Biochimica et Biophysica Acta, 469 (1977) 350—354

BBA Report

BBA 71309

EVIDENCE FOR THE PRESYNAPTIC LOCATION OF ADENYLATE CYCLASE AND THE CYCLIC AMP-STIMULATED PROTEIN KINASE WHICH IS BOUND TO SYNAPTIC MEMBRANES

MALCOLM WELLER

Neurochemistry Research Unit, Department of Chemical Pathology, University of Stellenbosch, P.O. Box 63, Tygerberg 7505 (Republic of South Africa)

(Received June 28th, 1977)

Summary

By comparison of activities measured with either intact or ruptured synaptosomes it was found that about half of the cerebral adenylate cyclase is presynaptic while all the membrane bound, cyclic AMP-stimulated protein kinase activity appears to be presynaptic with the cyclic AMP receptor facing inward.

It is well established that synaptosome plasma membranes from brain contain a bound protein kinase which catalyses the phosphorylation of endogenous proteins and which is stimulated by cyclic AMP [1,2,3]. In addition there is a bound protein phosphatase which catalyses the dephosphorylation of the proteins [4]. The bound protein kinase and its substrate protein(s) are located in the region of the synaptic junction [5] but until now it was not known whether the system was bound to the pre- or postsynaptic membrane. It has, however, been found that phosphorylation of protein(s) in the intact synaptosome lowers the permeability of the presynaptic membrane to Ca^{2+} [6]. While it is not known if the phosphorylation of these proteins is stimulated by cyclic AMP the results suggest the possibility that the cyclic AMP-stimulated intrinsic protein kinase activity of the synaptic membrane may be at least partially presynaptic. Based on results obtained with the superior cervical ganglion Greengard's group have, however, proposed a generalised hypothetical model in which a suitable neurotransmitter interacts with a receptor on the postsynaptic membrane and stimulates the postsynaptic production of cyclic AMP. The increased concentration of cyclic AMP is postulated as stimulating the phosphorylation of a postsynaptic membrane protein resulting in hyperpolarisation [7,8].

While it is undoubtedly true that cyclic AMP causes postsynaptic effects

in the superior cervical ganglion [7,8] there is no evidence that these effects are mediated by phosphorylation of postsynaptic membrane proteins [11]. In the case of Purkinje cells, however, where cyclic AMP also causes postsynaptic effects [9,10], there is indirect evidence that protein phosphorylation may be involved [12] and the increased protein phosphorylation seen in brain slices on exposure to certain neurotransmitters may also be, at least partially, postsynaptic [13].

In addition to these observations it has also been found that cyclic AMP can have presynaptic effects, stimulating the release of acetylcholine from the neuromuscular junction [14] and noradrenalin from the hypogastric nerve [15]. For these reasons initial experiments were carried out to determine what proportion (if any) of the adenylate cyclase activity of brain is presynaptic.

Synaptosomes were prepared from rat cerebral cortex as previously described [16] (their full characterisation being given in that paper) and the adenylate cyclase activity of the preparation measured either under isotonic (0.32 M sucrose) or hypotonic (0.05 M sucrose) conditions. In the former case the synaptosomes are intact [6] and any adenylate cyclase activity on the inner surface of the presynaptic membrane would, presumably, be inaccessible to ATP. In the latter case, however, the synaptosomes would be ruptured and presynaptic sites exposed. Table I shows that much more cyclic AMP was produced by ruptured than by intact, synaptosomes while the presence or absence of 0.32 M sucrose made no difference to the adenylate cyclase activity of preparations of synaptic plasma membrane (prepared as previously described [16]) showing that the enzyme was unaffected by sucrose as such.

It may thus be concluded that some 60% of the adenylate cyclase activity in synaptosomal preparations from rat cerebral cortex is located on the inner surface of the presynaptic membrane. This observation agrees with the finding that increased cyclic AMP phosphodiesterase activity is observed on rupturing synaptosomes, suggesting that part of this enzyme may also be presynaptic [17].

Experiments were next carried out to determine the location of the

TABLE I

EVIDENCE FOR THE PRESYNAPTIC LOCATION OF A PROPORTION OF CEREBRAL ADENYLATE CYCLASE

Samples of synaptosomes, or isolated synaptosome plasma membranes, (about 0.5 mg of protein) were incubated with 3 mM $MgCl_2$, 30 mM Tris · HCl (pH 7.4), 6 mM theophylline, 0.3 mM [γ-^{32}P]ATP (specific activity about $2 \cdot 10^7$ cpm/μmol) in a final volume of 0.3 ml for 10 min at 22° C. Reactions were stopped by the addition of 20 μl of 100 mM EDTA and the tubes heated at 95° C for 3 min. 10 μl of 100 μM cyclic AMP and 0.8 ml of 50 mM Tris · HCl, pH 7.6, were then added and the contents of the tubes passed through columns (0.5 × 3 cm) of aluminium oxide (Brockman grade 1, Active, neutral BDH laboratories, U.K.) which had been previously equilibriated with 50 mM Tris · HCl, pH 7.6. The eluents were collected and the rest of the cyclic AMP eluted from the columns with 2 ml of 50 mM Tris · HCl, pH 7.6. Aliquots (0.2 ml) of 0.25 M $ZnSO_4$ and 0.25 M $Ba(OH)_2$ were added to the column eluents which were then shaken and briefly centrifuged. The supernatants were added to 5 ml lots of INSTA-GEL (Packard Instrument Co., U.S.A.) and counted [20]. Results are shown as means ± standard deviations and are taken from 4 separate observations.

Starting material	Sucrose (M)	Cyclic AMP produced (pmol/mg of protein per min)
Intact Synaptosome	0.32	134 ± 15
	0.05	348 ± 34
Isolated synaptosome Membranes	0.32	423 ± 41
	0.05	444 ± 57

protein kinase which is bound to the synaptic membrane. Synaptosomes were incubated under either isotonic or hypotonic conditions as described above with [γ-^{32}P]ATP in the presence or absence of cyclic AMP and the amount of radioactive phosphate transfered to protein determined by a previously described method [2]. It may be seen from Table II that there was more protein phosphorylation in ruptured than in intact synaptosomes and that cyclic AMP only stimulated phosphorylation in the former case. These effects were not merely caused by the high concentration of sucrose since the presence or absence of 0.32 M sucrose had no effect on the intrinsic protein kinase activity of isolated synaptosome membranes.

TABLE II

EVIDENCE FOR THE PRESYNAPTIC LOCATION OF THE CYCLIC AMP-STIMULATED PROTEIN KINASE WHICH IS BOUND TO THE SYNAPTIC PLASMA MEMBRANE

Samples of synaptosomes, or synaptosomal plasma membranes, (about 200 μg of protein) were incubated for 30 s with 1 mM $MgCl_2$, 50 mM Tris·HCl, pH 7.4, 0.5 mM [γ-^{32}P]ATP (specific activity about $5 \cdot 10^6$ cpm/μmol) in the presence or absence of 10 μM cyclic AMP in a final volume of 0.5 ml at 37°C. Reactions were stopped by the addition of 2 ml of ice cold 15% trichloracetic acid and the amount of ^{32}P transferred to protein determined as previously described [2]. Results are shown as means ± standard deviations and are taken from 6 separate observations.

Starting material	Sucrose (M)	^{32}P transferred to protein (pmol/mg of protein)	
		–cyclic AMP	+cyclic AMP (10 μM)
Intact synaptosomes	0.32	34 ± 12	34 ± 17
	0.05	140 ± 13	222 ± 30
Isolated synaptosome membranes	0.32	83 ± 12	189 ± 25
	0.05	84 ± 14	200 ± 11

It is thus apparent that most of the sites of protein phosphorylation in the synaptosomes are only exposed after osmotic shock. The most plausible interpretation of this result is that it is the breaking open of the synaptosome and exposure of presynaptic sites which allows the increased phosphorylation and that the cyclic AMP-stimulated intrinsic protein kinase activity of synaptosome membranes is presynaptic with the cyclic AMP receptor facing inwards.

The fact that intact synaptosomes may be phosphorylated suggests that the membrane-bound kinase can use external ATP as a source of phosphate. The possibility arises, however, that the proteins which are phosphorylated in the intact synaptosomes are not the same as those phosphorylated in isolated synaptosome membranes.

In order to test this possibility, either intact synaptosomes or isolated synaptosome membranes were incubated with [γ-^{32}P]ATP and the phosphorylated proteins separated by polyacrylamide gel electrophoresis as described in Fig. 1. The pattern of protein phosphorylation was found to be the same for either type of sample, demonstrating that no new protein substrates are exposed on rupturing the synaptosomes and suggesting that phosphorylation may occur in intact synaptosome due to the membrane-bound protein kinase being able to utilise external ATP as phosphate donor. The pattern shown in Fig. 1 is similar to that found by Greengard's [3] and Dunkley's [18] groups. It would appear that my bands I, II and III correspond to bands α, β

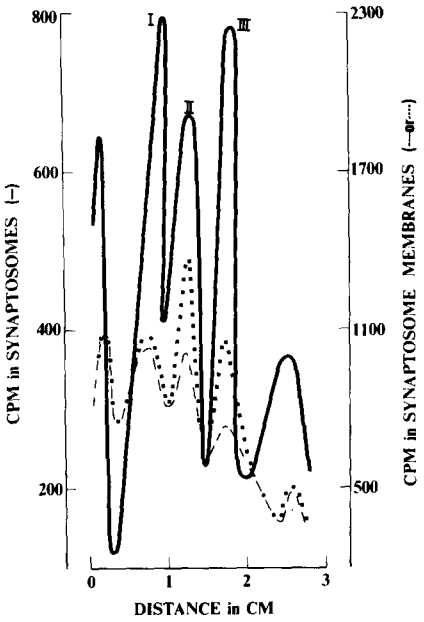

Fig. 1. Samples of synaptosomes (—) were incubated for 1 min with 50 mM Tris·HCl (pH 7.4) 1 mM $MgCl_2$, 0.32 M sucrose, 0.5 mM [γ-^{32}P]ATP (specific activity about $1 \cdot 10^8$ cpm/μmol) at a protein concentration of about 0.8 mg/ml in a volume of 0.15 ml at 37°C. Samples of synaptosome plasma membranes were incubated under similar conditions (but at a protein concentration of about 0.3 mg/ml) in the presence (• • •) or absence (– – –) of 10 μM cyclic AMP. Reactions were stopped by the addition of 0.15 ml of a mixture containing 2% sodium dodecyl sulphate, 2M urea and 1% mercaptoethanol and the proteins separated by polyacrylamide gel electrophoresis, the gels being cut into 2 mm lengths and radioactivity determined as previously described [5].

and γ of Dunkley et al. [18], while Greengard's bands I and II correspond, in terms of molecular weights and sensitivity to cyclic AMP, to my bands II and III.

In view of the presynaptic location of the cyclic AMP-stimulated intrinsic protein kinase and adenylate cyclase it seems reasonable to suggest that a presynaptic increase in the concentration of cyclic AMP will stimulate the phosphorylation of membrane proteins as shown in Fig. 2. The question, however, arises as to how the presynaptic production of cyclic AMP can be stimulated. This can scarcely be through the interaction of a neurotransmitter with a receptor site on the cyclase enzyme, as is postulated to occur for the postsynaptic enzyme. It could be that it is membrane depolarisation and, perhaps Ca^{2+} influx, which stimulates the presynaptic adenylate cyclase.

It has previously been demonstrated that phosphorylation of proteins in the presynaptic membrane can reduce the permeability of the membrane to Ca^{2+} [6]. The fact that the phosphorylation can be stimulated by cyclic AMP suggests that the Ca^{2+} permeability of the synaptic membrane may be reduced by agents or treatments which increase cyclic AMP concentration. Since an influx of Ca^{2+} into the synaptic terminal is essential for neurotransmitter release this suggests an important method of control of nervous activity.

While the results described above indicate that cyclic AMP can stimulate the phosphorylation of presynaptic membrane proteins the data of Rodnight's group [19] supported by the work of William's [13] and the indirect evidence

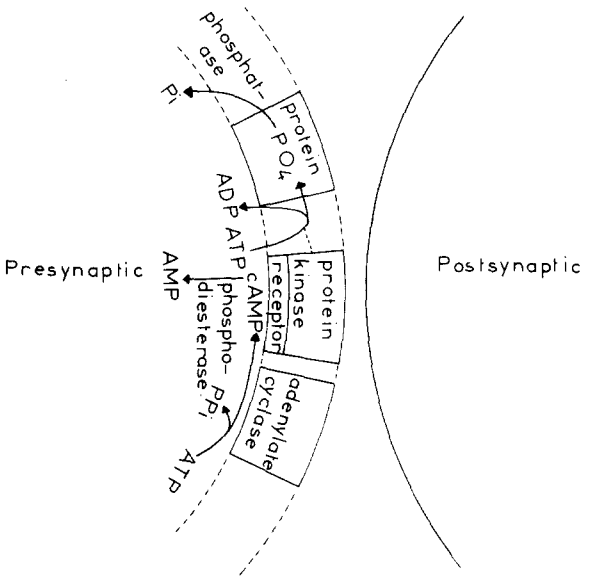

Fig. 2. A hypothetical scheme for the presynaptic location and interaction of adenylate cyclase and the membrane-bound protein kinase.

of Siggins and Henriksen [12] suggests that postsynaptic protein phosphorylation may also occur.

I would like to thank Miss W. Laing for her excellent technical assistance and I am indebted to the South African Medical Research Council for financial help during the course of this study.

References

1 Weller, M. and Rodnight, R. (1970) Nature 225, 187—188
2 Weller, M. and Morgan, I.G. (1976) Biochim. Biophys. Acta 436, 675—685
3 Ueda, T., Maeno, H. and Greengard, P. (1973) J. Biol. Chem. 248, 8295—8305
4 Weller, M. and Rodnight, R. (1971) Biochem. J. 124, 393—406
5 Weller, M. and Morgan, I.G. (1976) Biochim. Biophys. Acta 433, 223—228
6 Weller, M. and Morgan, I.G. (1977) Biochim. Biophys. Acta 465, 527—534
7 Greengard, P. and Kebabian, J.W. (1974) Fed. Proc. Fed. Amer. Soc. Exp. Biol. 33, 1059—1067
8 Greengard, P. (1976) Nature 260, 101—108
9 Siggins, G.R., Hoffer, B.J. and Bloom, F.E. (1969) Science 165, 1018—1020
10 Siggins, G.R., Hoffer, B.J. and Bloom, F.E. (1971) Brain Res. 25, 535—553
11 Weller, M. (1976) in Protein Phosphorylation, Pion Press, London, in press
12 Siggins, G.R., Henriksen, S.J. (1975) Science 189, 559—560
13 Williams, M. (1976) Brain Res. 109, 190
14 Singer, J.J. and Goldberg, A.L. (1971) Adv. Biochem. Psychopharmacol. (Greengard, P. and Costa, E., eds.), 3, 335—348, Raven Press, N.Y.
15 Wooten, G.F., Nguyen, B.T., Kopin, I.J. and Axelrod, J. (1973) Mol. Pharmacol. 9, 178—183
16 Morgan, I.G., Wolfe, L.S., Mandel, P. and Gombos, G. (1971) Biochim. Biophys. Acta 212, 515—517
17 Lust, W.D., Passonneau, S.V. and Goldberg, N.D. (1972) Fed. Proc. Fed. Amer. Soc. Exp. Biol. 31, 555
18 Dunkley, P.R., Holmes, H. and Rodnight, R. (1976) Biochem. J. 157, 661—666
19 Reddington, M., Rodnight, R. and Williams, M. (1973) 132, 475—482
20 White, A.A. and Zenser, T.V. (1971) Anal. Biochem. 41, 372—396