were stimulated directly through platinum electrodes with a Grass S44 stimulator. Stimulation frequency was either 66 or 100 Hz. No differences in results were found between these two frequencies. Muscle length was controlled with a Cambridge Technology dual-mode servo system (model 300 H). The switch from length control to force control was made with an appropriate timing circuit. Force was monitored by the Cambridge Technology system or a Kistler-Morse force transducer (model DSK-3), and both force and length signals were recorded on a Gould Brush recorder (model 440) and a Gould digital oscilloscope (model OS4000). Length and time data were transferred to a Tektronix storage oscilloscope for comparison.

9. All values given are means ± standard errors.
10. Myosin light chain phosphorylation was measured in muscles frozen with modified Wallenberger tongs cooled with liquid nitrogen. This method (*7*) involves extraction of the muscle with $HClO_4$ and isoelectric focusing of the proteins in a urea-containing polyacrylamide gel. The phosphorylated form of the 18,000-dalton light chain has a lower isoelectric point than does the nonphosphorylated form. The gels are stained with Coomassie blue and scanned at 570 nm. This method has been found to give results nearly identical to those obtained with the more common two-dimensional technique. In all experiments a rest period of at least 30 minutes was allowed between procedures on the same muscle. This period was sufficient for light chain phosphorylation to return to a value of less than 10 percent.
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12. Supported by NIH grant HL 15835 to the Pennsylvania Muscle Institute. T.M.B. is the recipient of NIH research career development award AM 00973.

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1 February 1983; revised 17 March 1983

Selective Reduction of Forskolin-Stimulated Cyclic AMP Accumulation by Inhibitors of Protein Synthesis

Abstract. *Inhibiting protein synthesis by incubating C6-2B rat astrocytoma cells with cycloheximide or emetine for periods up to 24 hours caused a progressive decrease in the accumulation of adenosine 3′,5′-monophosphate (cyclic AMP) when the cells were challenged for 30 minutes with 100* μM *forskolin. In contrast, cholera toxin–stimulated (6 n*M*, 3 hours) cyclic AMP accumulation was not diminished in cycloheximide-treated cells, and cyclic AMP was only minimally diminished in response to a 30-minute challenge with 10* μM *(−)-isoproterenol. These experiments suggest the presence of a previously unrecognized cyclase component, which is essential for forskolin-stimulated cyclic AMP accumulation and has a shorter half-life than the* β*-adrenergic receptor, the guanine nucleotide regulatory proteins, or the cyclase catalytic component.*

The mechanism of hormone-stimulated adenosine 3′,5′-monophosphate (cyclic AMP) accumulation in eukaryotic cells is currently thought of as involving three components of the membrane-bound adenylate cyclase system: hormone receptors, guanine nucleotide regulatory proteins (abbreviated G/F or N), and a catalytic component (*1*). Adenylate cyclase activity, although most commonly increased by hormonal interaction with receptors, can occur without hormone receptors—for example, by the direct action of cholera toxin on G/F (*2*) or by the action of the diterpene forskolin on the catalytic moiety (*3*). Cholera toxin is believed to act by catalyzing the adenosine diphosphoribosylation of G/F component peptides (*4*). Forskolin is believed to act by direct stimulation of the catalytic component, because it stimulates cyclase activity in G/F-deficient mutants of S49 murine lymphoma cells (*4*) and in other cyclase preparations apparently devoid of hormone receptors or G/F (*5*).

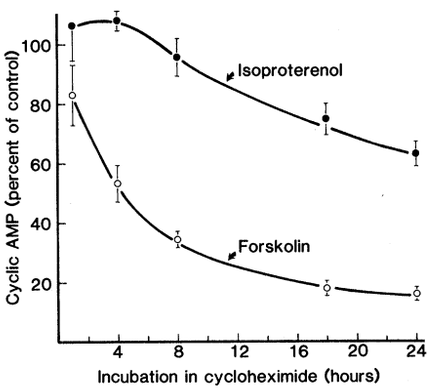

Fig. 1. Time course of the effect of cycloheximide (5 μg/ml) on a 30-minute challenge with 100 μ*M* forskolin or 10 μ*M* (−)-isoproterenol in C6-2B cells. Cells were treated with cycloheximide for the indicated times and intracellular cyclic AMP was measured after a 30-minute challenge with either 100 μ*M* forskolin or 10 μ*M* (−)-isoproterenol. Results are the means ± standard error (S.E.M.) of five to ten separate experiments, with three culture wells used at each time point. The results are expressed as the percentage of the cyclic AMP response in cells not incubated with cycloheximide.

We report that prolonged inhibition of protein synthesis in C6-2B rat astrocytoma cells reduces cyclic AMP accumulation stimulated by forskolin but does not reduce that stimulated by cholera toxin, and either does not reduce or minimally reduces that stimulated by catecholamines. These data suggest that forskolin acts on a rapidly turning over site of the adenylate cyclase system that is not necessary for cyclic AMP accumulation stimulated by cholera toxin or hormone but is essential for that stimulated by forskolin.

Confluent monolayers of C6-2B rat astrocytoma cells (passages 12 through 28) were grown (16-mm wells) in a humidified atmosphere at 37°C in Ham's F-10 medium (Gibco) supplemented with 10 percent donor calf serum (*6*). The cells were placed in serum-free medium 24 hours before the initiation of the experiments. Inhibitors of protein and RNA synthesis were added for various times up to 24 hours. At the end of these time periods the cells were stimulated with maximal concentrations of the following agents to test the functionality of cyclase components after inhibition of protein synthesis. The cells were challenged with either 100 μ*M* forskolin or 10 μ*M* (−)-isoproterenol for 30 minutes or with 6 n*M* cholera toxin for 3 hours. The responses of the cells were compared with those of cells not previously treated with the protein synthesis inhibitors. Cellular cyclic AMP content was determined as described (*7*, *8*). The efficacy of the protein synthesis inhibitors and of RNA synthesis inhibitors was tested by incubating cells with [^{3}H]leucine and [^{3}H]uridine, respectively, and then measuring radioactivity incorporated into cell homogenates precipitated with trichloroacetic acid.

Incubation of C6-2B cells with cycloheximide (5 μg/ml) caused a progressive and pronounced loss of forskolin-stimulated cyclic AMP accumulation (Fig. 1). After 8 hours of treatment with cycloheximide, the cells had lost more than 65 percent of their responsiveness to forskolin, and by 24 hours only 15 percent of the forskolin response remained. In contrast, the responsiveness to (−)-iso-

Table 1. Effect of 3-hour challenge with 6 n*M* cholera toxin on cyclic AMP accumulation (measured as picomoles per milligram of protein) in cells previously incubated with cycloheximide (5 μg/ml) for 24 hours. Cycloheximide (5 μg/ml) was included in the 3-hour challenge so that comparison could be made with cells incubated with cycloheximide for 24 hours. Earlier studies (*9*) have demonstrated that cycloheximide increases the response to cholera toxin after 3 hours. Cholera toxin increased cyclic AMP to 210 ± 10 after 3 hours in the absence of cycloheximide. As shown in Table 2, cycloheximide had no effect on basal levels of cyclic AMP.

Treatment	Cyclic AMP (pmole/mg)	
	Control	Cholera toxin
None	25 ± 3	1121 ± 189
Cycloheximide	31 ± 4	1551 ± 138

Table 2. In three separate experiments C6-2B cells were incubated with the protein or RNA synthesis inhibitors for 24 hours. The intracellular cyclic AMP accumulation in response to a 30-minute challenge with 100 μM forskolin or 10 μM (−)-isoproterenol is indicated. Results are means ± S.E.M. for three determinations.

Treatment	Cyclic AMP (pmole/mg)		
	Control	Forskolin	Isoproterenol
None	55 ± 6	4075 ± 277	5866 ± 110
Cycloheximide (5 μg/ml)	52 ± 4	831 ± 127	3951 ± 118
None	61 ± 9	4473 ± 177	5224 ± 210
Emetine (1 μg/ml)	55 ± 3	848 ± 71	3860 ± 238
None	24 ± 2	2686 ± 114	5572 ± 295
DRB (100 μM)	23 ± 8	3004 ± 30	5124 ± 374

proterenol was unaltered after 8 hours of cycloheximide treatment and was decreased about 35 percent after 24 hours. The responsiveness of the cells to cholera toxin was unaltered after 24 hours of cycloheximide treatment (Table 1).

Incubation of cells for 24 hours with emetine, another protein synthesis inhibitor, gave results similar to those obtained with cycloheximide (Table 2). However, treatment of the cells for up to 24 hours with the RNA synthesis inhibitor 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) caused no loss of cellular responsiveness to (−)-isoproterenol, cholera toxin, or forskolin (Table 2). Incubation of the cells with cycloheximide or emetine caused a 93 percent and 95 percent inhibition of [^{3}H]leucine incorporation, respectively, and had no effect on [^{3}H]uridine incorporation. An 85 percent inhibition of RNA synthesis, but no inhibition of protein synthesis, was observed with DRB treatment.

The more labile component of the cyclase system required for forskolin-stimulated cyclic AMP accumulation identified by these experiments is unlikely to be the β-adrenergic receptor, the guanine nucleotide regulatory component, or the catalytic component of cyclase. Cyclic AMP production stimulated by cholera toxin or the β receptor was either unaltered or slightly reduced after an 8- to 24-hour incubation with cycloheximide, yet these conditions reduced the responsiveness of the cells to forskolin by 65 to 85 percent. If it is assumed that forskolin directly stimulates the catalytic subunit of cyclase, it is difficult to reconcile a linear model of the hormone-sensitive adenylate cyclase with these data.

There are, however, several other ways to interpret these data. The cyclase catalytic unit involved in forskolin-stimulated cyclase activity may differ from the one necessary for hormone- or cholera toxin–stimulated cyclic AMP accumulation. Another possibility is that forskolin stimulates adenylate cyclase activity through a regulatory protein not necessary for hormone- or cholera toxin–stimulated cyclase activity. Our experiments do not differentiate between these two possibilities. However, we can conclude that the message for the putative protein is long-lived because inhibition of RNA synthesis for 24 hours did not reduce the responsiveness of forskolin, and the half-life of the protein is probably shorter than those of the β-adrenergic receptor, G/F, or catalytic cyclase.

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18 October 1982; revised 30 November 1982

In vivo One-Dimensional Imaging of Phosphorus Metabolites by Phosphorus-31 Nuclear Magnetic Resonance

Abstract. *A phosphorus-31 nuclear magnetic resonance imaging technique has been used to obtain information on phosphorus metabolites from different spatial regions of tissues in vivo. The technique for selection of planes through the tissue is based on phase-encoding of spin echoes and was used to obtain one-dimensional discrimination of phosphorus-31 spectra from different parts of the tissue simultaneously. Specimens were resolved into 16 distinct slices and a signal-to-noise ratio of about 20 to 1 was obtained in ½ hour. Results are presented for phantoms, rat legs, and gerbil heads.*

Phosphorus-31 muclear magnetic resonance (^{31}P NMR) is a valuable noninvasive method for investigating the metabolic state of tissues in vivo (*1–3*). In order to realize the full potential of the method, it will be important to be able to discriminate different spatial regions of the tissue. Topical magnetic resonance (*4*) with surface coils has been used for this purpose (*5*), but a more versatile approach would be to use NMR imaging techniques to discriminate spatially different regions of the tissue simultaneously. Although there have been speculations, based on images of phantoms, about the possibility of using ^{31}P NMR imaging in vivo (*6, 7*), to our knowledge this is the first report of experimental in vivo ^{31}P NMR imaging.

There are two main differences between ^{31}P NMR imaging and the rather well-developed techniques of proton imaging which prevent the direct use for ^{31}P measurement of the techniques developed for ^{1}H. First, the concentration of ^{31}P metabolites in tissues is about 10^4 to 10^5 times lower than the proton concentration. Second, the biochemical information in ^{31}P signals can be obtained only under "high-resolution" conditions; that is, one must be able to distinguish the ^{31}P NMR signals from different metabolites, say by chemical shift differences. Spatial and spectral resolution of the "high-energy" phosphate metabolites, such as adenosine triphosphate (ATP) and phosphocreatine (PCr), and the "low-energy" phosphate metabolites, such as inorganic phosphate (Pi) and sugar phosphates (SP), will yield valuable information about the state of the tissue. Hence it is of practical importance to study the whole spectrum at each part of the tissue. We describe here a technique for simultaneously recording the ^{31}P NMR spectra from different planes through the tissue and present our results for rat legs and gerbil heads.

Nuclear magnetic resonance spectra at 1.4 T were recorded with a John-