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Changes in Catecholamine- and Glucagon-Responsive Adenylate Cyclase Activity in Preneoplastic Rat Liver

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SUMMARY

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Increases in basal and catecholamine-responsive adenylate cyclase activity have been observed in livers of rats treated with the carcinogen 3′-methyl-4-dimethylaminoazobenzene (3′-MeDAB). These increases are accompanied by a decrease in glucagon responsiveness of the enzyme. Binding of 3′-MeDAB metabolites reaches a peak at 3 weeks, considerably earlier than the peak increases in adenylate cyclase activity at 6 weeks. By the time tumors are detected in the tissue (10–20 weeks), adenylate cyclase activity and response to catecholamine stimulation are back to or below normal. The greatly increased stimulation of adenylate cyclase activity by catecholamines is a *beta* receptor effect; however, inhibition of the enzyme by catecholamines is also seen when there is elevated basal activity and may be an *alpha* adrenergic effect. That glucagon and catecholamine effects are additive at certain concentrations of catecholamine, but only partially so at others, indicates that the two receptor-cyclase systems are not entirely separate. The preneoplastic rat liver would appear to be a suitable tissue to study hormone-receptor interactions at both molecular and physiological levels. It is the first broken cell preparation in which inhibitory effects of catecholamines on adenylate cyclase activity have been observed. The relationship between the observed changes and the induction of cancer is not yet clear.

INTRODUCTION

During the preneoplastic stages of carcinogenesis in rat liver with 3′-methyl-4-dimethylaminoazobenzene and 2-acetylaminofluorene, there is an increase in the basal activity and isoproterenol responsiveness of the membrane-bound enzyme adenylate cyclase. This phenomenon has been seen in crude membrane preparations (1, 2), and also with 2-acetylaminofluorene in intact cells and tissue slices (3, 4). The statistical validity of the alterations observed with 3′-MeDAB[3] was established in our study (1) by the use of large numbers of rats, which required freezing of homogenates for subsequent batch assays. It was noted in these experiments that one effect of freezing was to decrease the elevated activity of the enzyme, indi-

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[3] The abbreviations used are: 3′-MeDAB, 3′-methyl-4-dimethylaminoazobenzene; cAMP, adenosine cyclic 3′,5′-monophosphate.

cating that, although qualitatively sound, our data may not have been quantitatively representative of the changes which occurred. We were also aware that the changes in adenylate cyclase activity may have reflected a pharmacological action of the 3'-MeDAB metabolites present in the tissue rather than a change in the tissue itself. We have therefore repeated our time study, assaying fresh preparations of washed particles and correlating the total concentrations of 3'-MeDAB metabolites present in the preparations with the biochemical changes. These findings are reported in the present paper.

This report contains also the findings made in our first pharmacological studies of adenylate cyclase activation in this tissue with respect to *alpha* and *beta* adrenergic receptors and glucagon receptors.

MATERIALS AND METHODS

Chemicals. The sources of chemicals and biochemicals used were as follows: 3'MeDAB, Koch-Light Laboratories, Colnbrook, England; the L isomers of isoproterenol, epinephrine, and norepinephrine bitartrates, Sigma Chemical Company; ATP (disodium salt), Boehringer/Mannheim, Mannheim, Germany; phosphoenolpyruvate, Boehringer/Mannheim and Fine Chemicals of Australia; pyruvate kinase, Boehringer/Mannheim, theophylline and propranolol hydrochloride, Calbiochem; crystalline porcine glucagon, a gift from Eli Lilly and Company; bovine serum albumin, Cohn fraction V, Commonwealth Serum Laboratories, Melbourne; and [α-^{32}P]ATP, the Radiochemical Centre, Amersham, England.

Carcinogen diet. Sprague-Dawley albino rats weighing more than 100 g were fed ad libitum with crushed Barastoc dog food cubes mixed with 0.06% 3-'-MeDAB dissolved in corn oil (5). A control group of rats was fed the same diet without the carcinogen. At weekly intervals, up to 12 weeks, rats were killed in groups containing one control and two test animals.

Preparation of crude membrane fractions. Rats were killed by cervical dislocation. Livers were removed and inspected, and a portion was placed in formol-acetic acid fixative for hematoxylin and eosin staining and histological examination. Subsequent steps were carried out at 5°. The livers were rinsed in 0.9% NaCl, blotted, and transferred to homogenizing medium (Tris-HCl, pH 7.6, 50 mM; KCl, 30 mM; $MgSO_4$, 4.5 mM; dimethyl sulfoxide, 10%). Portions of each liver were homogenized with 10 volumes of medium with 10 strokes of a hand-held, loosely fitting Dounce homogenizer. The homogenate was filtered through four layers of cheesecloth, and the filtrate was centrifuged at 1100 $\times$ g_{max} for 10 min. The pellet was removed, washed once, recentrifuged, and resuspended gently in the same volume of homogenizing medium, and aliquots were used immediately in the adenylate cyclase assay. Aliquots of each preparation were immediately frozen and stored at $-90°$ for subsequent estimations of binding of 3'-MeDAB metabolites, and for studies of the effect of freezing and storage at this low temperature.

Adenylate cyclase activity. Adenylate cyclase activity was estimated by incubation of 50 μl of the homogenate (250 μg of protein) at 30° for 10 min with [α-^{32}P]ATP (2 mM, 10 cpm/pmole) in a buffer-electrolyte solution containing Tris-HCl, pH 7.6, 50 mM; KCl, 30 mM; $MgSO_4$, 4.5 mM; bovine serum albumin, 0.01%; and theophylline, 10 mM, in a final volume of 95 μl. A regenerating system of pyruvate kinase (0.154 mg/ml) and phosphoenolpyruvate (3 mM) was used. Hormones and/or drugs were added in 10 μl of the buffer-electrolyte solution. Catecholamines were used without preservative within 15 min of solution in the cold, and away from strong light. The reaction was begun by addition of enzyme and terminated by boiling for 5 min. ^{32}P-Labeled cyclic 3',5'-AMP was separated from the other components of the reaction mixture by chromatography on columns (6.0 $\times$ 0.6 cm) of neutral alumina (6) and eluted with 4.0 ml of Tris-HCl (50 mM, pH 7.4). Recovery was 70%. In some experiments tritiated cAMP was added in trace amounts (1 μM) to test recovery. The recovery from incubation and separation together was also 70%, indicating negligible phosphodiesterase activity. Protein

concentrations were estimated by the Hartree modification of the Lowry method (7), using bovine serum albumin as standard.

Estimation of 3'-MeDAB binding. Binding of 3'-MeDAB to the crude liver membrane preparations was measured with slight modifications of the method of Chauveau and Decloitre (8) as follows: 3-ml samples of membranes were taken, and 3 ml of cold 20% perchloric acid were added. The precipitates were washed once with cold 5% perchloric acid and twice with cold absolute ethanol before two successive extractions at 80° with an ethanol–ether mixture (3:1). After two washes with acetone, the precipitates were dried and dissolved in 3 ml of 85% formic acid. Differential spectrophotometry at 450 and 600 nm allowed calculation of the amount of azo dye by use of the formula of May (9). Results are expressed as nanograms of 3'-MeDAB per milligram of protein of crude liver membranes.

Experiments with fetal rats. In some experiments female rats were killed on the 17th day of pregnancy, and the livers of the fetuses were removed, pooled, and weighed. Homogenates were prepared and assayed for adenylate cyclase activity and hormone responsiveness as described for adult livers.

RESULTS

The increase in basal activity and isoproterenol responsiveness and depression of glucagon responsiveness of the adenylate cyclase in carcinogen-treated rats observed in these studies (Fig. 1) was qualitatively similar to that previously described (1). There were, however, some quantitative differences both in the magnitude of the effects and in the time at which they were seen. Changes in hormone responsiveness were clearly seen by the end of the first week. The isoproterenol response and basal activity rose sharply to a maximum at 6–7 weeks (previously 8–12 weeks), the former reaching a value of 750% (previously 450%) of that of the controls. The glucagon response decreased progressively to a value of 40% of the control at 12 weeks. There was much less scatter during the rising phase of the basal and isoproterenol curves than on the descending phase. Histological studies showed a similar pattern; all tissues examined up to 7 weeks showed minor changes (patches of pale, swollen cells), whereas after 7 weeks gross cellular abnormalities were seen in some animals, while in others the changes were still minor. In one animal, in which an area of cholangiocarcinoma appeared at 8 weeks, the basal activity and isoproterenol responses were almost back to normal.

As can be seen in Fig. 2, the peak of binding of metabolites of the carcinogen to the preparations was reached at 3–4 weeks, or 2–3 weeks earlier than the peak in the changes in cyclase activity.

Examination of the dose-response curves to isoproterenol and to glucagon obtained after 4 weeks of carcinogen feeding gives some information as to the nature of the changes observed. For isoproterenol, the K_m in both control and treated

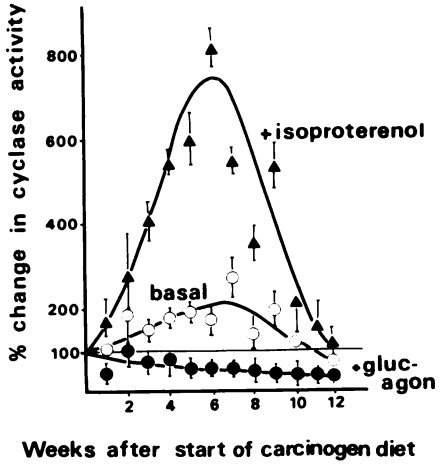

FIG. 1. *Time course of effect of 3'-MeDAB treatment on isoproterenol (100 μM) responsiveness (▲), basal activity (○), and glucagon responsiveness (1 μM) (●) of rat liver adenylate cyclase*

Liver cyclase activity from carcinogen-treated animals is expressed as a percentage of that found in livers from control animals under identical conditions of assay. Each point was calculated from the means of triplicate assays of fresh preparations from each of two test animals and one control animal. Standard error bars are shown. A value of 100% indicates no change, and deviations upward or downward indicate increases or decreases in cyclase activity, respectively.

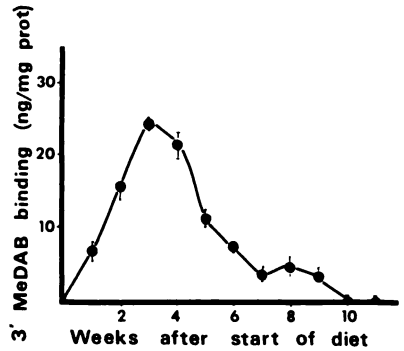

FIG. 2. *Binding of 3'-MeDAB and its metabolites to washed particles from livers of rats killed at weekly intervals throughout course of treatment and assayed for adenylate cyclase activity to give the results shown in Fig. 1*

The values of nanograms of 3'-MeDAB per milligram of protein were obtained from spectroscopic examination of extracts of the preparations as described under MATERIALS AND METHODS.

animals was difficult to ascertain, but some approximations can be made. The response of cyclase from control animals was small, and extremely variable. The curve in Fig. 3a was therefore computed from the means of results from 22 different experiments; the K_m is approximately 1 μM. On the other hand, the responsiveness of adenylate cyclase from carcinogen-treated animals was such that maximal stimulation was not reached and had to be determined by extrapolation. If this is done, it is seen that again the K_m for isoproterenol stimulation is roughly 1 μM (Fig. 3a). In the case of glucagon, the maximal effect could not be assessed in either treated or control animals, because the solubility properties of the hormone prevented our taking its concentration high enough (Fig. 3b). It can only be said that the response of adenylate cyclase to a given concentration of glucagon was greatly decreased in preparations from carcinogen-treated animals. Our most striking finding from these curves is that the V_{max} for isoproterenol stimulation was increased at least 4-fold by carcinogen treatment.

Studies of the combined effects of isoproterenol and glucagon on adenylate cyclase activity in normal liver are hampered by the fact that the normal response to catecholamines is very small (only about 1/10 of the magnitude of the response to glucagon) and variable. Because of the larger effect of isoproterenol on preparations from preneoplastic tissue, it became possible to investigate the question of the additivity of the effects of the two hormones (4, 10–12). We found that additivity occurred when a high concentration of glucagon (1 μM) was combined with low (1 nM) and high (100 μM) doses of isoproterenol. However, this was not complete throughout the isoproterenol concentration range and was much diminished between 0.1 and 10 μM (Fig. 4a).

The effects of the two hormones were studied similarly using fetal rat liver as the source of adenylate cyclase, since it had been reported by others (13, 14) that cyclase from fetal tissue also responded to a much higher degree to catecholamines than does adenylate cyclase from adult rats. We found this to be true; the responsiveness to the two hormones was similar to that described above (Fig. 4b), although in fetal tissue the combined effects were more nearly additive throughout the isoproterenol concentration range.

The greatly enhanced basal activity and catecholamine responsiveness of the enzyme from carcinogen-treated livers made possible a critical and comparative study of the effects of catecholamines on liver

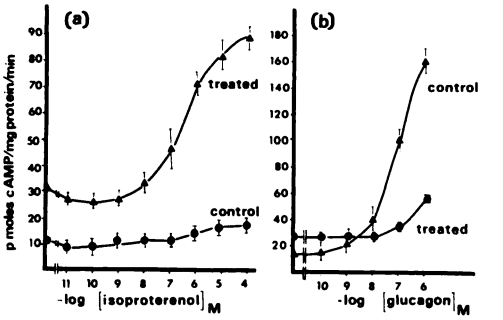

FIG. 3. *Basal activity (– – ‖) and hormone responsiveness of adenylate cyclase in washed particles from livers of control rats (●) and rats treated for 4 weeks with 3'-MeDAB (▲).*

a. Effects of isoproterenol. b. Effects of glucagon. In this and subsequent figures, adenylate cyclase activity is expressed as picomoles of cAMP produced per minute per milligram of protein (± standard errors). Assays were performed in triplicate.

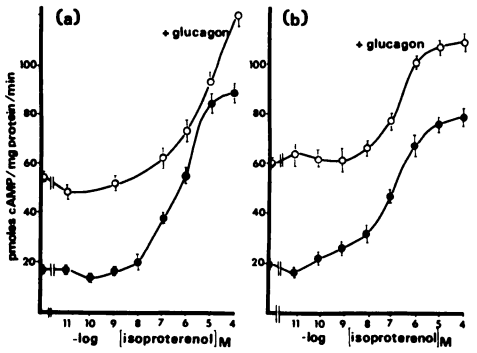

FIG. 4. *Effect of glucagon on isoproterenol stimulation of activity of adenylate cyclase from (a) carcinogen-treated (4 weeks) and (b) fetal (17 days) rat livers*

●, isoproterenol alone; ○, isoproterenol plus glucagon (1 μM).

adenylate cyclase not possible for normal livers. The relative potencies of the catecholamines in stimulating activity indicated that the enzyme has the properties of a *beta* adrenoreceptor; viz. the order of stimulation was isoproterenol>epinephrine>norepinephrine. However, it is interesting that at the lower catecholamine concentrations, an inhibition of cyclase activity was seen. Then the order of potency was norepinephrine>epinephrine>isoproterenol; i.e., the hormone inhibition of cyclase activity displayed characteristics of *alpha* adrenergic receptors (Fig. 5a). The inhibition of basal activity with isoproterenol was minor and variable, but statistically significant in 75% of the preparations. Epinephrine and norepinephrine almost always caused significant inhibition. Propranolol blocked the stimulatory effects of isoproterenol on the cyclase activity without altering basal activity or these inhibitory effects. Moreover, it unmasked inhibition at high catecholamine doses as well as increasing that due to low (Fig. 5b). Since the inhibitory effects on cyclase activity caused by low concentrations of catecholamines appeared to be *alpha* effects, we attempted to block them with the *alpha* blocking agent phentolamine. However, phentolamine (1–100 μM) depressed the basal activity of the enzyme and also the stimulatory effects of norepinephrine. Moreover, it reduced in a similar manner the responsiveness to glucagon. It would appear, therefore, that the effect of phentolamine was nonspecific and that the drug was not of use in giving information with respect to *alpha* receptors in this tissue.

The effects of freezing and storage on the elevated basal activity and catecholamine responsiveness of the enzyme from 3'-MeDAB-treated animals are shown in Fig. 6. Over a period of 10 days at −90°, responses to half-maximally effective concentrations (1 μM) of epinephrine and norepinephrine fell to about 50%, and the basal activity fell to about 75%, of those originally seen in fresh preparations.

DISCUSSION

The increases in basal activity and catecholamine responsiveness of washed particles from livers of rats treated with 3'-MeDAB seen in the earlier study with frozen preparations (1) were more marked in preparations from freshly killed animals. The current study showed increases in basal activity and catecholamine responsiveness as early as 1 week after commencement of the diet, and they reached a peak at 6 weeks. The changes were larger and occurred earlier than in the previous study. An increase in the degree of change seen in treated animals as compared to controls (from 450% to 750%) can be attrib-

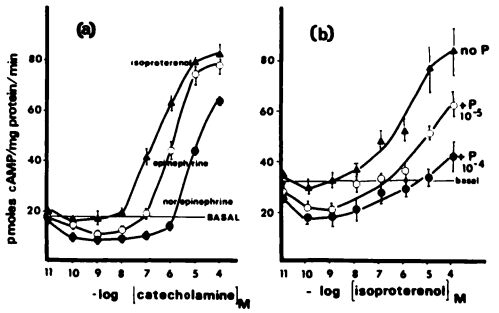

FIG. 5. *Receptor classification of effects of catecholamines on activity of adenylate cyclase from carcinogen-treated (4 weeks) rat liver*

a. Relative potencies of isoproterenol (▲), epinephrine (○), and norepinephrine (●). b. Effect of the beta adrenergic blocking agent propranolol (P) (○, 10 μM; ●, 100 μM) on the stimulation of cyclase activity by isoproterenol.

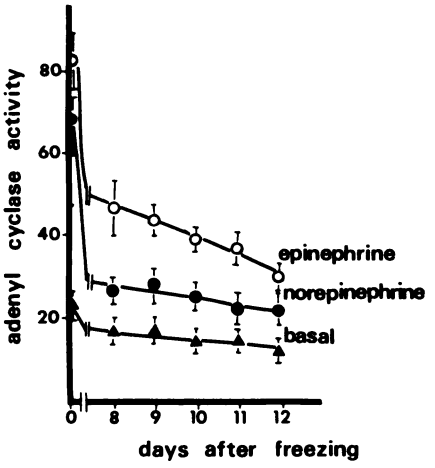

FIG. 6. *Effect of storage at −90° on elevated basal activity of adenylate cyclase in washed particles of liver of a rat treated for 4 weeks with 3'-MeDAB*

▲, basal activity; ●, norepinephrine stimulation; ○, epinephrine stimulation. The zero point was obtained *before* freezing. The dose of catecholamine used (1 μM) was the half-maximally effective concentration obtained from complete dose-response curves measured on the days shown. (This remains constant, as the V_{max}, but not the K_m, decreased.)

uted to the higher activity seen in fresh preparations from preneoplastic livers. The change in the time of the peak from 10 to 6 weeks and the earlier appearance of tumors in the second study must be due to some other factor. The animals used in the second study were younger, and it is possible that their faster growth was responsible for the more rapid changes, but this is not proven.

An important finding in this study is that the decline in elevated activity occurred before cessation of the diet; i.e., by the time the carcinogen was withdrawn at 12 weeks, the cyclase activity and catecholamine responsiveness had returned to normal levels. These findings, combined with the observation that the peak of dye binding occurred at least 2 weeks before the peak of cyclase changes, make it clear that it was not the presence of carcinogen metabolites in the liver tissue per se which caused the increase in activity observed. We conclude that some change takes place in liver cell membranes early in the course of carcinogen feeding, which leads to an altered state of activation and responsiveness of the adenylate cyclase complex. The significance of this change to neoplastic transformation of the liver cells remains obscure, although there seems no reason to change our earlier view (1) that increased adenylate cyclase activity bears little relation to the uncontrolled cell division of malignancy. The nature of the change which occurs is not understood.

Whereas the adenylate cyclase activity and catecholamine responsiveness of normal rat liver membranes was, in our hands, stable during prolonged storage at −85° after snap freezing in liquid nitrogen, this was not so with the enzyme from carcinogen-fed rats. It is possible that some rearrangement of the membrane components may indeed have occurred as a result of carcinogen treatment.

The studies of the effects of catecholamines on the elevated cyclase activity in preneoplastic livers give some interesting information about the nature of the adrenergic receptors in this tissue. The stimulation of adenylate cyclase by catecholamines certainly appears to be a *beta* phenomenon. On the other hand, the order of *alpha* potency of inhibition of its activity again raises the possibility that inhibition of adenylate cyclase activity by catecholamines may be mediated by *alpha* receptors (15, 16). Although lowering of intracellular levels of cAMP in response to *alpha* adrenergic agents (17, 18) has given credence to the suggestion that *alpha* adrenergic receptor activation may lead to inhibition of adenylate cyclase, to our knowledge such inhibition of adenylate cyclase activity has not previously been observed in broken cell preparations.

That "*alpha* and *beta* agents" have apparently opposed though overlapping effects is also consistent with previous findings that liver adrenergic receptors mediating glucose release defy strict classification as either *alpha* or *beta* (19–21). Haylett and Jenkinson (21) have shown that glucose release can be caused by either *alpha* or *beta* adrenergic agonists, while effects on potassium movement and membrane potential appear to be mediated only by *alpha* receptor stimulation. Stud-

ies with preneoplastic liver, where these phenomena are more marked, may yield more insight into these problems.

The lack of blockade by phentolamine of inhibition, which is apparently an *alpha* adrenergic effect, is at first sight confusing. However, since this agent antagonized the effects of glucagon and also lowered the basal activity of adenylate cyclase, it appears that its actions were not specific. Such nonspecificity of *alpha* adrenergic blocking agents has previously been observed in isolated organ systems (22, 23), where they antagonize acetylcholine and have anticholinesterase actions. Our observation of the nonspecific actions of an *alpha* blocking agent at the level of activity of a membrane-bound enzyme does, in fact, suggest that such agents may exert their actions by causing changes in the state of the membrane or in the catalytic activity of the enzyme rather than by competing with hormones for their receptors. We are left with the possibility that the inhibition we observed is an *alpha* receptor phenomenon. Further studies, which we anticipate will clarify this situation, are in progress.

The findings that the effects of glucagon and high doses of isoproterenol were additive is consistent with similar observations of some (11, 12) but not others (10). A finding of additivity has previously been interpreted to indicate that there are separate cyclase-receptor systems for each hormone. However, it is important that, in addition to the variability of results with broken cells, Christoffersen and Berg (4) have found that additivity did not occur in intact cells. The lack of parallelism in the upward shift of the dose-response curve to isoproterenol caused by glucagon in our experiments suggests that the additivity that we observed is a complex phenomenon and that there is some interaction between the two hormones. We could not conclude, as others have done (11, 12), that the two hormones act on entirely separate cyclase systems. The complexity may well be related to the mixed nature of the adrenergic effects on adenylate cyclase, and it may be this mixture of two opposing effects of catecholamines which contributed to the variable findings of others. The artifacts inherent in a broken cell system may also contribute to the variable additivity observed therein.

The finding that the dose-response curve to isoproterenol in the presence of a high dose of glucagon was affected similarly using preparations from fetal and preneoplastic livers, and that the orders of magnitude of the two hormonal responses were so similar and so different from those seen in normal tissue, points again to the similarity between fetal and preneoplastic tissues. It gives further support to the suggestion (24) that during carcinogenesis there emerges a population of cells with fetal characteristics, some of which give rise to cancer.

There are clearly many questions to be answered with respect both to preneoplastic changes in adenylate cyclase activity and to the nature of the molecular properties of adrenergic receptors. We feel that the findings reported here point to the importance of pursuing these questions, as well as providing a system in which to do so. The significance to our understanding of normal control of cell function and of mechanisms of action of drugs which affect this, and to such deviant states as cancer, which may come from such investigations is far-reaching.

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