for a major part, depend on the shortest incubation time used.

Under condition B the ATP/SPM ratio is approx. 140 times higher than under condition A. Here the disappearance of ATP from the medium is considerably slower (Figure 1B). This results in a much higher ATP concentration during the incubation. Accordingly, the incorporation of phosphate into proteins was linear during the first 20 sec. From this partial linearity of the curve (Figures 2B and 5B), it can be concluded that at least during the first 20 sec of the incubation the concentration of ATP is not a limiting factor in the phosphorylation reaction. The incorporation of phosphate finally resulted in a much higher maximum than when condition A was used (total SPM protein: 0.55 pmol/μg; band B50: 6.3 fmol/μg SPM protein). This maximum was reached only after 60 sec. A similar time course was found by Weller and Morgan (13), who studied the endogenous cAMP-stimulated phosphorylation of total SPM protein, using an incubation system containing 500 μM ATP and 0.2 μg SPM/μl in a final volume of 1 ml.

Further increase of the ATP/SPM ratio by a factor 2.5 (condition C) enhanced the initial velocity of the phosphorylation of total protein by the same factor (Figure 2B). This then points towards a stimulatory effect of ATP on the phosphorylation of total SPM protein as was also described by Rodnight et al. (3). Moreover, the maximal amount of phosphate incorporated into total protein increased proportionally indicating that acceptor proteins not saturated with phosphate were still present under these conditions (Figure 2B and C). However, such effects of ATP on the velocity and on the maximum of the phosphorylation were not found when phosphate incorporation into protein band B50 was determined (Figure 5). In this band, the maximum incorporation reached the same value under both conditions B and C. This indicates, in fact, a saturation of the phosphorylation system of this substrate protein under both conditions.

Taken together with the qualitative and quantitative differences found between scanning patterns of SPM material phosphorylated under low and high ATP/SPM ratio conditions, these data not only suggest the presence in SPM of protein kinases with different properties, but also stress the differences existing between acceptor proteins.

In conclusion, the data presented in this paper lend support to the notion that saturation of the acceptor proteins with phosphate can be a matter of minutes (cf. Weller and Morgan, reference 13) rather than seconds (cf. Ueda et al., reference 4) and will certainly not occur at limiting ATP concentrations. Therefore, and in view of the high ATPase activity in SPM preparations, it is essential to use high ATP concentrations in studies on the endogenous phosphorylation of total as well as of separated SPM proteins to avoid limiting conditions.

If the influence of experimental variables (drugs, ions, etc.) on the endogenous phosphorylation of synaptic plasma membrane proteins is studied, one should take into consideration the dependence of the phosphorylation profile and thus that of the kinase involved on the ATP/SPM ratios used.

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REFERENCES

1. Walton, K. G., DeLorenzo, R. J., Curran, P. F., and Greengard, P. 1975. Regulation protein phosphorylation and sodium transport in toad bladder. J. Gen. Phys. 65:153–177.
2. Greengard, P. 1976. Possible role for cyclic nucleotides and phosphorylated membrane proteins in postsynaptic actions of neurotransmitters. Nature 260:101–108.
3. Rodnight, R., Reddington, M., and Gordon, M. 1975. Methods for studying protein phosphorylation in cerebral tissue. *in* Marks, N., and Rodnight, R. (eds.), Vol. 3, Pages 325–367, Plenum, Press, New York.
4. Ueda, T., Maeno, H., and Greengard, P. 1973. Regulation of endogenous phosphorylation of specific proteins in synaptic membrane fractions from rat brain by adenosine 3′,5′-monophosphate. J. Biol. Chem. 248:8295–8305.
5. Routtenberg, A., and Ehrlich, Y. H. 1975. Endogenous phosphorylation of four cerebral cortical membrane proteins: Role of cyclic nucleotides, ATP and divalent cations. Brain Res. 92:415–430.
6. Zwiers, H., Veldhuis, D., Schotman, P., and Gispen, W. H. 1976. ACTH, cyclic nucleotides and brain protein phosphorylation in vitro. Neurochem. Res. 1:669–677.
7. Terenius, L. 1973. Stereospecific interaction between narcotic analgesics and a synaptic plasma membrane fraction of rat cerebral cortex. Acta Pharmacol. Toxicol. 32:317.
8. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265–275.
9. Glynn, I. M., and Chappell, J. B. 1964. A simple method for the preparation of ^{32}P-labeled adenosine triphosphate of high specific activity. Biochem. J. 90:147–149.
10. Hurst, R. O. 1964. The determination of nucleotide phosphorus with a stannous chloride-hydrazine sulphate reagent. Can. J. Biochem. 42:287–292.

11. LUGTENBERG, B., MEIJERS, J., PETERS, R., van der Hoek, P., and VAN ALPHEN, L. 1975. Electrophoretic resolution of the major outer membrane protein of Escherichia coli K12 into four bands. FEBS Lett. 58:254–258.
12. ZWIERS, H., WIEGANT, V. M., SCHOTMAN, P. and GISPEN, W. H. 1978. ACTH-induced inhibition of endogenous rat brain protein phosphorylation in vitro: Structure activity. Neurochem. Res. (this volume).
13. WELLER, M., and MORGAN, I. 1976. Distribution of protein kinase activities in subcellular fractions of rat brain. Biochim. Biophys. Acta 436:675–685.