a rebound phenomenon without substantial "overshoot." In each case, however, at least one pulse of $PGF_{2\alpha}$ preceded the first detectable elevation in the circulating LH. Further experiments will be required to establish the latter relationship statistically.

In the two animals that received only autologous plasma, there was no statistically significant alteration in the frequency or pulse height of the peaks of $PGF_{2\alpha}$. In one of the control experiments (not shown), plasma LH increased slightly (to 12 ng/ml) for a brief period, beginning about 4 hours after the infusion of autologous plasma ended.

Since cells are not thought to store preformed PG's in significant quantities (*11*), the Δ_{V-A} for $PGF_{2\alpha}$ was taken to represent net synthesis of $PGF_{2\alpha}$ by the brain. Using Δ_{V-A} as an index, our report shows that (i) synthesis of $PGF_{2\alpha}$ by the brain can be monitored with relative ease under physiological conditions in the conscious, resting animal; (ii) the release of $PGF_{2\alpha}$ by the brain occurs in pulses whose frequency approximates the circhoral rhythm of LH release in ovariectomized ewes (*12*); and (iii) after the infusion of 17β-E_2, the pulses of $PGF_{2\alpha}$ first decline and later return to control levels coincident with the LH surge.

These pulses might conceivably come from nonneural elements such as the cerebrovascular system. At present, it seems more likely that they arise from the brain itself because (i) central nervous tissue is known to synthesize PG's at several loci (*13*) and (ii) neurogenic stimuli can release PG's from the brain and spinal cord (*13*). Moreover, the system that produces the pulses of $PGF_{2\alpha}$ is apparently sensitive to estrogen, a property it shares with many central neurons. Because the system is estrogen-sensitive, measurement and comparison of the relative suppressive effects of intra-arterial and intraventricular infusions of 17β-E_2 on the pulses of $PGF_{2\alpha}$ might indicate whether the pulses arise from blood vessels or from neurons.

Since current evidence suggests that LH release is associated with elevated PG's, the initial decline in Δ_{V-A} for $PGF_{2\alpha}$ might at first sight seem paradoxical. However, in addition to its positive feedback actions, estrogen has well-known negative feedback effects on the secretion of LH. In particular, 17β-E_2 reduces the basal level of LH in the blood of cycling ewes (*14*) and eliminates the circhoral pulses of LH observed in ovariectomized ewes (*15*). Since the rate at which 17β-E_2 reduces LH in these instances approximates the rate at which we observed it to suppress Δ_{V-A} for $PGF_{2\alpha}$ in anestrous ewes, the declining pulses of $PGF_{2\alpha}$ may well be a manifestation of the negative feedback action of estrogen on LH release.

Similarly, the recurrence of large-amplitude pulses of $PGF_{2\alpha}$ immediately before the actual surge of LH may play a role in the positive feedback action of 17β-E_2. This conclusion is consistent with the finding that plasma LH increases shortly after intracarotid infusion of $PGF_{2\alpha}$ (*4*) and with the related observation that 17β-E_2 cannot induce LH release in sheep undergoing treatment with indomethacin (*5*).

Although these points are compatible with the hypothesis that PG's of central origin play a role in mediating the negative and positive feedback effects of estrogen on LH secretion, they do not prove that either the pulsatile pattern of $PGF_{2\alpha}$ synthesis or its modulation over periods of time are necessary conditions for the regulation of LH secretion. It is possible that these phenomena may reflect as yet unknown events in neuroendocrine control processes. Assessment of the significance of the pulses of $PGF_{2\alpha}$ and their apparent modulation by 17β-E_2 must therefore await further investigation.

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Cyclic AMP-Dependent Protein Kinase: Pivotal Role in Regulation of Enzyme Induction and Growth

Abstract. *Dibutyryl cyclic adenosine 3',5'-monophosphate (cyclic AMP) produces phosphodiesterase induction, growth arrest, and cytolysis in S49 lymphoma cells. The striking parallelism between protein kinase activity that is dependent on cytosol cyclic AMP and cellular responses to dibutyryl cyclic AMP in wild-type cells and three classes of clones resistant to cyclic AMP indicates that protein kinase mediates cyclic AMP regulation of growth and enzyme induction in S49 cells.*

The ways in which cyclic adenosine 3',5'-monophosphate (cyclic AMP) exerts its diverse regulatory effects in eukaryotic cells are still unknown. Investigations of enzymes in cell-free systems have established that cyclic AMP regulates lipolysis and glycogen metabolism by activating cyclic AMP-dependent protein kinase or kinases (*1*). Similar cyclic AMP-dependent kinases are ubiquitous in mammalian cells (*2*), implying that they are involved in other cyclic AMP-mediated processes as well, such as regulation of cell growth and differentiation.

Enzymologic approaches have not revealed these other roles of cyclic AMP-dependent protein kinase. We now report the results of a genetic approach for investigating the role of cyclic AMP-dependent protein kinase in cell regulation. Cultured S49

mouse lymphoma cells (*3*) respond to cyclic AMP by induction of cyclic AMP phosphodiesterase, cycle-specific growth arrest, and subsequent cytolysis (*4–6*). In contrast to their wild-type (WT) parents, a class of mutant S49 cells, completely unresponsive to cyclic AMP, also lacks cyclic AMP binding activity (*7*) and cyclic AMP–dependent kinase activity (*8*). We concluded on this basis that the kinase mediates cyclic AMP's actions in wild-type cells; however, we could not exclude the possibility that a mutation with pleiotropic effects simultaneously affected both kinase and cyclic AMP responsiveness in the resistant cells. The phenotypes of other variant S49 clones, reported here, provide compelling evidence for the pivotal role of the kinase in cyclic AMP–mediated regulation. Comparison of these phenotypes demonstrates not only that the kinase's affinity for cyclic AMP determines the molar potency of the cyclic nucleotide in regulating the intact cell, but also that the amount of cellular kinase activity available for stimulation by cyclic AMP apparently determines the cell's maximal response.

N^6,$O^{2'}$-dibutyryl cyclic AMP arrests wild-type S49 cells in the G_1 phase of the cell cycle (*5*); 48 to 72 hours later the cells die (*4, 7, 8*). This cytocidal effect allows the selection of clones of variant S49 cells that can proliferate in soft agar containing the dibutyryl derivative, as described (*7*). Such clones can be grown to mass culture in the absence of selective drugs. In this report we compare the wild-type parental line with three classes of dibutyryl cyclic AMP–selected clones [designated kin A, kin B, and kin C (*9*)], with respect to (i) activity in cell lysates of cyclic AMP–dependent protein kinase, assessed by binding of ^{3}H-labeled cyclic AMP (presumably to the enzyme's regulatory subunit) (Fig. 1a) or by cyclic AMP–stimulated phosphorylation of histone (Fig. 1b); and to (ii) the biologic effects of dibutyryl cyclic AMP in intact cells on phosphodiesterase induction, growth inhibition in G_1, and cellular proliferation (Fig. 2).

The concentrations of cyclic AMP required for half-maximal stimulation of histone phosphorylation or of specific ^{3}H-labeled cyclic AMP binding are approximately ten times higher in kin A than in wild-type cytosol (Fig. 1). Similarly, intact kin A cells require about ten times more dibutyryl cyclic AMP than do wild-type cells for equivalent growth inhibition and enzyme induction (Fig. 2). However, maximum effects of cyclic AMP and its dibutyryl derivative in cytosols and in intact cells, respectively, are similar in kin A and wild-type cells (Figs. 1 and 2).

Another variant, kin B cells, has a quite different lesion. In kin B cytosol maximal ^{3}H-labeled cyclic AMP binding and cyclic AMP stimulation of kinase activity are about half the value measured in wild-type cells (Fig. 1). Similarly, maximally effective concentrations of dibutyryl cyclic AMP produce in kin B cells only about half the phosphodiesterase induction and growth inhibition observed in wild-type cells (Fig. 2). However, cyclic AMP concentrations required for half-maximal stimulation are similar in kin B and wild-type cells.

Clone kin C (*5–7*) is completely resistant to dibutyryl cyclic AMP in culture (Fig. 2) and lacks both cyclic AMP–stimu-

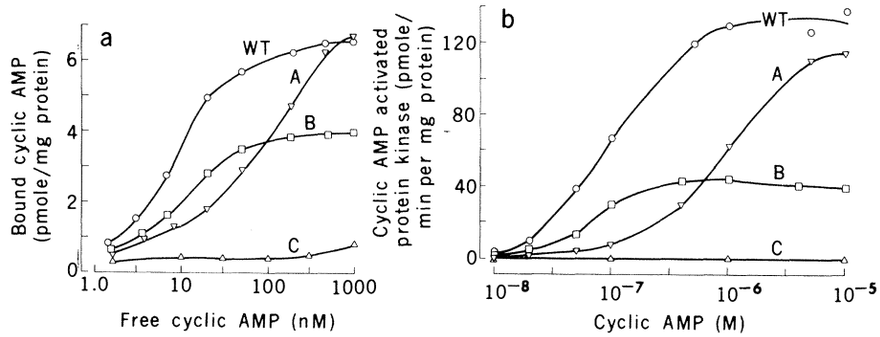

Fig. 1 (above). ^{3}H-Labeled cyclic AMP binding (a) and cyclic AMP stimulated histone phosphorylation (b) by cytosol extracts from wild-type (*WT*) and variant clones. (*A, B,* and *C* designate kin A, kin B, and kin C, respectively). Cells were sonicated, and cytosol extracts were prepared by centrifugation at 100,000*g* (*7, 8, 15*). ^{3}H-Labeled cyclic AMP binding was measured by Millipore filtration (*7, 16*) after incubation of 60 to 100 μg of cytosol protein with 50 m*M* sodium acetate (*p*H 6.5), and various concentrations of ^{3}H-labeled cyclic AMP (37.7 c/mmole, New England Nuclear) in a total volume of 0.12 ml. All values were corrected for nonspecific binding measured in the presence of nonradioactive 1.0 m*M* cyclic AMP and represent the means of duplicate determinations in a typical experiment. Protein was measured by the method of Lowry *et al.* (*17*). (b) Kinase reactions were initiated by adding 10 to 200 μg of cytosol protein to an incubation mixture containing 100 μg of f2b calf thymus histone (Sigma), 10 m*M* $MgSO_4$, 10 m*M* dithiothreitol, 50 m*M* sodium acetate (*p*H 6.5), 0.62 mg of bovine serum albumin, 20 μ*M* [γ-^{32}P]ATP (100 to 300 count/min per picomole (International Chemical Nuclear), and the indicated concentrations of cyclic AMP in a volume of 0.25 ml. After 5 minutes at 30°C, incubations were terminated with 4 ml of ice-cold 5 percent trichloroacetic acid containing 1 m*M* ATP, 2 m*M* sodium pyrophosphate, and 5 m*M* sodium phosphate. Tubes were centrifuged at 2000*g* for 5 minutes, and pellets were redissolved in 0.1 ml of 1*N* NaOH. After reprecipitation with 4 ml of ice-cold 5 percent trichloroacetic acid, the samples were filtered over GF-C (Whatman) filters and washed with 12 ml of cold trichloroacetic acid. The radioactivity on the filters was then counted by the liquid scintillation method. The phosphorylation of histone increased linearly with time (up to 10 minutes) and with increasing amounts of cytosol protein (up to 200 μg). All values were corrected for activity in the absence of cyclic AMP and represent the mean values of at least four separate experiments.

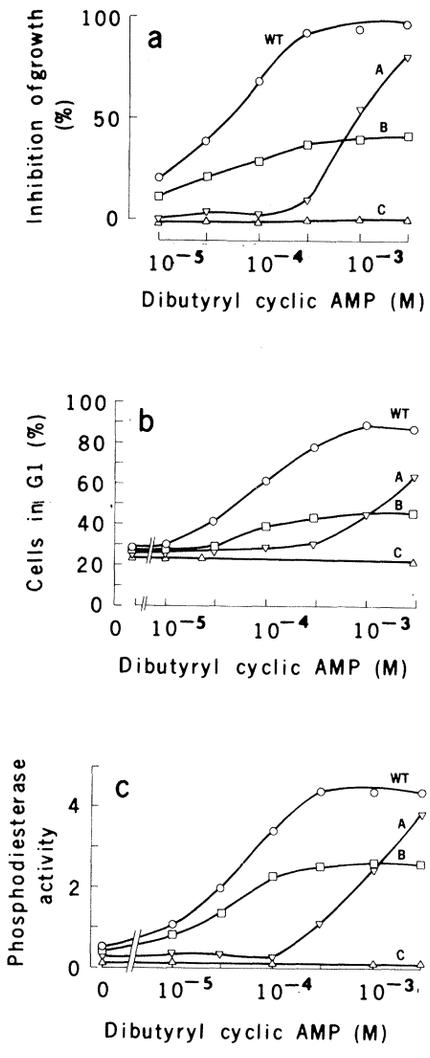

Fig. 2 (right). Biologic effects of dibutyryl cyclic AMP on intact wild-type and variant S49 cells. The indicated concentrations of dibutyryl cyclic AMP were added to cells growing logarithmically under standard growth conditions (*3–8*). (a) Reduction of viable cell number (reflecting cell death and inhibition of cell proliferation) was assessed by counting cells that excluded trypan blue after 72 hours (four doubling times) (*7*). (b) The percentage of cells in the G_1 phase of the cell cycle after 24 hours in the presence or absence of dibutyryl cyclic AMP was measured by flow microfluorimetry of cells stained for DNA (*5*). (c) Phosphodiesterase induction was measured (*18*) by determining enzyme activity in crude sonicated cell extracts (nanomoles per 40 minutes per milligram of protein) after 8 hours of incubation of intact cells with dibutyryl cyclic AMP, as described (*6, 7*).

lated kinase activity and ^{3}H-labeled cyclic AMP binding (Fig. 1).

Thus, the dose-response relationships for all four types of clones determined in vitro by assay of catalytic or binding activities of cyclic AMP–dependent protein kinase accurately predict the response of the corresponding intact cells to exogenous dibutyryl cyclic AMP. The differing properties of the variant enzymes are also reflected by the responses of the corresponding clones to endogenous cyclic AMP, elevated within the cell in response to stimulators of adenylate cyclase, such as cholera enterotoxin (results not shown).

In addition to establishing the role of cyclic AMP–dependent protein kinase in S49 cells, these results offer insight into the quantitative relationship between cyclic AMP concentrations and biologic effects. In several tissues, hormones can stimulate the accumulation of more cyclic AMP than necessary for a maximum biologic response (*10*). It is not known which cellular components limit maximum responses to cyclic AMP. Clone kin B contains reduced kinase activity and exhibits a proportional decrease in biologic response to cyclic AMP. Thus, at least in the S49 cell, the cellular content of cyclic AMP–dependent kinase determines responsiveness to the cyclic nucleotide.

Kuo and Greengard (*2*) have proposed that in animal cells all cyclic AMP effects are mediated by protein kinase. Our results are consistent with this hypothesis. However, we cannot formally exclude the possibility that these actions of cyclic AMP are mediated by the enzyme's cyclic AMP–binding subunit alone, in an analogous fashion with the cyclic AMP receptor protein of *Escherichia coli* (*11*), rather than by phosphorylation of specific protein substrates.

The precise biochemical lesions of the clones which we describe remain to be defined. Mixing experiments, with cytosol extracts of the wild type and the three variants, provide no evidence for an aberrant activator or inhibitor of cyclic AMP–dependent kinase in any of the clones (data not shown). The cyclic AMP–stimulated enzymes of all three cyclic AMP–responsive cell lines show similar requirements for adenosine triphosphate (ATP), histone, and magnesium (*12*).

The phenotype of each cyclic AMP–resistant clone has been stable in the absence of dibutyryl cyclic AMP for more than 200 generations in culture. A working hypothesis, supported by genetic evidence (*13*), is that cyclic AMP resistance represents a mutation. Further studies that indicate that the kin A lesion can be specifically assigned to a structural alteration in one of the subunit peptides for the cyclic AMP–dependent kinase (*14*) provide more direct evidence for the mutational origin of at least some of these variants. The kin B and kin C lesions have not been defined in detail; either or both could represent alterations in cellular concentration of the kinase, because of regulatory rather than structural mutations.

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Collagen Biosynthesis in Blood Vessels of Brain and Other Tissues of the Hypertensive Rat

Abstract. *It was previously shown that hypertension elevates collagen biosynthesis and increases collagen deposition in peripheral arteries and that antihypertensive agents prevent and reverse the increased synthesis. These findings have now been extended to the microvessels of the central nervous system and to other small vessels.*

In a previous report we demonstrated that collagen biosynthesis is increased in a large artery (aorta) and in arteries of medium caliber (mesenteric arteries) of rats made hypertensive by treatment with deoxycorticosterone acetate (DOCA) and in spontaneously hypertensive rats (*1*). We further showed that treatment with the antihypertensive drugs chlorothiazide or reserpine can prevent or reverse this hypertension-induced biosynthesis of vascular collagen.

Recently Brendel *et al.* (*2*) reported a simple procedure for the isolation of metabolically active brain microvessels. We have isolated brain microvessels (arterioles, capillaries, and venules) from DOCA-salt hypertensive rats and have shown elevations in their prolyl hydroxylase activity and in their ability to synthesize collagen in vitro. These markers of collagen biosynthesis were also found to be elevated by hypertension in the pial arteries, in the circle of Willis, and in testicular arteries. Treatment of the DOCA-salt rats with reserpine prevented the increase in blood pressure as well as the increase in vascular prolyl hydroxylase.

Hypertension (*3*) was produced in uninephrectomized, 8-week-old, male Wistar