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A Temperature-Sensitive Change in the Energy of Activation of Hormone-Stimulated Hepatic Adenylyl Cyclase

(epinephrine/glucagon/prostaglandin E_1/fluoride)

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ABSTRACT **We describe an abrupt increase (at 32°) in the energy of activation for the reaction of hepatic adenylyl cyclase in the presence of glucagon or epinephrine. This increase is not seen in the presence of fluoride, prostaglandin E_1, or 1-propanol, or in the absence of cyclase stimulators. The change in energy of activation found with hormones is abolished by 1-propanol. This change does not represent differences in hormone or substrate binding at different temperatures, but seems to reflect interactions among elements of the cyclase stimulation sequence. Similar changes in energy of activation were not observed for alkaline phosphatase, cyclic AMP-phosphodiesterase, 5′-nucleotidase, or ouabain-sensitive ATPase. Since the mole fraction of cholesterol in liver membranes is sufficiently high to preclude a phase change in bulk membrane lipids, our observation suggests either that cyclase is restricted to cholesterol-poor membrane regions or that the change in its energy of activation is largely restricted to protein components of the cyclase apparatus. The data are compatible with fundamental differences in the stimulation process(es) for the hormones (glucagon and epinephrine) as compared with those for fluoride and prostaglandin E_1.**

The mechanism by which hormones and other regulators stimulate adenylyl cyclase (cyclase) is obscure in part because of the possible existence of unidentified participants in the stimulation sequence. One proposed mechanism is based on the observation that magnesium can mimic hormone stimulation of adipocyte cyclase (1). This observation led to the suggestion that hormones facilitate binding of magnesium to an activator site, distinct from the binding site for the substrate, ATP-Mg^{++}. This model, however, does not adequately explain stimulation of cyclase in liver (2) and heart (3), where hormones stimulate in the presence of saturating magnesium concentrations. A constraint model (4, 5) depicts cyclase as intrinsically competent but inhibited, and assigns to the hormone the role of releasing the cyclase catalytic moiety from its physiological constraint. This model is suggested by the observation in several different cells that chemical or mechanical traumata can produce a cyclase that is fully active but no longer responsive to hormones. This result has been found for cyclase in rat brain (6, 7) salmon testis (8), bovine photoreceptor (4), and mouse testicular and pituitary carcinomas (unpublished observations).

The mechanism by which hormonal signals are communicated from receptor to the catalytic moiety is unknown. Factors that participate in the stimulation sequence include calcium [for melanocyte stimulating hormone (9) and ACTH (10)] and GTP [for glucagon, (11), epinephrine (12), and prostaglandins (13)]. Phospholipids also appear to play a role, since hormone stimulation of cyclase is diminished or absent in membranes exposed to phospholipase and may be partially restored by addition of specific phospholipids (14, 15). Observations of basal cyclase activity, and the effects of trauma, calcium, GTP, and phospholipase on cyclase stimulation, may ultimately permit distinctions to be made between different functional (and structural) components of the cyclase apparatus. In this paper we describe an effect that may provide an additional experimental "handle" on the mechanism by which hormones stimulate cyclase. More specifically, experiments with hepatic glucagon- and epinephrine-responsive cyclase systems are presented in which we have observed a profound effect of temperature on the energy of activation of the hormone-stimulated cyclase reaction. This effect is not observed for activation by fluoride, prostaglandin E_1, or 1-propanol.

MATERIALS AND METHODS

Washed membrane particles from livers of normal and adrenalectomized weanling (50 g) female Sprague–Dawley rats were prepared (16). Porcine crystalline glucagon and L-epinephrine bitartrate were from Sigma. Prostaglandin E_1 was a gift from Drs. Caldwell and Speroff of Yale University.

Cyclase activity was assayed as described (17), with these modifications: [8-^{14}C]ATP was used as substrate. Labeled cyclic AMP was purified by descending thin-layer chromatography (20 × 40 cm × 0.4 mm thick layers) on polyethyleneimine (PEI) cellulose (two parts E. Merck polyethyleneimine cellulose and one part Macherey Nagel cellulose powder, molecular weight 300) for 8 hr with 1 M ammonium acetate–methanol 2:5. The origin was removed by shaving just behind the cyclic AMP marker. The plates were then dried, rewicked, and rechromatographed (descending) for 12 hr with 1-butanol–acetic acid–water 2:1:1. Chromatography in the first solvent was at 31°. The purified cyclic AMP, still on PEI cellulose, was shaved directly into scintillation vials, dispersed by sonication, and counted with toluene–2,5-diphenyloxazole (PPO) in the presence of Cabosil in a Beckman LS 200 liquid scintillation spectrometer. In later experiments [^{3}H]ATP was used, and the thin layers were processed with an Oxymat (Intertechnique), thus eliminating sonication and Cabosil. Cyclase was assayed with a 3-min incubation at the temperatures indicated, in the presence of 1.6 mM [8-^{14}C]ATP (45 Ci/mol) or [^{3}H]ATP diluted to the same specific activity, 7 mM aminophylline, and an ATP regenerating system (38 mM phosphocreatine and 80 μg/ml of creatine phosphokinase) in buffer 1 [3 mM $MgSO_4$–0.4 mM EDTA–32 mM glycylglycine (pH 7.4)]. 3′,5′-Nucleotide phosphodiesterase was assayed by incubation in the presence of 1.0 mM [8-^{3}H]cyclic AMP (diluted to 50 Ci/mol) in buffer 1, and measurement of the disappearance of substrate in 1 min. Alkaline phosphatase was assayed with *p*-nitrophenyl phosphate (18). Creatine phosphokinase was assayed in the presence of 38 mM phosphocreatine and 1 mM ADP in

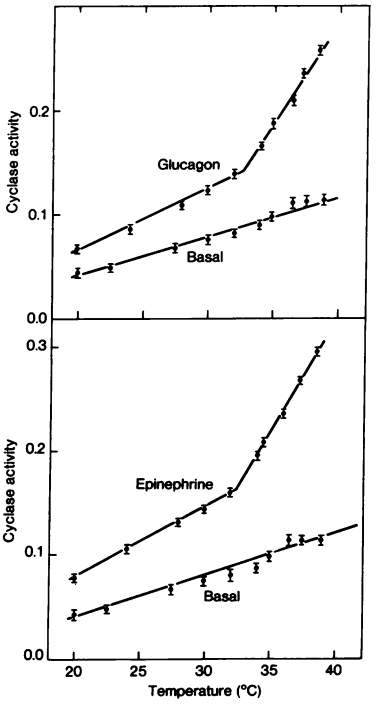

FIG. 1. Cyclase activity (nmol of cAMP per min per mg of protein) of liver washed particle. Data shown are for one of several experiments, with mean and extreme values of a triplicate determination. *Top:* With no added stimulator (basal) or with 16 μM glucagon. *Bottom:* With no added stimulator or with 25 μM epinephrine.

buffer 1 by following the consumption of H^+. Protein concentrations were determined by the method of Lowry *et al.* (19). Steady-state kinetic parameters for the cyclase reaction were determined by incubation at several concentrations of [δ-^{14}C]ATP and fitting of the data to the Michaelis–Menten equation with a linear regression on $v = V_{max} - (v/s)\ K_m$

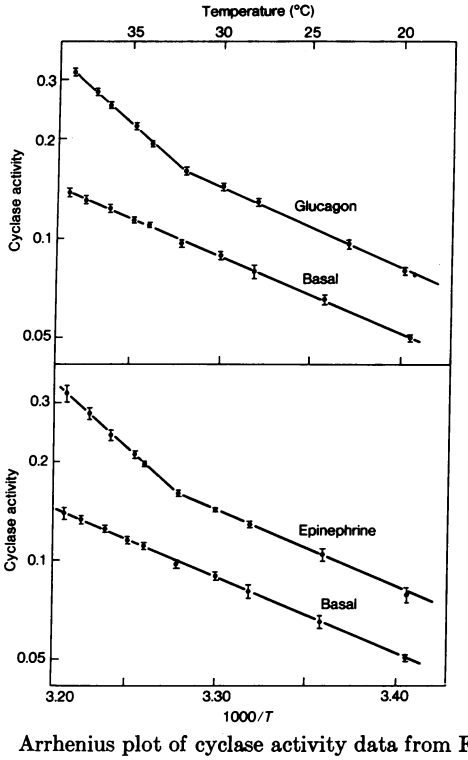

FIG. 2. Arrhenius plot of cyclase activity data from Fig. 1.

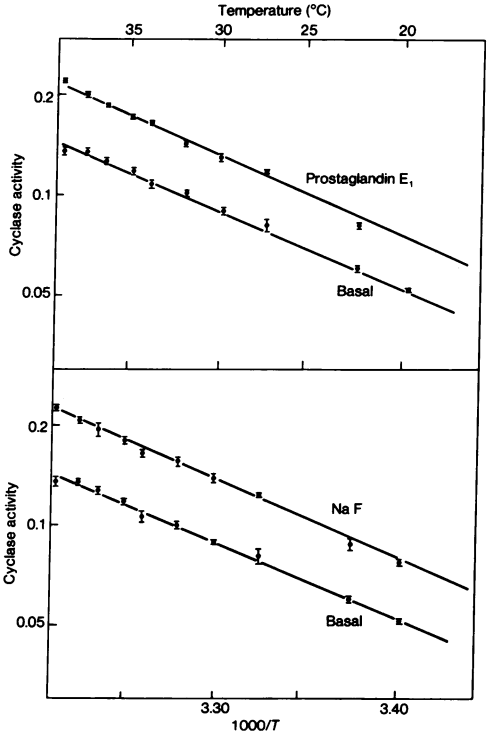

FIG. 3. Cyclase activity (nmol of cAMP per min per mg of protein) of liver-washed particle. *Top:* With no added stimulator (basal) or with 28 μM prostaglandin E_1. *Bottom:* With no added stimulator or with 10 mM NaF.

(20, 21). The slopes for the Arrhenius plots and the existence and location of break points were determined by linear regression analysis. The fit for a single line (from 23–39°) was compared to various possible two-line fits (e.g., one line from 23°–30°, a second from 30°–39°; one line from 23°–32°, a second from 32°–39°, etc.). The fit that gave the smallest value for the sum of the squares of the deviations in activity was selected. The energy (or energies) of activation was determined from the slope(s); for a two-line fit, the temperature at which the two lines intersect was determined.

RESULTS

Fig. 1 shows the temperature dependence for both epinephrine- and glucagon-stimulated hepatic cyclase. Examination of the Arrhenius plots of these data (Fig. 2) indicates that in the presence of hormones, there is a change in the thermodynamic properties of the enzymic mechanism that is characterized by an abrupt increase in the energy of activation at 32°. For both hormone-stimulated reactions, the energy of activation is about twice as great above 32° as below. This difference in energy of activation is statistically significant ($P < 0.001$). The temperature at which this change is observed is very reproducible [e.g., for glucagon the mean value for seven different temperature curves is 32.2 ± 0.5°; for epinephrine the mean value for five different curves is 31.8 ± 0.7° (Table 1)]. The abrupt increase in energy of activation, henceforth referred to as temperature effect, is not observed in the fluoride-, prostaglandin E_1-, or 1-propanol-stimulated enzymes or in the absence of stimulators (basal cyclase activity) (Fig. 3). When the best two-line fit with a break at 32° was found for the basal an fluoride-, 1-propanol-, or prostaglandin-stimulated enzymes, an insignificant change in energy of activation of the fluoride- and 1-

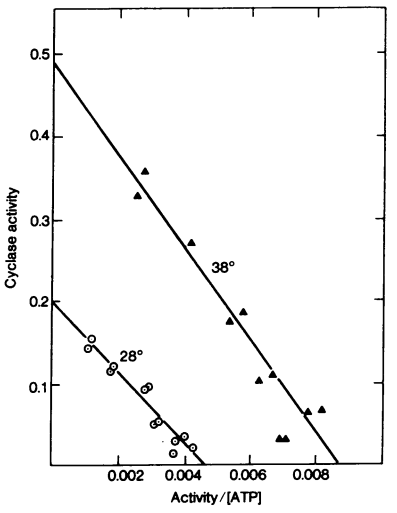

FIG. 4. Eadie plot of hepatic cyclase activity in the presence of 16 μM glucagon at 28° (●) or 38° (▲). Cyclase activity, nmol of cAMP per min per mg of protein; ATP concentration, mM.

propanol-stimulated enzymes was found as a function of temperature between 23 and 39°. A small decrease in the energy of activation (in contrast to the marked increase seen with the glucagon- or epinephrine-stimulated enzyme) was found for basal and prostaglandin-stimulated cyclase activities. This small decrease in activation energy might reflect denaturation at higher temperatures. For data obtained with the hormone-stimulated enzymes, a two-line model with a sharp break gave a much better fit than a one-line model (sum of the squares of the deviations at least 3-times greater for the one-line model). For data obtained with basal, prostaglandin-, 1-propanol- or fluoride-stimulated enzymes, there was no significant advantage to a two-line fit. It is interesting that the energies of activation for the basal cyclase system and for the fluoride- or prostaglandin-stimulated enzymes are similar to the low temperature values for the hormone-stimulated enzymes. Above 39° there is a fall in basal and epinephrine-stimulated cyclase activity, presumably due to thermal denaturation. A fall in glucagon-stimulated activity during the assay is only apparent above 50°. The serine protease inhibitor, phenylmethylsulfonyl fluoride (0.1 mM), does not significantly enhance the enzyme activity (in the presence or absence of hormones) at higher temperatures.

The temperature effect appears to be reversible, since membrane particles that were first incubated at different temperatures (30° or 37° for 5 min) or at one temperature for different times (30° for 1 or 10 min) exhibit the same temperature–activity relationships for basal or glucagon-stimulated cyclase as membrane particles that were kept at 0°. The apparent change in energy of activation with temperature is not explained by changes in the duration of a "lag phase" [which has been described in association with stimulation by hormones (28)] as a function of temperature. The reactions were linear during the entire 3 min of incubation at temperatures above, at, and below the break point. The increased energy of activation for the reaction found with hormones at higher temperatures is accounted for by an increased V_{max}, since, for the glucagon-stimulated cyclase, the K_m for ATP increases slightly at higher temperatures (0.66 ± 0.11 mM at 28° and 0.90 ± 0.17 mM at 38°) (Fig. 4). The effect of temperature on cyclase activity cannot be explained by a change

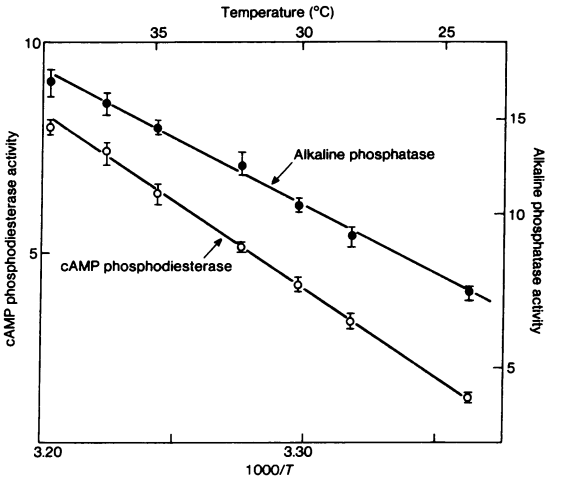

FIG. 5. Alkaline phosphatase activity (●) (nmol of P_i per min per mg of protein) and cAMP phosphodiesterase activity (○) (nmol of cAMP per min per mg of protein) of liver washed particle.

in the pH of the assay mixture with temperature. There is a pH change from 7.4 at 23° to 7.0 at 39°, but the activity of the enzyme is almost constant in this pH range.

The temperature effect with glucagon is not explained by increased glucagon solubility at higher temperatures, since it is observed at several glucagon concentrations below the solubility limits for this peptide. In addition, the tem-

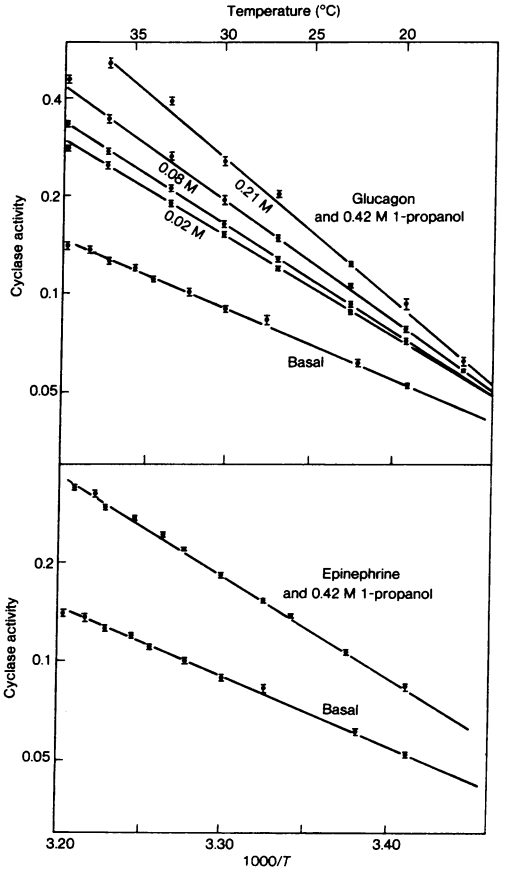

FIG. 6. Cyclase activity (nmol of cAMP per min per mg of protein) in the presence of 1-propanol and hormone. *Top:* With no added stimulator (basal) or with 16 μM glucagon and 0.42, 0.21, 0.08, or 0.02 M 1-propanol. *Bottom:* With no added stimulator or with 25 μM epinephrine and 0.42 M 1-propanol.

TABLE 1. *Effects of stimulators and 1-propanol on energy of activation for the cyclase reaction*

Stimulator	No. of temp. curves	Temp. at which energy of activation breaks (range 23°–39°)	Energy of activation (kcal/mol)		
			Below 32°		Above 32°
None (basal)	9	No break	—	9.8 ± 0.5	—
Glucagon (16 μM)	7	32.2 ± 0.5°	10.0 ± 0.7		17.5 ± 1.1
Epinephrine (25 μM)	5	31.8 ± 0.7°	9.4 ± 0.5		17.6 ± 1.7
Prostaglandin E_1 (28 μM)	4	No break	—	11.1 ± 1.3	—
Fluoride (10 mM)	4	No break	—	11.0 ± 1.0	—
Glucagon (16 μM) and 1-propanol					
(0.42 M)	3	No break	—	20 ± 1.8	—
(0.21 M)	2	No break	—	18 ± 1.3	—
(0.08 M)	2	No break	—	16 ± 1.9	—
(0.02 M)	2	No break	—	15.4 ± 1.6	—
Epinephrine (25 μM) and 1-propanol (0.42 M)	2	No break	—	14 ± 1.6	—
1-Propanol (0.42 M)	4	No break	—	12 ± 1.6	—

Each temperature curve consists of triplicate determinations for each of ten temperatures. Errors shown are standard deviations of the mean for the number of curves indicated.

perature effect was observed for the epinephrine-stimulated enzyme, where saturating concentrations of hormone are well below its solubility limit. A change in the energy of activation (at 32°) was not found for the membrane-associated fraction of cyclic AMP phosphodiesterase (Fig. 5) or for creatine phosphokinase (a component of the ATP regenerating system). Several other membrane marker enzymes do not show this change in energy of activation, including alkaline phosphatase (Fig. 5), 5′-nucleotidase, and Na^+,K^+-activated ATPase (22).

Short-chain alcohols produce a marked increase in glucagon-stimulated cyclase activity and a small increase in basal and epinephrine-stimulated activity (2). The temperature effect in the presence of both epinephrine and glucagon was abolished by 1-propanol (Fig. 6). In the presence of 1-propanol, the energy of activation for the glucagon-stimulated liver cyclase reaction varies depending on the concentration of 1-propanol, but is similar to the value at high temperature in the absence of 1-propanol. For the epinephrine-stimulated enzyme, in the presence of 1-propanol (0.42 M), the energy of activation is intermediate between those found above and below the break point in the absence of 1-propanol (Table 1).

DISCUSSION

These experiments indicate that hepatic cyclase, in the presence of epinephrine and glucagon, exhibits an abrupt increase at 32° in the slope of the Arrhenius plot. The abruptness of the change in slope permits determination of well-defined energies of activation at high and low temperatures, and suggests a cooperative change similar to the "phase transitions" seen in some lipid membranes. The cyclase measured in this experiment is probably almost entirely derived from hepatocytes (23, 24). Modest contributions from Kupfer cells, bile duct, or vascular endothelium (25), however, cannot be entirely excluded. The data obtained cannot be explained in terms of the solubility of activators or by thermal effects on the enzymes responsible for product destruction (phosphodiesterase) or substrate availability (creatine phosphokinase). The fact that changes of the energy of activation are reliably observed with less than saturating concentrations of the hormones argues against the explanation that higher temperatures provide an increase in the number of their respective binding sites. The change in the energy of activation is not explained by enhanced binding of stimulators at higher temperatures since glucagon binds with maximum efficacy at 0° (26). Also, both hormones show the change at the same temperature (an unlikely situation were the effect really a function of enhanced binding). The effect is not a reflection of enhanced substrate binding, since the K_m for ATP actually increases somewhat at higher temperatures.

Plasma membranes form vesicles (which retain or reverse normal inside-outside relationships) under various conditions of homogenization and buffer composition (27). Our data do not appear to reflect changes in the rate of vesicle formation since (*a*) four other membrane enzymes do not exhibit this temperature effect, (*b*) the basal activity and the fluoride- or prostaglandin-stimulated activities do not exhibit the effect, (*c*) the observed change in energy of activation occurs abruptly rather than gradually (as would be more likely with vesicle formation), and (*d*) the effects are reversible. Also, the data are not a reflection of temperature-dependent time lags in the onset of hormonal stimulation.

In view of the large mole fraction of cholesterol in most mammalian tissues, it is suggested that the changes in energy of activation observed here are probably occurring in protein components rather than lipid components of cell membranes. The magnitude of thermal transitions in membrane lipids is markedly diminished when the mole fraction of cholesterol is above 0.20, and transitions are absent by 0.33 (29–31). The mole fraction of cholesterol in the purified preparations of liver plasma membranes is about 0.30 (32, 33), so that thermal transitions in bulk lipid would not occur. Under these conditions temperature-sensitive protein–protein or protein–lipid interactions are more likely to be involved in the cyclase stimulation sequence. However, we cannot exclude the possibility that cyclase resides exclusively in cholesterol-poor

regions of the membrane and is affected by thermal transitions in the lipids of these regions. The ability of propanol to abolish the temperature effect and itself alter the energy of activation, and the absence of a temperature effect in the stimulation of cyclase by fluoride, prostaglandin E_1, and 1-propanol, suggest that the temperature effect is not an obligatory event in the cyclase stimulation sequence, but rather is a feature of the interaction of some components of this sequence.

Possibly related examples of an abrupt change in energies of activation may be seen in the activities of several bacterial membrane enzymes unrelated to cyclase (34, 35). In bacterial enzymes, however, the changes in energy of activation are correlated with a thermal transition in bulk membrane lipid, a correlation that does not appear to obtain for the transition in cyclase activity. Also, for rat hepatic cyclase, the slope of the Arrhenius plot is greater at temperatures above the break, while in the bacterial enzymes the slope is less at higher temperatures.

The fact that the hormone-stimulated cyclase and not the basal cyclase exhibits the temperature effect further suggests that basal activity is in some fundamental way different. Basal activity may simply reflect damage to the cyclase system, resulting in loss of constraint by some portion of the catalytic moieties. An alternative explanation for basal activity, i.e., that it represents the presence of endogenous hormone and thus is really indistinguishable from the hormone-stimulated cyclase, appears less likely in view of the absence of the temperature effect in the basal system. However, the existence of basal activity as part of the undamaged cell cyclase apparatus, its origin in cell-types other than those responsive to hormones, or its reflection of the effects of endogenous prostaglandins are not excluded.

Our data suggest differences in the stimulation sequence for glucagon and epinephrine as compared to fluoride and prostaglandin E_1. If one assumes that all four stimulators may act on the same catalytic moieties, then since stimulation by the two hormones but not by prostaglandin E_1 or fluoride shows the temperature effect, the temperature effect does not depend on events that occur at the catalytic moiety or at the locus of stimulation by fluoride or prostaglandin E_1.

The normal body temperature of the Sprague–Dawley rats used in these experiments is between 40 and 41°. Clearly the rats' core temperature does not normally fluctuate between 30° and 41°. In this species the temperature effect cannot have physiological significance. Further, in hibernating species, norepinephrine appears necessary for utilization of brown fat at temperatures around 10° (36, 37). Thus, stimulation of cyclase can clearly occur at lower temperatures (and does to some extent even in rat liver), but below the temperature at which the energy of activation changes, the magnitude of this stimulation for liver is significantly reduced.

The present data, however, provide an additional experimental feature of cyclase that may assist in elucidation of its mechanisms of stimulation. The structurally diverse stimulators are presumably interacting with the cyclase at different loci. Yet the common temperature at which the temperature effect for two of these activators occurs suggests that they initiate consequences that could converge at some common thermally sensitive locus. The abrupt change in the energy of activation for the cyclase reaction seen in association with hormone stimulation is a striking and unique characteristic of hepatic cyclase that is not exhibited by other liver-cell membrane enzymes.

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