Protein Phosphorylation in Respiring Slices of Guinea-Pig Cerebral Cortex

EVIDENCE FOR A ROLE FOR NORADRENALINE AND ADENOSINE 3':5'-CYCLIC MONOPHOSPHATE IN THE INCREASED PHOSPHORYLATION OBSERVED ON APPLICATION OF ELECTRICAL PULSES

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1. Exposure of slices of cerebral cortex from guinea pigs to electrical pulses for 10s or to noradrenaline, 5-hydroxytryptamine or histamine increases the rate of phosphorylation of unidentified proteins in the tissue; the increases in protein phosphorylation due to electrical pulses and noradrenaline were non-additive, whereas the increases due to pulses and 5-hydroxytryptamine or histamine were additive. 2. The stimulating effects of electrical pulses and noradrenaline on protein phosphorylation were antagonized by the β -adrenergic blocking agents L-propranolol, dichloroisoprenaline, practolol and ICI 66082, but not by the α -adrenergic blocking agents, phentolamine and phenoxybenzamine. 3. The increase in protein phosphorylation associated with electrical pulses was antagonized by 10 μ M-trifluoperazine and 0.5 μ M-prostaglandin E₁. 4. It is postulated that under the experimental conditions used the action of electrical pulses on protein phosphorylation is mediated by noradrenaline acting through a β -adrenergic receptor mechanism probably involving adenylate cyclase.

It has been suggested by Reddington & Rodnight (1972), and independently by Greengard & McAfee (1972), that the physiological effects of some neurotransmitters in the central nervous system may be mediated by cyclic AMP-dependent protein kinase activity catalysing the phosphorylation of specific proteins in the post-synaptic cell. The main supporting evidence for this idea comes from studies *in vitro* of intact and subcellular preparations of mammalian cerebral cortex, and may be summarized as follows.

(1) The application of electrical pulses to respiring slices of cerebral cortex from the guinea pig for periods up to 10s and under conditions now known to result in the release of neurotransmitters (Katz & Chase, 1970) promotes the net phosphorylation of protein located in a membrane fraction of the tissue (Heald, 1957; Trevor & Rodnight, 1965; Jones & Rodnight, 1971; Williams & Rodnight, 1975). (2) The putative neurotransmitters noradrenaline, 5-hydroxytryptamine and histamine also increase protein phosphorylation in respiring slices (Reddington *et al.*, 1973). (3) Preparations of synaptic-membrane fractions from cerebral cortex are rich in a cyclic AMP-stimulated protein kinase enzyme which mediates the phosphorylation of specific membrane proteins (Weller & Rodnight, 1970, 1971, 1973; Johnson *et al.*, 1971; Ueda *et al.*, 1973). Such

preparations also contain protein phosphatase activity towards labelled membrane protein (Weller & Rodnight, 1971; Maeno & Greengard, 1972).

The involvement of cyclic AMP in these protein-phosphorylation responses is suggested by observations showing that exposure of respiring cerebral-cortex slices to electrical pulses (Kakiuchi *et al.*, 1969; Rall, 1972), noradrenaline, 5-hydroxytryptamine or histamine (Kakiuchi & Rall, 1968*a,b*; Sattin & Rall, 1970; Chasin *et al.*, 1971; Rall, 1972) all increase the steady-state content of cyclic AMP in the tissue.

Taken together these findings have led us to formulate the following working hypothesis to explain the mechanism of the protein-phosphorylation response induced in slice tissue by electrical pulses. An unknown neurotransmitter released from synapses by the stimulus activates adenylate cyclase in the post-synaptic cell to increase the local concentration of cyclic AMP in the region of the post-synaptic membrane; the newly synthesized cyclic AMP stimulates in turn the phosphorylation of membrane protein by activating a contiguous membrane-bound protein kinase, thus modifying some property of the membrane and consequently some aspects of neuronal function. Dephosphorylation of the protein eventually returns the membrane to its original state.

The object of the present work, of which a preliminary account has appeared (Williams & Rodnight,

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1974), was to identify the neurotransmitter involved in the protein-phosphorylation response to electrical pulses and to obtain firmer evidence for the involvement of cyclic AMP in the process.

Methods

Preparation of brain slices

Guinea pigs weighing 200–400 g were stunned, rapidly exsanguinated, and the forebrain was removed and divided into four parts along the sagittal and coronal planes. One superficial slice of neocortex was prepared from each quarter by the method of Jones & Rodnight (1971) by using a recessed glass guide of depth 0.35–0.38 mm. Slices weighed between 20 and 40 mg and were approx. 0.3 mm thick; they were capable of actively extruding Na^+ to the surrounding medium and were found to take up radioactive phosphate uniformly. Slices were removed from the cutting blade, weighed and floated into a bath containing approx. 50 ml of incubation medium at 37°C which had been well gassed with oxygen. The incubation medium contained (final concentrations) 128 mM-NaCl, 6.3 mM-KCl, 0.75 mM- CaCl_2 , 25 mM-Tris/HCl, pH 7.4, and 10 mM-glucose. The slices were positioned in quick-transfer electrodes (McIlwain & Rodnight, 1962b) and placed in an incubation vessel containing 5 ml of oxygenated medium at 37°C. The time elapsing between the death of the animal and placing the last slice in the incubation vessel was 3–4 min. The slices were incubated with continuous oxygenation for 30 min to allow time for the resynthesis of energy-rich phosphates; this was termed the preincubation period and was common to all the experiments described below. The slices were then labelled with [^{32}P]P_i (16 $\mu\text{Ci/slice}$) and generally transferred to 5 ml of fresh medium with two washes in oxygenated medium at 37°C. In a few experiments (Table 5) slices were not transferred to fresh medium, but were treated throughout in the same medium. Reddington *et al.* (1973) found that this procedure is valid provided that extra washes with medium and acid were introduced. At the end of the experiment the slices were released from the holders into approx. 20 ml of ice-cold incubation medium, washed twice with 10 ml of ice-cold 0.3 M- HClO_4 containing 1 mM-EDTA (HClO_4 /EDTA), drained of excess of fluid and homogenized in closely fitting all-glass homogenizers with 2 ml of HClO_4 /EDTA.

Extraction of tissue protein

Tissue protein was extracted from the homogenates as previously described (Reddington *et al.*, 1973) with slight modifications. The homogenates were left in ice for 30 min before centrifuging at 2500g for 15 min at 0°C. The supernatants were poured off and kept

in ice for the determination of acid-soluble phosphates, and the pellets were transferred to polypropylene centrifuge tubes (50 ml) with 10 ml of 10% (w/v) trichloroacetic acid and centrifuged for 5 min at 2500g. The pellets were washed once more with 10% trichloroacetic acid and the lipids extracted with two washes (each 20 ml) of ethanol/diethyl ether (1:1, v/v), with centrifugation for 5 min at 2500g between each wash. The final pellets were carefully drained and excess of fluid was evaporated in a boiling-water bath. The pellet was then assayed for radioactivity in the alkali-labile phosphate fraction as described below.

Measurement of changes in protein phosphorylation

This was done by determining the alkali-labile phosphate in the lipid-free protein residues. The residues were heated with 1 ml of M-NaOH at 100°C in a boiling-water bath for 12 min; the tubes were stoppered at this stage. They were then cooled in ice and 1.6 ml of silicotungstate reagent (McIlwain & Rodnight, 1962a) was added dropwise with shaking. The tubes were left in ice for a further 10 min to complete the precipitation of tissue protein and then centrifuged at 2500g for 15 min. After equilibration to room temperature (18°C) samples (2 ml) were transferred to stoppered tubes and 0.3 ml of ammonium molybdate (5%, w/v) followed by 2.5 ml of 2-methylpropan-1-ol/benzene (1:1, v/v) were added. After vortex mixing for 15 s the tubes were briefly centrifuged in a bench centrifuge and 2 ml of the organic layer was added to 6 ml of 95% (v/v) ethanol in a scintillation vial.

To correct for variation in the uptake of radioactivity by different slices, the procedure adopted by Reddington *et al.* (1973) was used. The total radioactivity in the alkali-labile phosphate fraction was divided by the amount of radioactivity in the acid-soluble phosphate fraction derived from the same quantity of tissue (determined in a sample of the supernatant from the original tissue homogenate). The resulting quotient is termed the 'relative radioactivity of the alkali-labile phosphate'; its validity compared with other procedures for measuring protein phosphorylation was discussed by Rodnight *et al.* (1975).

All measurements of radioactivity were made in a Packard 3003 scintillation spectrometer by using Čerenkov radiation; the efficiency was 45% and sufficient counts were collected to give a standard error of 2.4% (Wang & Willis, 1965).

Statistical treatment of results

All results are given as the mean $\pm$ S.E.M. The standard deviation of the relative radioactivity of the alkali-labile phosphate in any one experimental series was usually less than 10% of the mean.

Significance was calculated by using a two-tailed paired Student 't' test (Bailey, 1959). Control and test values were compared and the test values expressed as a percentage of controls. Normally eight separate observations from four independent experiments were required for satisfactory statistical analysis of the effect of a given drug or chemical, such experiments being carried out on separate days. In some instances, however, four or six observations were found to be adequate.

Experimental treatment of neocortical slices

Slices were stimulated with exponential pulses of peak voltage 15V, frequency 50Hz and time-base 0.4ms, by using equipment described by McIlwain & Rodnight (1962b).

Materials

All reagents were of the highest grade commercially available and were made up in deionized water.

Carrier-free [32 P]P_i was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. L-Noradrenaline bitartrate was from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K., and histamine acid phosphate and 5-hydroxytryptamine (serotonin) creatine sulphate were from British Drug Houses, Poole, Dorset, U.K.

The following were gifts: phenoxybenzamine hydrochloride, phentolamine hydrochloride, dichloroisoprenaline and isoprenaline bitartrate from Dr. J. D. Stephenson, Department of Pharmacology,

Institute of Psychiatry; practolol [4-(2-hydroxy-3-isopropylaminopropoxy)acetanilide] and ICI 66082 [4-(2-hydroxy-3-isopropylaminopropoxy)phenylacetamide] from Dr. A. R. Somerville, Pharmaceuticals Division, I.C.I. Ltd., Macclesfield, Cheshire, U.K.; prostaglandin E₁ from Professor H. Baum, Department of Biochemistry, Chelsea College, University of London; and trifluoperazine from Smith, Kline and French, Welwyn Garden City, Herts., U.K. I.C.I. 66082 and practolol, which were supplied as the base, were dissolved in dilute HCl and the pH was adjusted to 7.4 with 1M-Tris base.

All amine solutions were prepared immediately before use and contained ascorbic acid (0.1mg/ml) to prevent spontaneous oxidation. Ascorbate had no effect on protein phosphorylation at this concentration (M. Williams, unpublished work).

Results

Previous work showed that the increase in protein phosphorylation induced by electrical pulses applied for 10s is additive to that caused by exposure to histamine for 10min (Reddington *et al.*, 1973). This result was confirmed in the present study and found also to apply to 5-hydroxytryptamine (Table 1), suggesting that under the conditions of the experiment the phosphorylation system activated by pulses is distinct from those activated by these two amines. With noradrenaline, however, addition of responses was not observed (Table 1), suggesting that noradrenaline may mediate the response induced by

Table 1. Combined effects of electrical pulses and putative neurotransmitters on protein phosphorylation

Slices were prepared and labelled as described in the Methods section and then transferred to fresh medium containing the amine at the concentration indicated. After incubation for a further 10min the slices were electrically stimulated for 10s (see the Methods section) and then released into ice-cold medium to terminate the experiment. Values quoted are means $\pm$ S.E.M. of 16 observations for noradrenaline and 5-hydroxytryptamine and eight observations for histamine. Relative radioactivity is defined in the Methods section.

Treatment with pulses or amine	10 ³ $\times$ Relative radioactivity of alkali-labile phosphate		
	Noradrenaline (50 μ M)	5-Hydroxytryptamine (1 μ M)	Histamine (100 μ M)
(a) Neither	12.63 $\pm$ 0.27	12.16 $\pm$ 0.22	11.60 $\pm$ 0.33
(b) Pulses only	14.08 $\pm$ 0.35	13.66 $\pm$ 0.32	13.07 $\pm$ 0.18
(c) Amine only	14.59 $\pm$ 0.29	13.14 $\pm$ 0.30	13.13 $\pm$ 0.57
(d) Pulses+amine	14.37 $\pm$ 0.29	14.44 $\pm$ 0.37	14.32 $\pm$ 0.32
Parameters compared	Percentage changes and significance (control = 100)		
	Noradrenaline	5-Hydroxytryptamine	Histamine
(a) versus (b)	112 $\pm$ 2, $P < 0.001$	113 $\pm$ 2, $P < 0.001$	113 $\pm$ 4, $P < 0.02$
(a) versus (c)	117 $\pm$ 3, $P < 0.001$	108 $\pm$ 2, $P < 0.001$	114 $\pm$ 4, $P < 0.02$
(a) versus (d)	115 $\pm$ 3, $P < 0.001$	119 $\pm$ 3, $P < 0.001$	124 $\pm$ 3, $P < 0.001$
(c) versus (d)	103 $\pm$ 3, $P > 0.2$	110 $\pm$ 3, $P < 0.005$	110 $\pm$ 3, $P < 0.005$
(b) versus (d)	99 $\pm$ 3, $P > 0.7$	107 $\pm$ 3, $P < 0.02$	110 $\pm$ 3, $P < 0.05$

Fig. 1. Relation between increase in protein phosphorylation and concentration of noradrenaline

After labelling, slices were transferred to fresh medium containing noradrenaline at the concentration indicated and incubation was continued for 6 min. Results are expressed as percentages of the relative radioactivity of alkali-labile phosphate in control slices incubated for the same time. The control value for the relative radioactivity of the alkali-labile phosphate was 10.53×10^{-3} . Points are means $\pm$ S.E.M. of six or eight observations.

pulses. To investigate this possibility further, the effects of adrenergic blocking agents were investigated.

To establish the optimum conditions for these studies, the time-course of the protein-phosphorylation response to noradrenaline was determined. This showed that with $500 \mu\text{M}$ -noradrenaline a minimum incubation period of 6 min was required for the maximum increase in protein phosphorylation. By using this incubation period the concentration-response relationship was then studied (Fig. 1). The maximum phosphorylation response was given by $10 \mu\text{M}$ -noradrenaline, but owing to the large variance in the results, a concentration of $50 \mu\text{M}$ was used as a routine for subsequent studies.

In the first experiments with blocking agents the effects of isomers of the β -adrenergic antagonist propranolol were examined. The protein-phosphorylation responses to both electrical pulses and $50 \mu\text{M}$ -noradrenaline were effectively blocked by $50 \mu\text{M}$ -DL($\pm$)-propranolol, but interpretation of the result with electrical pulses was complicated by the well-known membrane-stabilizing properties of propranolol (Barrett & Cullum, 1968). It was desirable therefore to examine the effects of the D(+) and L(-) isomers of propranolol separately, since both are equipotent in their membrane-stabilizing properties, whereas in cardiac tissue at least (Barrett & Cullum, 1968) the L(-) isomer is 100 times more effective in blocking β -receptors. The D(+) and L(-) isomers ($50 \mu\text{M}$) completely blocked the increase in protein labelling given by electrical pulses, but only the L(-) isomer antagonized the response to $50 \mu\text{M}$ -noradrenaline. By lowering the concentrations of the

Table 2. Effect of isomers of propranolol on the protein-phosphorylation response induced by electrical pulses

Slices were prepared and labelled as described in the Methods section and then transferred to fresh medium containing propranolol isomers at $10 \mu\text{M}$ concentration. Electrical pulses (for 10 s) were applied 1 min after transfer. Values quoted are means $\pm$ S.E.M. of eight observations.

Treatment with pulses or amine	$10^3 \times$ Relative radioactivity of alkali labile phosphate		
	DL($\pm$) Racemate	D(+) Isomer	L(-) Isomer
(a) Neither	7.58 ± 0.39	7.85 ± 0.19	7.66 ± 0.27
(b) Pulses only	8.75 ± 0.32	9.33 ± 0.56	9.23 ± 0.21
(c) Propranolol only	7.72 ± 0.27	8.53 ± 0.28	8.51 ± 0.25
(d) Pulses and propranolol	8.67 ± 0.44	9.45 ± 0.45	9.03 ± 0.31

Parameters compared	Percentage changes and significance (control = 100)		
	DL($\pm$) Racemate	D(+) Isomer	L(-) Isomer
(a) versus (b)	$116 \pm 3, P < 0.005$	$118 \pm 2, P < 0.02$	$119 \pm 1, P < 0.001$
(a) versus (c)	$103 \pm 1, P > 0.50$	$106 \pm 2, P > 0.50$	$110 \pm 2, P < 0.01$
(a) versus (d)	$115 \pm 2, P < 0.01$	$120 \pm 3, P < 0.02$	$114 \pm 2, P < 0.02$
(c) versus (d)	$112 \pm 1, P < 0.01$	$113 \pm 2, P < 0.01$	$104 \pm 1, P > 0.10$

Table 3. Effect of α - and β -adrenergic blocking agents on the increase in protein phosphorylation induced by electrical pulses and noradrenaline

Slices were prepared and labelled as described in the Methods section and then transferred to fresh medium containing the blocking agent at the concentration indicated. Pulses (for 10s) were applied 10min after transfer; noradrenaline (to a concentration of $50\mu\text{M}$) was added 4min after transfer and incubation continued for 6min. Values quoted are means $\pm$ S.E.M. of eight observations, except for the first line, where the control observations for all agents are combined. The control values for relative radioactivity of the alkali-labile phosphate ranged from 7.84×10^{-3} to 12.63×10^{-3} . The experimental design included controls for the effects of the agents alone, which in no case gave a change greater than $\pm 3\%$ of control. * $P < 0.01$ compared with control slices.

Agent	Stimulation of protein phosphorylation, as percentage of control (=100), by:	
	Pulses	Noradrenaline
None	115 $\pm$ 3*	117 $\pm$ 3*
Phenoxybenzamine ($50\mu\text{M}$)	117 $\pm$ 4*	119 $\pm$ 6*
Phentolamine ($50\mu\text{M}$)	113 $\pm$ 5*	116 $\pm$ 2*
Dichloroisoprenaline ($50\mu\text{M}$)	103 $\pm$ 4	103 $\pm$ 3
Practolol ($5\mu\text{M}$)	101 $\pm$ 2	98 $\pm$ 3
ICI 66082 ($5\mu\text{M}$)	103 $\pm$ 2	101 $\pm$ 2

two isomers to $10\mu\text{M}$, their actions on the pulse-induced response were differentiated; antagonism was observed with the L(-) isomer but not with the D(+) isomer (Table 2). This suggested that at this concentration the effect of the L(-) isomer was due to its ability to antagonize β -receptors rather than to its membrane-stabilizing properties. Nevertheless some doubt remained because L(-) propranolol was found to stimulate protein phosphorylation on its own (Table 2) and if this increased phosphorylation was occurring in different proteins from those associated with the pulse-induced response, it might conceivably be masking the effect of pulses. Further observations with more specific agents were therefore required.

The results of a series of experiments with other adrenergic blockers are summarized in Table 3, where for conciseness percentage changes only are quoted. The protein-phosphorylation responses to both electrical pulses and noradrenaline were blocked by the β -adrenergic antagonists dichloroisoprenaline, ICI 66082 and practolol, but not by the α -antagonists phenoxybenzamine and phentolamine. At the concentrations used none of these compounds affected protein phosphorylation on their own, although at a concentration of $50\mu\text{M}$ both ICI 66082 and practolol, like $10\mu\text{M}$ -L(-)-propranolol, increased incorporation in the absence of pulses or noradrenaline. With

Fig. 2. Relation between increase in protein phosphorylation and concentration of isoprenaline

For details see Fig. 1. The control value for the relative radioactivity of the alkali-labile phosphate was 9.67×10^{-3} .

regard to the specificity of these antagonisms, some local, anaesthetic activity has been described for dichloroisoprenaline (Somani & Lum, 1965), but both ICI 66082 and practolol are considered to be pure β -adrenergic blockers (Barrett *et al.*, 1973; Dunlop & Shanks, 1968).

Since it appeared that a β -adrenergic system was involved in the protein-phosphorylation response to noradrenaline and pulses, the action of the β -agonist isoprenaline was studied (Fig. 2). With this amine the maximum increase in protein phosphorylation was approximately the same as that observed with noradrenaline, but the concentration required for the half-maximal response was considerably lower. From Hill plots (Reuter, 1974) of the data in Figs. 1 and 2 this was found to be $0.3\mu\text{M}$ for isoprenaline, compared with $1.5\mu\text{M}$ for noradrenaline. Isoprenaline is therefore the more potent stimulator of the two and confirms the suggestion that a β -adrenergic receptor is involved.

Unequivocal evidence for the involvement of cyclic AMP in the protein-phosphorylation response in slices has proved difficult to obtain. However, mediation by cyclic AMP is consistent with the observations given in Table 4, which shows that the responses to both pulses and noradrenaline were blocked by prostaglandin E_1 and trifluoperazine.

Both these agents are known to inhibit catecholamine-sensitive adenylate cyclase systems in certain situations, but for reasons discussed below this result cannot be taken as definitive evidence.

The protein-phosphorylation responses to electrical pulses and noradrenaline were also alike in that neither was inhibited by adenosine (Table 5), a substance shown by Reddington *et al.* (1973) to inhibit completely the analogous response to histamine. The effect on histamine-induced phosphorylation was ascribed in the previous work to the ability of adenosine to inhibit cyclic AMP-stimulated protein kinase activity in membrane fragments (Weller & Rodnight, 1973), but it is clear from Table 5 that the phosphorylation systems in the intact tissue responsive to the other agents are insensitive to inhibition by externally applied adenosine.

Discussion

Involvement of noradrenaline

Under the experimental conditions used in this study the protein-phosphorylation response to electrical pulses appears to be mediated by nor-

adrenaline acting on a β -adrenergic receptor. This conclusion accords well with the results of other experiments in which the increases in protein phosphorylation resulting from exposure of slices to both electrical pulses and noradrenaline were located in a neuronal cell-body fraction prepared from the incubated tissue, whereas the phosphorylation responses to 5-hydroxytryptamine and histamine occurred in a cell fraction consisting of neuropil, glia and unidentified products of the fractionation procedure (Williams *et al.*, 1974a,b).

The marked difference in the time-courses of the effects of electrical pulses and noradrenaline on protein phosphorylation requires comment; it will be recalled that the effect of pulses is maximal within 2 s of application (Williams & Rodnight, 1975), whereas the maximum effect of noradrenaline is not seen until 6 min after exposure to the amine. The discrepancy is probably related partly to the time required for exogenously applied amine to diffuse to its site of action in sufficient concentration to activate adenylate cyclase and partly to the cellular uptake of noradrenaline by neuronal and possibly glial structures. With regard to the former factor it is relevant to note that Schultz & Daly (1973a) found that the half-life of the diffusion of [3 H]sorbitol into cortical slices of roughly the same dimensions as those used in the present study was approx. 3 min. The relative rapidity of the phosphorylation response to electrical pulses is presumably due to the release of endogenous noradrenaline proximal to its site of action.

Although under the particular conditions of our experiments it seems clear that the increase in protein phosphorylation induced by electrical pulses is mediated by noradrenaline, this does not exclude the possibility that under different conditions a protein-phosphorylation response to endogenously released 5-hydroxytryptamine or histamine may prove demonstrable. Applied exogenously, both these amines increase the rate of protein phosphorylation in cortex slices (Reddington *et al.*, 1973) and in rat neural tissue evidence for a receptor for adenylate cyclase activated

Table 4. *Effect of trifluoperazine and prostaglandin E₁ on the increase in protein phosphorylation induced by electrical pulses and noradrenaline*

Experimental details are as in Table 3. The control values for relative radioactivity of the alkali-labile phosphate ranged from 8.55×10^{-3} to 10.6×10^{-3} . * $P < 0.01$ compared with control.

Agent	Stimulation of protein phosphorylation as percentage of control (=100), by:	
	Pulses	Noradrenaline
None	115 $\pm$ 3*	115 $\pm$ 2*
Trifluoperazine (10 μ M)	98 $\pm$ 4	101 $\pm$ 4
Prostaglandin E ₁ (0.5 μ M)	102 $\pm$ 3	104 $\pm$ 2

Table 5. *Effect of adenosine on the protein-phosphorylation response to electrical pulses and putative neurotransmitters*

Slices were prepared and labelled as described in the Methods section. In the experiment using amines, the slices were transferred after labelling to fresh medium containing amine with or without adenosine. In experiments with pulses the slices were not transferred to fresh medium: adenosine was added after the 30 min preincubation period; [32 P]P_i was added 7 min later and pulses were applied for 10 s after a 3 min labelling period. The control values for relative radioactivity of the alkali-labile phosphate ranged from 9.15×10^{-3} to 12.32×10^{-3} . Values quoted are means $\pm$ S.E.M. for eight observations. * $P < 0.02$ compared with control (=100); † $P < 0.02$ compared with value for histamine alone.

Addition	Stimulation of protein phosphorylation, as percentage of control (=100), by:			
	Pulses	Histamine (100 μ M)	Serotonin (1 μ M)	Noradrenaline (500 μ M)
None	111 $\pm$ 3*	119 $\pm$ 5*	110 $\pm$ 4*	119 $\pm$ 4*
Adenosine (50 μ M)	112 $\pm$ 4*	91 $\pm$ 7†	111 $\pm$ 3*	119 $\pm$ 6*

by 5-hydroxytryptamine has been reported (Nathan-son & Greengard, 1974).

Role of cyclic AMP

This rests largely on the inhibitory effect of trifluoperazine and a low concentration of prostaglandin E_1 . However, although trifluoperazine inhibits the catecholamine-stimulated formation of cyclic AMP in preparations from mammalian brain (Uzonov & Weiss, 1971; Miller *et al.*, 1974) and prostaglandin E_1 has a similar blocking effect on the adenylate cyclase system in isolated fat-cells (Butcher & Baird, 1968), both these compounds exhibit non-specific effects on cell membranes. Thus phenothiazines are potent membrane stabilizers (Seeman, 1972) and prostaglandins have a variety of non-specific effects on membrane-bound enzymes (Johnson & Ramwell, 1973). Further, a direct inhibitory effect of prostaglandin E_1 on noradrenaline-sensitive adenylate cyclase in neural tissue does not appear to have been demonstrated. The justification for its use in the present work rests on its ability to block in the mammalian nervous-system processes that are believed to be cyclic AMP-dependent: the dopamine-dependent hyperpolarization of post-ganglionic neurons in the superior cervical ganglion of the rabbit (McAfee & Greengard, 1972), the noradrenaline-dependent inhibition of the spontaneous discharge rate of Purkinje cells in the rat cerebellum (Siggins *et al.*, 1971) and pyramidal cells in the rat hippocampus (Segal & Bloom, 1974), and the dopamine-dependent inhibition of prolactin release in ovariectomized oestrogen-treated rats (Ojeda *et al.*, 1974).

Two facts tend to argue against mediation by cyclic AMP: the lack of any effect of adenosine on protein phosphorylation in slices (Reddington *et al.*, 1973) and the involvement of a β -adrenergic receptor. Adenosine is generally considered to be the main endogenous mediator of the increase in cyclic AMP induced by electrical pulses applied to guinea-pig cortical slices (Sattin & Rall, 1970; Zanella & Rall, 1973; Rall, 1975) and the greater part of the cyclic AMP produced in response to adrenergic agents in this tissue appears to be mediated by α -adrenergic receptors (Chasin *et al.*, 1971; Schultz & Daly, 1973b). With regard to the negative results obtained with adenosine, Daly *et al.* (1972) showed that this compound increases the cyclic AMP content of cortical slices through an adenylate cyclase system distinct from systems responsive to other putative transmitters; therefore the adenosine-sensitive system may have a physiological function which does not involve detectable protein phosphorylation under the conditions of our experiments. The β -nature of the response also does not rule out mediation by cyclic AMP. A small component of the increase in cyclic AMP in guinea-pig neocortical slices by combinations

of adenosine and noradrenaline is apparently mediated by a β -receptor (Schultz & Daly, 1973b), and more recent work (Sattin *et al.*, 1975) has demonstrated that noradrenaline alone is capable of stimulating adenylate cyclase in this system, despite earlier reports to the contrary (Schultz & Daly, 1973a,b). It may also be relevant that in Purkinje cells of the rat cerebellum (Hoffer *et al.*, 1972; Bloom *et al.*, 1973) and in pyramidal cells of the rat hippocampus (Segal & Bloom, 1974) the hyperpolarization evoked by noradrenaline is inhibited by the β -adrenergic blocking agent Sotolol (MJ 1999). Further, in turkey erythrocytes, Rudolph & Greengard (1974) have shown that a β -adrenergic adenylate cyclase system apparently regulates the phosphorylation state of a membrane protein.

Location of the protein phosphorylation responses

Trevor & Rodnight (1965) found that the phosphorylation response to electrical pulses occurred in a membrane fraction of the tissue, and Williams *et al.* (1974a,b) showed that the responses to both pulses and noradrenaline were confined to a neuronal cell-body fraction of the tissue. The subcellular localization of the response to noradrenaline has not been determined. However, a location in synaptic structures seems likely and the crucial question is whether this is situated in the pre-synaptic structures or in the post-synaptic membrane. This may be difficult to establish by direct observation, but it is relevant to note that, in the rat at least, noradrenaline-sensitive adenylate cyclase in the neocortex appears to be located exclusively on post-synaptic sites. Thus treatment of rats with 6-hydroxydopamine or reserpine does not affect the responsiveness of noradrenaline-sensitive adenylate cyclase (Palmer, 1972; Huang *et al.*, 1973) and leads to a selective increase in the response of the β -adrenergic component of the adenylate cyclase system (Dismukes & Daly, 1974).

It is not known whether the acceptor protein or proteins phosphorylated on exposure of the tissue to electrical pulses or noradrenaline are enzymic or structural in nature. Phosphorylation of a membrane-bound enzyme involved in the turnover of some other membrane components such as lipids is one possibility; there is, for example, evidence (Gullis & Rowe, 1975a,b) that the activity of the phospholipase A2-acylation system of synaptic membranes is controlled by cyclic phosphorylation and dephosphorylation mediated by neurotransmitters and cyclic AMP. Of the structural proteins present in the central nervous system, tubulin is known to be phosphorylated by a cyclic AMP-dependent mechanism (Lagnado *et al.*, 1975; Sloboda *et al.*, 1975). Further, there are reports of the occurrence of a tubulin-like protein in the synaptic complex (Banker *et al.*, 1974; Walters & Matus, 1975) which is appar-

ently phosphorylated by intrinsic cyclic AMP-stimulated protein kinase activity (Lagnado *et al.*, 1975). Tubulin-like protein prepared from unfractionated tissue is also phosphorylated during incubation of respiring slices of guinea-pig cerebral cortex with [32 P]P_i (Reddington & Lagnado, 1973).

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