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Turnover of Protein-Bound Serine Phosphate in Respiring Slices of Guinea-Pig Cerebral Cortex

EFFECTS OF PUTATIVE TRANSMITTERS, TETRODOTOXIN AND OTHER AGENTS

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1. The effect of various agents on the turnover of protein-bound phosphorus in respiring slices of cerebral cortex was studied. 2. Confirming previous work turnover was increased by the application of electrical pulses for 10s to the tissue. 3. Turnover was also increased by exposure of the slices for 10min to noradrenaline (0.5mM), 5-hydroxytryptamine (1 μM) and histamine (0.1mM). 4. When slices were stimulated by electrical pulses in the presence of histamine the increase in turnover was the sum of the responses given by each agent above, suggesting that different phosphorylating systems were involved. 5. Tetrodotoxin (0.5 μM) blocked the increased turnover due to electrical pulses, but not that due to histamine. Tetrodotoxin also prevented the increase in tissue cyclic AMP content caused by the application of electrical pulses. 6. Phosphoprotein turnover was not affected by adenosine, despite the increase in tissue cyclic AMP content given by this agent. 7. Adenosine blocked the phosphoprotein response to histamine, but did not affect the response to electrical pulses. 8. The results are discussed in relation to the hypothesis that the stimulation of protein phosphorus turnover by electrical pulses is secondary to the release of cyclic AMP in the tissue.

The brief application of electrical pulses to respiring slices of guinea-pig cerebral cortex increases the rate of incorporation of [^{32}P]P_i into protein-bound phosphoserine (Heald, 1957; Trevor & Rodnight, 1965; Jones & Rodnight, 1971). Studies on the subcellular distribution of the serine residues involved indicate that the increased turnover occurs in membrane-bound proteins (Trevor & Rodnight, 1965). Two later observations have led us to suggest that this particular response to electrical pulses may be mediated through the action of cyclic AMP (Weller & Rodnight, 1970). First, electrical pulses applied for longer periods to brain slices maintained under similar conditions increase the cyclic AMP content of the tissue (Kakiuchi *et al.*, 1969). Secondly, membrane fragments prepared from ox cerebral cortex (Weller & Rodnight, 1970, 1971, 1973; Rodnight & Weller, 1971) or rat cerebrum (Johnson *et al.*, 1971) contain a tightly bound protein kinase that catalyses the phosphorylation of intrinsic membrane protein and is stimulated by cyclic AMP.

Apart from electrical excitation, a number of other agents increase the formation of cyclic AMP or its content in respiring brain slices. These include the putative transmitters noradrenaline, 5-hydroxytryptamine and histamine (Kakiuchi & Rall, 1968*a*,*b*; Kakiuchi *et al.*, 1969; Chasin *et al.*, 1971), adenine ribose compounds (Sattin & Rall, 1970) and certain depolarizing agents (Shimizu *et al.*, 1970). Further excitation of slices with electrical pulses results in a release of neurohumoural agents from the tissue including noradrenaline, 5-hydroxytryptamine (Katz & Chase, 1970) and adenosine (McIlwain, 1971), any of which may act as intermediates in the effect of electrical pulses on cyclic AMP content. We therefore considered it of interest to examine the effects of certain of these substances on protein P turnover in slices maintained under conditions similar to those used in our previous studies on the response to electrical stimuli. A preliminary report of this work has appeared (Reddington & Rodnight, 1972).

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Experimental

Materials

All reagents were of the greatest purity available. Noradrenaline (bitartrate), histamine (dihydrochloride) and 5-hydroxytryptamine (creatinine sulphate) were obtained from British Drug Houses Ltd., Poole, Dorset, U.K. Histamine (acid phosphate) was obtained from Burroughs Wellcome Ltd., Beckenham, Kent, U.K.; both salts of histamine gave the same results. Histamine and noradrenaline were dissolved in 50mM-HCl; ascorbic acid (0.1mg/ml) was incorporated in the noradrenaline solutions. All amine solutions were made up freshly immediately

before use. Tetrodotoxin was obtained from Sigma (London) Chemical Co., London S.W.6, U.K. Carrier-free [^{32}P]P_i was obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

Methods

Preparation and incubation of slices. The procedure for preparing slices was that described by Jones & Rodnight (1971) with a blade and guide. Only superficial slices were used. The slices were weighed immediately after cutting and floated in 50ml of oxygenated incubation medium at 37°C. They were then mounted in the transfer-electrode holders described by McIlwain & Rodnight (1962). The incubation medium contained (final concns.): 128mM-NaCl; 6.3mM-KCl; 0.75 or 2.8mM-$CaCl_2$; 1.3mM-$MgSO_4$; 25mM-Tris–HCl buffer, pH7.4; 10mM-glucose. The $CaCl_2$ concentration used is indicated in the Tables; the protein P response to electrical pulses and to added histamine was independent of Ca^{2+} concentration within this range (M. Reddington & M. Williams, unpublished work). After the usual preincubation period of 30min, the slices were labelled with [^{32}P]P_i (16μCi/slice) for 3min and transferred to fresh medium as described by Trevor & Rodnight (1965) and Jones & Rodnight (1971). To terminate the experiment the slices were released from their holders into 20ml of ice-cold medium, transferred with a rider to 20ml of ice-cold 0.3M-$HClO_4$ containing 1mM-EDTA and then to a glass homogenizer tube containing 2ml of the same $HClO_4$–EDTA mixture. The tissue was thoroughly homogenized by hand with a closely fitting glass pestle.

In some experiments designed to examine the combined effects of added substances and electrical pulses on protein P turnover transfer to the second medium was omitted. The substances were added to the medium after preincubation for 30min and before labelling with ^{32}P. Because the slices on removal from the medium were more heavily contaminated with ^{32}P they were given one extra rinse in 20ml of ice-cold medium and one extra rinse in 20ml of $HClO_4$–EDTA mixture before homogenization.

Electrical stimulation. In all experiments involving electrical stimulation the pulses were exponential with peak voltage 15V, frequency 50Hz and timebase 0.4ms.

Determination of cyclic AMP. The method of Weller *et al.* (1972) was used, except that the tissue was extracted with cold 0.1M-HCl instead of HCl at 100°C.

Extraction of tissue. The homogenates were left in ice for 1h before centrifuging at 2500*g* for 30min at 0°C. The supernatants were poured off and kept in ice for the determination of acid-soluble phosphates. The pellets were transferred to 50ml polypropylene tubes with 10ml of 10% trichloroacetic acid and centrifuged at 2500*g* for 5min at 0°C. After a further wash with 10ml of 10% trichloroacetic acid, the lipids were extracted with 20ml, then with 10ml of ethanol–diethyl ether (1:1, v/v) and spun as above. The resulting pellet was drained before analysis for radioactivity in the alkali-labile P as described below.

Determination of radioactivity in the alkali-labile P. Phosphoserine P was released from the extracted tissue as P_i by incubation with 1ml of 1M-NaOH for 18h at 37°C. The hydrolysates were cooled in ice and 1.6ml of 1.33M-$HClO_4$ was added, followed by 0.6ml of silicotungstate reagent (McIlwain & Rodnight, 1962), added drop by drop with shaking. After standing in ice for 10min the precipitated protein was centrifuged at 2500*g* for 10min. The supernatants were poured into test tubes and 2ml was taken for determination of the radioactivity of the P_i released by conversion into phosphomolybdate and extraction into organic solvent (see below).

Determination of radioactivity in total acid-soluble fraction. A sample (0.25ml) of the supernatant from the original tissue homogenate was mixed with 6ml of 95% ethanol in a glass scintillation vial and Čerenkov radiation was measured in a scintillation spectrometer (Packard model 3003).

Determination of the specific radioactivity of the γ-P of ATP. The remainder of the acid-soluble fraction was fractionated on deactivated charcoal as described by Threlfall (1957) except that the charcoal was supported on a Millipore filter (0.45UP, 2.5cm diam.) without the admixture of Celite. The pyridine eluate was freeze-dried and the γ-P of ATP released by a Na^+,K^+-activated adenosine triphosphatase preparation made from a microsomal or synaptosomal membrane fraction from ox brain (Pumphrey, 1969) as follows. The residue was dissolved in 2.5ml of a medium containing 100mM-NaCl, 5mM-KCl, 0.01mM-$MgCl_2$ and 30mM-Tris–HCl buffer (pH7.6). A sample (2ml) was taken and incubated at 37°C with 0.5mg of membrane protein for 1min. The reaction was stopped by adding 0.1ml of 12M-$HClO_4$ and 0.2ml of silicotungstate reagent. After being left in ice for 10min the specific radioactivity of the P_i released was measured as described below.

Determination of P_i. The procedure used was a modification of the method of Martin & Doty (1949). The ice-cold samples (2ml) were left at room temperature for 10min before adding 2.5ml of 2-methylpropan-1-ol–benzene (1:1, v/v). After addition of molybdate, mixing and centrifuging, 2ml of the organic layer was taken. If total radioactivity was required as in the determination of the relative radioactivity of the alkali-labile P, then 2ml of organic layer was mixed with 6ml of 95% ethanol in a scintillation vial. If the specific radioactivities were being determined the organic layer was added to

1ml of 3% (v/v) H_2SO_4 in ethanol followed by 0.3ml of $SnCl_2$. After being left at room temperature for 15min the extinction was read at 725nm with a 2cm light-path. A sample of the solution was then mixed with 6ml of 95% ethanol and radioactivity determined as described above.

Expression of results. Considerable variation was observed in the uptake of ^{32}P by the slices. To correct for this the total radioactivity of the alkali-labile P and the ATP (γ-P) specific radioactivity were divided by the total acid-soluble radioactivity in the slice. Thus the relative radioactivity of the alkali-labile P = c.p.m. in alkali-labile P/c.p.m. in acid-soluble P and the relative specific radioactivity of the ATP (γ-P) = c.p.m. per μmol of ATP (γ-P)/c.p.m. in acid-soluble P.

Statistical treatment of results. All results are given as means ± S.E.M. The standard deviation in any one experiment (four control and four test samples) was less than 10% of the mean.

Significance levels of the effects of various agents were calculated by using a paired Student's *t* test. Control and treated samples were taken from equivalent slices cut from opposite hemispheres and the test values expressed as a percentage of the controls. The significance of the mean per cent difference between control and test samples was calculated.

Results

Electrical pulses and protein P turnover

A variety of methods have been used to study factors affecting the turnover of protein P in respiring slices. Heald (1957) described the effects of electrical pulses on the specific radioactivity of the alkali-labile P related to the specific radioactivity of P in the preliminary incubation medium. Trevor & Rodnight (1965) measured the specific radioactivity of protein-bound phosphoserine P isolated by chromatography on Dowex 50 resin, but the phosphoserine obtained by this method is contaminated with unlabelled phosphates (Jones & Rodnight, 1971), the concentrations of which decrease on electrical stimulation giving an anomalously high increase in phosphoserine P specific radioactivity. Jones & Rodnight (1971) described a more-specific method for determining phosphoserine P radioactivity by using high-voltage electrophoresis, but in practice this proved too time-consuming to enable sufficient results to be gathered for satisfactory statistical analysis. When evidence was obtained (C. N. Cook & R. Rodnight, unpublished work; Rodnight, 1971) that the alkali-labile P of lipid-free protein residues of cerebral tissue was approximately equivalent to phosphoserine P, we decided to develop the present procedure. This was based on determination of the total radioactivity rather than specific radioactivity because (*a*) experience showed that determination of the latter introduced an unacceptable degree of error into the results and (*b*) previous work (Heald, 1957; M. Reddington, unpublished work) showed that no detectable change occurred in the concentration of alkali-labile P in the tissue as a result of stimulation. A comparison of the effects of electrical pulses on protein P labelling by using the various methods has already been published (Rodnight & Weller, 1971); results obtained with the present method were comparable with those reported previously. Typical results are given in Table 1.

To check on any gross changes in precursor radioactivity the relative specific radioactivity of ATP (γ-P) was determined in a few cases. The value of this determination is questionable since what is obtained is an average value for the radioactivities of the various ATP pools in the tissue, whereas what is required is the radioactivity of the ATP pool that phosphorylates the membrane protein that responds to stimulation. Nevertheless it was thought that if an

Table 1. *Effect of electrical pulses on the relative radioactivity of alkali-labile phosphate*

Slices of cerebral cortex were incubated for 30min, labelled with ^{32}P for 3min and then transferred to fresh medium for 2min before stimulation for 10s. Further details are given in the Experimental section. In Experiment 3 the $CaCl_2$ concentration in the medium was 0.75mM. Results quoted are means ± S.E.M. with the numbers of observations in parentheses in the last column; relative radioactivity is defined in the text. Significance was determined by paired *t* tests.

	$10^3 \times$ Relative radioactivity of alkali-labile P			
Experiment	Without electrical pulses	With electrical pulses	% change	Significance
1	10.8 ± 0.60	13.0 ± 0.80	+20	$P<0.01$ (16)
2	10.35 ± 0.64	11.48 ± 0.80	+11	$P<0.01$ (8)
3	8.30 ± 0.50	10.20 ± 0.60	+22	$P<0.01$ (20)

Table 2. *Effect of high K^+ concentration on the radioactivity of alkali-labile phosphate and ATP*

After incubation for 30min and labelling for 3min with ^{32}P slices were transferred to fresh medium containing normal (6.3mM) or high (56.3mM) concentrations of KCl. Incubation was continued for 2min. Further details are given in Table 1. Results quoted are means±S.E.M. with the numbers of observations in parentheses in the last column.

$10^3 \times$ Relative radioactivity of alkali-labile P			
Control	Test	% change	Significance
11.97±0.66	9.61±0.28	−19	$P<0.01$ (8)
Relative specific radioactivity of ATP			
Control	Test		
1.24±0.16	0.71±0.08	−43	$P<0.025$ (8)

Table 3. *Effect of amines on relative radioactivity of alkali-labile phosphate*

After incubation for 30min and labelling with ^{32}P for 3min slices were transferred to fresh medium. Incubation was continued for 10min in the presence or in the absence of the amines at the concentrations shown. Results are means±S.E.M. with the numbers of observations in parentheses in the last column.

	$10^3 \times$ Relative radioactivity of alkali-labile P			
Agent	Control	Test	% change	Significance
Noradrenaline (0.5mM)	16.30±0.22	18.04±0.28	+11	$P<0.01$ (16)
5-Hydroxytryptamine (1 μM)	15.26±0.26	16.69±0.28	+10	$P<0.01$ (8)
Histamine (0.1mM)	15.27±0.54	17.60±1.22	+15	$P<0.01$ (8)

Table 4. *Combined effects of electrical pulses and histamine on the relative radioactivity of alkali-labile phosphate*

In this experiment slices were not transferred to fresh medium. Histamine (0.1mM) was added to the medium after incubation for 30min and ^{32}P after 37min. Where stated electrical pulses were applied for 10s at 40min. Results quoted are means±S.E.M. with the numbers of observations in parentheses in the last column.

	$10^3 \times$ Relative radioactivity of alkali-labile P			
Agent	Control	Test	% change	Significance
A Electrical pulses	7.89±0.51	9.33±0.47	+19	$P<0.01$ (8)
B Histamine	6.79±0.51	7.60±0.27	+12	$P<0.05$ (8)
C Electrical pulses+histamine	6.84±0.25	8.97±0.37	+31	$P<0.01$ (8) A versus C $P<0.05$ B versus C $P<0.05$

increase in the average ATP (γ-P) specific radioactivity of the slice on stimulation could be excluded the results could, with reservation, be more readily interpreted in terms of change in protein P turnover.

In several experiments the specific radioactivity of ATP (γ-P) either declined or did not change on stimulation for 10s, confirming the observations of Trevor (1963) with a less specific method.

Table 5. *Effect of tetrodotoxin on the alkali-labile phosphate response to electrical pulses or histamine*

The experimental design was the same as in Tables 1 and 3. After preincubation and labelling slices were transferred to medium containing as indicated, 0.1 mM-histamine and/or 0.5 μM-tetrodotoxin. In experiments A and B the time of exposure was 10 min. In experiments C and D electrical pulses were applied for 10s after incubation in fresh medium for 2 min. Results are means ± S.E.M. with the numbers of observations in parentheses in the last column.

Agent used in test	10^3 × Relative radioactivity of alkali-labile P: Control	Test	% change	Significance
A Histamine	13.39±0.77	15.87±0.62	+19	$P<0.05$ (8)
B Histamine+tetrodotoxin	12.71±0.68	15.18±0.42	+19	$P<0.01$ (8)
C Electrical pulses	10.32±0.64	11.87±0.88	+15	$P<0.01$ (8)
D Electrical pulses+tetrodotoxin	10.05±0.84	10.38±1.12	+3	$P=0.20$ (7)
				A versus B $P=0.30$
				C versus D $P<0.05$

Table 6. *Inhibition by tetrodotoxin of the increase in cyclic AMP content of slices given by electrical stimulation*

After preincubation of the slices for 30min in media with 0.75mM-$CaCl_2$, tetrodotoxin (to a final concn. of 0.5 μM) was added where indicated and the slices were stimulated for 10min with electrical pulses. Each slices was then homogenized in 2ml of 0.1M-HCl and cyclic AMP determined on the extract. Results are given with the standard error of the determination (see Weller *et al.*, 1972).

Treatment	Tissue cyclic AMP content (pmol/mg of tissue)
(None, control)	4.4±1.6
Electrical pulses	18.0±4.0
Electrical pulses+tetrodotoxin	3.6±2.1

High K^+ concentration and protein P turnover

Incubation of respiring slices of cerebral cortex in media containing high K^+ concentrations increases the cyclic AMP content of the tissue (Sattin & Rall, 1967; Shimizu *et al.*, 1970). In the present work incubation of slices for 2min with 56.3mM-KCl decreased the labelling of both protein P and ATP (γ-P) relative to the normal control medium (Table 2). If it is assumed that the changes in the average specific radioactivity of the ATP in the slices reflect a similar change in the precursor pool for protein phosphorylation, the actual change in alkali-labile P could have been an increase of the order of 40%. At any rate, in view of the large decrease in average ATP radioactivity, it seems unlikely that the turnover of the precursor pool increased. The results may therefore be taken as suggestive of some increase in protein P turnover as a result of exposure to high K^+ concentrations.

Biogenic amines and protein P turnover

Kakiuchi & Rall (1968*b*) found that exposure of rabbit cerebral cortex slices to 0.1mM-noradrenaline or histamine for 10min resulted in increases in the cyclic AMP content of the tissue of 77% and 1600% respectively; similar results were obtained by Kakiuchi *et al.* (1969) in cortex slices from the guinea pig except that the response of histamine was rather less. In the present work exposure of guinea-pig cerebral-cortex slices to the amines resulted in significant increases in the relative radioactivity of the alkali-labile P (Table 3). Also, electrical pulses applied during exposure to histamine resulted in an increase in the alkali-labile P labelling that was approximately double that observed with either electrical pulses or histamines alone (Table 4). Kakiuchi *et al.* (1969) found that the effect of combined application of electrical pulses and histamine on the cyclic AMP content of the slices was synergistic rather than additive.

No changes were observed in any of these experiments in the relative specific radioactivity of ATP in the tissue.

A low concentration (0.5 μM) of tetrodotoxin had no effect on the increased protein P labelling given by histamine, but it completely blocked the same response to electrical pulses (Table 5). Tetrodotoxin also prevented the increase in cyclic AMP content of the tissue after electrical stimulation for 10min (Table 6).

Adenosine and protein P turnover

Adenosine and adenine nucleotides are another group of compounds that increase the cyclic AMP

Table 7. *Effect of adenosine and 5'-AMP on the relative radioactivity of alkali-labile phosphate*

The experimental design was the same as in Table 3 except the slices were exposed to the agents for the times stated in column 2. Results quoted are means±S.E.M. with the number of observations in parentheses in the last column.

		$10^3 \times$Relative radioactivity of alkali-labile P			
Agent	Time (min)	Control	Test	% change	Significance
Adenosine (125 μM)	5	14.32±0.56	14.02±0.44	−2	$P = 0.15$ (8)
5'-AMP (125 μM)	6	12.57±0.73	12.95±0.48	+4	$P = 0.15$ (8)

Table 8. *Effect of adenosine on the relative radioactivity of the alkali-labile phosphate in relation to cyclic AMP content*

The experimental design was the same as in Table 3 except that the slices were exposed to adenosine for 5 min and the Ca^{2+} concentration was 0.75 mM. To enable cyclic AMP to be determined at the end of the experiment the slices were first homogenized in 2 ml of cold 0.1 M-HCl for 1 min, the homogenate was centrifuged at 2500*g* for 10 min and 1 ml of the supernatant was removed for cyclic AMP determination. To the remainder of the supernatant 2 ml of 0.3 M-$HClO_4$ containing 1 mM-EDTA was added, then the residue was homogenized again and analysed for alkali-labile P as described in the Experimental section. Results are means±S.E.M. with the numbers of observations in parentheses.

	$10^3 \times$Relative radioactivity of alkali-labile P				Tissue cyclic AMP content (pmol/mg)	
Agent	Control	Test	% change	Significance	Control	Test
Adenosine (10 μM)	5.55±0.18	5.09±0.79	−8	$P = 0.90$ (7)	2.7±0.35	6.4±0.5
Adenosine (50 μM)	5.23±0.28	5.07±0.48	−3	$P = 0.75$ (8)	2.1±0.4	14.8±1.8

Table 9. *Effect of adenosine on changes in the relative radioactivity of the alkali-labile phosphate in response to electrical pulses or histamine*

The effect of adenosine on the response to histamine was investigated by transferring the slices after preincubation and labelling with ^{32}P to fresh medium containing histamine (0.1 mM) or histamine (0.1 mM)+adenosine at the concentration indicated. In experiments in which electrical pulses were applied there was no transfer to fresh medium: adenosine was added after incubation for 30 min and ^{32}P after 37 min with stimulation for 10 s at 40 min. In experiments C and F the Ca^{2+} concentration in the medium was 0.75 mM. Results are means±S.E.M. with the numbers of observations in parentheses in the last column.

	$10^3 \times$Relative radioactivity of alkali-labile P			
Agent	Control	Test	% change	Significance
A Histamine	15.27±0.54	17.60±1.22	+15	$P<0.01$ (8)
B Histamine+adenosine (125 μM)	16.43±0.57	16.91±0.56	+3	$P>0.1$ (8)
C Histamine+adenosine (10 μM)	9.13±0.32	8.35±0.92	−9	$P>0.1$ (8)
D Electrical pulses	7.89±0.51	9.33±0.47	+19	$P<0.01$ (8)
E Electrical pulses+adenosine (125 μM)	6.61±0.40	7.59±0.58	+15	$P<0.025$ (8)
F Electrical pulses+adenosine (10 μM)	4.25±0.39	4.90±0.43	+16	$P<0.05$ (8)
				A versus B and C $P<0.01$
				D versus E and F $P = 0.20$

content of respiring slices of cerebral cortex (Sattin & Rall, 1970). In the latter study the maximum effect of adenosine, amounting to a 30-fold increase in cyclic AMP content, was obtained by exposure to a concentration of about 0.125mM for 5min; 10μM-adenosine gave a 10-fold increase. Surprisingly, over this concentration range adenosine had no effect on the relative radioactivity of the alkali-labile P, even though in the same experiments it increased the tissue cyclic AMP content (Tables 7 and 8). 5'-AMP, which also increases the tissue cyclic AMP, was also without effect on protein P labelling (Table 7). Neither compound changed the relative specific radioactivity of the γ-P of the tissue ATP.

In view of these results, and of the known effect of adenosine in inhibiting intrinsic protein kinase activity in membrane fragments (Weller & Rodnight, 1973) we decided to examine whether adenosine would block the action of histamine or electrical pulses on protein P labelling in the slices. Table 9 shows that 0.125mM-adenosine did indeed inhibit the response to histamine, but not to electrical pulses. In other experiments lower concentrations of adenosine also had no effect on the response to electrical pulses.

Discussion

Inhibition by tetrodotoxin of the protein P response to electrical pulses is convincing confirmatory evidence that the phosphoprotein concerned is involved in some aspect of neuronal activity. Tetrodotoxin is well known to block impulse conduction by preventing the influx of Na^+ that accompanies depolarization of the neuronal membrane (Kao, 1966; McIlwain *et al.*, 1969). In blocking protein P turnover during excitation the drug is probably acting remotely from the site of the response, assuming that this has a location in or close to synapses. [We now discount a previous suggestion (Rodnight & Weller, 1971) that the response may be involved in conduction since we have been unable to find protein kinase activity in fragments of the axolemma (Weller & Rodnight, 1973).] A remote action is concluded, since tetrodotoxin is without effect on transmitter release (Bloedel *et al.*, 1966) or on spontaneous or evoked activity in the neuromuscular end plate (Furukawa *et al.*, 1959; Katz & Miledi, 1967) or on spontaneous activity in spinal motor neurons (Colomo & Erulkar, 1968; Blankenship, 1968). Similar considerations apply to the inhibition of cyclic AMP release by tetrodotoxin, which we interpret as being caused by interruption of synaptic events secondary to the blockage of axonal transmission.

As a working hypothesis to explain the link between electrical pulses and protein P turnover we have assumed that the response is mediated through the release by electrical pulses of a neurohumoural agent that stimulates adenylate cyclase to increase the synthesis of cyclic AMP, which in turn activates a contiguous membrane-bound protein kinase. Actually, stimulation for 10s (the time required to elicit the protein P response) is insufficient to produce any detectable change in the content of cyclic AMP in the tissue, so it is necessary to assume in the hypothesis that the time is sufficient to produce enough cyclic AMP locally to stimulate kinase activity. Of the two classes of chemical agent examined, both known to increase the cyclic AMP content of the tissue, only the biogenic amines also increased the turnover of protein P. Histamine was chosen for detailed investigation in the first instance since previous work (Chasin *et al.*, 1971) showed it to be the most effective of the three amines in increasing cyclic AMP content in slices of guinea-pig cerebral cortex. Assuming that histamine actually reaches the sites of phosphorylation affected by electrical pulses, the present evidence suggests that this amine is not involved in the response, since it appears to activate a different protein-phosphorylating system. This is shown by the observations that the effect of the amine is additive with that of electrical pulses (Table 4) and is inhibited by adenosine, an agent that does not affect the response to electrical pulses (Table 9). It may be added that there is no information at present as to whether the phosphoprotein that responds to histamine is located in the neuronal membrane, as appears to be the case for the protein responding to electrical pulses (Trevor & Rodnight, 1965), or in some other part of the cell. A neuroglial location must also be considered: adenylate cyclase activity in cultured human astrocytoma cells is greatly increased by additions of noradrenaline or histamine (Clark & Perkins, 1971), although histamine has no effect on cyclase activity in cultured glial cells from the rat (Schultz *et al.*, 1972).

The failure of adenosine to increase the turnover of protein P was surprising in view of the potency of adenosine in increasing the cyclic AMP content of brain slices (Sattin & Rall, 1970). In general, this negative result might be ascribed, as previously suggested (Reddington & Rodnight, 1972), to the property that adenosine possesses of inhibiting the intrinsic protein kinase activity of preparations of membrane fragments from brain. In the study of Weller & Rodnight (1973) this action was only evident at concentrations of adenosine greater than 50μM; yet this concentration gave a sevenfold increase in the cyclic AMP content of the tissue, but no effect on protein phosphorylation (Table 8). Uptake by the tissue of adenosine in the medium to give a local tissue concentration sufficient to inhibit the kinase seems unlikely, since Shimizu *et al.* (1972) have found that adenosine uptake into

brain slices occurs by diffusion followed by phosphorylation by adenosine kinase. Therefore if inhibition of protein kinase activity is a factor in the negative response to adenosine it must be assumed that the particular kinase involved is more sensitive to inhibition by adenosine *in situ* than in the subcellular preparation. This seems possible in the case of the protein phosphorylating system stimulated by histamine, which was fully inhibited by 10μM-adenosine (Table 9).

The lack of any effect of adenosine on the protein P response to electrical pulses is more difficult to understand. First, it cannot be entirely excluded on present evidence that the protein kinase concerned in the response to electrical pulses is distinct from the enzyme studied by Weller & Rodnight (1971, 1973) and is not inhibited by adenosine. Alternatively added adenosine may not reach the phosphorylation sites sensitive to electrical pulses. There is insufficient evidence at this stage to choose between these suggestions, but it does seem unlikely that the supposed failure of adenosine to reach the sites would stem from an inability of adenosine to penetrate the synaptic areas where adenosine is released by electrical pulses, since isotopic experiments have shown that both externally added adenosine and adenosine released by the action of electrical pulses are converted in part into newly synthesized cyclic AMP (Shimizu & Daly, 1970; Pull & McIlwain, 1972). On present evidence, therefore, it seems reasonable to conclude that adenosine is probably not the neurohormonal agent involved in the protein P response to electrical pulses. The possibility that an adenylate cyclase receptor specific to some other neurohormone is involved needs to be investigated.

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