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Phosphorylation of Ribosome-Associated Protein by an Adenosine 3′:5′-Cyclic Monophosphate-Dependent Protein Kinase: Location of the Microsomal Receptor and Protein Kinase

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ABSTRACT **In the adrenal cortex, the adenosine 3′:5′-cyclic monophosphate (cAMP)-receptor protein and the cAMP-dependent protein kinase are located in both the cytosol and endoplasmic reticulum. The cAMP-dependent protein kinase from the cytosol catalyzes the phosphorylation of ribosome-associated protein. Ribosomes washed in 0.5 M NH_4Cl retain the substrate of the protein kinase reaction, but are dependent on the NH_4Cl extract for *in vitro* protein synthesis. Dissociation of the 80S ribosomes by 0.88 M KCl, however, releases the ribosome-associated (protein) substrate of the phosphorylation reaction.**

In the adrenal cortex, ACTH activates adenylate cyclase to catalyze the formation of cAMP (1, 2). The nucleotide mediates intracellularly the action of the hormone (3). The stimulation of cAMP formation is not dependent upon protein synthesis (4), while the induction of steroidogenesis by cAMP does require protein synthesis (5, 6); RNA synthesis, however, is not required (6, 7). We postulated that cAMP regulates adrenal function by modulating protein synthesis at the level of mRNA translation (6, 8, 9). Recently, we identified a specific receptor for cAMP in the cytosol of the adrenal cell (10) and demonstrated its association with protein kinase in a regulatory complex (11, 12). The cAMP receptor inhibits the activity of the protein phosphokinase (EC 2.7.1.37) when complexed to the enzyme; cAMP activates the enzyme by binding to the receptor, causing it to dissociate from the enzyme moiety.

The present investigation demonstrates that the cAMP receptor and protein kinase activities are located in the endoplasmic reticulum. Also, ribosome-associated protein is shown to serve as a substrate for the cAMP-dependent protein kinase reaction.

Methods

cAMP Binding Assay. cAMP binding was determined by isolation of the receptor–cAMP complex on cellulose-ester membrane filters (13).

Protein Kinase Assay. Protein kinase was determined by a modified procedure of DeLange *et al.* (14). Where indicated, the first 5% trichloroacetic acid (TCA) wash was heated to 90°C for 15 min (15). Although the microsomal protein kinase catalyzed the phosphorylation of both histone and protamine, the activity was higher with protamine; hence, the latter was used as substrate in the studies with the microsomal enzyme. Under the assay conditions described for microsomal activity, reaction rates were linear for up to 10 min with the amount of protein used. A substantial amount of ^{32}P incorporation into microsomes was observed in the absence of exogenous substrate, but this was generally less than 20% of the incorporation observed with protamine.

Abbreviations: TCA, trichloroacetic acid; TKM buffer, 50 mM Tris·HCl (pH 7.5)–25 mM KCl–10 mM $MgCl_2$.

Materials

Preparation of [γ-^{32}P]ATP. [γ-^{32}P]ATP was prepared (16) from 10 mCi of [^{32}P]orthophosphate and was purified according to Walsh, *et al.* (17); it was diluted immediately prior to use to a specific activity of 7–8000 cpm/nmol.

Salmon protamine sulfate, phosphoserine, and phosphothreonine were purchased from Sigma Chemical Co. cAMP was obtained from P-L Biochemicals, Inc., and cGMP, cIMP, and cCMP were from Boehringer Mannheim Corp.

Preparation of subcellular fractions

Microsomes. Subcellular fractionation of bovine adrenal glands was as described (10), except that microsomes were washed by resuspension in 10 volumes of 50 mM Tris·HCl (pH 7.5)–25 mM KCl–10 mM $MgCl_2$ (TKM buffer) and centrifuged at 100,000 × *g* for 1 hr. Submicrosomal fractions were prepared on a discontinuous sucrose gradient from twice-washed microsomes (18).

Ribosomes. Ribosomes were prepared from microsomes by precipitation with $MgCl_2$ (19), and further purified by suspension in TKM, containing 0.5 M NH_4Cl or 0.88 M KCl, at a concentration of 30–40 A_{260} units/ml; they were then centrifuged at 100,000 × *g* for 2 hr. The pellets were resuspended and dialyzed against 2 mM Tris·HCl (pH 7.5)–0.25 mM $MgCl_2$–10 mM β-mercaptoethanol. Immediately before use, ribosomal suspensions were centrifuged at 27,000 × *g* for 20 min to remove aggregates.

Ribosomes were isolated from sucrose density gradients by layering 60–80 A_{260} units, in 1 ml, on a 37-ml linear 10–30% sucrose gradient containing 50 mM Tris·HCl (pH 7.5)–1 mM $MgCl_2$. Centrifugation was for 15 hr at 22,000 rpm (4°C) in a SW 27 rotor (Beckman Instruments). Ribosomal subunits were prepared as described by Martin and Wool (20), except

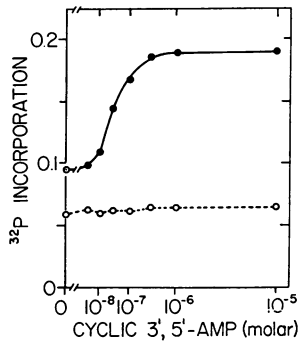

FIG. 1. Effect of cAMP concentration on the phosphorylation of protamine catalyzed by the microsomal protein kinase. Microsomes were washed twice, and protein kinase activity was determined as described in *Methods*. Reaction mixtures, in a total volume of 0.25 ml, contained 50 mM glycerol phosphate (pH 6.0), 20 mM NaF, 4 mM theophylline, 10 mM $MgCl_2$, 0.6 mM[^{32}P]-ATP, 250 μg of protamine sulfate (when added), 210 μg of microsomal protein, and cAMP as indicated. Protein kinase-dependent phosphorylation of protamine, ●—●. Endogenous ^{32}P incorporation into microsomes without the addition of protamine, O—O. Activity in nmol/mg per min.

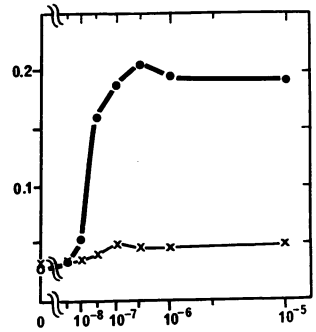

FIG. 2. The effect of cAMP concentration (molar) on ribosomal phosphorylation. Reaction mixtures as in Fig. 1, except 40 μg of protein kinase, 0.20 mg of ribosomes, and no protamine added. Kinase-catalyzed ^{32}P incorporation into ribosomal protein, ●—●. Endogenous phosphorylation of enzyme, ×—×. The figures on the ordinate indicate the total ^{32}P incorporated, less the endogenous phosphorylation of ribosomes, in nmol/mg per min.

that a linear sucrose density gradient was used and centrifugation was at 25,000 rpm for 15 hr at 4°C. The A_{260} was monitored with a flow cell and spectrophotometer (Gilford). Ribosomal fractions were pooled and dialyzed against 50 mM Tris·HCl (pH 7.5)–1 mM $MgCl_2$, and were concentrated to about 50 A_{260} units/ml with a PM-30 membrane and ultrafiltration cell (Amicon Corp.).

Ribosomes had a 260/235 nm absorbance ratio of 1.4–1.6; the amount of ribosomes was calculated on the basis of an E 1% (1 cm) at 260 nm of 135 (21). Protein was determined (22) with bovine serum albumin as a standard. RNA was determined by a modification (23) of the Schmidt-Tannhauser procedure.

Enzyme Preparation. cAMP-stimulable protein kinase from the cytosol of bovine adrenal cortex was purified as described (11, 12), and represents pooled fractions of peaks I and III from DEAE-cellulose chromatography.

RESULTS

Subcellular distribution of the cAMP-receptor protein and cAMP-dependent protein kinase

As previously demonstrated with the cAMP-receptor protein (10), protein kinase activity was observed in the microsomal and cytosol fractions of the adrenal cell; the cAMP receptor and protein kinase activities of the microsomes remained associated with this fraction even after repeated washings (Table 1). A similar localization of protein kinase in the brain has been reported recently (24). The microsomes were further fractionated by centrifugation through a discontinuous sucrose density gradient into the following fractions: smooth membrane (endoplasmic reticulum), rough membrane (ribosomes bound to endoplasmic reticulum), and free ribosomes. The cAMP receptor and protein kinase activities were both mainly associated with the endoplasmic reticulum; only minimal activities were observed in the fraction containing free ribosomes (Table 1). Solubilization of the endoplasmic reticulum with 0.25% sodium deoxycholate concomitantly solubilized cAMP receptor and protein kinase activities. 71% of both activities were solubilized without inhibition or stimulation by the detergent. At 0.5% sodium deoxycholate, however, the detergent caused a 72% and a 24% inhibition of kinase and receptor activities, respectively. The association of both receptor and kinase activities in the endoplasmic reticulum suggests that they exist in a similar regulatory complex in this organelle, as we have demonstrated for the enzyme purified from the cytosol (11, 12).

With protamine as substrate, increasing concentrations of cAMP stimulate microsomal protein kinase activity, demonstrating a half-maximal saturation concentration of 5×10^{-8} M (Fig. 1). Maximum stimulation is obtained in response to about 5×10^{-7} M cAMP, which provides a 100% increase in enzyme activity. In this experiment, cAMP failed to stimulate endogenous protein kinase activity (i.e., without the addition of the protamine as substrate for the reaction).

TABLE 1. *Protein kinase and cAMP binding in adrenal cortex fractions*

Fraction	RNA/ protein ratio	^{32}P Incorporation (nmol/mg per min)	cAMP binding (pmol/mg)
Postmitochondrial supernatant	—	0.48	2.9
Soluble	—	0.25	2.7
Microsomes, 1st wash	—	0.43	2.5
Microsomes, 2nd wash	—	0.37	2.1
Microsomes, 3rd wash	—	0.36	2.0
Smooth membrane	0.06	0.73	3.2
Rough membrane	0.16	0.46	1.7
Free ribosomes	0.56	0.09	0.5

Protein kinase and cAMP binding were determined from duplicate assays. Kinase activity was determined in a total volume of 0.125 ml, containing 50 mM glycerol phosphate (pH 6.0), 20 mM NaF, 4 mM theophylline, 25 μM cAMP, 0.6 mM [^{32}P]ATP, 5 mM $MgCl_2$, 250 μg of protamine sulfate (when added), and 50–150 μg of subcellular protein. Phosphorylation of protamine is corrected for the ^{32}P incorporated when no substrate was added.

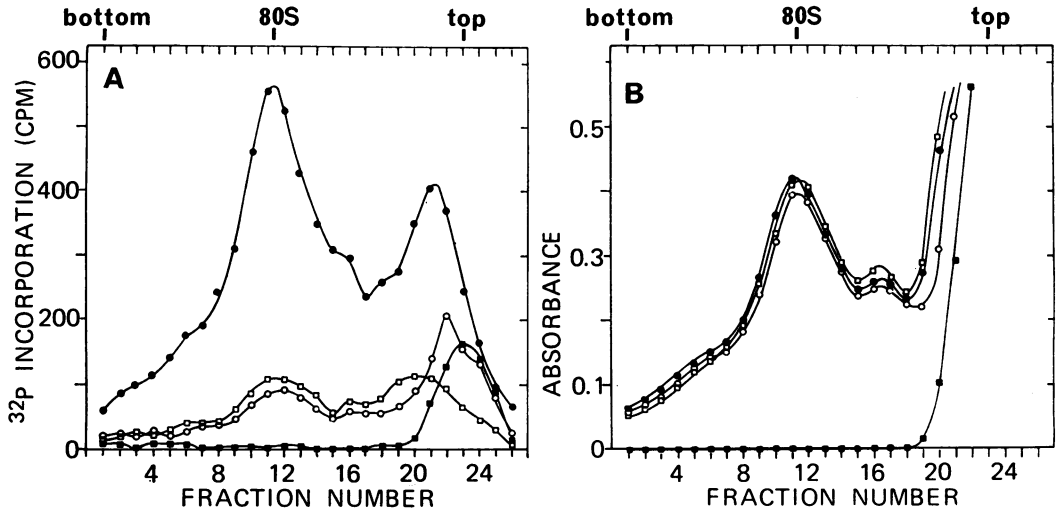

FIG. 3. cAMP-stimulated, protein kinase-dependent, ^{32}P incorporation into ribosomes that have been sedimented through sucrose gradients. The reaction mixture was as described under Fig. 2, except that 5 mM $MgCl_2$ (5 μM cAMP, when added), 3.7 A_{260} units of ribosomes, and 61 μg of enzyme were used. The reaction mixture was incubated for 15 min at 25°C, stopped with 0.75 ml of H_2O, immediately layered on 11 ml of a linear 10–30% sucrose gradient in 50 mM Tris·HCl (pH 7.5)–1 mM $MgCl_2$, and centrifuged at 20,000 rpm for 15 hr at 4° C in a SW 40 rotor. Fractions were collected, diluted to 1 ml with water, and absorbance at 260 nm was determined. Carrier protein was added and precipitated with 10% TCA. The precipitates were washed with hot TCA. Reaction with the addition of all the components, ●—●. Reaction with all the components except cAMP, ○—○. Reaction with all the components except ribosomes, ■—■. Reaction with all the components except enzyme, □—□.

Ribosomes as an endogenous substrate of cAMP-dependent protein kinase

Although cAMP was reported to stimulate protein kinase activity in microsomes from brain without the addition of exogenous substrate (25), we were unable to consistently demonstrate a cAMP activation of protein kinase without the addition of exogenous substrate (Fig. 1). Since the background of microsomal phosphorylation not dependent on cAMP was high, this may have obscured the cAMP-dependent reaction.

TABLE 2. *Dependence of in vitro protein synthesis on NH_4Cl extract*

Ribosomes	0.5 M NH_4Cl extract added (μg)	pmoles of [^{14}C]Met per mg of ribosomal protein
$MgCl_2$ washed	0	7.8
NH_4Cl washed	0	0.3
NH_4Cl washed	12	2.9
NH_4Cl washed	24	3.7
NH_4Cl washed	36	4.7
NH_4Cl washed	48	5.8
None	48	0.0

$MgCl_2$- and 0.5 M NH_4Cl-washed ribosomes were prepared as in *Methods*. [^{14}C]Methionyl-tRNA was prepared from adrenal cortex (31). Data represent the average of several assays done in duplicate. Zero-time blanks were subtracted. The reaction mix of 125 μl, incubated 30 min at 30°C, contained 0.19 M sucrose, 38 mM KCl, 27 mM Tris·HCl (pH 7.5), 3 mM β-mercaptoethanol, 0.055 mM EDTA, 80 mM NH_4Cl, 1 mM ATP, 0.25 mM GTP, 5 mM phospho(enol) pyruvate, 1.5 units of pyruvate kinase, 5 mM $MgCl_2$, 16 μg of transferase I, and 57 μg of tRNA labeled with [^{14}C]methionine. Where indicated, 1.0 A_{260} units of ribosomes was added. 100 μl of reaction mix was counted on Whatman 3 MM paper discs (32).

Because the enzymic activity of the microsomal fraction is in the endoplasmic reticulum, we considered the ribosomes as a possible endogenous substrate of the reaction. To pursue this further, extensively purified adrenal cortical ribosomes, without associated protein kinase activity, were prepared as substrate; the partially purified protein kinase of the cytosol, without significant endogenous phosphorylation activity, was used as the enzyme. The phosphorylation of ribosomes was shown to be dependent upon both cAMP and protein kinase.

The dependence on cAMP of the protein kinase catalysis of ribosomal phosphorylation in response to increasing concentrations of cAMP is depicted in Fig. 2; a half-maximal saturation concentration of about 4×10^{-8} M cAMP is observed.

TABLE 3. *Protein kinase-dependent phosphorylation of ribosome-associated protein*

Substrate	Protein (μg)	^{32}P Incorporation (nmol/mg per min) − cAMP	+ cAMP
$MgCl_2$-precipitated ribosomes	219	0.05	0.28
80S ribosomes	260	0.05	0.26
0.5 M NH_4Cl extract	202	<0.01	<0.01
0.5 M NH_4Cl-washed ribosomes	192	0.09	0.58
0.88 M KCl extract	236	0.09	0.21
0.88 M KCl-washed ribosomes	196	0.10	0.59
60S subunit	175	<0.01	<0.01
40S subunit	188	<0.01	<0.01

The reaction mixture was the same as described in Fig. 2, except for the substrate as indicated above and 5 μm cAMP, when added. The reaction mixtures were washed with hot TCA. Values represent the total amount of ^{32}P incorporated in the presence of enzyme minus the sum of that observed without enzyme and with enzyme without substrate.

This constant is similar to that obtained with the cAMP activation of protein kinase when protamine is the substrate. The reaction was shown to be dependent upon increasing amounts of ribosomes, with saturation occurring at about 0.15 mg of ribosomes; cAMP increased the incorporation of ^{32}P into ribosomes, at saturation, by as much as 5-fold. Phosphorylation of the ribosomes without the addition of the protein kinase was minimal and was not stimulated by cAMP.

The specificity of cAMP was compared with other cyclic nucleotides for activating the phosphorylation of ribosomes. Substitution of cGMP, cIMP, and cCMP for cAMP revealed that only cIMP significantly stimulated protein kinase activity. At 1×10^{-7} M cIMP (the maximal saturation concentration of cAMP), the stimulation was only 20% of that observed with the same concentration of cAMP.

The following experiments indicate that the substrate phosphorylated is a protein associated with the 80S ribosomes. The ribosomal fraction was phosphorylated and the reaction mix was sedimented through a 10–30% sucrose gradient. The fractions from the gradient were analyzed for both absorbance at 260 nm and ^{32}P incorporation into hot-TCA-precipitable protein. The peak of radioactivity (Fig. 3*A*) sediments in parallel with the ribosomal peak, at 80 S (Fig. 3*B*). The phosphorylation of the ribosomal peak appears to be almost entirely dependent upon cAMP, since only minimal phosphorylation is observed without the addition of the nucleotide.

Phosphorylation of ribosome-associated protein

Ribosomes were phosphorylated and the RNA was solubilized by heating in TCA at 90°C for 15 min. The ^{32}P incorporated into ribosomes remains associated with the precipitated protein; it was not released with the solubilized RNA. The protein was hydrolyzed and the amino acids were separated by high-voltage electrophoresis. The radioactivity cochromatographed with authentic samples of phosphoserine and phosphothreonine (Fig. 4).

Wash with high-salt buffers and protein synthesis

Factors regulating initiation and polypeptide chain elongation are adsorbed onto ribosomes and are released by incubation in high-salt buffers (28–30). Therefore, to further characterize the ribosomal substrate of the cAMP-dependent phosphorylation reaction, the ribosomes were washed with 0.5 M NH_4Cl. As shown in Table 2, factors essential for *in vitro* ribosomal protein synthesis were eluted from the ribosomes. The amino acid-incorporating system contains aminoacyl-tRNA and highly purified transferase I. This indicates that the NH_4Cl wash removes other factors from the ribosomes that are essential in protein synthesis, presumably, as shown in other mammalian systems, involved in initiation (29, 33) and polypeptide chain elongation (28). Nevertheless, this treatment failed to diminish the effectiveness of the ribosome as a substrate for the cAMP-dependent protein kinase reaction. Indeed, the salt wash only diminished the content of ribosomal protein relative to RNA, thus producing a more-purified ribosome. When the ribosomes were further washed in 0.88 M KCl, and sedimented through a 15–30% sucrose gradient, a portion of the ribosomes placed on the gradient were dissociated into 60S and 40S subunits (20). As illustrated in Table 3, the subunits sedimenting at 60S and 40S failed to serve as a substrate for the protein kinase reaction; the substrate, however, now appeared in the 0.88 M KCl extract; i.e., the supernatant after centrifugation. Also, ribosomes that failed to dissociate into subunits in response to the 0.88 M KCl wash still contained the substrate of the protein kinase reaction.

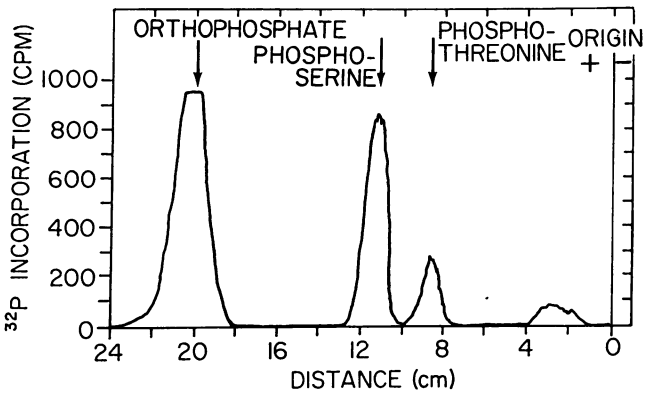

FIG. 4. Radiochromatogram of an acid hydrolysate of phosphorylated ribosomal protein. Protein kinase-dependent ^{32}P incorporation of ribosomes was determined as described in the legend to Fig. 3. The reaction mixture, with 5 μM cAMP, was increased proportionally to contain 44 A_{260} units of ribosomes. The reaction was stopped by the addition of 7.5 ml of cold H_2O, layered on 0.5 M sucrose in TKM buffer containing 0.5 M NH_4Cl, and centrifuged at 100,000 $\times$ *g* for 2 hr at 4°C. The ribosomal pellet was resuspended in 1.5 ml of 10% TCA and protein was isolated by the hot-TCA wash procedure. The ribosomal protein was hydrolyzed in 1 ml of 6.7 M HCl for 7 hr at 108°C in a sealed, evacuated ampule. The HCl was removed under reduced pressure, and aqueous solutions of the residue were paper electrophoresed (26). The ^{32}P-labeled hydrolysate was coelectrophoresed with phosphoserine, phosphothreonine, and orthophosphate. Compounds were visualized with either 0.1% ninhydrin in ethanol or a phosphate spray (27). Radioactive spots were located with a radiochromatogram scanner (Packard Instrument Co., Inc.).

DISCUSSION

The present investigation demonstrates that the cAMP receptor and cAMP-dependent protein kinase are both found in the endoplasmic reticulum of the adrenal cell, suggesting the presence of a receptor–enzyme regulatory complex in this subcellular fraction similar to that previously demonstrated in the cytosol (11, 12).

The cAMP-dependent protein kinase catalyzes the phosphorylation of a protein associated with ribosomes. Serine and threonine residues are phosphorylated, as observed with other substrates of this enzymic reaction (26). Loeb (34) has shown that cAMP stimulated a protein kinase from rat liver to phosphorylate ribosomal protein, and Kabat (35) has demonstrated *in vivo* a phosphorylation of reticulocyte ribosomes not dependent on cAMP.

The substrate of the protein kinase reaction remained associated with ribosomes despite several washes in $MgCl_2$ during the ribosomal purification procedure, and sedimented with the ribosome peak through a sucrose gradient. NH_4Cl, which removed factors from the ribosomes that are required in *in vitro* protein synthesis, also failed to elute the substrate of the cAMP-dependent reaction. The substrate was eluted from the ribosomes by 0.88 M KCl only when the ribosomes were dissociated into subunits; the subunits were not phosphorylated in the protein kinase reaction. Although the function of the ribosome-associated protein is not defined by these studies, the removal from the ribosomes of the substrate of the cAMP-

dependent protein kinase reaction by concentrated KCl indicates that the ribosome-associated protein is relatively specific for the enzymic reaction. This is of interest since the cAMP-dependent protein kinase catalyzes the phosphorylation of numerous substrates, such as histone (36), protamine, casein, and phosphorylase kinase (14), a lipoprotein lipase (37, 38), glycogen synthetase (39), and RNA polymerase (40). In our study, the ribosomal protein of the subunits, as well as the factors eluted with NH_4Cl, failed to serve as substrate in the cAMP-dependent protein kinase reaction. Studies are in progress to isolate the protein(s) extracted from the ribosomes by 0.88 M KCl, i.e., the substrate of the protein kinase reaction, and to identify its function in ribosomal protein synthesis.

The fact that the cAMP receptor and cAMP-dependent protein kinase are located in the endoplasmic reticulum of the microsomes may be of importance in the cAMP-dependent phosphorylation of ribosomes. When we consider the numerous proteins that have been demonstrated to be a substrate for this enzyme, perhaps some specificity is obtained from the intracellular localization of both the enzyme and its substrate.

We suggested that ACTH, acting through cAMP, regulates adrenal function by modulating protein synthesis at the level of the translation of mRNA (6, 8, 9). The presented data demonstrate that the adrenal cell contains a cAMP-dependent protein kinase that phosphorylates protein tightly associated with ribosomes. We postulate that this event has a regulatory role in the hormone-mediated translational control of adrenal cortical protein synthesis.

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