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Effects of Electroshock and Drugs Administered *In Vivo* on Protein Kinase Activity in Rat Brain

H. HOLMES, R. RODNIGHT AND R. KAPOOR

Department of Biochemistry, Institute of Psychiatry
de Crespigny Park, Denmark Hill, London, SE5 8AF, U.K.

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HOLMES, H., R. RODNIGHT AND R. KAPOOR. *Effects of electroshock and drugs administered in vivo on protein kinase activity in rat brain.* PHARMAC. BIOCHEM. BEHAV. 6(4) 415–419, 1977. – The effect of electroshock and treatment with reserpine, amphetamine or lithium chloride on protein kinase activity in synaptic membrane fragments prepared from rat brain was investigated. Naive rats subjected to electroshock procedures showed significant increases in both basal and cyclic AMP-stimulated activity irrespective of whether the treatment was sham, acute or chronic. These increases did not occur in animals which had been tamed by daily handling for 15 days prior to treatment, suggesting that the response was induced by the stress of an unfamiliar situation. Administration of lithium chloride and reserpine caused a small but significant increase in the stimulated activity. Doses of d-amphetamine of 5 mg/kg had no effect on either basal or stimulated activity, but higher doses (up to 15 mg/kg) resulted in a pronounced increase in both activities, which may have been related to drug-induced stress.

Electroshock Protein kinase activity

MANY of the actions of cyclic AMP in the central nervous system appear to be mediated through the phosphorylation of specific neuronal proteins. Cyclic AMP-stimulated protein kinases occur in the neuronal cell membrane [12,26], in the cytosol [19] and in association with neurotubular protein [15]. The functional significance of these and other protein kinases in brain is poorly understood, but there is evidence that protein phosphorylation mediated by cyclic AMP may be concerned in synaptic transmission [11,21].

A variety of environmental influences such as electroshock, drugs and nonspecific stress alter the concentration of cyclic AMP in the brain [5, 6, 17] but the extent to which these changes in cyclic AMP modify protein kinase activity over a period of time has not been investigated. Two reports, however, suggest that drug treatment may induce detectable changes in protein phosphorylation in the brain. Clark *et al.* [3] found that the activity of an intrinsic protein phosphorylating system in cerebral microsomes was significantly decreased in chronically morphinized rats and increased during withdrawal from the drug. Ehrlich and Brunngraber [8] in a brief report showed that treatment of rats with chlorpromazine increased the cyclic AMP-stimulated phosphorylation of a specific protein in synaptic membrane preparations from cortex and neostriatum. In the present work we have examined the activity of the intrinsic protein kinase system present in membrane fragments of synaptic origin following exposure of rats in vivo to electroshock and three psychotropic drugs. These membrane-bound enzymes differ in many respects from the soluble protein kinases of brain which catalyse the phosphorylation of histones and phosvitin. They are tightly bound to the membrane fragments and cannot be solubilised by hypotonic washes, by exposure to solutions of high ionic strength or by detergents that solubilise up to 40% of the membrane protein [7, 23, 24, 25].

METHOD

Animals

Wistar albino rats (80–100 g) of both sexes were used. Animals subjected to amphetamine treatment (higher doses) were housed individually; the remainder including controls for amphetamine were kept in groups of 2–4 in standard plastic cages. The colony room was maintained on a 12 hr on/12 hr off light–dark cycle. All animals were allowed ad lib access to food and water.

Electroshock Treatment (ES)

Rats of 80–100 g were used. The ears were cleaned with acetone and then rubbed with electrode jelly. Electrodes were slipped on to the base of the ears and a shock of frequency 150 Hz, delay 0.01 msec, duration 1.5 msec and voltage 85 V was administered for 1–3 sec with an S_4-Stimulator (Grass Medical Instruments). Controls were subjected to the same treatment as the chronically treated animals except that no current was passed (Sham ES). For

chronic treatment an electric shock was administered once a day for 5 days. For investigations of protein kinase activity the final treatment was given 10–30 min before killing the animals; when cyclic AMP concentrations were being studied the animals were killed immediately (10–20 sec) after the last treatment. Acute ES consisted of a single treatment 10–30 min before killing the animal.

Drug Treatment

All drugs were dissolved in minimum volume of 95% ethyl alcohol (to give 0.5% in final solution) and then made up to required volume with 0.9% NaCl (saline). Administration was by intraperitoneal injection. Table 1 shows doses, frequency of administration and length of treatment. Doses of lithium chloride were gradually increased from 1 to 2 meq/kg in order to maintain constant blood levels. Doses higher than 2 meq/kg were found to be toxic. With regard to amphetamine a number of workers [4, 10, 16] stated that doses of about 5 mg/kg are required before significant changes in noradrenaline and 5-hydroxytryptamine stores in brain are observed. However, as these doses did not have any effect on the membrane-bound protein kinases investigated in this study we decided to examine higher doses.

TABLE 1

CHRONIC TREATMENT OF RATS WITH DRUGS

	Dose of Drug				
	1st Day	2nd Day	3rd Day	4th Day	5th Day
Lithium Chloride meq/kg	1	1.5	1.5	2	2
Reserpine mg/kg	5	5.0	5.0	5	10
d-Amphetamine sulphate mg/kg	5	5.0	5.0	5	5
d-Amphetamine sulphate administered twice daily mg/kg	10	10.0	15.0	15	60

Controls set up for each drug were injected with saline, the volume and frequency of injections being the same as for the test groups.

Determination of Cyclic AMP

Sample preparation. Rats of 80–100 g were killed by total immersion in liquid N_2 10–20 sec after the last ES. Brains were transected immediately behing the occipital cortex and the rostral portion (forebrain) transferred to a mortar (already cooled and containing liquid N_2) and crushed to a coarse powder with a pestle. Portions of the powder (50–80 mg) were extracted with trichloroacetic acid according to the method of Albano *et al.* [1].

Cyclic AMP assay. Samples (20–50 μl) of reconstituted brain extract were assayed by the protein binding technique of Brown *et al.* [2]. Brain extracts were found to exert a significant effect on the standard response curve due to factor(s) interfering with the binding of cyclic AMP to the adrenal cortex binding protein, as was the case with the binding protein from skeletal muscle [27]. To correct for this, standards were made up in an extract free of endogenous cyclic AMP prepared from brain tissue which had been allowed to stand at room temperature for 48 hr. The standards were then taken through the same trichloroacetic acid extraction procedure as for test samples.

Preparation of Synaptic Membrane Fragments

The method of Jones and Matus [13] with the following modifications was used: rats were decapitated using a guillotine, and the forebrains rapidly removed and transferred to ice. Pyramidal tracts, hypothalamus, thalamus and basal ganglia were dissected out and the remainder including some periventricular white matter used for the preparation of the membrane fragments. All isolation procedures were performed at 4 °C and all sucrose solutions were made in 5 mM-Tris-citrate buffer (pH 7.4) containing 50 μM Ca^{2+}. The density gradients were centrifuged at 95000 G for 120 min in a Beckman L265B ultracentrifuge using a No. S.W. 27 swing-out head. The fraction floating at the 34%/28.5% (w/w) sucrose interface was collected, adjusted to 10% (w/w) sucrose and centrifuged at 80000 G for 30 min. The pellet was washed once in 4 mM-imidazole buffer (pH 7.4) and resuspended in this buffer at a concentration of 10 mg/ml. Until use these synaptic membrane preparations were stored at −20 °C.

Determination of Protein

The method of Miller [18] was used with bovine plasma albumin as standard.

Determination of Endogenous Protein Kinase Activity

The standard procedure of Rodnight *et al.* [22] was used with an ATP/protein ratio of 0.25 nmol/μg, and an incubation time of 10 sec at 37 °C.

Statistical Methods

Significance of the difference between means was assessed by a Student's *t*-test.

RESULTS

Gross Effects of Treatment on Health and Behaviour

High levels of anxiety in naive rats used for ES studies were manifested as a struggle to escape, aggresiveness and tachycardia. This behaviour was absent in rats (4–6 weeks old) which had been tamed by being picked up, the ears rubbed and the fur stroked for 2–3 min twice daily for 15 days. At the end of this period the animals had become accustomed to being handled as shown by their docile behaviour. Administration of acute, chronic or sham ES did not leave any noticable effects on the animal's behaviour.

In lithium treated animals polyurea and sedation were seen at about the 3rd day of the treatment. Reserpinised rats developed ptosis and diarrhea on the second day of treatment. The higher doses of d-amphetamine were accompanied by anorexia, hyperthermia and hypermobility. None of the rats died during any of the treatments.

Effect of ES on Protein Kinase Activity and Cyclic AMP Content

When naive animals were used to investigate the effect of ES on protein kinase activity in synaptic membrane fragments, significant increases in both the basal and stimulated activities were obtained, irrespective of whether the treatment was sham, acute or chronic ES (Table 2). Variation in the interval between the last treatment and killing the animals from 10–30 min did not have any effect on the increase. As indicated above naive rats exhibited a

TABLE 2

EFFECT OF ELECTRO-SHOCK (ES) FOR 5 DAYS ON CYCLIC AMP CONTENT OF RAT BRAIN

Treatment	Cyclic AMP (pmol/mg tissue)	Significance (P)
Naive Controls (6)	0.98 ± 0.09	
Chronic Sham ES (3) (naive rats)	0.44, 0.64, 0.52	
Chronic ES (3) (naive rats)	1.39, 0.84, 1.36	
Chronic Sham ES (11) (handled rats)*	0.84 ± 0.09	NS
Chronic ES (10) (handled rats)*	1.48 ± 0.15	< 0.001

The number of rats is indicated in parentheses.

Naive rats were not handled before treatment.

*Rats were handled for 15 days before starting ES treatment.

high degree of anxiety when taken from their cages, and it was to eliminate the possible effect of this factor that rats were handled for 15 days prior to treatment with ES. Handling abolished the increase in protein kinase activity of the membrane fragments observed in naive rats subjected to the ES-procedure: the mean values for both the sham and chronic ES-groups were not significantly different from the mean value for naive controls (Table 3).

In view of the lack of any effect of ES on protein kinase activity in handled rats it was clearly of interest to determine whether the cerebral content of cyclic AMP was increased by ES in animals tamed by this procedure. A significant increase over the values for control naive animals was observed in handled rats submitted to chronic ES but no to sham chronic ES (Table 3). In small groups of naive animals the same trend was observed but the values were too few for statistical analysis; however, there are several reports in the literature which show that ES increases the cyclic AMP content of the brain in normal rats [5,17].

TABLE 3

EFFECT OF ES ON PROTEIN KINASE ACTIVITY IN MEMBRANE FRAGMENTS FROM RAT BRAIN

Treatment	Protein Kinase Activity (pmol of ^{32}P/0.4 mg protein/10s) With Cyclic AMP	Significance P	Without Cyclic AMP	Significance P
Naive Controls (8)	128 ± 5		98 ± 4	
Chronic Sham ES (18) (naive rats)	216 ± 11	<0.001	173 ± 12	<0.002
Chronic ES (18) (naive rats)	201 ± 7	<0.001	157 ± 7	<0.001
Acute ES (15) (naive rats)	225 ± 9	<0.001	182 ± 10	<0.001
Chronic Sham ES (12) (handled rats)*	140 ± 7	NS	97 ± 5	NS
Chronic ES (12) (handled rats)*	128 ± 6	NS	88 ± 2	NS

Numbers of animals in parentheses. Values quoted are means ± S.E.M..

*Rats were handled for 15 days before starting ES treatment.

Effect of Some Drugs on Protein Kinase Activity

Lithium chloride and reserpine caused a small but significant increase in the stimulated activity; doses of d-amphetamine of 5 mg/kg had no effect on either basal or stimulated activities, whereas higher doses (see Table 1) resulted in a pronounced increase in both activities (Table 4). This increase in the cyclic AMP stimulated kinase activity was maintained after withdrawal of d-amphetamine for 24 hr from chronically treated animals.

TABLE 4

EFFECT OF CHRONIC TREATMENT OF RATS WITH CHEMICAL AGENTS ON PROTEIN KINASE ACTIVITY

Chemical agent	Protein Kinase Activity (pmol ^{32}P/0.4 mg protein/10s) With Cyclic AMP	Significance (P)	Without Cyclic AMP	Significance (P)
Saline (12)	140 ± 3		107 ± 7	
Lithium Chloride (8)	160 ± 3	<0.001	114 ± 7	NS
Reserpine (8)	152 ± 6	<0.001	115 ± 6	NS
d-Amphetamine (6) (low doses)	140 ± 9	NS	106 ± 5	NS
d-Amphetamine (12) (high doses-twice daily)	197 ± 8	<0.001	141 ± 5	<0.002
d-Amphetamine (8) (withdrawal)	171 ± 8	<0.001	120 ± 8	NS

Number of animals in parentheses.

Values quoted are means ± S.E.M.

See table 1 for drug dosages.

DISCUSSION

It appears from these results that in naive rats the anxiety accompanying an electroshock procedure can induce an increase in the in vitro activity of a membrane-bound protein phosphorylating system, probably involved in synaptic function. There is no evidence in this work that ES per se increases protein kinase activity, which contrasts with the clear cut consequences which this treatment has on certain other enzyme systems in brain [9].

The specificity of this stress response to an unfamiliar situation has not yet been investigated. It would be of interest, for instance, to compare the effects of maintaining animals at low temperatures with the stress resulting from immobilization. Further, it cannot be excluded that the increased protein kinase activity observed in animals treated with the relatively high doses of reserpine, lithium chloride and amphetamine used in this work (Table 4) is due to the stress associated with the behavioural effects of the drugs. This comment particularly applied to animals subjected to the higher doses of amphetamine, since in this case pronounced hypermobility and hyperthermia was present. Animals treated with reserpine and lithium chloride were sedated, but nevertheless a small but significant increase in protein phosphorylating activity was noted. Further, chlorpromazine, another drug with tranquillizing properties apparently increases protein kinase activity [8]. By contrast, Clark *et al.* [3] found protein kinase activity was decreased in animals sedated with morphine, although a marked increase in activity was observed on withdrawal from the drug when the animals become excited. Clearly there is no pattern in these results suggesting an association between enhanced protein kinase activity and a particular behavioural consequence. More detailed studies using a range of doses and exploring the time course of the changes are obviously needed.

The mechanism(s) involved in these changes of protein kinase activity are unknown. The major factor in the brain modulating the activity of this membrane-bound enzyme is cyclic AMP, but the increases observed in the present work cannot be due to enhanced cyclic nucleotide synthesis. If this were the case no increase would be expected in the

total activity of the enzyme (i.e. the stimulated plus basal components); rather, by analogy with the mechanism by which cyclic AMP stimulates the soluble protein kinase in brain [28] the basal activity should have increased at the expense of the stimulated activity. In fact in all cases the total kinase activity was found to increase, although in the ES experiments over 80% of the increase was confined to the basal component. However, even here measurable increases in cyclic AMP concentrations did not appear to play a part, since sham ES, which raised kinase activity, had no effect on the nucleotide content. In other circumstances it appears that stress may affect cyclic nucleotide concentrations: thus restraining rats by wrapping them firmly in wire mesh for 2 hr such that head, tail and limb movements are minimal but breathing is not restricted, results in a 67% increase in the concentration of cyclic AMP in the septum [6].

The possiblity that the increase in the membrane-bound enzyme activity results from the translocation of a protein phosphorylating system from the cytoplasm to the synaptic membrane has to be considered. For a number of reasons we consider this possibility unlikely. The substrates for the normal membrane bound kinase are intimately associated with the membrane structure and preliminary results (H. Holmes, unpublished) show that their pattern is unchanged after a treatment which enhances kinase activity. Thus if translocation does occur it must be either of a kinase which attaches to a membrane protein, or also involve the migration of a protein substrate common to both the membrane and the cytosol. Moreover, one of the criteria used by Keely [14] to determine translocation was the occurrence of changes in the ratio of protein kinase activity measured with and without cyclic AMP, and such changes were not observed in the present work.

The increase in cyclic AMP content of the brain following ES observed in our experiments confirms other work using mice [17]. However, in the present work the post-ES increase was rather smaller than in the study [17] on mice. This may be due to the fact that in the latter work cerebral cortex rather than whole forebrain was analysed. There was no reason to suspect that our control values for cyclic AMP were significantly influenced by postmortem ischaemia: the figures quoted in Table 2 compare favourably with those of 0.78 ± 0.05 nmol/g reported for rats by Nahorski and Rogers [20] using an ultra rapid freeze blowing technique for killing the animals.

It is known that at least 5 proteins are phosphorylated in synaptic membrane fragments by ATP and the endogenous protein kinase system acting in vitro [7]. A preliminary study in this laboratory has shown that treatment of animals with ES or drugs affects the phosphorylation of only certain of these proteins. For example chronic treatment of rats with high doses of amphetamine or acute ES (sham or actual) to naive rats increased the phosphorylation of a protein of molecular weight 80×10^3. By contrast the phosphorylation of this protein was significantly decreased in rats which had been habituated to handling for 15 days before subjecting them to the ES treatment. These findings are currently under further investigation.

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REFERENCES

1. Albano, J. D. M., G. D. Barnes, D. V. Maudsley, B. L. Brown and R. P. Etkins. Factors affecting the saturation assay of cyclic AMP in biological systems. *Analyt. Biochem.* **60:** 130–141, 1974.
2. Brown, B. L., J. D. B. Albano, R. P. Etkins, A. M. Sgherzi and W. Tampion. A simple and sensitive saturation assay method for the measurement of adenosine 3':5'-cyclic monophosphate. *Biochem. J.* **121:** 561–562, 1971.
3. Clark, A. G., R. Jovic, M. P. Ornellas and M. Weller. Brain microsomal protein in the chronically morphinized rat. *Biochem. Pharmacol.* **21:** 1989–1990, 1972.
4. Costa, E. and A. Groppetti. Biosynthesis and storage of catecholamines in tissues of rats injected with various doses of d-amphetamine. In: *Amphetamines and Related Compounds*, edited by E. Costa and S. Garattini. New York: Raven Press, 1970, pp. 231–255.
5. Daly, J. Role of cyclic nucleotides in the nervous system. In: *Handbook of Psychopharmacology V*, edited by L. L. Iversen, S. D. Iversen and S. H. Snyder. New York: Plenum Press, 1975, pp. 47–130 and 361–374.
6. Delapez, R. L., S. R. Dickman and B. I. Grosser. Effects of stress on rat brain adenosine 3',5'-monophosphate *in vivo*. *Brain Res.* **85:** 171–175, 1975.
7. Dunkley, P. R., H. Holmes and R. Rodnight. Phosphorylation of synaptic membrane proteins from ox cerebral cortex *in vitro*. Partition of substrates and protein kinase activities with Triton X-100. *Biochem. J.* **157:** 661–666, 1976.
8. Ehrlich, Y. H. and E. G. Brunngraber. cAMP-stimulated phosphorylation of proteins after chronic chlorpromazine. *Abstracts of 7th Annual Meeting of the American Society for Neurochemistry*, p. 109, 1976.
9. Essman, W. B. *Neurochemistry of Cerebral Electroshock*. Flushing, New York: Spectrum Publication, Inc., 1973.
10. Fuxe, K. and U. Ungerstedt. Histochemical biochemical and functional studies on central monoamine neurons after acute and chronic amphetamine administration. In: *Amphetamines and Related Compounds*, edited by E. Costa and S. Garattini. New York: Raven Press, 1970, pp. 257–288.
11. Greengard, P. Possible role of cyclic nucleotides and phosphorylated membrane proteins in post-synaptic actions of neurotransmitters. *Nature, Lond.* **260:** 101–108, 1976.
12. Johnson, E. M., H. Maeno and P. Greengard. Phosphorylation of endogenous protein of rat brain by cyclic adenosine 3',5'-monophosphate-dependent protein kinase. *J. biol. Chem.* **246:** 7731–7739, 1971.
13. Jones, D. H. and A. I. Matus. Isolation of synaptic plasma membrane from brain by combined flotation-sedimentation density gradient centrifugation. *Biochim. Biophys. Acta* **356:** 276–287, 1974.
14. Keely, S. L., Jr., J. D. Corbin and C. R. Park. On the question of translocation of heart cAMP-dependent protein kinase. *Proc. Natn. Acad. Sci.* **72:** 1501–1504, 1975.
15. Lagnado, J., L. P. Tan and M. Reddington. The *in situ* phosphorylation of micro-tubular protein in brain cortex slices and related studies on the phosphorylation of isolated brain tubulin preparations. *Ann. N. Y. Acad. Sci.* **253:** 577–597, 1975.
16. Leonard, B. E. and S. A. Shallice. Some neurochemical effects of amphetamine, methylamphetamine and p-bromomethylamphetamine in the rat. *Br. J. Pharmac.* **41:** 198–212, 1971.
17. Lust, W. D., N. D. Goldberg and J. V. Passonneau. Cyclic nucleotides in murine brain: the temporal relationship of changes induced in adenosine 3',5'-monophosphate and guanosine 3',5'-monophosphate following maximal electroshock or decapitation. *J. Neurochem.* **26:** 5–10, 1976.

18. Miller, G. L. Protein determination for large numbers of samples. *Analyt. Chem.* **31**: 964, 1959.
19. Miyamoto, E., J. F. Kuo and P. Greengard. Cyclic nucleotide-dependent protein kinases. III. Purification and properties of AMP-dependent protein kinase from bovine brain. *J. biol. Chem.* **244**: 6395–6402, 1969.
20. Nahorski, S. R. and K. J. Rogers. The adenosine 3′5′-monophosphate content of brain tissue obtained by an ultra-rapid freezing technique. *Brain Res.* **51**: 332–336, 1973.
21. Rodnight, R. Cyclic AMP and protein phosphorylation in the central nervous system in relation to synaptic function. In: *Metabolic Compartmentation and Neurotransmission*, edited by S. Berl, D. D. Clarke and D. Schneider. New York: Plenum Press, 1975, pp. 205–228.
22. Rodnight, R., M. Reddington and M. Gordon. Methods for studying protein phosphorylation in cerebral tissues. *Research Methods in Neurochemistry* **3**: 325–367, 1975.
23. Weller, M. A study of protein kinase and phosphatase enzyme activities in preparations of membrane fragments from brain and other tissue. *Ph D. Thesis*, University of London, London, 1972.
24. Weller, M. and I. G. Morgan. Localisation in synaptic junction of the cyclic AMP stimulated intrinsic protein kinase activity of synaptosomal plasma membranes. *Biochim. Biophys. Acta* **433**: 223–228, 1976.
25. Weller, M. and R. Rodnight. Turnover of protein-bound phosphoserine in membrane preparations from ox brain catalysed by intrinsic kinase and phosphatase activity. *Biochem. J.* **124**: 393–406, 1971.
26. Weller, M. and R. Rodnight. Protein kinase activity in membrane preparations from ox brain. Stimulation of intrinsic activity by adenosine 3′,5′-cyclic monophosphate. *Biochem. J.* **132**: 483–492, 1973.
27. Weller, M., R. Rodnight and D. Carrera. Determination of adenosine 3′,5′-cyclic monophosphate in cerebral tissues by saturation analysis. Assessment of a method using a binding protein from ox muscle. *Biochem. J.* **129**: 113–121, 1972.
28. Witt, J. J. and R. Roskoski, Jr. Bovine brain adenosine 3′,5′-monophosphate dependent protein kinase. Mechanism of regulatory subunit inhibition of the catalytic submit. *Biochemistry* **14**: 4503–4507, 1975.