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EPINEPHRINE- AND GLUCAGON-SENSITIVE ADENYLATE CYCLASES OF RAT LIVER DURING AGING

EVIDENCE FOR MEMBRANE INSTABILITY ASSOCIATED WITH INCREASED ENZYMATIC ACTIVITY

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Summary

The influence of post-maturational aging on the activity and stability of rat liver epinephrine- and glucagon-sensitive adenylate cyclases (ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1) was studied in liver homogenates from male and female Wistar rats ages 3, 12, and 24 months. Enzyme activity was measured in the unstimulated (basal) state and with fluoride activation (5 mM) as well as with a range of concentrations of glucagon and epinephrine. Basal activity was the same at 3 and 12 months but at 24 months was 1.4 times higher in both sexes. With fluoride and glucagon (1 nM—10 μM) activities were slightly but significantly greater (1.2 times) at 24 months vs. 3 and 12 months in females but not in males. In contrast, epinephrine-stimulated activity doubled from 3 to 24 months in both males and females, with most of the increase occurring between 12 and 24 months. The concentration of epinephrine necessary for 50% activation was the same (0.25 μM) at all ages, showing that increased epinephrine response during aging is not due to altered affinity of receptors for hormone. Increased epinephrine-sensitive activity was due either to increased numbers of enzyme catalytic units or to an increase of V but not to altered K_m for substrate which was unchanged with age for both hormone- and fluoride-activated enzyme. Stability of the membrane-bound enzyme was examined in homogenates kept at 0°C for 24 h in the presence of protease and esterase inhibitors. Loss of adenylate cyclase activity was significantly correlated with increasing age. At each age epinephrine activity was least stable, glucagon activity was most stable, and fluoride activity was intermediate. Glucagon-sensitive

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activity was similar in fresh homogenates from animals of all three ages; only homogenates prepared from 24-month-old animals showed gross instability when kept at 0° C for 24 h. These experiments suggest that the increased activity of hormone-sensitive adenylate cyclase with aging may be related to an alteration of the cell membrane.

Introduction

Although age is a known determinant of adenylate cyclase (ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1) activity in a variety of animal tissues, most previous reports relating adenylate cyclase activity to animal age have dealt with early development and maturation [1—6]; only a few have been concerned with post-maturational changes or with senescence in such tissues as fat, brain, and aorta [7—10]. More systematic measurements of hormone-stimulated adenylate cyclase made over the entire life span may provide a basis for understanding age-related changes of hormone sensitivity. Moreover, since adenylate cyclase is an enzyme which is a component of the plasma membrane, quantitative and qualitative studies of enzyme activity changes with age would also point to age-related alterations of membrane structure and function.

Several studies of the distinct glucagon- and epinephrine-sensitive adenylate cyclases of liver have reported that the two enzymes exhibit decreases of activity during development and that the change is greater for epinephrine- than for the glucagon-stimulated enzyme [1—3]. One recent study has indicated a postnatal increase of glucagon-sensitive adenylate cyclase [6]. Such observations have not previously been extended to include later times in the life span. This paper describes the relationship of maturation and senescence to epinephrine- and glucagon-sensitive adenylate cyclases of rat liver. We also present evidence suggesting that the observed changes of adenylate cyclase with age may be due to alterations of plasma membrane structure. The age-related changes of glucagon- and epinephrine-sensitive adenylate cyclases of liver are strikingly different from those recently seen for the same enzymes from adipose tissue of the same species [7].

Materials and Methods

Materials. Crystalline glucagon (mixed porcine-bovine), L-epinephrine bitartrate, glycylglycine, Tris base, Tris · HCl, theophylline, ATP (disodium salt), cyclic AMP (sodium salt), GTP (sodium salt), creatine phosphate (disodium salt), creatine phosphokinase (Type 1, rabbit muscle), sodium lauryl sulfate, and diisopropylfluorophosphate (DFP) were from Sigma. Reagent grade sodium fluoride (F^-) was from Fisher. [α-^{32}P]ATP, tetra (triethylammonium) salt (10—20 Ci/mmol), and cyclic [G-^{3}H]AMP, ammonium salt (20—30 Ci/mmol), were from New England Nuclear. Cyclic [8-^{14}C]AMP (35—50 Ci/mol) was from Schwarz/Mann. Trasylol (aprotinin) was obtained from FBA Pharmaceuticals. Pepstatin was a gift from H. Umezawa (Institute of Microbial Chemistry, Tokyo, Japan). 3-, 12-, and 24-month-old male and female rats were from an outbred Wistar strain raised at the Gerontology Research Center.

Preparation of tissue. Rats were narcotized with CO_2. Livers were excised and quickly placed into ice-cold homogenizing medium containing 2 mM glycylglycine, 20 mM KCl, 20 mM NaCl, and 1 mM $MgSO_4$, pH 7.4. 6 g of an individual liver were weighed, added to 20 volumes of medium, and homogenized for 10 s with a Virtis homogenizer. Tissue from pooled animals of the same age and sex was used whenever available; in these experiments 2 g of liver from each of three animals were pooled and homogenized in 20 volumes of homogenizing medium. For statistical calculations each experiment was weighted equally (i.e. assigned an $n = 1$) whether tissue was derived from single or pooled animals. Homogenates were assayed for adenylate cyclase activity immediately after preparation.

Particulate ("membrane") preparations were made from fresh homogenates by centrifuging measured volumes of homogenates at 30 000 × *g* for 5 min. The supernatant fractions were discarded and the pellets resuspended in homogenizing medium to the same volume as the original homogenates. Particulates were twice recentrifuged and resuspended in the same volume, rehomogenized for 10 s to insure uniform suspension, and assayed immediately for adenylate cyclase.

Assay of adenylate cyclase activity. Adenylate cyclase activity was assayed by the method of White and Zenser [11] or, in kinetic studies requiring an assay of lower blank and higher sensitivity, the method of Salomon et al. [12]. The latter was also used to study the effect of GTP. Both methods quantitate the formation of cyclic [^{32}P]AMP from [α-^{32}P]ATP. Reactions were initiated by adding 20 μl tissue homogenate (110—150 μg protein) or particulate (50—60 μg) to 30 μl of assay medium. Hormones or F^- were added to the assay medium prior to addition of enzyme. The final 50 μl volume contained 20 mM Tris · HCl buffer at pH 7.6, 5 mM $MgCl_2$, 2 mM theophylline, 1 mM ATP, 1 μCi [α-^{32}P]ATP, 2 mM cyclic AMP, 2.5 μCi cyclic [^{3}H]AMP, + ATP-regenerating system consisting of 20 mM creatine phosphate and 1 mg/ml creatine phosphokinase. Incubations (10 or 20 min) were in duplicate at 30° C in a shaking water bath and were terminated by the addition of 100 μl of a pH 7.6 stopping solution (34 mM sodium lauryl sulfate, 40 mM ATP, 12 mM cyclic AMP) followed by 50 μl containing 0.005 μCi cyclic [8-^{14}C]AMP. The total 200 μl volume was then placed in boiling water for 5 min, cooled and applied to the columns for isolation of cyclic AMP. Identity of the product of liver adenylate cyclase activity was verified as cyclic [^{32}P]AMP by its destruction with cyclic AMP phosphodiesterase. Recoveries of cyclic [^{3}H]- and cyclic [8-^{14}C]AMP in any single tube were essentially identical, indicating that there was no destruction of cyclic AMP product during the incubations and that all product loss (25—50%) occurred during chromatographic isolation.

Tissue protein concentrations determined by the method of Lowry et al. [13] were within a range where adenylate cyclase activity was linear with respect to protein concentration. Enzyme activity was also linear with respect to incubation times up to 20 min. Linearity with protein concentration and time was seen with both homogenate and particulate preparations from rats of all ages. The rationale for use of protein content of homogenates as a denominator in the determination of enzyme specific activities is the presence of equal concentrations of protein (per g wet weight tissue) in livers of animals of all ages studied.

Analysis of results. Age comparisons were always made with simultaneous assay of liver enzyme activity from 3-, 12-, and 24-month-old rats of the same sex. Particulate preparations were assayed for adenylate cyclase activity along with the parent homogenates. Age-, tissue preparation-, and stability-related comparisons of basal and stimulated adenylate cyclase activities were analyzed by the paired Student's *t*-test; male and female comparisons were unpaired. In stability experiments where enzyme preparations were assayed before and after 24 h at 0°C, significance of percentage activity losses after 24 h was analyzed by comparing percentage losses with 0% loss using the Student's *t*-test (one sample). Calculations were facilitated by the use of a Fortran program written in our laboratory and performed in a Raytheon Computer Model 706.

Results

Quantitation of liver adenylate cyclase activities in homogenate and particulate preparations

Adenylate cyclase is usually determined in washed particulate or purified membrane preparations. However, our results consistently showed greater total adenylate cyclase activity per starting wet weight of tissue in homogenates than in particulates prepared from the same homogenates. Adenylate cyclase activity was not lost mechanically during preparation of particulates, since supernatant fractions showed no detectable basal, hormone-, or F^--stimulated enzyme activity. Table I compares homogenate and particulate enzyme activities at each rat age tested. Epinephrine-, glucagon-, and F^--stimulated activities were all considerably and significantly greater in homogenates than in particulates except for epinephrine-stimulated activities of the 3-month-old rats. Furthermore, homogenate response to epinephrine stimulation was significantly greater in the 24-month- than in the 3-month-old rats while particulate activity showed

TABLE I

HOMOGENATE VS. PARTICULATE DIFFERENCES OF ADENYLATE CYCLASE ACTIVITIES FROM RATS OF THREE AGES

Homogenate and particulate preparations were assayed as described in Materials and Methods. Each activity represents the mean ± S.E. of six animals. Three experiments used males, and three used females. *P* values from paired Student's *t*-test; n.s., not significant ($P > 0.05$).

Age (months)		Adenylate cyclase activity ± S.E. (pmol cyclic AMP/20 min per 20 μl tissue equivalent)			
		Basal	Epinephrine (0.1 mM)	Glucagon (10 μM)	F^-(5 mM)
3	Homogenate	14.8 ± 2.3	39.7 ± 5.1	116.3 ± 15.2	86.8 ± 11.1
	Particulate	11.7 ± 1.5	31.8 ± 3.4	77.7 ± 6.3	51.3 ± 7.0
	P	n.s.	n.s.	<0.05	<0.05
12	Homogenate	16.5 ± 2.9	61.0 ± 10.3	125.5 ± 14.4	94.2 ± 7.5
	Particulate	12.8 ± 1.8	37.7 ± 5.4	76.5 ± 8.7	52.7 ± 8.1
	P	n.s.	<0.02	<0.01	<0.005
24	Homogenate	17.3 ± 3.6	72.8 ± 10.2	128.7 ± 15.9	93.7 ± 9.0
	Particulate	10.2 ± 2.4	38.7 ± 5.9	69.3 ± 12.0	49.0 ± 7.6
	P	n.s.	<0.005	<0.001	<0.005

no significant age difference with epinephrine stimulation at any age tested (see below). Age differences were thus obliterated by use of particulates.

Several other considerations preclude the use of particulates or purified membranes for quantitation of adenylate cyclase activity in liver. Recent work in our laboratory indicates that the supernatant fraction of homogenized rat liver contains a factor which is necessary for full expression of hormone- and fluoride-stimulated adenylate cyclase activities. Particulate fractions, when resuspended in supernatant, exhibit activities which are restored nearly to those of the original homogenates (Katz, M.S., Piñeyro, M.A., Kalish, M.I. and Gregerman, R.I., unpublished). Others have independently noted similar effects with supernatants from a variety of tissues tested on a membrane preparation from heart muscle [14]. A soluble supernatant factor has also been described in brain [15]. This issue is further discussed below (see Effect of age on stability of liver homogenate adenylate cyclase).

Another consideration precluding the use of purified liver membranes is that in our hands and those of others, such membranes are poorly responsive to catecholamines [16]. Although glucagon responsiveness is well preserved, qualitative alteration of the membranes may be inferred from their altered dose vs. response curves [17]. A recent study of the simultaneous effects of endotoxin and epinephrine on adenylate cyclase in mouse liver homogenates and purified membranes clearly demonstrates a gross blunting of responsiveness in the latter [18]. Thus, at the present time at least, adenylate cyclase of liver is clearly better quantitated in homogenates than in particulates or purified membranes.

Adenylate cyclase in males vs. females

Comparisons of males versus females at any single age tested (3, 12, or 24 months) showed no statistically significant sex differences of basal, glucagon-, or F^--stimulated homogenate adenylate cyclase activities. However, males and females did show different age-related changes of glucagon and F^- activities (Fig. 1, Table II and below). Epinephrine-stimulated values at any single age showed no sex differences at epinephrine concentrations up to 10 nM, but at 1 μM and 0.1 mM epinephrine 3-month females showed significantly greater activities than 3-month males ($P < 0.025$; data not shown). Because of these sex differences, enzyme changes with age were examined separately for males and females.

Effect of age on basal and F^--stimulated adenylate cyclase

Basal and F^--stimulated adenylate cyclase activities of liver homogenates from 3-, 12-, and 24-month-old rats are shown in Table II. The 24-month basal activities were significantly greater than those at 3 months (male, $P < 0.02$; female, $P < 0.01$) and 12 months (male, $P < 0.001$; female, $P < 0.01$). F^--stimulated activity was significantly greater in 24-month-old females than in 3- and 12-month-old females ($P < 0.01$), but F^- stimulation of 24-month-old males was not significantly increased.

Effect of age on hormone-sensitive adenylate cyclase

Glucagon- and epinephrine-sensitive homogenate adenylate cyclases are shown in the dose vs. response curves of Figs. 1 and 2. There was no significant

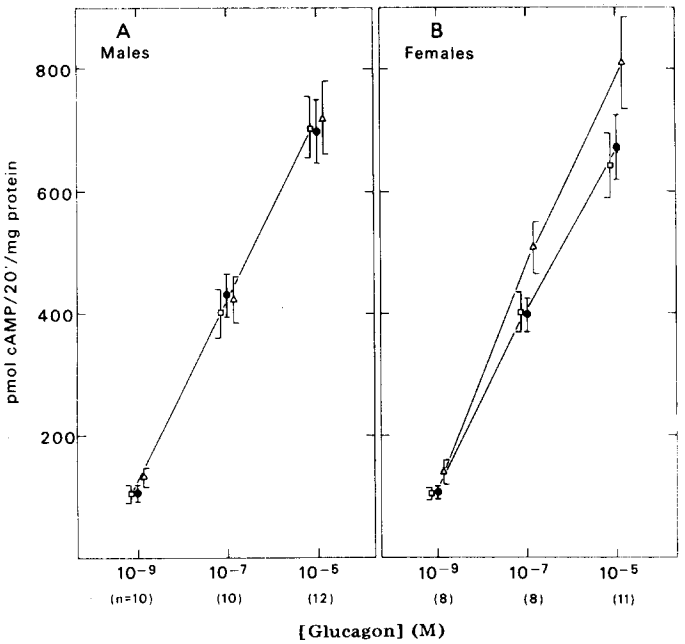

Fig. 1. Glucagon stimulation of liver homogenate adenylate cyclase from 3-, 12-, and 24-month-old rats, males (A) and females (B). Each plotted value represents the mean stimulated activity ± S.E. of *n* experiments each comparing liver homogenates from three single rats ages 3 (●———●), 12 (□———□), and 24 (△———△) months. Glucagon titration in males (A) does not show significant age differences except for the lowest concentration (10^{-9} M) where the 24-month values are slightly increased (12- vs. 24-month males; $P < 0.05$). In the females (B) values for the 24-month animals are higher at all concentrations: 12- vs. 24-month females, $P < 0.02$, < 0.005, < 0.005 for 10^{-9}, 10^{-7} and 10^{-5} M, respectively. Comparable significance of differences was found for 3- vs. 24-month animals. Basal values, not shown, are given in Table II.

change of glucagon-stimulated activity between 3 and 12 months in animals of either sex. Glucagon-sensitive enzyme activity (Fig. 1) of female rats increased modestly but significantly from 12 to 24 months for all hormone concentrations tested. In males there was no significant increase of glucagon-stimulated activity except at the threshold dose (Fig. 1). This single increase in males may merely reflect the significant increase of basal activity between 12 and 24

TABLE II

BASAL AND F^--STIMULATED LIVER HOMOGENATE ADENYLATE CYCLASE FROM 3-, 12-, AND 24-MONTH-OLD RATS

Rat liver homogenates were assayed as described in Materials and Methods. Each activity represents the mean ± S.E. of 11 experiments (except for males (basal) where $n = 12$).

Age (months)	Adenylate cyclase activity ± S.E. (pmol cyclic AMP/20 min per mg protein)			
	Males		Females	
	Basal	F^-(5 mM)	Basal	F^-(5 mM)
3	81.6 ± 12.0	528.7 ± 33.4	87.4 ± 8.7	615.4 ± 66.5
12	79.8 ± 9.0	513.8 ± 32.8	81.1 ± 7.3	532.6 ± 42.5
24	107.5 ± 9.4	564.4 ± 34.8	134.3 ± 20.5	718.1 ± 85.0

months (Table II and above) rather than a true increase of glucagon-sensitive activity at low concentrations of hormone.

Epinephrine-sensitive adenylate cyclase activity (Fig. 2) increased strikingly between 12 and 24 months in both males and females and at every hormone concentration tested. Furthermore, in both sexes epinephrine activity at 24 months (1 μM and 0.1 mM) was twice as great as that at 3 months. Mean activities for both sexes were greater at 12 than at 3 months at the latter concentrations of epinephrine, but the differences were not statistically significant. However, when the data were pooled for males and females, activities at 12 months were significantly higher than those at 3 months ($P < 0.05$). No other differences between 3- and 12-month animals were seen for basal, glucagon-, or F^--stimulated activities even when male and female activities were pooled.

The striking increase of epinephrine response between 12 and 24 months shown in Fig. 2 is not obvious from age-related comparisons of homogenates in Table I. However, this difference is undoubtedly due to the smaller number of experiments in Table I than in Fig. 2. In fact, the results from Table I were expressed per mg protein and are included in the data for Fig. 2.

Dose vs. response curves for epinephrine

Epinephrine stimulation of adenylate cyclase of 3-, 12-, and 24-month-old female rats was determined as a function of concentration of hormone and plotted as percent of maximum response to determine whether any of the observed results could be due to displacements of the dose vs. response relationships. Fig. 3 shows essentially the same curve for epinephrine-stimulated adenylate cyclase activity at all three ages, eliminating this possibility as an explanation for the age differences.

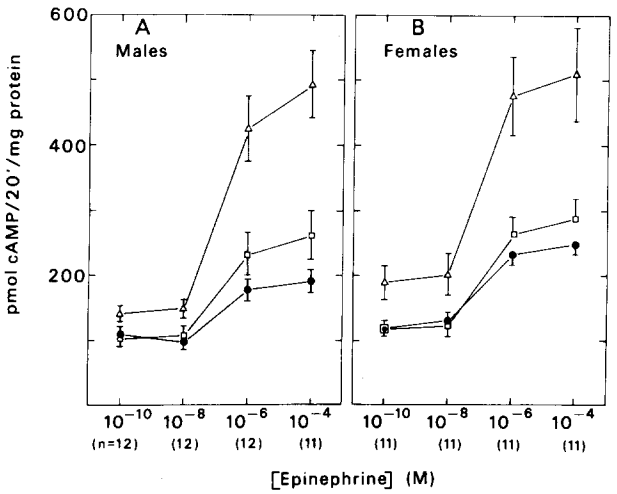

Fig. 2. Epinephrine stimulation of liver homogenate adenylate cyclase from 3-, 12-, and 24-month-old rats, males (A) and females (B). Each plotted value represents the mean stimulated activity ± S.E. of n experiments each comparing liver homogenates from three single rats ages 3 (●———●), 12 (□———□), and 24 (△———△) months. For males 12 vs. 24 months, $P < 0.02$, < 0.01, < 0.005, < 0.005 for 10^{-10}, 10^{-8}, 10^{-6} and 10^{-4} M, respectively. For females 12 vs. 24 months, $P < 0.005$, < 0.005, < 0.001, < 0.005. Significance of differences between 3 and 24 months is comparable. Basal values, not shown, are given in Table II.

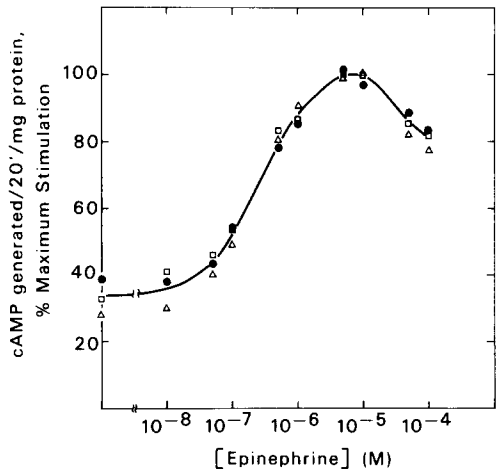

Fig. 3. Epinephrine stimulation of liver homogenate adenylate cyclase from 3-, 12-, and 24-month-old female rats. Each plotted value of percent of maximum epinephrine response represents the mean of duplicate determinations from a single experiment comparing three liver homogenates each pooled from three rats ages 3 (●———●), 12 (□———□), or 24 (△———△) months.

Repeat dose vs. response curves gave similar results to Fig. 3, and both experiments showed slightly less stimulation by 0.1 mM epinephrine than by 1 μM epinephrine. This is in contrast to the slight increase in epinephrine stimulation from 1 μM to 0.1 mM shown for each age in Fig. 2. The apparent discrepancy may be due to preparation for logistical reasons of epinephrine dilutions nearly 1 h in advance of initiation of adenylate cyclase assays in the experiments for Fig. 2, whereas epinephrine was prepared immediately before assay for Fig. 3. Because epinephrine is readily oxidized on exposure to air, concentrations prepared for 1 μM and 0.1 mM but used after 1 h were probably lower than recorded. The results in Fig. 2 remain valid as relative responses because all epinephrine concentrations in each experiment were used at the same time after preparation and were therefore subject to the same degree of inactivation. Furthermore, the shape of the curves in Figs. 2 and 3 indicates that at the highest epinephrine concentration used, maximal or near maximal stimulation must have occurred.

Ratios of stimulated : basal activities

Age-related changes of glucagon- and epinephrine-stimulated adenylate cyclase activities were further examined by comparisons of ratios of stimulated to basal (relative) activities. Although often used in the past, such ratios of hormone-stimulated to basal activities ("fold-stimulation") are by some workers no longer considered meaningful indices of hormone stimulation because of uncertainty over the significance of basal activities and the acknowledged complexity of interactions between enzyme, substrate, and activators [19,20]. Nonetheless, the ratios are included here because they do emphasize a major difference between stimulation by epinephrine as compared to other agents.

Table III shows that relative glucagon (10 μM) stimulation decreased from 12 to 24 months in rats of both sexes and from 3 to 24 months in males only.

TABLE III

RATIOS OF STIMULATED TO BASAL ACTIVITIES OF LIVER HOMOGENATE ADENYLATE CYCLASE FROM 3-, 12-, AND 24-MONTH-OLD RATS

Ratios of stimulated to basal activities are calculated from the same experiments as in Table II and Figs. 1 and 2. Significant differences between 12 and 24 months ratios are shown by * ($P < 0.05$, by paired Student's *t*-test) and ** ($P < 0.01$). Significant differences between 3 and 24 months ratios are shown by ‡ ($P < 0.05$) and ‡‡ ($P < 0.01$). Ratios in this table show small numerical differences from the same ratios calculated directly from Table II and Figs. 1 and 2 because of different calculation methods; values in this table are means of stimulated/basal ratios ± S.E., whereas calculations from Table II and Figs. 1 and 2 give ratios of mean absolute activities.

Age (months)	Males			Females		
	Glucagon (10 μM)	Epinephrine (0.1 mM)	F^- (5 mM)	Glucagon (10 μM)	Epinephrine (0.1 mM)	F^- (5 mM)
3	10.8 ± 2.2	2.8 ± 0.6	8.1 ± 1.7	8.3 ± 0.9	3.2 ± 0.4	7.5 ± 0.9
12	9.7 ± 1.0	3.3 ± 0.4	7.3 ± 1.0	8.2 ± 0.5	3.8 ± 0.4	6.9 ± 0.6
24	7.3 ± 0.9 **,‡	4.9 ± 0.7 ‡ ‡	5.9 ± 0.8 **	6.8 ± 0.6 *	4.2 ± 0.4 ‡	5.4 ± 0.4 *,

On the other hand, relative epinephrine (0.1 mM) stimulation increased from 3 to 24 months in both sexes and from 12 to 24 months in males only. F^- stimulation relative to basal declined with age. Changes of activity from 3 to 12 months were not statistically significant. These reciprocal age changes of relative glucagon and epinephrine activities provide indirect evidence for the concept of two distinct hormone-sensitive adenylate cyclase enzymes in rat liver [21]. The conceptual problems of viewing adenylate cyclase activities in terms of "fold-stimulation" as opposed to absolute activities seen after stimulation are further considered in Discussion.

Effect of age on stability of liver homogenate adenylate cyclase

The activity of adenylate cyclase could reflect the integrity or structure of the plasma membrane to which the enzyme is bound rather than the number of enzyme units (see Discussion). To examine possible age-related changes in the cell membranes which might account for our observed increases of adenylate cyclase activity, stability of activity, a measure of membrane integrity, was assessed by assaying for losses with time. The loss of epinephrine- and glucagon-responsive activities during preparation of particulates and membranes precluded use of purified membranes for these stability experiments (see Quantitation of liver adenylate cyclase activities in homogenate and particulate preparations).

Homogenates from rats of different ages were allowed to remain at 0°C for 24 h. Losses of activity in homogenates from 3-, 12-, and 24-month-old animals are shown in Fig. 4. The magnitude of the loss of basal and stimulated activities was directly related to increasing age. Loss of basal activity was significant between 3 and 24 months ($P < 0.02$). Losses of stimulated activity were significant between 12 and 24 months, but in no case was there significant change from 3 to 12 months.

Glucagon-stimulated adenylate cyclase activity was the most stable. Losses with preparations from 3- and 12-month animals were not significant while greater than 50% decrease was seen at 24 months ($P < 0.005$). Epinephrine-stimulated adenylate cyclase was the most unstable activity, and significant

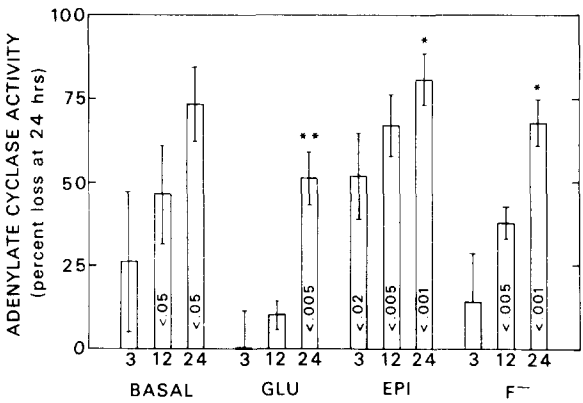

Fig. 4. Stability of adenylate cyclase activity in rat liver homogenates. Preparations were assayed before and after incubation for 24 h at 0°C. Bars represent means percent loss of activity, ± S.E. (five experiments; three with male animals, two with females). Each experiment used three animals (3, 12, 24 months). Concentrations of glucagon (GLU), 10 μM; epinephrine (EPI), 0.1 mM; F^-, 5 mM. *P* values within bars show levels of significance of activity loss after 24 h. Symbol above bar gives significance of difference from preceding bar (*, $P < 0.05$; **, $P < 0.02$; no symbol, $P > 0.05$).

losses of activity were seen in homogenates from 3-, 12-, and 24-month animals. Basal and F^--stimulated activities were of intermediate stability with significant activity losses after 24 h in 12- and 24-month animals but not in those of 3 months.

In comparing stability of stimulated activities at a given age, the order was generally glucagon > F^- > epinephrine. Exceptions were lack of significant differences of stabilities between glucagon and F^- at 3 months and between F^- and epinephrine at 12 months. Fig. 4 suggests a difference between F^- and epinephrine stabilities at 12 months, but this difference was not statistically significant by the paired Student's *t*-test used for stability comparisons. An unpaired comparison, on the other hand, shows F^- activity to be significantly more stable than epinephrine activity at 12 months ($P < 0.05$).

Liver content of lysosomal enzymes is known to increase with age [22], and higher protease or esterase activities in the liver of old rats might conceivably therefore account for the decreased stability of adenylate cyclase in 24-month liver homogenates compared to 3- and 12-month preparations. To examine this possibility, losses of basal and stimulated adenylate cyclase activities in a homogenate pooled from two 24-month-old male rats were compared in the presence and absence of several protease and esterase inhibitors. Tissue aliquots were homogenized in standard medium or medium containing pepstatin (10 μM), Trasylol (1000 kallikrein inactivator units/g tissue), or diisopropylfluorophosphate (2.7 mM); aliquots were then assayed for adenylate cyclase activity immediately after preparation and again after 24 h at 0°C. None of the three inhibitors tested prevented the 24-h losses of activity seen in Fig. 4. The decreased stability of adenylate cyclase from 24-month-old rats was therefore probably not due merely to increased protease or esterase activity in preparations from the old animals.

As discussed earlier, the supernatant fraction of liver homogenates appears to contain a factor which increases adenylate cyclase activity (see Quantitiation of

liver adenylate cyclase activities in homogenate and particulate preparations). However, the supernatant factor does not appear to be involved in our observed age-related instability of adenylate cyclase during incubation at 0°C for 24 h. In separate experiments we have shown that the supernatant fraction is stable under these conditions. Furthermore, the ability of supernatant factor to restore hormone responsiveness of particulate preparations is not related to the age of the animal from which the supernatant is derived. Increased hormone-responsiveness of the homogenates of 24-month-old animals is clearly related in particulate-supernatant cross-over experiments to the membrane-bound enzyme rather than to any alteration of activity of the soluble supernatant factor (Katz, M.S., Pineyro, M.A., Kalish, M.I. and Gregerman, R.I., unpublished).

Effect of GTP on adenylate cyclase activity

GTP activates rat liver membrane adenylate cyclase presumably by interacting with either the catalytic subunit or a separate regulatory subunit of the enzyme complex [23,24]. The demonstration of a requirement for GTP usually involves conditions other than those used in these experiments, i.e. lower concentrations of ATP, purified membranes, or the use of highly purified, GTP-free ATP [25]. However, we wished to be certain that our observed age-related increase of enzyme activity was not due to relative lack of available GTP in homogenates from young animals. A liver homogenate preparation pooled from three 3-month-old female rats was assayed for adenylate cyclase in the absence and presence of added 0.1 mM GTP. Basal and stimulated activities were unaffected by addition of GTP. Addition of GTP to a pooled homogenate from 24-month animals was also without effect.

Age and kinetic characteristics of adenylate cyclase

Kinetic analyses of enzyme were performed to examine possible evidence for qualitative age effects on the adenylate cyclase complex. Using homogenates from 3-, 12-, and 24-month-old animals, each prepared and pooled from three females, basal and stimulated activities were measured at 10 min with ATP concentrations varied from 10 μM to 1 mM and excess (5 mM) Mg^{2+}. Kinetic data were fitted to Eadie-Hofstee plots of v vs. v/S, which have been shown to be superior to Lineweaver-Burk determinations of kinetic parameters [26]. Linear regression, resultant slopes (slope by Eadie-Hofstee plot = $-K_m$), and standard errors of slopes were calculated. K_m values with epinephrine (0.1 mM)-, glucagon (10 μM)-, and F^- (5 mM)-stimulated adenylate cyclase showed no statistically significant variation with age. Mean K_m values from 3-, 12-, and 24-month homogenates were 0.33 mM for basal, 0.26 mM with epinephrine, 0.18 mM with glucagon, and 0.22 mM with F^-. These K_m values approximate those previously calculated for basal and stimulated liver adenylate cyclase in several assay systems [16,23]. The K_m for basal adenylate cyclase activity appeared to be significantly lower at 12 than at 3 months in this experiment ($P < 0.01$). However, fits of the data of the linear regression lines for the basal activities were adequate (r equal to or better than 0.85) only to provide approximations of K_m. For this reason establishment of significance for suspected differences of basal K_m values would require a large number of assays and would apparently serve to establish at most only small differences. However, the present data are

sufficient to indicate that the age-related changes observed for basal and stimulated adenylate cyclase activities are not due to alterations of K_m. Moreover, the K_m data support the validity of the routine assays since the latter were performed at concentrations of substrate (1 mM ATP) which were 3—5 times those of the apparent K_m values.

Discussion

In this study the most striking age-related change of adenylate cyclase of rat liver was the post-maturational increase of epinephrine-responsive activity, which approximately doubled between 3 and 24 months of age (Fig. 2). Animals 12 months old showed only slightly greater epinephrine response than did those of 3 months. Most of the increase of epinephrine-responsive activity therefore occurs during senescence rather than during earlier adult aging. Although changes with senescence of hormone-sensitive adenylate cyclase have been seen earlier, no previous studies have observed increased catecholamine-sensitive enzyme. This age-related increase of epinephrine-responsive adenylate cyclase of liver is, in fact, in striking contrast to the decline with senescence of catecholamine-responsive activity previously noted in fat [7] and brain [8].

Basal, F^--, and glucagon-stimulated adenylate cyclase activities also showed significant increases in senescent rats without changes between 3 and 12 months (Table II; Fig. 1). However, increases at 24 months were smaller than those for the epinephrine response and were sex dependent. While basal activity was increased in both 24-month-old males and females, F^-- and glucagon-stimulated activities showed significant increases only in senescent females.

The age-related increases of basal, F^--, and glucagon-responsive adenylate cyclase activities in rat liver are in contrast to adenylate cyclase changes with aging found in other tissues. In epididymal fat basal, F^-, and glucagon activities all decrease between 2 and 24 months of age [7]. In the cerebral cortex and hippocampus basal activities are unchanged between 3 and 24 months but increase in the caudate and cerebellar regions of the 24-month animals. F^--stimulated activity also decreases at advanced age in brain caudate but increases in the cerebral cortex and is unchanged in the cerebellum and hippocampus [8]. In the rabbit thoracic aorta basal and F^--stimulated adenylate cyclase is unchanged with age, while F^--stimulated activity increases in the abdominal aorta [10].

Our conclusions concerning age-related alterations of liver adenylate cyclases are based on enzyme activities expressed as absolute activities following hormone (and F^-) stimulation and not on ratios of stimulated to basal activities, or "fold-stimulation". Unfortunately, many previous investigators have used fold-stimulation ratios synonymously with total enzyme responsiveness, an approach which is clearly misconceived and confusing. Some of the problems of expressing activities as fold-stimulation have been mentioned previously (see Results). Although ratios of stimulated to basal activities have usefulness as measures of the presence in a particular tissue of hormone-sensitive activity, there is in fact no way of quantitating total enzyme activities by such ratios. Any change of the number or activity of hormone-sensitive adenylate cyclase catalytic units would not only be accompanied by a change of stimulated activ-

ity but might also be paralleled by a change of basal activity. However, under these circumstances, the ratio of stimulated to basal activities would not be expected to change. Our observed age-related increases of basal activities may in fact be specifically due to increased amounts or activity of epinephrine-sensitive enzyme. In this case, expressing our results merely as fold-stimulation would obscure significant age differences. As shown in Table III, the increases with age of such ratios for epinephrine-sensitive activity are less striking than the absolute increases although still statistically significant. For glucagon- and F^--stimulated activities the ratios actually decrease with age.

The post-maturational changes we have found in rat liver differ from the developmental changes reported in earlier studies [1—6]. Two reports show decreased basal adenylate cyclase activity during development to 3 months of age [3,4]; in contrast, basal activity increases in our older animals. One study of "old adult" Sprague-Dawley rats noted a decline of basal, epinephrine-, and glucagon-stimulated activity but reported age in terms of body weight [1]. One can estimate from the reported body weights and published growth curves for this strain [27] that the "old" rats were probably no more than 2—5 months of age. Also in contrast to our results with liver homogenates are the large decreases of epinephrine-responsive liver particulate adenylate cyclase which have been descirbed during development. In addition, by 40—90 days of age particulates were noted to be nearly unresponsive to epinephrine [2,3]. On the other hand, our particulates prepared from 90-day-old animals showed nearly 3-fold stimulation (Table I). The limitations of studies based only on the use of particulates have been discussed (see Results).

Recent studies from several laboratories have indicated that aging may be associated with the accumulation of altered enzyme molecules. Examples of this phenomenon include fructose-1,6-diphosphate aldolase from liver and muscle of the mouse and aldolase, isocitrate lyase, enolase, and phosphoglycerate kinase from a free living nematode [28—33]. In all cases studied to date, enzyme specific activities have decreased because of the synthesis of partially or completely inactive enzyme molecules [28,29,33]. Although possible, it seems unlikely that our results are related to such a phenomenon, since it is difficult to conceive of an alteration of the protein portion of adenylate cyclase which would result in enhanced rather than decreased enzymatic activity.

The mechanism of the increase with age of hormone-sensitive adenylate cyclase activity does not appear to involve gross qualitative change of the enzyme as reflected by kinetic parameters. Similar dose vs. response curves for the epinephrine-sensitive enzyme in preparations from rats of different ages (Fig. 3) suggest that the affinity of the hormone receptors for epinephrine does not change with age. Similarly, K_m values for hormone- and F^--stimulated adenylate cyclase do not appear to change significantly with age. Thus, in the absence of evidence for age-related change of enzyme characteristics to account for enhanced activities, our results would appear to be compatible with an increase with age in the number of epinephrine-sensitive adenylate cyclase complexes and a smaller, sex-dependent increase in the numbers of glucagon-sensitive complexes *.

* The data permit no distinction, of course, between the possibilities of increased numbers of hormone receptors versus catalytic subunits. Since F^- is thought to stimulate adenylate cyclase direct-

However, an alternative explanation could account for the observed increase in the amount of enzyme activity; the V of adenylate cyclase could increase. At least one definite qualitative change of the adenylate cyclase complex appears to occur with aging. Fig. 4 shows that the stability of basal, hormone-, and F^--stimulated adenylate cyclase activities decreases with increasing age. These findings may well be based in an age-related change of the structure or composition of the cell membrane to which adenylate cyclase is bound. It is now established that alterations of phospholipids in membranes can affect the activity of enzymes associated with these lipids. In the well-studied case of glucose-6-phosphatase of liver microsomes, phospholipids have been shown to provide an environment which constrains maximum enzyme activity [36]. Age-related alterations of enzyme activities in rat liver microsomal enzymes have also been noted in association with altered concentrations of phospholipids [37]. Recent work published since completion of our own shows that plasma membranes of old rats show 2-fold increases of activities of several ATPases associated with increased thermal instability (37° C) of these enzymes. These changes are also accompanied by decreased phospholipid content which can be reversed by feeding "essential" phospholipids, a maneuver which also increases the stability of the ATPases in the membranes from the old animals [38]. A role for phospholipids in the regulation of adenylate cyclase, probably at the level of the "transducer", is well recognized. Moreover, "constraint" of adenylate cyclase activity by membrane lipids has been discussed in terms of the mechanism by which certain agents, including detergents, activate the enzyme [39,40]. The recent studies of age-related changes in cell membranes of ATPase activity and phospholipids [38] therefore appear to have direct implications for the interpretation of our own results. The complexities of this area are, however, already clear. Other work has shown that, in very young animals, dietary depletion of essential fatty acids produced increased ATPase activity but decreased glucagon- and F^--sensitive adenylate cyclase activity in liver membranes [41]. Thus, membrane-bound ATPase and adenylate cyclase activity need not change in parallel. Nevertheless, in explaining changes of adenylate cyclase activity generally, altered composition and structure of the plasma membrane with a resulting change of enzyme activity (V) must now be considered, in addition to the usually invoked possibilities of altered numbers of receptors and/or catalytic units.

Our results suggest that age-related alterations of hormone responsiveness, especially to epinephrine, may be demonstrable for rat liver in vivo, although direct experimental evidence is not yet available. In several other situations positive correlations have already been made between changes of adenylate cyclase activity and hormone responsiveness. For example, decreased glucagon-sensitive adenylate cyclase activity in liver plasma membranes, produced during

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ly at the catalytic subunit rather than through receptors [34], one might consider that changes of F^--stimulated activity could be used to estimate altered total numbers of catalytic units if, as some workers have assumed, F^- causes full expression of the enzyme activity [35]. However, Figs. 1 and 2 and Tables I and II show that F^- activity is not only less than total hormone-sensitive activity (glucagon plus epinephrine) at each age tested but is also less than glucagon activity alone. Less F^- activity than glucagon activity has been previously reported for liver membranes [16]. These findings indicate that age-related change of F^--stimulated liver enzyme activity does not adequately reflect alterations of total activity or numbers of catalytic subunits.

experimental deficiency of essential fatty acids, appears to correlate with decreased hormone responsiveness in vivo [42]. Moreover, during dietary manipulations altered fat cell adenylate cyclase activity is similarly associated with parallel changes of hormone-induced lipolysis [43].

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References

1 Bitensky, M.W., Russell, V. and Blanco, M. (1970) Endocrinology 86, 154—159
2 Christoffersen, T. and Øye, I. (1974) Acta Endocrinol. Suppl. 191, 67—71
3 Bär, H-P. and Hahn, P. (1971) Can. J. Biochem. 49, 85—89
4 Williams, R.H. and Thompson, W.J. (1973) Proc. Soc. Exp. Biol. Med. 143, 382—387
5 Forn, J., Schonhoffer, P.S., Skidmore, I.F. and Krishna, G. (1970) Biochim. Biophys. Acta 208, 304—309
6 Blazquez, E., Rubalcava, B., Montesano, R., Orci, L. and Unger, R.H. (1976) Endocrinology 98, 1014—1023
7 Cooper, B. and Gregerman, R.I. (1976) J. Clin. Invest. 57, 161—168
8 Walker, J.B. and Walker, J.P. (1973) Brain Res. 54, 391—396
9 Zimmerman, I.D. and Berg, A.P. (1975) Mech. Ageing Dev. 4, 89—96
10 Schonhoffer, P.S., Skidmore, I.F., Forn, J. and Fleisch, J.H. (1971) J. Pharm. Pharmacol. 23, 28—31
11 White, A.A. and Zenser, T.V. (1971) Anal. Biochem. 41, 372—396
12 Salomon, Y., Londos, C. and Rodbell, M. (1974) Anal. Biochem. 58, 541—548
13 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
14 Sanders, R.B. (1976) Fed. Proc. 35, 455
15 Cheung, W.Y., Bradham, L.S., Lynch, T.J., Lin, Y.M. and Tallant, E.A. (1975) Biochem. Biophys. Res. Commun. 66, 1055—1062
16 Pohl, S.L., Birnbaumer, L. and Rodbell, M. (1971) J. Biol. Chem. 246, 1849—1856
17 Kalish, M.I., Katz, M.S., Piñeyro, M.A. and Gregerman, R.I. (1975) Program 57th Annual Meeting of the Endocrine Society, New York, N.Y., p. 159
18 Donlon, M.A. and Walker, R.I. (1976) Experientia 32, 179—181
19 Rodbell, M. (1975) J. Biol. Chem. 250, 5826—5834
20 Londos, C. and Rodbell, M. (1975) J. Biol. Chem. 250, 3459—3465
21 Bitensky, M.W., Russell, V. and Robertson, W. (1968) Biochem. Biophys. Res. Commun. 31, 706—712
22 Wilson, D. (1973) Gerontologia 19, 79—125
23 Hanoune, J., Lacombe, M.-L. and Pecker, F. (1975) J. Biol. Chem. 250, 4569—4574
24 Rodbell, M., Birnbaumer, L., Pohl, S.L. and Krans, H.M.J. (1971) J. Biol. Chem. 246, 1877—1882
25 Kimura, N., Nakane, K. and Nagata, N. (1976) Biochem. Biophys. Res. Commun. 70, 1250—1256
26 Dowd, J.E. and Riggs, D.S. (1965) J. Biol. Chem. 240, 863—869
27 Poiley, S.M. (1972) Lab. Anim. Sci. 22, 759—779
28 Gershon, H. and Gershon, D. (1973) Proc. Natl. Acad. Sci. U.S. 70, 909—913
29 Gershon, H. and Gershon, D. (1973) Mech. Ageing Dev. 2, 33—41
30 Zeelon, P., Gershon, H. and Gershon, D. (1973) Biochemistry 12, 1743—1750
31 Reiss, U. and Rothstein, M. (1975) J. Biol. Chem. 250, 826—830
32 Sharma, H.K., Gupta, S.K. and Rothstein, M. (1976) Arch. Biochem. Biophys. 174, 324—332
33 Gupta, S.K. and Rothstein, M. (1976) Arch. Biochem. Biophys. 174, 333—338
34 Birnbaumer, L., Pohl, S.L. and Rodbell, M. (1971) J. Biol. Chem. 246, 1857—1860
35 Perkins, J.P. (1973) in Advances in Cyclic Nucleotide Research (Greengard, P. and Robison, G.A., eds.), Vol. 3, pp. 1—64, Raven Press, New York
36 Zakim, D. (1970) J. Biol. Chem. 245, 4953—4961
37 Grinna, L.S. and Barber, A.A. (1972) Biochim. Biophys. Acta 288, 347—353
38 Hegner, D. and Platt, D. (1975) Mech. Ageing Dev. 4, 191—200
39 Pohl, S.L., Krans, H.M.J., Kozyreff, V., Birnbaumer, L. and Rodbell, M. (1971) J. Biol. Chem. 246, 4447—4454
40 Levey, G.S. (1975) Recent Prog. Horm. Res. 29, 361—386
41 Brivio-Haugland, R.P., Louis, S.L., Musch, K., Waldeck, N. and Williams, M.A. (1976) Biochim. Biophys. Acta 433, 150—163
42 Louis, S.L., Brivio-Haugland, R.P. and Williams, M.A. (1976) J. Supramol. Struct. 4, 487—496
43 Gorman, R.R., Tepperman, H.M. and Tepperman, J. (1973) J. Lipid Res. 14, 279—285