Development of Guanylylimidodiphosphate-dependent Activation of Adenylate Cyclase by Glucagon in the Neonatal Rat Heart

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Extract

The basal adenylate cyclase activity of the rat heart increases with the age of the animal. By itself, 10^{-5} M glucagon activates only adenylate cyclase activity from adult rat hearts. In contrast, 10^{-5} M glucagon in the presence of 10^{-4} M 5'-guanylylimidodiphosphate (GMP-PNP) clearly activates adenylate cyclase activity in the 14-day-old rat heart, with some activation being evident in hearts of 7-day-old animals. GMP-PNP, 10^{-4} M, activates adenylate cyclase activity by itself at ages of 14 days and older, but to a far lesser degree than in combination with 10^{-5} M glucagon. Activity elicited by NaF increases throughout the neonatal period. The ratio of NaF-stimulated activity to basal activity increases from 6.3 at 2 days to 10.0 in the adult, a change which is not statistically significant.

We conclude that a cardiac receptor for glucagon is present early in neonatal period of the rat, but this receptor cannot effect activation of adenylate cyclase and an increase in heart rate, or depletion of glycogen. Even in the presence of 10^{-4} M GMP-PNP, the response to glucagon by cardiac adenylate cyclase depends on the age of the rat. In heart cells from a 7-day-old rat, the response is barely measurable but the magnitude of the response increases each week.

Speculation

This study demonstrates that GMP-PNP, an analog of GTP, facilitates the activation of adenylate cyclase by glucagon in the neonatal rat heart. It remains to be determined whether GMP-PNP facilitates glucagon binding per se or the interaction of glucagon with adenylate cyclase. The effect of the naturally occurring GTP on newborn cardiac adenylate cyclase activity is unknown.

We have previously shown that glucagon does not stimulate adenylate cyclase activity in neonatal rat heart (5). Furthermore, when administered to the neonatal rat, glucagon neither depletes myocardial glycogen nor stimulates heart rate (5, 18). Myocardial adenylate cyclase from adult rats is responsive to glucagon (5). Since guanine nucleotides have been reported to enhance the responsiveness of some adenylate cyclase systems to certain hormones, including glucagon (14), we have examined the effect of the GTP analog, 5'-GMP-PNP on the responsiveness to glucagon of the neonatal rat cardiac adenylate cyclase. We report here that the adenylate cyclase activity in heart cells isolated from 7-day-old rats is responsive to glucagon in the presence of 10^{-4} M GMP-PNP.

METHODS AND MATERIALS

Pregnant rats were purchased to provide young rats of known age (19). Female adult and weanling rats were used; younger animals of either sex were used.

Isolated heart cells were prepared from animals newly born to 21 days of age as previously described (6). Cells were isolated from hearts of adult rats by the technique of Berry *et al.* (1) using the reagents described in reference (6).

Adenylate cyclase activity was determined by the appearance of cyclic [32 P]AMP according to the method of Pohl *et al.* (13). The assay solution (50 μ l final volume) included 0.4 mM [α - 32 P]ATP, 10^6 cpm/reaction; 8.1 mM MgCl_2 , 23.4 mM Tris-HCl, pH 7.6; 0.94 mM EDTA, 0.5 mM cyclic AMP, 11.1 mM phosphoenolpyruvate, and 4 μ U/ml pyruvate kinase. The reaction was started by the addition of the heart cells which lyse immediately on addition to the hypotonic assay solution. (Lysis was assumed from the immediate uptake of trypan blue by cells exposed in preliminary experiments.) The reaction was incubated at 30° for 10 or 15 min and stopped by adding 50 μ l of a recovery solution (40 mM ATP, 12.5 mM cyclic AMP, and 50,000 cpm cyclic [3 H]AMP) and by heating for 3.5 min in a boiling water bath. Cyclic [32 P]AMP was separated from [α - 32 P]ATP on Dowex 50-X4 and alumina columns as described by Salomon *et al.* (15) and ^3H and

Fig. 1. Effect of increasing concentrations of guanylylimidodiphosphate (GMP-PNP) on the adenylate cyclase activity of adult heart cells in the presence of (○) and absence (●) of 10^{-5} M glucagon. Each point represents the mean of three replicates. Standard errors are shown except where they fall within the points. Assay conditions are described in the text. cAMP: cyclic AMP.

^{32}P were measured by liquid scintillation counting. Final values for cyclic AMP formed were calculated from the efficiency of counting, recovery of cyclic ^3H AMP, subtraction of "blanks" (cyclic AMP formed in absence of cells) and specific activity of $[\alpha\text{-}^{32}\text{P}]\text{ATP}$. Protein was determined by the Biuret method (8) and adenylate cyclase activity was expressed as pmoles cyclic AMP formed per milligram of protein per minute of incubation.

The formation of cyclic AMP was linear for 10–15 min at 30° with heart cells from all ages studied. This linearity was maintained in the presence of 10^{-5} M glucagon, 10^{-4} M GMP-PNP and 10^{-5} M glucagon, and 10^{-2} M NaF. In the presence of these compounds the amount of cyclic AMP formed in 10 min was also linear with the amount of protein per assay tube over the range of 40–200 μg protein. The exception was in the presence of 10^{-2} M NaF when the reaction was linear only to 70 μg protein. The time course showed a lag in cyclic AMP formation in the presence of 10^{-4} M GMP-PNP alone. This lag time was not characterized for these studies. A substrate concentration of 0.4 mM ATP was chosen for all assays because preliminary experiments showed that higher concentrations of ATP in the assay with adult heart cells produced substrate inhibition.

Collagenase, type I, was purchased from Worthington Biochemical Corp. (20). Hyaluronidase, type I, from bovine testes; pyruvate kinase, type 2 or 3; phosphoenolpyruvate, cyclic AMP, and ATP were purchased from Sigma Chemical Company (21). ICN Pharmaceutical, Inc. (22) was the source of GMP-PNP and $[\alpha\text{-}^{32}\text{P}]\text{ATP}$, 10–16 Ci/mmol. Tritiated cyclic AMP was purchased from New England Nuclear (23). Glucagon was a gift of Dr. William Bromer (24). All other chemicals were analytical grade.

RESULTS

The effect of increasing concentrations of GMP-PNP on the adenylate cyclase activity of adult rat heart cells is shown in Figure 1. Stimulation of adenylate cyclase activity was seen at concentrations of GMP-PNP greater than 10^{-5} M. With the addition of 10^{-5} M glucagon, a much greater degree of activation was seen at even lower concentrations of GMP-PNP. Maximal effects of GMP-PNP were seen at 10^{-5} M in the presence of 10^{-5} M glucagon, whereas in the absence of glucagon no plateau was seen at the concentrations of GMP-PNP studied.

The effect of increasing concentrations of glucagon alone on adenylate cyclase activity in heart cells from rats of different ages is shown in Figure 2. Glucagon stimulated only the adenylate cyclase activity in heart cells from adult rats. However, when these studies were performed in the presence of 10^{-4} M GMP-PNP,

Fig. 2. Effect of increasing concentrations of glucagon on the adenylate cyclase activity of heart cells obtained from rats of different ages. Each point represents the mean of three replicates. Standard errors are shown except where they fall within the points. Assay conditions are described in the text. cAMP: cyclic AMP.

Fig. 3. Effect of increasing concentrations of glucagon in the presence of 10^{-4} M guanylylimidodiphosphate on the adenylate cyclase activity of heart cells obtained from rats of different ages. Each point is the mean of three replicates. Standard errors are shown except where they fall within the points. Assay conditions are described in the text. At 10^{-5} M glucagon, the points for the 14 and 21 day adult activities are significantly different than the control value. The activity from the 7-day heart at 10^{-5} M is not significantly different than its control but the activity at 10^{-4} M glucagon is different ($P < 0.001$). The value for the 2-day heart at 10^{-4} M glucagon is not significantly different than its control value. cAMP: cyclic AMP.

stimulation by 10^{-5} M glucagon could be seen in heart cells from animals as young as 1 week (Fig. 3). The degree of activation increased at both 14 days and 21 days. At 21 days, the activation was about half that seen in heart cells from adult animals.

The development of rat heart adenylate cyclase activity and its activation is summarized in Figure 4. The basal adenylate cyclase activity of the rat heart increases with the age of the animals. By itself, 10^{-5} M glucagon activates only adenylate cyclase from adult rats. In contrast, 10^{-5} M glucagon in the presence of 10^{-4} M GMP-PNP clearly activates adenylate cyclase activity in the 14-day-old rat heart, with some activation being evident in hearts of 7-day-old animals (Fig. 3). GMP-PNP, 10^{-4} M, activates adenylate cyclase activity by itself at ages of 14 days and older, but to a far lesser degree than in combination with 10^{-5} M glucagon. Activity elicited by NaF increases throughout the neonatal period. The ratio of NaF-stimulated activity to basal activity increases from $6.3 \pm \text{SD } 3.2$ at 2 days to $10.0 \pm \text{SD } 2.8$ for the adult, a change which is not statistically significant.

DISCUSSION

Kohrman (10) reported that rat heart adenylate cyclase activity falls and rises from the prenatal through adult period whether

basal norepinephrine stimulated or fluoride-stimulated activity is measured (10). Our results agree more with those of Brus (3) and Brus and Hess (4) which show a 2-fold increase in cardiac adenylate cyclase activities during the neonatal to adult period. We see a 3- to 4-fold increase in basal and fluoride-stimulated cardiac adenylate cyclase activity during development. The greater increase may reflect the use of isolated cells rather than washed particles if it is assumed that washed particles are a more "damaged" membrane system than are the isolated cells.

The adult rat heart responds to either epinephrine or glucagon with positive chronotropic and inotropic effects and an increase in percentage of total phosphorylase activity which is in the active or "a" form (9, 12, 17). The mechanism by which epinephrine and glucagon appear to act is the activation of membrane-bound adenylate cyclase and a consequent increase in intracellular concentration of cyclic AMP. In the neonatal rat, glucagon activates adenylate cyclase and depletes glycogen in the liver but not in the heart, although epinephrine is active in both neonatal tissues (18). We have suggested previously that the glucagon receptor for the adenylate cyclase system either has not developed or is inoperative in the neonatal rat heart, whereas the epinephrine receptor is already functional (5). We find in the present studies that glucagon can activate cardiac adenylate cyclase in the presence of the GTP analog, GMP-PNP, as early as 7 days after birth. We conclude that a receptor for glucagon is present in neonatal rat heart but this receptor cannot effect activation of adenylate cyclase, an increase in heart rate, or depletion of glycogen. Moreover, even in the presence of 10^{-4} M GMP-PNP, the magnitude of response to glucagon by cardiac adenylate cyclase depends on the age of the rat. In heart cells from a 7-day-old rat the response is barely measurable but the degree of the response increases each week (Fig. 3).

Guanine nucleotides can regulate activity in many adenylate cyclase systems (2). Since GTP and GDP have opposing effects on

some adenylate cyclase systems, and since GTP is readily hydrolyzed to GDP, GMP-PNP, which is resistant to hydrolysis at its terminal phosphate, has been used as a GTP analog (16). GMP-PNP differs from GTP in that GMP-PNP has proven to be a more potent activator of many adenylate cyclase systems than GTP (11) and, unlike GTP, to be an irreversible activator (7). Activation is seen in the absence of hormones but activation is cooperative with hormones. From detailed kinetic analyses of fat cell and liver plasma membranes, Rodbell (14) has proposed that the binding of GMP-PNP causes the enzyme to exist in a state that has a higher substrate turnover (V_m), but is highly susceptible to inhibition by protonated ATP. Binding of both hormone and GMP-PNP causes the enzyme to exist in a state that has the higher substrate turnover (V_m), but which is no longer susceptible to inhibition by protonated ATP. Cuatrecasas *et al.* (7) have suggested that both GMP-PNP and GTP pyrophosphorylate the enzyme but that the enzyme-PNP is stable whereas the enzyme-pyrophosphate is rapidly hydrolyzed stepwise (7). They also demonstrated a lag time for GMP-PNP activation of adenylate cyclase.

The results reported here are consistent with a role of GMP-PNP which facilitates the action of glucagon either by inducing a receptor site for glucagon so that glucagon may bind and act or by stabilizing an enzyme conformation with which glucagon can now effectively interact. Since the effect of GMP-PNP on the binding of glucagon to liver plasma membranes is to decrease the amount of glucagon bound (12), we favor the hypothesis that GMP-PNP induces a structural change in a protein or in adenylate cyclase such that adenylate cyclase is activated when glucagon is bound to its receptor.

SUMMARY

Adenylate cyclase activity can be readily measured in cells isolated from hearts of rats of different ages. Cardiac adenylate cyclase activity, expressed on a protein basis, increases throughout the neonatal period. In isolated cells, as in homogenates, 10^{-5} M glucagon does not stimulate adenylate cyclase activity in hearts of preweanling rats. However, with the addition of 10^{-4} M GMP-PNP, 10^{-5} M glucagon can clearly stimulate cardiac adenylate cyclase activity in rats as young as 7 days.

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Fig. 4. Basal and activated adenylate cyclase activities of heart cells as a function of the age of the rats from which the cells were obtained. Activators tested were 10^{-4} M guanylylimidodiphosphate (GMP-PNP), ▲; 10^{-2} M NaF, ■; 10^{-5} M glucagon, ○; and 10^{-4} M GMP-PNP + 10^{-5} M glucagon, △. Each point is the mean of three replicates. Standard errors are shown except where they fall within the points. Assay conditions are described in the text.

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Bilirubin
hyperbilirubinemia
newborn
phototherapy
riboflavin

Riboflavin and Bilirubin Response during Phototherapy

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Extract

Twenty-four jaundiced neonates were studied, 12 in the treatment group and 12 in the untreated group. Patients were randomly selected to receive oral riboflavin. The mean 24-hr bilirubin decrease was determined during phototherapy. Blue light (420-470 nm) energy ranged from 6-10 $\mu\text{W}/\text{cm}^2$. The observed 24-hr bilirubin decrease was compared with the expected decrease based on an energy-dose-response relationship. Riboflavin-treated infants received either 6-7 $\mu\text{W}/\text{cm}^2$ blue light energy or 8-10 $\mu\text{W}/\text{cm}^2$ (same as control group). Those infants receiving less energy than the control group (8-10 $\mu\text{W}/\text{cm}^2$) had a mean 24-hr bilirubin decrease (3.05 mg/100 ml/24 hr) equal to the control group (3.09 mg/100 ml/24 hr). Those riboflavin-treated infants receiving energy equal to the control group showed a greater decline (5.2 mg/100 ml/24 hr) in their mean 24-hr bilirubin. Although effective, additional *in vivo* studies are required to clarify the full effects, especially on DNA, of using photosensitizers such as riboflavin in the presence of bilirubin and blue light energy (420-470 nm).

Speculation

Riboflavin may become an important adjunct to phototherapy for neonatal hyperbilirubinemia. As a producer of singlet oxygen it is capable of transferring enough energy to overcome the oxygen quenching effect of bilirubin in its rapid degradation.

Riboflavin (6',7-dimethyl-9-(D-1'-ribityl) isoalloxazine is an essential dietary constituent for mammals (9). The mono- or dinucleotide form functions as the prosthetic group of a number of

important enzymes. Flavin mononucleotide, riboflavin 5'-phosphate is formed from riboflavin and ATP catalyzed by flavokinase. Further addition of ATP with the mononucleotide forms PP_i and FAD, an important nucleotide and coenzyme in cellular respiration. All biologic functions of riboflavin appear to be limited to its contribution to the synthesis of these two coenzymes. In man, ingested riboflavin is excreted largely unchanged or as riboflavin 5'-phosphate (FMN).

Recent *in vitro* (7) and *in vivo* (4) evidence indicates that riboflavin enhances the photodecomposition of bilirubin. Application of this principle to phototherapy for neonatal hyperbilirubinemia using blue light energy in a predictable dose-response relationship (5) forms the basis for this report.

MATERIALS AND METHODS

All infants studied were at term (38-40 weeks) and predominantly Caucasian. Assignment to the study was made only after the infants were committed to phototherapy for hyperbilirubinemia by their private pediatricians (10).

Study subjects included 24 infants born at St. Francis Hospital, Tulsa, Oklahoma. There were 12 males and 12 females. Hyperbilirubinemia was defined as a serum bilirubin concentration greater than 9 mg/100 ml and phototherapy was initiated at or above this level (range 9-18 mg/100 ml). Blue light energy was administered and measured as described in a previous report (5). Serum bilirubin was collected and measured. All infants studied had developmental (physiologic) jaundice with no laboratory or clinical evidence of illness or hemolytic process. A system of random numerical selection was used to determine which patients