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Protein Kinase Activity in Membrane Preparations from Ox Brain

STIMULATION OF INTRINSIC ACTIVITY BY ADENOSINE 3′:5′-CYCLIC MONOPHOSPHATE

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1. Properties of the stimulation by cyclic AMP of the intrinsic protein kinase activity of membrane fragments from ox brain were studied. 2. Stimulation of activity declined from about 100% at 1min to less than 20% at 10min. The time-course was explained by the observation that cyclic AMP did not stimulate turnover of protein-bound serine phosphate once the membrane protein was fully phosphorylated. 3. Cyclic AMP accelerated the activity of a component of the basal activity rather than activating a different kinase. 4. The pH optimum for both the stimulated and basal activities was 7.2–7.4. NaCl (100mM) and KCl (10–100mM) inhibited the stimulated activity but did not affect the basal activity. 5. Strychnine and theophylline inhibited both activities equally, but the stimulated activity was more sensitive to inhibition by adenosine, bicuculline, vinblastin, veratrine, *N*-ethylmaleimide and cysteine. 6. No firm evidence for a role for endogenous cyclic AMP in the basal activity was found, but the possibility was not excluded. 7. Some 90% of both the stimulated and basal activities remained in an insoluble form after treatment of the membrane fragments with Triton X-100 (0.5%).

Membrane fragments prepared from several mammalian sources contain intrinsic protein kinase activity that is stimulated by cyclic AMP (Weller & Rodnight, 1970, 1971; Rodnight & Weller, 1971; Johnson *et al.*, 1971). (In this context the term intrinsic is used to indicate that the acceptor protein for the kinase enzyme constitutes part of the membrane structure to which it is tightly bound.) The activity is relatively high in cerebral cortex (Rodnight & Weller, 1971) and within that tissue in membrane fragments of synaptic origin (Johnson *et al.*, 1971; D. Luxton & R. Rodnight, unpublished work). This suggests that the kinase activity in membrane fragments may correspond to that involved in the increased turnover of protein and membrane-bound serine phosphate which occurs when respiring slices of cerebral cortex are depolarized with electrical pulses (Heald, 1957; Trevor & Rodnight, 1965; Jones & Rodnight, 1971), a procedure that also increases the cyclic AMP content of the tissue (Kakiuchi *et al.*, 1969). The physiological function of this kinase system is unknown. Cyclic AMP has been implicated both in the release of transmitter from presynaptic stores (Thoa *et al.*, 1972) and in transmembrane properties in the postsynaptic area (Siggins *et al.*, 1971; Kebabian & Greengard, 1971). The activation of a protein kinase by cyclic AMP synthesized as a result of the release of a neurohumoural agent could conceivably lead to altered permeability to cations, for example, in either of these areas.

The present paper is concerned with properties of the cyclic AMP-stimulated component of the total kinase activity in membrane fragments from ox brain. In particular we have sought to explain the unusual time-course of the stimulated activity and the possible role of endogenous cyclic AMP in the membrane preparations in activating the kinase in the absence of added cyclic AMP. The effects of cations and substances known to affect membrane function have also been examined. A preliminary account of part of this work has appeared (Rodnight & Weller, 1971).

Experimental

Materials

Phosvitin was obtained from Nutritional Biochemicals Corp. (Cleveland, Ohio, U.S.A.) and purified by the procedure of Rose & Heald (1961). Casein (Hammarsten purified grade) was obtained from Hopkin and Williams (Chadwell Heath, Essex, U.K.) and veratrine (mixture of alkaloids) from British Drug Houses Ltd. (Poole, Dorset, U.K.). Histones (type II) and [lysine]-vasopressin (grade 1-S) were obtained from Sigma (London) Chemical Co. (London S.W.6, U.K.). Prostaglandins were a gift from Dr. John Pike of the Upjohn Co. (Kalamazoo, Mich., U.S.A.); bicuculline was given by Dr. D. Greenwood and vinblastin by Dr. J. Lagnado. All other chemicals were of the purest grade available. Solutions were made up in deionized distilled water.

Methods

Membrane preparations. Ox brain microsomal fractions were prepared by the method of Rodnight (1970); crude synaptosomal-membrane fragments from the same species were prepared by the method of Rodnight *et al.* (1969). Unless otherwise stated frozen grey matter from the cerebral cortex was used as the source of tissue. The preparations were normally frozen, stored at −20°C and used within 40 days; freezing and storage for this period did not affect protein kinase activity in the presence or in the absence of cyclic AMP. Some membrane preparations were treated with NaI as described by Weller & Rodnight (1971).

Isotopically labelled ATP. [γ-^{32}P]ATP was prepared as the Tris salt by the procedure described by Rodnight & Lavin (1966).

Determination of protein. The method of Miller (1959) was used with bovine plasma albumin as standard.

Extrinsic protein kinase activity. The method of Rodnight & Lavin (1964) was used. When determining extrinsic protein kinase activity in membrane preparations controls were always done in the absence of added phosphoprotein to allow for incorporation of ^{32}P into intrinsic membrane protein; the amount of ^{32}P incorporated into the added protein was determined by difference. Samples were counted for radioactivity by dissolving the protein precipitates in 0.5M-NaOH and measuring Čerenkov radiation in a scintillation spectrometer (Packard model 3003)

Intrinsic protein kinase activity. This was determined as described previously (Weller & Rodnight, 1971) except that two washes of the protein precipitate with 10% (w/v) trichloracetic acid containing 1M-H_3PO_4 and one wash with ethanol–diethyl ether (1:1, v/v) were found sufficient.

Determination of cyclic AMP. A modification of the method of Gilman (1970) employing internal standards was used (Weller *et al.*, 1972). The modified method was developed because it was observed that extracts of the membrane preparations contained a diffusible factor that altered the binding of cyclic AMP by the muscle binding protein. Cyclic AMP was extracted from membrane preparations by adding HCl to a final concentration of 0.1M and boiling for 2min. Denatured protein was removed by centrifugation; the supernatant was then adjusted to pH4.0 by the addition of 1M-sodium acetate and cyclic AMP was determined in samples of the supernatant.

Results

Time-course of the stimulation by cyclic AMP of protein kinase activity

Examination of a number of different membrane preparations confirmed the observation (Weller & Rodnight, 1970) that the stimulation of protein kinase activity in membrane preparations from cerebral-cortex tissue decreases from about 100% after incubation for 1min to less than 20% after 10min and to an insignificant value at 30min (Table 1). The decline in stimulation occurs although the maximum incorporation of ^{32}P into the membrane protein is only reached under these conditions after incubation for 60min (Weller & Rodnight, 1971). Further, since the decline is independent of the concentration of cyclic AMP in the medium (Weller & Rodnight, 1970) it cannot be due to hydrolysis of the nucleotide by membrane-bound phosphodiesterase activity. An alternative explanation relates it to the same factors that determine the curvilinear time-course of phosphorylation observed in the absence of added cyclic AMP. These were shown by Weller & Rodnight (1971) to be (a) the partially dephosphorylated state of the membrane proteins and (b) the rate-limiting action of membrane-bound protein phosphatase activity on ^{32}P incorporation once the membrane protein is fully phosphorylated. Rephosphorylation of vacant serine-OH was found to be largely complete after incubation for about 15min with

Table 1. *Stimulation of protein kinase activity in membrane preparations by cyclic AMP*

Protein kinase activity was determined at 20°C by the procedure of Weller & Rodnight (1971). The values quoted are means±S.D. with the number of experiments in parentheses. The concentration of cyclic AMP used was 10μM.

Membrane preparation	Incubation time ...	Protein kinase activity in the absence of cyclic AMP (pmol of ^{32}P in phosphoserine/mg of protein)			Stimulation by cyclic AMP (%)		
		1min	10min	30min	1min	10min	30min
Microsomal (7)		76±27	350±90	—	126±16	16±5	—
Synaptosomal (9)		62±20	300±95	—	90± 7	9±3	—
Synaptosomal (1)		70, 80	—	690, 640	88	—	3

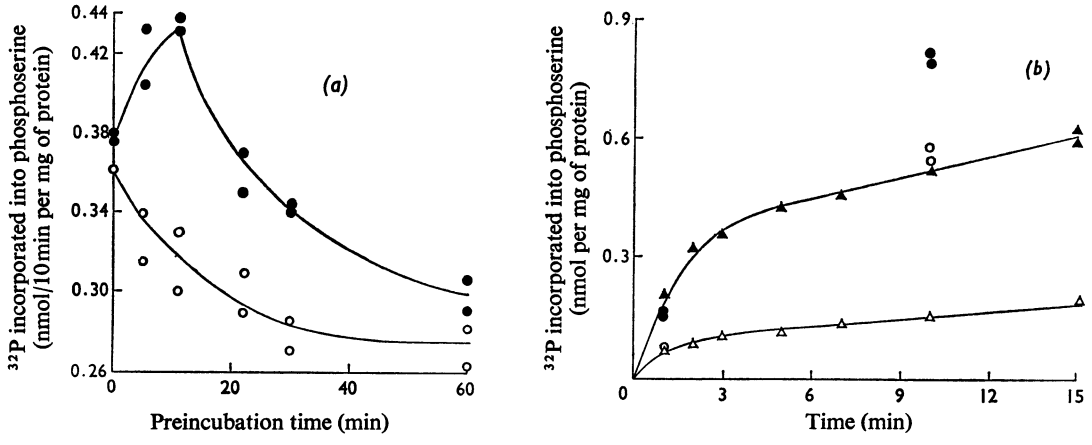

Fig. 1. *Effect of preincubation of a membrane preparation under dephosphorylating conditions on intrinsic kinase activity*

A sample of synaptosomal-membrane fragments was preincubated for various times at a protein concentration of 0.5mg/ml in 33mM-Tris–HCl buffer (pH7.4) containing 1.25mM-$MgCl_2$ at 37°C. Kinase activity was determined at 20°C on 2ml portions after addition of 0.25ml of 10mM-[^{32}P]ATP and 0.25ml of water or 100μM-cyclic AMP. (*a*), Kinase activity measured 10min after addition of [^{32}P]ATP in samples preincubated for various times: ○, without cyclic AMP; ●, with cyclic AMP (10μM). (*b*), Time-course of phosphorylation after preincubation for 10min: △, without cyclic AMP; ▲, with cyclic AMP (10μM); ● and ○, control samples not preincubated, with and without cyclic AMP respectively.

[^{32}P]ATP. It follows, therefore, that as phosphatase activity is not affected by cyclic AMP, stimulation of kinase activity would be expected to be greatest in the initial stages of the reaction and subsequently to decline. Further, if this interpretation is correct the magnitude of the response to cyclic AMP should depend upon the state of phosphorylation of the membrane protein. To test this suggestion we measured kinase activity in preparations pretreated under conditions designed to vary the amount of protein-bound P in the membrane.

To examine the effect of dephosphorylation the membrane-protein preparations were preincubated at 37°C for various time-intervals under conditions shown by Weller & Rodnight (1971) to result in a net loss of protein-bound P; kinase activity was then determined with and without added cyclic AMP. As predicted the treatment increased the magnitude of the stimulation given by cyclic AMP (Figs. 1*a* and 1*b*). The time of preincubation was critical, however, the maximum enhancement of the ^{32}P incorporated at 10min being obtained after pretreatment for 5–10min (Fig. 1*a*). Time-courses for ^{32}P incorporation in a preparation dephosphorylated for 10min are shown in Fig. 1(*b*). These are strikingly different from those reported by Weller & Rodnight (1970) for an untreated preparation in that there was no decrease with time in the magnitude of the stimulation given by cyclic AMP. The preincubation time was critical because, as noted by Weller & Rodnight (1971), preincubation in the absence of ATP is accompanied by loss of kinase activity. From Fig. 1 it seems that the loss of kinase activity commences sooner than suspected by Weller & Rodnight (1971) and that preincubation decreases the kinase activity observed in the absence of cyclic AMP to a greater extent than the stimulated activity.

When the protein-bound P in the membrane fragments was increased by preincubation with unlabelled ATP (see Weller & Rodnight, 1971) stimulation of ^{32}P incorporation by cyclic AMP after incubation for 1min progressively decreased with increasing time of preincubation (Fig. 2). In another approach it was found that addition of cyclic AMP to the normal reaction mixture after incubation for 10min did not stimulate ^{32}P incorporation over the period 10–11min (Table 2); this result incidentally confirmed that the decrease in stimulation is not due to hydrolysis of added cyclic AMP.

We have shown that treatment of membrane preparations with NaI destroys about 90% of their intrinsic protein kinase activity in the sedimentable product (Weller & Rodnight, 1968, 1971). In the present study it was found that the residual activity is still sensitive to stimulation by cyclic AMP, but with the difference that during phosphorylation the magnitude of the stimulation increased with time (Fig. 3). Since preparations treated with NaI possess very

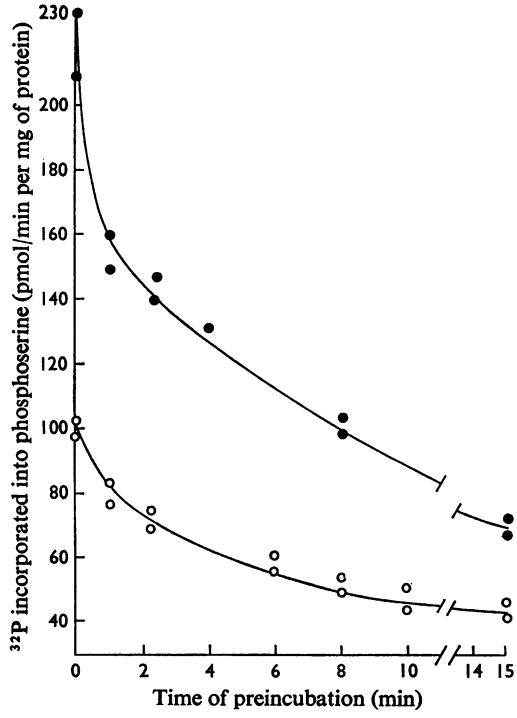

Fig. 2. *Effect of preincubation of a membrane preparation with non-radioactive ATP on the initial rate of kinase activity*

Separate samples of a synaptosomal preparation were preincubated for various times in 30mM-Tris–HCl buffer (pH7.4)–1mM-ATP (Tris salt)–1mM-$MgCl_2$ at 20°C in a volume of 2.25ml. Kinase activity was then determined over 1min in the same tube by the addition of 0.25ml of 10mM-[^{32}P]ATP with or without 100μM-cyclic AMP, thus giving in the final mixture 1.8–2.0mM-ATP (depending on the amount already hydrolysed), 0.9mM-$MgCl_2$ and 10μM-cyclic AMP. The exact initial specific radioactivity of the [^{32}P]ATP was calculated in each case in parallel experiments by determining the P_i released during preincubation. o, Without cyclic AMP; ●, with cyclic AMP.

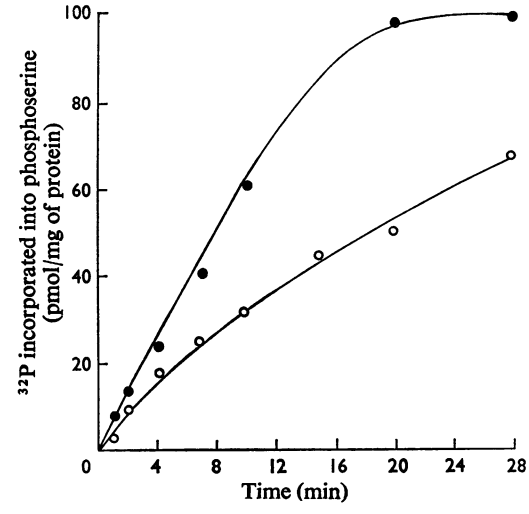

Fig. 3. *Time-course of intrinsic kinase activity in a synaptosomal-membrane preparation after treatment with NaI*

Standard reaction conditions were used (see the text). o, Without cyclic AMP; ●, with cyclic AMP (100μM).

little protein phosphatase activity (Weller, 1972), it seems that the residual activity incorporating ^{32}P consists predominantly of a phosphorylation of vacant serine-OH without ensuing turnover. This suggests that in the disruption by NaI of the kinase–phosphatase complex mediating turnover only a small number of units comprising kinase and dephosphorylated acceptor protein remain intact. However, it must be emphasized that the residual kinase activity constitutes only a very small proportion of the original and there is no evidence that the treatment results in a major dephosphorylation of the membrane protein. In fact the alkali-labile P content of a treated preparation was slightly higher than before treatment (Weller, 1969).

Effect of cations and pH on protein kinase activity stimulated by cyclic AMP

Kinase activity was measured from 0–1min during which ^{32}P incorporation largely consists of phosphorylation of vacant serine-OH. The optimum concentration of Mg^{2+} for the activity in the presence of cyclic AMP was similar to that observed in its absence, but the percentage stimulation was greater at 0.5mM-$MgCl_2$ and concentrations above the optimum depressed the stimulation (Fig. 4). Concentrations of Ca^{2+} above 50μM inhibited both the basal and stimulated activities to the same degree.

The stimulated activity was markedly depressed by K^+ and to a lesser extent by Na^+ (Fig. 5). This result contrasts with the effect of K^+ and Na^+ on the basal activity measured over short periods; confirming previous observations (Weller & Rodnight, 1971), we found that without cyclic AMP Na^+ had no effect and that of K^+ was minimal.

The pH optimum for the stimulated activity was identical with that of the basal activity (Fig. 6).

Table 2. *Effect of cyclic AMP added after phosphorylation for 10 min on protein kinase activity*

Kinase activity was determined at 20°C by the procedure of Weller & Rodnight (1971). A synaptosomal preparation was used.

Experiment	Period over which phosphorylation was measured (min)	Time of addition of cyclic AMP (min)	Cyclic AMP (100 μM)	...	Protein kinase activity (pmol of ^{32}P in phosphoserine/mg of protein) Absent	Present	Stimulation (%)
1	0–1	0			96, 80	136, 146	62
	10–11	10			50, 60	48, 50	0
2	0–1	0			52	92, 93	79
	10–11	10			38, 32	31, 25	0

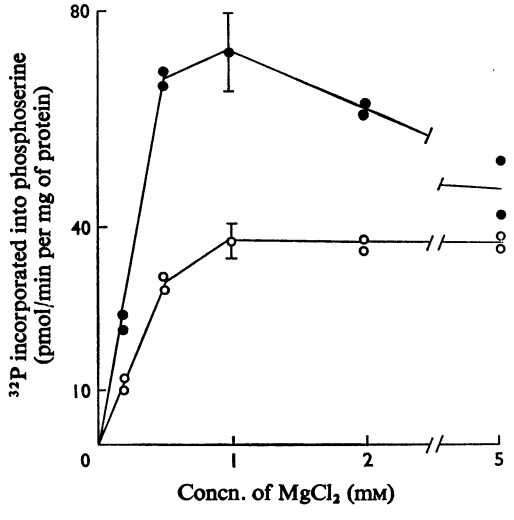

Fig. 4. *Effect of Mg^{2+} concentration on intrinsic kinase activity with and without cyclic AMP*

Kinase activity was determined over 1 min by the standard procedure (see the text) but at various Mg^{2+} concentrations. o, Without cyclic AMP; •, with cyclic AMP (10 μM). The bars represent ± S.D.

Effects of some chemical agents on the stimulated activity

The effects of a number of substances are given in Table 3. Strychnine and theophylline inhibited the basal and stimulated activities to about the same degree, but adenosine (0.1 mM), bicuculline, vinblastin, crude veratrine alkaloids, *N*-ethylmaleimide and cysteine all gave a more pronounced inhibition of the stimulated activity. The effect of adenosine was of particular interest in view of the fact that it increases the cyclic AMP content of respiring slices of brain tissue (Sattin & Rall, 1970), but does not in the same preparation stimulate protein P turnover (Reddington *et al.*, 1973). Substances not mentioned in Table 3 and lacking any effect on either the basal or stimulated activity included atropine (0.1 mM), acetylcholine (0.1 mM with and without 0.1 mM-eserine), nicotine (0.1 mM), D-tubocurare (0.1 mM), colchicine (0.1 mM), valinomycin (0.1 mM) and papaverine (0.1 mM).

Prostaglandins (E_1, E_2, F_1 and F_2) were examined because of their ability in some tissues to activate adenylate kinase (Ramwell & Rabinowitz, 1971). In general these substances tended to inhibit ^{32}P incorporation at a concentration of 10 μg/ml. The inhibition was just statistically significant ($P<0.05$) for prostaglandins E_2 and F_2 in the absence of cyclic AMP and for prostaglandins E_1, E_2, F_1 and F_2 in the presence of 10 μM-cyclic AMP [Weller (1972) gives further details]. Only in the case of prostaglandin E_2 with cyclic AMP, however, did the inhibition exceed 25% of control and in general these effects seem of doubtful physiological significance.

[Lysine]-vasopressin (0.01 i.u./ml), which stimulates adenylate cyclase activity in intact kidney-cortex tissue (Brown *et al.*, 1963), had no effect on the basal or stimulated activities. F^- also stimulates adenylate cyclase in broken-cell preparations (Sutherland *et al.*, 1962). In the present work the Tris salt of F^- had no effect on the basal activity in the concentration range 1–100 mM, but NaF at concentrations of 50 mM or above was markedly inhibitory. The percentage inhibition given by 100 mM-NaF was about 90%, which is much higher than given by the same concentration of NaCl (Weller & Rodnight, 1971).

Hormones that stimulate adenylate cyclase in cell-containing preparations from brain were found by Weller & Rodnight (1970) to have no effect on kinase activity in membrane preparations from ox brain that had been stored frozen. We thought it advisable to repeat these observations with freshly prepared microsomal fractions from guinea-pig cerebral cortex. The results confirmed that adrenaline, noradrenaline

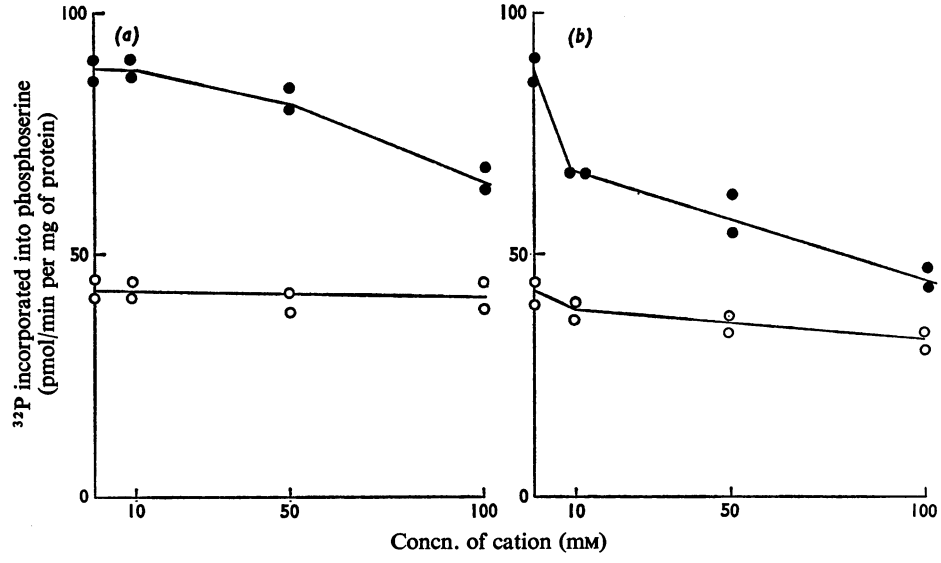

Fig. 5. *Effect of Na^+ (a) and K^+ (b) concentrations on intrinsic kinase activity without and with cyclic AMP*

Kinase activity was determined over 1 min by the standard procedure (see the text), but with the addition of NaCl or KCl. o, Without cyclic AMP; •, with cyclic AMP (10μM).

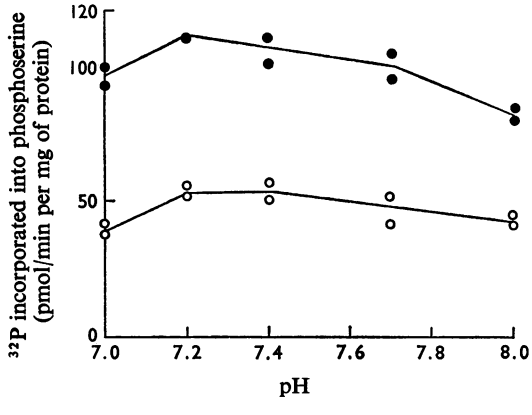

Fig. 6. *Effect of pH on intrinsic kinase activity with and without cyclic AMP*

Kinase activity was determined over 1 min by the standard procedure (see the text), but with the pH varied. The usual buffer of Tris–HCl was used. o, Without cyclic AMP; •, with cyclic AMP (10μM).

and histamine (all at 1 mM concentration) have no effect on the protein kinase activity measured under our conditions.

Possible role of endogenous cyclic AMP in the basal kinase activity

The membrane fragments contain traces of bound cyclic AMP and also adenylate cyclase activity that produces some cyclic AMP on incubation with ATP under the conditions used to measure kinase activity. These facts led us to suggest that kinase activity observed in the absence of added cyclic AMP might be to a greater or lesser extent dependent on endogenous cyclic AMP. We have now investigated this possibility quantitatively.

The rate of cyclic AMP formation in synaptosomal-membrane preparations under standard conditions of kinase assay amounted to 18 pmol/min per mg of protein, giving a medium concentration after incubation for 1 min of 7.2 nM, ignoring for the moment any binding of cyclic AMP to the membrane fragments. In other experiments (Weller *et al.*, 1972) 1 mM-papaverine, an inhibitor of phosphodiesterase, increased the yield of cyclic AMP by about 50%. The lowest concentration of added cyclic AMP stimulating phosphorylation is 20 nM (Weller & Rodnight, 1970). Clearly therefore, endogenously produced cyclic AMP can only be expected to stimulate the kinase reaction if part or all of it is bound by the kinase receptor without release to the medium. This point is considered further in the Discussion section.

With regard to the question of bound cyclic AMP,

Table 3. *Inhibitory effect of some substances on protein kinase activity in the presence and in the absence of cyclic AMP*

Kinase activity was determined at 20°C by the procedure of Weller & Rodnight (1971). The substances were dissolved in the medium before addition of synaptosomal-membrane protein to start the reaction. Where mean values are quoted they are given ± S.D. with the number of observations in parentheses.

Compound	Concentration	Protein kinase activity over period of 0–1 min (% of control) Cyclic AMP (100 μM) ... Absent	Present
None	—	100	100
Strychnine	1 mM	68 ± 10 (6)	59 ± 14 (6)
Strychnine	0.1 mM	99 ± 1 (3)	94 ± 2 (3)
Theophylline	5 mM	53, 50	55, 58
Adenosine	1 mM	39 ± 4 (6)	41 ± 1 (3)
Adenosine	0.5 mM	50, 52	49 ± 2 (3)
Adenosine	0.1 mM	80 ± 9 (4)	57 ± 3 (5)
Adenosine	0.05 mM	—	85, 91
Bicuculline	1 mM	88, 82	66, 60
Vinblastin	0.1 mM	72, 74	64, 63
Veratrine	0.07%	95, 88	66, 70
N-Ethylmaleimide	1 mM	100, 100	75 ± 13 (4)
Cysteine	1 mM	119 ± 1 (4)	74 ± 5 (4)

Table 4. *Lack of effect of cyclic AMP on extrinsic protein kinase activity in the membrane preparations*

Kinase activity was determined by the procedure of Decsi & Rodnight (1965) at a pH of 7.4 and with 1 mM-ATP as donor. The concentration of cyclic AMP was 200 μM.

Substrate	Preparation	Bound extrinsic protein kinase activity (nmol of P/h per mg of protein) Without cyclic AMP	With cyclic AMP
Phosvitin	Synaptosomal 1	16, 13	16, 11
Phosvitin	Synaptosomal 2	22,19	22, 23
Phosvitin	Microsomal	5, 8	8, 5
Casein	Synaptosomal	0	0
Histone	Synaptosomal	0	0

analysis of several membrane preparations by the procedure of Weller *et al.* (1972) indicates that the preliminary value of 10 pmol of cyclic AMP/mg of membrane protein quoted by Weller & Rodnight (1971) is too high. The discrepancy arose because the original analyses were conducted by the procedure of Gilman (1970) with external standards, which we later found to be unreliable when determining very small quantities of the nucleotide in extracts made from relatively high amounts of tissue (Weller *et al.*, 1972). Details are given by Weller *et al.* (1972); the values for synaptosome membrane fragments ranged from <1 to 3.5 pmol/mg of protein. No correlation between content of bound cyclic AMP and kinase activity in the absence of added nucleotide was observed.

Lack of effect of cyclic AMP on the phosphorylation of extrinsic proteins by the membrane preparations

Cyclic AMP was found by Weller & Rodnight (1970) to have no effect on the phosphorylation of casein and phosvitin by a soluble protein kinase prepared from ox brain by the procedure of Rabinowitz & Lipmann (1960). In the present study the nucleotide also failed to stimulate the membrane-bound kinase activity phosphorylating phosvitin described by Decsi & Rodnight (1965); the preparations examined included synaptosomal and microsomal membrane fragments (Table 4). It is noteworthy that under the same conditions of assay histones and casein did not function as acceptors, either in the presence or in the absence of cyclic AMP. This observation contrasts with that of Maeno *et al.* (1971),

who reported that similar membrane preparations contained kinase activity stimulated by cyclic AMP with histone and casein as substrate. However, there are major differences in the assay systems used in the two studies: Maeno *et al.* (1971) used an ATP concentration of 5 μM with a $[Mg^{2+}]/[ATP]$ ratio of $2\times10^3:1$; their reaction mixture also contained NaF, theophylline and ethanedioxybis(ethylamine)tetra-acetate, and had a pH of 6.0 instead of 7.4 used in the present work.

Intrinsic kinase activity in peripheral nerve

To gain information on the possible role of this enzyme activity in the nervous system it seemed desirable to examine a purely conducting tissue lacking synaptic structures. The unmyelinated nerves of crab leg muscles were chosen. Crabs (*Cancer paragus*) were obtained locally and brought to the laboratory packed in ice. Leg nerves were dissected out, homogenized in 0.25 M-sucrose and the homogenate spun at 10^4g for 15 min. The supernatant was collected at 1.5×10^5g for 30 min and the pellet resuspended in 4 mM-imidazole–HCl buffer, pH 7. The resulting membrane fragments contained $Na^+ + K^+$-activated adenosine triphosphatase activity but no protein kinase activity either in the presence or in the absence of cyclic AMP. A similar membrane preparation from human vagus nerve also exhibited no kinase activity.

Treatment with Triton X-100

As briefly mentioned by Weller & Rodnight (1971), the intrinsic kinase activity measured in the absence of cyclic AMP is tightly bound to the membrane fragments and resists disruption by detergents. The effect of Triton X-100 was of particular interest since with this detergent full recovery of activity was obtained. The cyclic AMP-stimulated activity has now been found to possess identical properties on exposure to Triton X-100 (Table 5). In both cases less than 10% of the activity was solubilized and the specific activity of the enzyme in the pellet was increased by approximately the same amount.

Table 5. *Effect of Triton X-100 on protein kinase activity*

A synaptosomal-membrane preparation was homogenized with 0.5% (v/v) Triton X-100 at a protein concentration of 10 mg/ml at 0°C. The suspension was centrifuged at 1×10^5g for 60 min, the supernatant removed and the pellet resuspended in 4 mM-imidazole–HCl buffer, pH 7.2, containing 0.5% Triton X-100. Protein kinase activity was determined at 20°C by the method of Weller & Rodnight (1971).

Treatment	Fraction	Protein (%)	Cyclic AMP (100 μM)	Protein kinase activity (pmol of ^{32}P in phosphoserine/min) (activity/mg of protein)		(mean total activity)		Distribution (%)	
				Absent	Present	Absent	Present	Absent	Present
None	Original sample	100	...	60, 53	120, 125	565	1225	—	—
Triton X-100	Before centrifuging	100		65, —	133, 135	650	1350	—	—
	Supernatant	33		16, 17	35, 36	55	117	8	9
	Pellet	67		88, 96	190, 165	617	1188	95	88

Discussion

The present results support the suggestion made previously (Weller & Rodnight, 1971) that the total intrinsic protein kinase activity of the membrane fragments is derived predominantly from one enzyme–substrate complex which is stimulated by on cyclic AMP. Thus it can be seen that at least part of the basal activity is due to the same enzyme as that stimulated by cyclic AMP. This is concluded for two reasons: (*a*) by the time rephosphorylation in the absence of cyclic AMP is nearly complete addition of cyclic AMP promotes no extra

incorporation (Table 2; the same tendency is seen in Fig. 2); (*b*) the two time-courses converge and eventually meet (Table 1; convergence is also evident in Fig. 1 of Weller & Rodnight, 1970). Therefore added cyclic AMP accelerates the basal reaction rather than activates a different kinase, so that even in the absence of added nucleotide the membrane protein is eventually phosphorylated. A similar conclusion was reached by Weller (1972) by a mathematical analysis of the time-courses of phosphorylation in the presence and in the absence of cyclic AMP. The observation that the stimulated activity shows greater sensitivity to certain agents (e.g. Na^+ and K^+ in Fig. 5 and some of the compounds tested in Table 3) may be due to an interference by the agents with the regulatory action of added cyclic AMP rather than a direct action on a distinct enzyme.

A role for endogenous cyclic AMP in the basal reaction has to be reconsidered in the light of results obtained by the more accurate method for determining cyclic AMP developed by Weller *et al.* (1972). These show that native bound cyclic AMP is not involved because its content is very low and varies from one preparation to another. Endogenous adenylate cyclase activity, however, might influence the kinase reaction if it is assumed that a part of the cyclic AMP synthesized during incubation is bound to kinase receptors without being released to the medium. This is possible since the membrane fragments are capable of binding low concentrations of cyclic AMP (M. Weller & R. Rodnight, unpublished work), but firm evidence that this occurs to a sufficient degree to stimulate the reaction is lacking. The fact that the phosphodiesterase inhibitor, papaverine, did not stimulate the basal reaction is not a serious objection, since the drug may only be inhibiting the hydrolysis of cyclic AMP that escapes binding by the kinase system. In other systems it has been shown that bound cyclic AMP is not hydrolysed by phosphodiesterase action (Cheung, 1972).

It is clear from these results that cyclic AMP does not stimulate the turnover of protein-bound phosphoserine that continues in the presence of ATP after the membrane has been fully phosphorylated. It follows that cyclic AMP can only play a significant role in regulating the coupled system in the intact tissue if the membrane protein which acts as acceptor undergoes a cycle of phosphorylation and dephosphorylation in relation to cell function. Apart from the availability of cyclic AMP, other regulatory factors in this process could be the local concentrations of ATP and Ca^{2+}. The concentration of ATP (1 mM) used in the present study is close to the mean ATP concentration in the brain *in vivo* and results in full phosphorylation of the membrane. It is possible, however, that the actual concentration in the local pool available to the kinase is lower, particularly since another membrane enzyme utilizing ATP, the Na^++ K^+-activated adenosine triphosphatase, has a turnover rate many times higher than the membrane-bound kinase system (Weller & Rodnight, 1971). The concentration of Ca^{2+} ions in the cell (<10 μM; see Rasmussen, 1970) would appear to be several times too low to serve as a controlling factor by inhibiting kinase action, since the minimum concentration having this effect was 50 μM.

Although the precise location of the cyclic AMP-stimulated kinase activity studied here is unknown, its relative enrichment in membrane fragments derived from synaptosomes (D. Luxton & R. Rodnight, unpublished work) and its absence from axonal fragments prepared from crab nerve are consistent with an involvement in some aspect of synaptic function. A synaptic location would accord well with the observation that adenylate cyclase occurs predominantly in membranes of synaptic origin (Robison *et al.*, 1970) and with the suggestion (Weller & Rodnight, 1970; Kebabian & Greengard, 1971) that membrane-bound protein kinase activity may either mediate or modulate permeability changes in the receptor cell membrane that occur on release of neurotransmitters. Because of the slow rate of turnover of the system (Weller & Rodnight, 1971) the consequent changes in the properties of the postsynaptic membrane may be relatively long-lasting and may serve to modify the response of the cell to stimuli from other sources. Here the work of Libet & Tosaka (1970) is possibly relevant: small doses of dopamine were found to induce changes in the postsynaptic membrane in superior cervical ganglia which selectively facilitated excitatory responses to cholinergic agents for periods greater than 1 h. It was suggested that the facilitation may be due to a long-lasting structural change induced in the receptor neuron by dopamine.

A cyclic AMP-stimulated phosphorylation of intrinsic protein in membrane fractions from rat brain has also been described by Johnson *et al.* (1971). There are, however, major differences in technique between this work and ours: in that of Johnson *et al.* (1971) the reaction mixture had a pH of 6.0 and contained Triton X-100, theophylline, F^- and ethanedioxybis(ethylamine)tetra-acetate; the ATP concentration was 5 μM and the $[Mg^{2+}]/[ATP]$ ratio was 2×10^3:1. These differences make it difficult to say whether the same enzyme activity is being measured in the two studies.

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