THE EFFECT OF DIGESTION WITH PHOSPHOLIPASE C ON INTRINSIC PROTEIN PHOSPHORYLATION IN SYNAPTIC PLASMA MEMBRANE FRAGMENTS

Ayşe DÖŞEMECI and Richard RODNIGHT*
Department of Biochemistry, Institute of Psychiatry, De Crespigny Park, London SE5 8AF, England

Received 11 Januari 1982

1. Introduction

The synaptic plasma membrane is an exceptionally rich source of intrinsic protein phosphorylation systems which catalyze the phosphorylation and dephosphorylation of a range of membrane proteins [1–4]. The role of cyclic AMP, Ca^{2+} and calmodulin in regulating the initial kinase reaction has been extensively studied, but relatively little is known about the influence of the membrane environment. In analogy to many membrane enzyme systems, it is possible that some of these protein phosphorylation systems have a specific requirement for phospholipids.

In this study chemical cleavage of membrane phospholipids with phospholipase C has been chosen as a method to investigate the role of the membrane environment in maintaining and modulating protein phosphorylation. Phospholipase C acts to remove the phosphate ester head groups from phospholipids leaving diacylglycerols. By this property it is an especially advantageous tool in studying the importance of polar or ionic interactions of proteins with phospholipids which may serve to maintain their correct conformation and/or orientation. Further the possibility must be considered that the diacylglycerols resulting from phospholipase C digestion may have physiologically significant actions on protein kinase activity. Diacylglycerols, which are believed to be produced as a result of receptor-activated phosphatidylinositol breakdown [5] have been shown in soluble systems to activate a new kind of kinase called protein kinase C [6].

We show that direct digestion of synaptic plasma membranes with phospholipase C results in changes in the phosphorylation of certain proteins and some of these effects are mimicked by the addition of diacylglycerol-containing lipid fractions. A synapse-specific protein called protein I [7] seems to require an intact phospholipid environment for maximum phosphorylation. The phosphorylation of 2 other bands of M_r 82 000 and 254 000 is altered either by the production or addition of diacylglycerols. Finally the data on a 45 000 M_r polypeptide (or B-50; see [8]) suggests that a kinase analogous to the protein kinase C [6] is involved in its phosphorylation. This conclusion is in agreement with [8].

2. Materials and methods

Phospholipase C (*C. perfringens,* type X) and cyclic AMP were purchased from Sigma (London) Chemical Co. The ammonium salt of [γ-^{32}P]ATP (17–21 Ci/mmol) was obtained from Amersham Intl., Bucks. Calmodulin was prepared as in [9] omitting the final DEAE-chromatography step.

2.1. *Preparation of synaptic plasma membrane (SPM) fragments*

Essentially the method in [10] was used, but omitting Ca^{2+} from the sucrose solutions. The synaptic plasma membrane fragments recovered from the gradient were centrifugally washed twice with 4 mM imidazole–HCl (pH 7.4) and resuspended in the same buffer. The suspension was diluted with an equal volume of glycerol and mixed thoroughly. After determination of protein [11] preparations were stored at −20°C.

2.2. *Digestion of synaptic plasma membrane fragments with phospholipase C*

Synaptic plasma membrane fragments were incubated with phospholipase C at room temperature in

* To whom correspondence should be addressed

Published by Elsevier Biomedical Press
00145793/82/0000–0000/$02.75 © 1982 Federation of European Biochemical Societies

4 mM imidazole–HCl buffer (pH 7.4) containing 0.5 mM $CaCl_2$. The final protein concentration was 5 mg/ml and unless indicated otherwise, phospholipase C was ~6 enzyme units/ml. The control samples were incubated under exactly the same conditions but without phospholipase C.

2.3. *Labelling of membrane proteins with [γ-^{32}P]ATP*

The labelling of proteins under basal conditions and in the presence of cyclic AMP, Ca^{2+} and calmodulin or Ca^{2+} only was studied. The basal incubation mixture contained 30 mM Tris–HCl (pH 7.4), 1 mM $MgSO_4$ and EGTA as indicated for particular experiments. For phosphorylation under basal or cyclic AMP-containing conditions, ATP was 20 μM with spec. act. 500–1500 cpm/pmol, whereas for phosphorylation in the presence of Ca^{2+}, ATP was 100 μM with 90–150 cpm/pmol spec. act. The other additions were as indicated for individual experiments. The labelling reaction was started by the injection of 20 μl synaptic plasma membrane preparation (5 mg protein/ml) to make up a total volume of 80 μl. After 10 s incubation with continuous shaking 20 μl SDS-containing stop reagent [12] was added to terminate the reaction and solubilize the proteins. In experiments where phospholipase C digestion was carried out prior to labelling an equivalent amount of phospholipase C was added to the labelling medium of control samples.

2.4. *SDS–polyacrylamide slab gel electrophoresis*

The labelled and solubilized proteins were separated on the discontinuous SDS–polyacrylamide slab gel electrophoresis system in [12]. Either 10% or 7% resolving gels were used and 30 μl samples containing 30 μg protein were applied to the tracks. Following electrophoresis, the gels were stained, destained, dried under vacuum and exposed to Kodak 'no screen' X-ray film. After developing the films were scanned in a 'Chromoscan' densitometer to give a semiquantitative profile of the ^{32}P-labelled polypeptide bands. The extent of ^{32}P-label incorporated was estimated by measuring peak heights from an arbitrary baseline. Apparent M_r-values were estimated by determining electrophoretic mobility on gels of different acrylamide concentration [4].

2.5. *Extraction of lipids from synaptic plasma membrane fragments*

Essentially the method in [13] was applied. However a 0.04 M $CaCl_2$ solution, or in experiments where the extracted lipid fraction was to be added to the labelling medium, 0.01 M $MgCl_2$ solution was used in the washing phase instead of water.

2.6. *Estimation of phosphate in lipid extracts*

Suitable volumes of the lipid extracts were dried under a stream of nitrogen. When all the organic solvent was removed, the samples were digested by heating in perchloric acid. The amount of phosphate was subsequently determined as in [14].

3. Results and discussion

The extent of hydrolysis of phospholipids due to phospholipase C treatment was measured as the amount of phosphate loss from lipid fractions extracted from membranes. The lipid extracts from synaptic plasma membranes incubated with phospholipase C showed a maximum phosphate loss of ~75% in 20 min whereas the controls incubated under the same conditions without the enzyme showed a loss of only ~10%.

When synaptic plasma membranes were digested with phospholipase C prior to labelling with [γ-^{32}P]-ATP, an increase in basal phosphorylation and a decrease in cyclic AMP-dependent phosphorylation of some bands was observed (fig.1). However certain

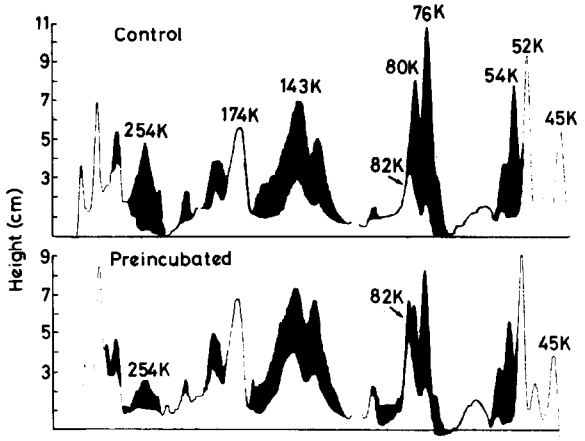

Fig.1. Densitometric scans (expanded × 3) of autoradiographs prepared from ^{32}P-labelled SPM fragments preincubated for 15 min with or without phospholipase C. Labelling with [γ-^{32}P]ATP, electrophoresis, autoradiography and densitometric scanning were done as in section 2. EGTA at 1 mM was used to chelate almost completely the 0.125 mM final [Ca^{2+}] derived from the preincubation medium. The black areas indicate the extent of the stimulation of phosphorylation given by 50 μM cyclic AMP.

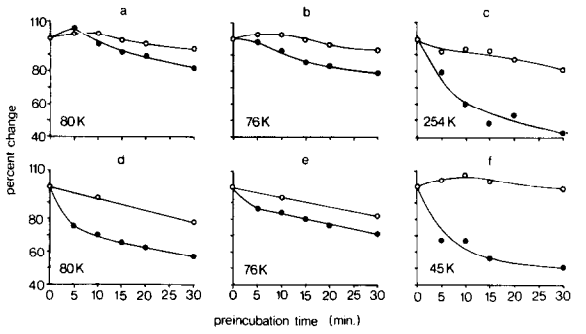

Fig.2. Phosphorylation of certain polypeptides as a function of preincubation time. Following incubation with or without phospholipase C for the indicated time intervals, 20 μl samples were taken with syringe and labelled with [γ-^{32}P]ATP. The conditions for labelling in the presence of cyclic AMP (a–c) were as in fig.1. The Ca^{2+} and calmodulin labelling medium contained ~4 μM calmodulin and 0.5 mM Ca^{2+} in the presence of 0.25 mM EGTA (d–f). Electrophoresis, autoradiography and densitometric scanning were done as in section 2. Each point on the graphs is the mean of 3 or 4 measurements corresponding to separate preincubation; (•) samples preincubated with phospholipase C; (o) control samples. The difference between controls and phospholipase C-treated samples were found to be significant according to statistical testing by 2 way analysis of variance in the 5–30 min incubation time range, in all cases except in (a) where the difference became significant only in the 10–30 min range.

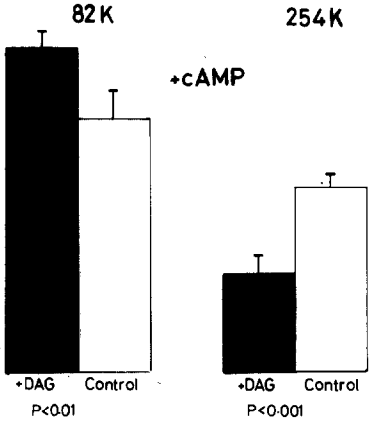

Fig.3. Effect of the addition of lipid fractions containing diacylglycerols (DAG) on the phosphorylation of 82 000 and 254 000 M_r bands in cyclic AMP-containing medium. Lipids were extracted from intact or phospholipase C-digested membranes. After drying under a stream of N_2 they were resuspended in buffer by sonication and samples from the suspensions were added to intact SPM fragments. The amount of phospholipid or phospholipid + diacylglycerol added was roughly equivalent to 40% of the total phospholipid content of the intact membranes. Labelling, electrophoresis, autoradiography and densitometric scanning were done as in section 2: control, addition of lipid fractions extracted from intact SPM; +DAG, addition of lipid fractions extracted from SPM digested with phospholipase C.

bands showed selective or more significant changes due to the treatment; these will be described in more detail in the rest of this section.

Both the cyclic AMP and Ca^{2+}- and calmodulin-dependent phosphorylation of the 76 000 and 80 000 M_r bands were decreased by phospholipase C digestion (fig.2a,b,d,e). Since this change is not observed upon the addition of diacylglycerol containing lipid fractions (not shown) it is probably the breakdown of phospholipids which is responsible for the inhibition. These 2 bands are the subunits of a synapse-specific protein generally called protein I [7] which is phosphorylated at distinct sites by 3 different kinases, one cyclic AMP-stimulated and 2 others, Ca^{2+}- and calmodulin-stimulated [15,16]. Since the phosphorylation mediated by both types of kinases is decreased it is probable that the substrate protein itself loses its specific conformation or orientation due to the removal of phosphate ester head groups of surrounding phospholipids.

Preincubation of synaptic plasma membranes with phospholipase C causes a marked increase in the phosphorylation of an 82 000 M_r band in cyclic AMP-containing medium (fig.1). This effect is mimicked by the addition of diacylglycerol containing lipid fractions on intact synaptic plasma membranes (fig.3). In [17] a synaptic membrane protein referred to as the '85 K band' was described whose phosphorylation is stimulated by a heat-stable factor from synaptosomal cytosol. This effect was independent of Ca^{2+}. It would be interesting to know whether this 85 000 M_r band is the same protein as the 82 000 M_r band described here.

The decrease observed in the phosphorylation of the 254 000 M_r protein upon direct digestion of synaptic plasma membranes by phospholipase C (fig.2c) is also produced by the addition of diacylglycerol containing lipid fractions (fig.3). Although it might be proposed that the inactivation due to digestion is mediated via diacylglycerols, it is less likely than in the activation case and the possibility of unspecific perturbations of the system leading to inactivation must be considered.

The phosphorylation of the 45 000 M_r band in

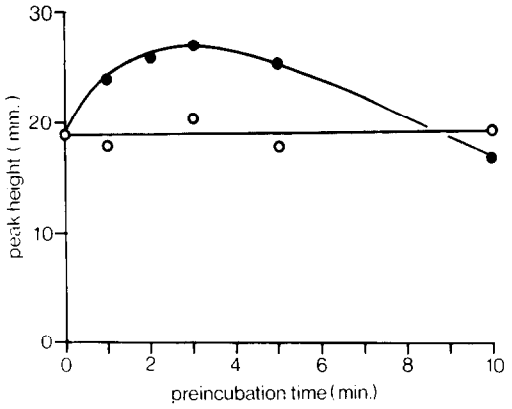

Fig.4. A typical plot for the phosphorylation of the 45 000 M_r polypeptide as a function of preincubation time. The procedure outlined in fig.2 was applied except that no calmodulin was added, phospholipid hydrolysis was more limited in that a smaller amount of phospholipase C was used (3 enzyme units/ml) and a time range of 1–10 min was taken instead of 5–30 min. Also [Ca^{2+}] in the labelling medium was much lower (0.2 mM in presence of 0.25 mM EGTA, estimated to be ~250 nM-free Ca^{2+}).

medium containing a high [Ca^{2+}] showed a significant decrease upon phospholipase C digestion (fig.2f). However, when a limited phospholipase C digestion was done as in fig.4 and the labelling medium contained a very low amount of Ca^{2+} (0.2 mM in the presence of 0.25 mM EGTA) a different pattern of change in phosphorylation as a function of digestion time was obtained (fig.4). This type of behaviour suggests the involvement of a kinase analogous to the protein kinase C in [6] which requires Ca^{2+} and phospholipids for activity and is activated by diacylglycerols. Thus the initial increase in phosphorylation could be explained by the limited hydrolysis producing enough diacylglycerols to activate the system while leaving the bulk of phospholipids intact, whereas the subsequent decrease upon further incubation would be due to an extensive hydrolysis of phospholipids leading to inactivation of the kinase. Also the initial increase in phosphorylation would be expected to be more pronounced in labelling medium containing a limited [Ca^{2+}] since diacylglycerols decrease the K_a of the enzyme for Ca^{2+}.

We have thus far interpreted the results only in terms of the possible changes in kinases or substrate proteins due to the treatments. Inhibition or activation of phosphatases might also contribute to the changes observed. Since phosphatase activity is much slower than kinase activity in synaptic plasma membranes [18] changes in phosphatase activity will not have significant effects on ^{32}P-incorporation in the 10 s labelling period unless there is a drastic activation of the enzyme. Preliminary experiments (not shown) suggest that there is no such drastic change. On the other hand an inhibition or activation of the phosphatase during preincubation of synaptic plasma membranes with phospholipase C might lead, respectively, to a smaller or greater number of vacant substrate sites thus yielding a decrease or an increase in the final ^{32}P-incorporation. An effect due to the inhibition of phosphatase can be ruled out because phosphatase does not seem to be active in our preincubation conditions anyway (as seen from fig.2 there is no significant increase in phosphorylation in control samples as a function of incubation time). Activation of phosphatase leading to increased labelling might also be ruled out for the bands discussed since in the only case where there is an increase in phosphorylation (82 000 M_r band), the effect is mimicked by the addition of diacylglycerol containing lipid fraction.

Studies using other types of phospholipases might give new and complementary information on the role of membrane phospholipids in protein phosphorylation.

Acknowledgement

We are grateful to the British Council for a Scholarship to A. D.

References

[1] De Blas, A. L., Wang, Y.-S., Sorensen, R. and Mahler, H. R. (1979) J. Neurochem. 33, 647–659.
[2] Dunkley, P. R. (1981) in: New Approaches to Nerve and Muscle Disorders. Basic and applied contributions (Kidman, A. D. et al. eds) pp. 38–51, Excerpta Medica, Amsterdam.
[3] Greengard, P. (1981) Harvey Lectures, Academic Press, New York.
[4] Gower, H. and Rodnight, R. (1982) Biochim. Biophys. Acta in press.
[5] Hawthorne, J. N. and Pickard, M. R. (1979) J. Neurochem. 32, 5–14.
[6] Kishimoto, A., Takai, Y., Mori, T., Kikkawa, U. and Nishizuka, Y. (1979) J. Biol. Chem. 255, 2273–2276.
[7] Bloom, F. E., Ueda, T., Battenberg, E. and Greengard, P. (1979) Proc. Natl. Acad. Sci. USA 76, 5982–5986.
[8] Aloyo, V. J., Zwiers, H. and Gispen, W. H. (1982) Prog. Brain Res. in press.

[9] Walsh, M. and Stevens, F. C. (1978) Can. J. Biochem. 56, 420–429.
[10] Jones, D. H. and Matus, A. L. (1974) Biochim. Biophys. Acta 356, 276–287.
[11] Peterson, G. L. (1977) Analyt. Biochem. 83, 346–356.
[12] Holmes, H. and Rodnight, R. (1981) Dev. Neurosci. 4, 79–88.
[13] Bligh, E. G. and Dyer, W. J. (1959) Can. J. Biochem. 37, 911–917.
[14] Bartlett, G. R. (1959) J. Biol. Chem. 234, 466–468.
[15] Huttner, W. B., De Gennaro, L. J. and Greengard, P. (1981) J. Biol. Chem. 256, 1482–1488.
[16] Kennedy, M. B. and Greengard, P. (1981) Proc. Natl. Acad. Sci. USA 78, 1293–1297.
[17] O'Callaghan, J. P., Juskevich, J. and Lovenberg, W. (1980) Biochem. Biophys. Res. Commun. 95, 82–89.
[18] Weller, M. and Rodnight, R. (1971) Biochem. J. 124, 393–406.