Differences between Male and Female Rats in the Regulation of Hepatic Glycogenolysis

THE RELATIVE ROLE OF CALCIUM AND cAMP IN PHOSPHORYLASE ACTIVATION BY CATECHOLAMINES*

Rebecca K. Studer and André B. Borle‡
From the Department of Physiology, University of Pittsburgh, Pittsburgh, Pennsylvania 15261

The relative importance of α- and β-adrenergic pathways and of their respective intracellular mediators, calcium and cAMP, in the stimulation of phosphorylase α induced by catecholamines was studied in hepatocytes isolated from mature male and female rats. The fractional efflux of 46Ca was used as an index of intracellular calcium activity. Our results show that, in females: 1) the activation of phosphorylase α induced by 10^{-4} to 10^{-3} M epinephrine correlates with a rise in cellular cAMP as well as with an increase in 46Ca fractional efflux, 2) both α- and β-agonists stimulate phosphorylase, 3) neither α- nor β-agonists effectively block the rise in phosphorylase caused by epinephrine, and 4) propranolol suppresses the rise in cAMP while phenoxybenzamine blocks the rise in calcium efflux. On the other hand, we found that in the male: 1) phosphorylase α activity is exclusively correlated with a rise in fractional calcium efflux, 2) epinephrine (10^{-4} to 10^{-3} M) does not increase cAMP and it causes a greater rise in calcium efflux than in the female at all concentrations, 3) phenylephrine increases calcium efflux and phosphorylase activity without affecting cAMP, 4) phenoxybenzamine totally blocks epinephrine action, and 5) β-agonists and β-antagonists are without effects. We conclude that, in females, epinephrine utilizes both α- and β-adrenergic pathways which activate phosphorylase by calcium or cAMP, respectively, while, in adult male rats, epinephrine increases phosphorylase α activity by an α-mediated, calcium-dependent and cAMP-independent pathway.

Since 1958, when Sutherland and Rall (1) discovered that cAMP was an intracellular messenger mediating epinephrine activation of phosphorylase in liver, the scientific literature has slowly shifted from the view that hepatic glycogenolysis induced by catecholamines occurs mainly through a β-adrenergic pathway which increases cAMP, to the recent consensus that, in rat liver at least, catecholamines act through a cAMP-independent α-adrenergic pathway involving a rise in cytosolic calcium (2-5). As early as 1961, Hynie et al. (6) had concluded from their data that, in the rat, epinephrine acted as an α-agonist to increase glycogenolysis. However, their work was overshadowed by the studies of Sutherland's group (7) which demonstrated the relationship between the rise in cAMP induced by catecholamines and glycogenolysis in liver slices and homogenates. The subsequent studies of Exton et al. (8) in perfused livers of immature male rats still supported the concept that cAMP was the mediator of catecholamine actions and, the relative potency of the agonists tested established the β-adrenergic nature of the response. The first study to question this relationship appeared in 1972: Sherline et al. (9) showed that epinephrine could stimulate glycogenolysis by a cAMP-independent α-adrenergic pathway as well as by a β-receptor-mediated increase in cAMP. These studies were done with perfused livers of more mature rats and the question was raised of a possible α-adrenergic-activated vasoconstric-

* This work was supported by United States Public Health Service Grants 1 R01 AM26889 and 2 R01 AM07867 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom all correspondence should be addressed.

(Received for publication, January 12, 1982)
Male-Female Differences in Phosphorylase Activation by Catecholamines

EXPERIMENTAL PROCEDURES

Hepatocyte Preparation—Hepatocytes were isolated from 250- to 300-g female or male Sprague-Dawley rats by a modification of the collagenase perfusion method of Berry and Friend (21). The livers were perfused in situ through the portal vein at a rate of 10 ml/min with 150 ml of Ca2+-free Krebs-Henseleit bicarbonate buffer containing 5 mM glucose and 2 units/ml of heparin, followed by 150 ml of Krebs-Henseleit bicarbonate buffer containing 1.3 mM Ca2+, 5 mM glucose, 1% bovine serum albumin (KHBG) and supplemented with 100 units/ml of collagenase (Sigma Type IV), and the amino acids and vitamins contained in Eagle’s Minimum Essential Medium. The hepatocytes were washed with ice-cold KHBG and incubated at 37°C in this same medium with a gas phase of 95% O2 and 5% CO2 with gentle stirring. The cell concentration was 5 to 10 mg of protein/ml. Viability was assayed by trypan blue staining and by the succinate stimulation of cell oxygen consumption according to the method of Bauer et al. (22). Routinely, we used only preparations with a succinate respiratory ratio of less than 1.2 and a trypan blue staining of less than 15%. Hepatocytes were incubated for 20 min, washed, and the incubation continued in fresh medium as dictated by the specific experimental protocol. 45Ca efflux, hepatocyte phosphorylase activity, and CAMP were determined after the same time of incubation on separate aliquots of the same liver cell preparation. Viability after 3.5 h of incubation or of perfusion was decreased by no more than 10%. 45Ca Fractional Efflux Ratio—Hepatocytes were preincubated for 45 min, labeled for 60 min with 5 μCi/ml of 45Ca, and desaturated for 110 min with nonradioactive medium. The labeled cells were separated from the 45Ca medium by centrifugation and washed once with 40 ml of Ca2+-Mg2+-free phosphate-buffered saline containing glucose (GKN). 60 μl of the packed hepatocyte pellet were placed in each of the four cells of a Lucite flow-through chamber (23). The cells were placed into a loosely packed glass wool plug laying over a 10-μm mesh nylon net placed at the outlet of the chamber perfused at a rate of 0.6 ml/min. The dead space of the system from medium reservoir to collection vials was 1.5 ml. The effluent was collected in scintillation minivials at intervals ranging from 30 s to 5 min, the volumes were equalized to 3 ml with nonradioactive medium to which 4 ml of scintillator were added (New England Nuclear Formula 963). At the end of the experiment, the cells were removed from the chamber, solubilized in 0.5 x NaOH, sonicated, neutralized with 0.5 x HCl, and aliquots analyzed for radioactivity and protein. Data are expressed as the fractional efflux ratio according to the method of Bories et al. (23). The fractional efflux is the radioactivity released per unit of time expressed as the percentage of the mean cell radioactivity during the collection period. The fractional efflux ratio is the fractional efflux of stimulated cells divided by the fractional efflux of unstimulated controls perfused concurrently. Increases in 45Ca fractional efflux can be caused by a rise in the cytosolic free calcium concentration or by a stimulation of the active calcium transport mechanism across the plasma membrane. In these experiments, we have assumed that catecholamines do not affect the transport mechanism and that the changes observed in 45Ca efflux reflect fluctuations in cytosolic calcium activity. Thus, 45Ca fractional efflux ratio is an indirect and probably slightly delayed index of cytosolic calcium.

Hepatocyte Phosphorylase Activity—Aliquots of 0.5 ml of the cell suspensions were added to 1.3 ml of the ice-cold buffer described by Hutson et al. (11). The mixture was rapidly homogenized and frozen until it was assayed by the method of Stalmans and Herens (24) in the presence of 0.5 mM caffeine to minimize any contribution of phosphorylase b to the activity measured. The [14C]glucose 1-phosphate converted to glycogen was determined by precipitation of the labeled glycogen on filter paper with 66% ethanol according to the method of Gilboe et al. (25). 60 μl of the packed hepatocyte pellet were placed in each of the control and experimental chambers was established by the initial 50-min perfusion with KHBG. From 50 to 80 min, the medium perfusing the experimental cells contained 10-15 m epinephrine. No changes in the fractional calcium efflux ratio were observed when the medium was changed back to the standard KHBG at 80 min, thus only the data showing the initial response to epinephrine are given. As the maximum concentration of epinephrine in the solution perfusing the cells is not attained for 3 min, time zero on the abscissa is actually equivalent to min 52 of perfusion, or when 30% of the maximum dose is reached. Values are expressed as picomoles mg-1 of cell protein.

Concentration of CAMP in Hepatocytes—Aliquots of 0.2 ml of the cell suspensions were added to 10 ml of ice-cold GKN containing 2 mM 3-isobutyl-1-methylxanthine and quickly centrifuged. The pellet was resuspended in 1 ml of 50 mM acetic acid buffer, pH 4.0 at 95°C, homogenized with an ultrasonic probe, and maintained at 95°C for 15 min. Samples were further homogenized in a Potter-Elvehjem tissue grinder with a Teflon pestle, held on ice for 80 min, and the protein precipitated by centrifugation at 2000 x g for 15 min at 4°C. The supernatants were analyzed for CAMP by radiomunoassay (27) using the acetylation procedure of Harper and Brooker (28) to enhance the sensitivity of the method. Values are expressed as picomoles mg-1 of cell protein.
Male-Female Differences in Phosphorylase Activation by Catecholamines

Materials—Minimal Essential Medium amino acids (Eagle), vitamins, and glutamine were obtained from Microbiological Associates, Walkersville, MD. 3-Isobutyl-1-methylxanthine, L-epinephrine bitartrate, L-phenylephrine/HCl, dl-propranolol/HCl, and isoproterenol/HCl were from Sigma. Phenoxybenzamine was from Smith, Kline and French, Sunnyvale, CA. $^{32}$P-$2\cdot O$-succinyl adenosine 3',5'-cyclic phosphoric acid $^4$CaCl$_2$, and $[^4\text{C}]$glucose 1-phosphate were from New England Nuclear. cAMP antibody was generously provided by Dr. F. DeRubertis, Veterans Administration Hospital, Pittsburgh, PA. Stock solutions of adrenergic agonists and antagonists were prepared in 0.01 N HCl.

RESULTS

Effects of Epinephrine on Calcium, cAMP, and Phosphorylase a Activity in Males and Females—Fig. 1 shows that $10^{-5}$ M epinephrine increased the cellular concentration of cAMP, the fractional efflux of $^4$Ca, and phosphorylase a activity in less than 30 s. In females, there was a good quantitative and temporal correlation among the three parameters: cAMP and phosphorylase a activity increased 3.2-fold while calcium efflux rose 4.2-fold. In male rats, on the other hand, the same concentration of epinephrine increased cAMP only 1.6-fold while phosphorylase a activity and the fractional efflux of calcium rose 8.2-fold and 6.3-fold, respectively. These results suggested to us that in female rat hepatocytes, epinephrine might stimulate both a- and $\beta$-receptors, but it was impossible to decide whether phosphorylase activation was due to calcium since there was no good quantitative or temporal relationship between cAMP and phosphorylase activity. Four differences could be observed between males and females: 1) the resting level of phosphorylase a activity was 3

![Graph](image-url)
Male-Female Differences in Phosphorylase Activation by Catecholamines

**Fig. 4.** Effects of α- and β-antagonists on the response of hepatocytes from male and female rats to $10^{-8}$ M epinephrine. Cells were incubated as described for Fig. 1 with (A) $10^{-6}$ M propranolol or (B) $10^{-7}$ M phenoxybenzamine added 10 min before epinephrine. Values are mean ± S.E.; $n = 4$ to 10 (cAMP or phosphorylase a activity) or 2 to 6 ($^{45}$Ca efflux).

Times lower in males than in females (10.94 versus 2.98 nmol min$^{-1}$ mg$^{-1}$ of protein), 2) the rise in phosphorylase a activity expressed as percentage of resting levels was greater in males than in females but declined more rapidly, 3) the increase in cAMP induced by epinephrine was much smaller in males than in females, and 4) the rise in calcium efflux was significantly greater in males than in females.

These results were confirmed when the effects of epinephrine were studied at concentrations ranging from $10^{-8}$ to $10^{-5}$ M. Fig. 2 shows that in female rat hepatocytes, the maximal stimulation of cAMP obtained with $10^{-7}$ M epinephrine represented a 5-fold increase over baseline level, whereas, in the male, the same concentration of epinephrine increased cAMP only 2-fold. The concentration of hormone giving half the maximal stimulation is not markedly different in males and females ($K_a = 2.5 \times 10^{-7}$ M and $5 \times 10^{-7}$ M, respectively). With the highest concentration of epinephrine of $10^{-5}$ M, phosphorylase a activity is greater in females than in males when expressed in absolute terms: 51.2 versus 33.4 nmol min$^{-1}$ mg$^{-1}$ of protein. Since the basal level of the enzyme was lower in males than in females (4.00 versus 12.2 nmol min$^{-1}$ mg$^{-1}$ of protein) the relative increase was twice as large in males: 8.4-fold versus 4.3-fold increase, respectively. The $K_a$ was around $3 \times 10^{-7}$ M in females and $6 \times 10^{-8}$ M in males. The maximal stimulation of the $^{45}$Ca fractional efflux was 6.2-fold in males and 4-fold in females with respective $K_a$ of $6 \times 10^{-8}$ M and $1.4 \times 10^{-7}$ M. Thus, the differences between male and female rat hepatocytes were evident at all concentrations of epinephrine. Moreover, the $K_a$ of phosphorylase activation in females matches the $K_a$ of cAMP stimulation, $3 \times 10^{-7}$ and $5 \times 10^{-7}$ M, respectively, while, in males, the $K_a$ of phosphorylase activation is identical with that of the stimulation of the fractional calcium efflux, $6 \times 10^{-8}$ M.

**Time Course of Epinephrine Action**—Fig. 3 shows the time course of the changes in cAMP, phosphorylase a activity, and calcium efflux induced by $10^{-6}$ to $10^{-4}$ M epinephrine in male and female rat hepatocytes, expressed as the ratio of experimental to control values. There was a good correlation between cAMP and phosphorylase with regard to the rapidity of the stimulation (less than 30 s), the magnitude of the effects, and the time course of the decline during the 15 min following epinephrine addition. It is more difficult to precisely correlate phosphorylase activity and calcium efflux.

**Fig. 5.** Effects of phenylephrine on cAMP, phosphorylase a activity, and calcium efflux in hepatocytes from male and female rats. Cells were incubated as in Fig. 1 with $10^{-7}$ to $10^{-5}$ M phenylephrine added at 150 min. Data are plotted as the ratio of the average stimulated values to the pooled basal values for all experiments, with $n = 4$ to 6 (cAMP and phosphorylase) or 2 to 6 ($^{45}$Ca efflux).
Because the latter is an indirect index of cytosolic calcium activity and because the experimental system in which it is measured differs. In the perfusion system, the concentration of the hormone progressively rose to its maximal concentration, whereas cAMP and phosphorylase α activity are determined after a stepwise addition of the hormone which instantaneously reached its maximal concentration. In addition, the declining phase of the 45Ca fractional efflux depends not only on the cytosolic free calcium concentration but also on the cell specific activity which constantly decreases and on the possible saturation of the plasma membrane active transport mechanism. In the male, phosphorylase α activity correlated well with the fractional efflux of calcium. At physiological concentrations of epinephrine, 10⁻⁸ and 10⁻⁷ M, there was no increase in cAMP while both calcium efflux and phosphorylase α activity were stimulated. Cyclic AMP was slightly elevated at the high epinephrine concentrations of 10⁻⁶ and 10⁻⁵ M, while calcium efflux and phosphorylase α activity were markedly stimulated. This suggests again that, in males, the intracellular signal mediating epinephrine action on liver glycogenolysis is cytosolic calcium and not cAMP.

Effects of α- and β-Antagonists in Males and Females—
We studied the relative importance of α- and β-receptors in males and females by adding propranolol or phenoxybenzamine (10⁻⁵ M) 10 min before the addition of epinephrine (10⁻⁶ M). Fig. 4A shows that, in females, the β-blocker propranolol totally suppressed the rise in cAMP usually induced by epinephrine; in males, cAMP was still slightly elevated. On the other hand, the fractional calcium efflux and phosphorylase α activity were markedly stimulated both in males and in females. Phosphorylase activation was slightly less with the β-blocker and, in females, the decline in activity was significantly faster (4-min point, p < 0.05 compared to Fig. 1).

The α-blocker phenoxybenzamine completely suppressed the rise in fractional efflux of calcium usually induced by epinephrine both in males and females (Fig. 4B). In females, however, cAMP and phosphorylase α activity were increased to the levels obtained without the α-blocker. In males, there was only a slight stimulation of cAMP and no increase in phosphorylase α.

Thus, it appears that epinephrine, in the female, acts through α- and β-receptors and phosphorylase activation is mediated by cAMP as well as by calcium. In contrast, phosphorylase activation by epinephrine in the male is exclusively mediated through α-receptors and calcium.

Effects of α- and β-Agonists in Males and Females—These results were corroborated by experiments using 10⁻⁷ to 10⁻⁵ M phenylephrine and isoproterenol (Figs. 5, 6, and 7). In females, phenylephrine was a much weaker agonist than epi-
neprine. Both cAMP and the fractional efflux of calcium were stimulated 2-fold by $10^{-5} \text{ M}$ and 1.5-fold by $10^{-6} \text{ M}$ phenylephrine; a concentration of $10^{-7} \text{ M}$ was without effects. Phosphorylase $\alpha$ activity was stimulated 2- to 3-fold at the higher concentrations (Fig. 5). The enzyme may have been activated by cAMP, by Ca or by both. Fig. 6 shows that phosphorylase $\alpha$ activity can be activated by calcium alone; in the presence of the $\beta$-bloker propranolol, phenylephrine stimulated the fractional efflux of calcium and phosphorylase without any rise in cellular cAMP.

In the male, the other hand, hepatocytes are much more responsive to phenylephrine (Fig. 5). The 8- to 9-fold stimulations of phosphorylase activity were observed at high concentrations of the agonist. The enzyme activation correlated well with the rise in fractional calcium efflux and there was no significant rise in cellular cAMP.

FIG. 7 shows the effects of the $\beta$-agonist isoproterenol in male and female rat hepatocytes. In females, phosphorylase $\alpha$ activity was increased 2.5-fold and there was a 2- to 3-fold rise in cAMP. The fractional calcium efflux was hardly affected. In the male, isoproterenol had practically no effect on cAMP, or on the fractional efflux of calcium, and there was no stimulation of phosphorylase activity.

**Correlation among cAMP, Fractional Ca Efflux, and Phosphorylase $\alpha$ Activity**—Fig. 8 presents the correlations obtained among the three parameters measured in both male and female rat hepatocytes. In females, there was a positive and significant correlation between the rise in fractional calcium efflux and phosphorylase activation. There was also a significant and positive correlation between the rise in cAMP and the enzyme activity. There was no significant correlation between cAMP and calcium, whether cAMP or Ca was assumed to be the independent parameter. In males, correlation between fractional efflux of calcium and the activation of the enzyme was positive and highly significant. There was no correlation between cAMP and phosphorylase $\alpha$. The correlation between cAMP and calcium efflux, although graphically significant, has no obvious physiological meaning.

**DISCUSSION**

Our studies establish that there are clear differences between adult male and female rats in the control of hepatic glycogenolysis by catecholamines. In the male, epinephrine stimulates glycogen breakdown by interacting with hepatocyte $\alpha$-receptors and subsequently produces a change in cell calcium which increases glycogen phosphorylase activity. In females, on the other hand, epinephrine appears to act through both $\alpha$- and $\beta$-receptors with both cAMP and calcium inducing phosphorylase activation. The difference may not appear before the full maturity of the animals. Indeed, in male and female immature rats, as well as in adult females, responses to catecholamines may be mediated by both $\alpha$- and $\beta$-receptor interactions (16–18). However, as the male ages, the $\beta$-component is lost or suppressed (18). The plasticity of the hepatocyte adrenergic receptors is not restricted to the age of the rats; it may also depend on other alterations in the physiological state of the animals. For example, in calcium-depleted hepatocytes isolated from adult males, epinephrine (or phenylephrine) stimulates a substantial $\alpha$-mediated increase in cAMP (29). Hepatocytes from hypothyroid rats respond to epinephrine with a significant increase in cAMP (30) as do hepatocytes from adrenalectomized rats (31). Thus, the relative contributions of cAMP and calcium to the control of glycogenolysis may be a function of sex, age, and of the endocrine state of the animal. The concept that adrenergic agonists activate glycogenolysis solely by CaAMP-independent, calcium-dependent mechanisms may be tenable only for a healthy, mature, male rat; the extension of this concept to females appears to be unwarranted. Its validity for animals of other species, age, and endocrine state remains to be tested.

Our data suggest a difference between male and female rats with respect to the interaction of epinephrine with $\alpha$- and $\beta$-receptors on liver cells. Many investigators have attempted to measure or estimate $\alpha$- or $\beta$-receptor binding on the plasma membrane of hepatocytes. The binding of $[1^H]$epinephrine to $\alpha$-receptors has been measured in males only (32, 33). $\beta$-Receptor binding on the other hand has only been estimated, in both males and females, from labeled $\beta$-antagonist-binding studies (34–36). From experiments using epinephrine displacement of antagonist binding, it has been estimated that in immature female rat liver epinephrine binds to $\beta$-receptors with an affinity 10 times greater than to $\alpha$-receptors (35). However, since adrenergic antagonists bind to a substantially greater number of sites than epinephrine, the physiological significance of these results is questionable (32, 33). A direct comparison of epinephrine binding to hepatocytes from males and females, and an assessment of the relative affinity of the $\alpha$- and $\beta$-receptors in males and females for epinephrine has
Male-Female Differences in Phosphorylase Activation by Catecholamines

not been done. Also, recent studies (3) suggest that, depending on the incubation condition used, α2-receptor activation in isolated plasma membranes may inhibit or activate adenylyl cyclase. Some of the male/female differences we observed could be due to differences in α2-activation by epinephrine. For example, in females, cAMP is greater than 2 to 15 min after stimulation in the presence of phenoxybenzamine and epinephrine than in the presence of epinephrine alone (Fig. 1 versus Fig. 4). We detected no influence of phenoxybenzamine on cAMP in the males. These results are consistent with an α-mediated inhibition of adenylyl cyclase in hepatocytes from females but not males. It is clear that in adult male rats, the stimulation of phosphorylase induced by catecholamines is correlated only with changes in calcium and not with cAMP (Fig. 8). The stimulations of phosphorylase activity and of calcium efflux by epinephrine have identical Ka of 6 x 10^{-8} M. Assuming that the calcium fractional efflux reflects changes in cytosolic free calcium, our results agree with those of Murphy et al. (37) who estimated cytosolic calcium activity in digitonin-treated hepatocytes. Our data also support the inference made by many other investigators who measure a net loss of calcium from hepatocytes after adrenergic stimulation (3-5). The stimulations of phosphorylase activity and of calcium efflux by epinephrine are blocked by propranolol, the increase in phosphorylase activity is reduced (Figs. 8 and 9), and it could be due to differences in cy2-activation by epinephrine.

We conclude that the relative importance of α- and β-adrenergic pathways and of intracellular calcium and cAMP in the regulation of phosphorylase activity of hepatocytes depends on the sex of the animals, and a review of the literature suggests that it may also depend on the age, the species, and the endocrine state of the animals. In hepatocytes isolated from adult female rats, epinephrine utilizes both α- and β-adrenergic pathways which activate phosphorylase α by calcium or cAMP, respectively. In intact, mature male rats, epinephrine increases phosphorylase α activity by an α-mediated, calcium-dependent, and cAMP-independent pathway.

Acknowledgments—The technical assistance of Pamela Cardillo and Philip Messer is gratefully acknowledged. The PROPHET computer system which was used extensively in these investigations was made available by the Division of Research Resources of the National Institutes of Health. We also thank Dr. Victor Stolc for his expert advice.

REFERENCES