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STUDIES ON PLASMA MEMBRANES

XIV. ADENYL CYCLASE IN PLASMA MEMBRANES ISOLATED FROM RAT AND MOUSE LIVERS AND HEPATOMAS, AND ITS HORMONE SENSITIVITY

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SUMMARY

1. For a proper assay of the adenyl cyclase of isolated plasma membranes using [8-^{14}C]ATP as substrate complete separation of the cyclic adenosine 3',5'-monophosphate (cyclic AMP) from labeled adenine, an ATP degradation product, is essential. Otherwise erroneously high basal activity and low hormone stimulation are recorded. This may explain certain discrepancies found in the literature on this liver enzyme activity.

2. Rat-liver plasma membranes formed 2.3 ± 1.0 nmoles cyclic AMP from ATP per mg membrane protein per h. This activity was some 20-fold increased by 10^{-5} M glucagon, 5-fold by 10^{-4} M epinephrine and 10-fold by 10^{-2} M fluoride under conditions in which ATP was not a limiting factor.

3. The basal adenyl cyclase activity displayed by the plasma membranes isolated from the rather anaplastic rat hepatoma-484A was less than half that of the liver membranes, the relative stimulation by fluoride was about the same, but that of glucagon was lower than in the case of liver membranes, whereas epinephrine was virtually without effect. By contrast, plasma membranes isolated from mouse liver and the well differentiated mouse hepatoma-147042 did not differ much in basal, hormone- and fluoride-stimulated adenyl cyclase activity.

4. The significance of the adenyl cyclase system for growth control and neoplasia is briefly discussed.

INTRODUCTION

The important mediatory role of cyclic adenosine 3',5'-monophosphate (cyclic AMP) in cell metabolism is becoming increasingly clear[1-3]. Of particular interest are the findings that adenyl cyclase, the enzyme catalyzing the formation of cyclic AMP from ATP, appears to be located in the plasma membrane of many cell types, and that the enzyme is activated by various hormones according to cell type. As far as we are aware, two groups of investigators[4-7] have reported on the adenyl cyclase activity in

Abbreviation: cyclic AMP, cyclic adenosine 3',5'-monophosphate.

isolated rat-liver plasma membranes and its hormone sensitivity, with widely divergent results. In these studies the experimental conditions for membrane isolation and enzyme assay differed. In the present investigation we have attempted to resolve the discrepancy between these data, using well characterized plasma membranes isolated from rat (mouse) liver and an accurate assay system. The basal and hormone-stimulated adenyl cyclase of plasma membranes isolated from liver tumors were also measured as a preliminary to more pertinent experiments which are being directed towards the evaluation of the hypothesis that the cyclic AMP control of the initiation of DNA synthesis and cell proliferation, known for at least certain cell types, is changed or abolished in the neoplastic state.

MATERIALS AND METHODS

Young adult male rats of the inbred strain R-Amsterdam and the transplanted rat hepatoma-484A, originally induced in a female rat of this strain by feeding 4-dimethylaminoazobenzene, were routinely used. Similar results were obtained with the hepatoma whether transplanted in male or female rats. Liver plasma membranes from CBA mice (9–12-month-old males) and the transplanted mouse hepatoma-147042, spontaneously arisen in an old CBA male, were also studied. Plasma membranes were isolated from livers and hepatomas as previously described[8,9].

Incubation was carried out with 0.15–0.2 (0.2–0.3) mg protein equivalents of liver (hepatoma) membranes in a total volume of 0.5 ml, containing final concentrations of 40 mM Tris–HCl of pH 7.5, 2 mM [8-^{14}C]ATP (1 μC/μmole; Amersham), 1 mM unlabeled cyclic AMP (Sigma), 5 mM $MgCl_2$, 10 mM theophylline, 10 mM phosphoenolpyruvate (Boehringer), 5 μl (7.5 units) pyruvate kinase (Boehringer), and, when added, 10 μM glucagon (Sigma), 0.1 mM L-epinephrine tartrate (Schuchart), 25 μg bovine growth hormone (Armour), 20 μM insulin (Sigma) or 10 mM NaF. After 15 min at 37°, 0.4 mg cyclic-[8-^{3}H]AMP (0.15 μC; Amersham) in 0.1 ml water was added and the reaction was stopped by keeping the reaction mixture for 3 min at 100°. After cooling and centrifugation (10 min at 1500 × *g*), 0.1 ml of the supernatant (together with 0.02 ml 5 mM adenine and hypoxanthine) was applied to Whatman 3 MM paper (20 cm × 20 cm) for 2-dimensional ascending chromatography with the solvents used by MARINETTI *et al.*[4] After the first chromatographic run (4.5 h, room temperature) with isopropanol–conc. NH_4OH–water (7:2:1, v/v), drying and ultraviolet inspection, a strip (broad 5 cm; 20 cm long) was cut off from the base of the paper, including the area of sample application where ATP (ADP and AMP) had remained. The solvent of chromatography in the second direction (7 h) was isopropanol–conc. HCl–water (65.0:16.7:18.3, v/v). In the first experiments the cyclic AMP spot was cut out after the second chromatographic run and counted. In all following experiments (see RESULTS), a third chromatographic run[10] was carried out in direction opposite to the second for 5 h using *n*-butanol saturated with water. Prior to this run, a strip parallel to (and containing) the second solvent front was cut off from the paper on a distance of 2 cm from the cyclic AMP spot, and a strip of fresh paper (9 cm broad) was sewn to the opposite side. The cyclic AMP (and adenine and hypoxanthine) spots were cut out, placed in counting vials, and 1 ml of water and 15 ml of Bray's scintillation fluid were added. ^{14}C and ^{3}H were measured (in triplicate) in a Packard Tri-Carb liquid scintillation spectrometer.

RESULTS

Assay system

The enzyme assay was carried out in the presence of the ATP regenerating system phosphoenolpyruvate and pyruvate kinase. Unless this was added, ATP was exhausted within 15 min by the action of the powerful[8] liver-membrane ATPase (EC 3.6.1.3) as shown by the amount of P_i liberated as a function of time. The regenerating system was also meant to counteract the degradation of ADP to AMP to adenosine by the adenosine diphosphatase and 5'-mononucleotidase[8] (EC 3.1.3.5) present in the membranes, and the further breakdown of adenosine. (The rat-liver plasma membranes used in this study contained appreciable adenosine diphosphatase activity, *i.e.* some 20 and 40 μmoles P_i released from ADP per mg protein per h in the presence of 1 mM Mg^{2+} and Ca^{2+} respectively; this activity—compare the high inosine diphosphatase activity reported previously[9]—is in accordance with the results of others[11].) Phosphodiesterase, also present in liver plasma membranes[8,9], which could act on the cyclic AMP formed, was counteracted by adding cold cyclic AMP and 0.01 M theophylline.

The adenyl cyclase activity of the plasma membranes was assayed with [8-^{14}C]-ATP as substrate, and following incubation for 15 min at 37°, but before termination of the reaction by heating, cyclic-[8-^{3}H]AMP was added to serve as a recovery marker. After two-dimensional paper chromatography, the cyclic AMP spot was well separated from adenosine, which was the only other compound, apart from theophylline, detectable by ultraviolet light. The cyclic AMP spot was cut out and differentially counted in a liquid scintillation spectrometer. After correction on the basis of ^{3}H recovery and blank values (blank = the same experiment carried out in the absence of membranes), the adenyl cyclase activity of rat-liver plasma membranes amounted to some 30 (22, 29, 39) nmoles cyclic AMP formed per mg membrane protein per h. Under these conditions glucagon increased the adenyl cyclase activity 100%, *e.g.* from 29 to 57 nmoles. However, when a strip of the paper joining the basis of the cyclic AMP spot (direction of the 2nd chromatographic run) was cut out and counted, it appeared to contain high ^{14}C but negligible ^{3}H activity. Marker adenine and hypoxanthine were shown to localize here. When unlabeled adenine was added after incubation, ultraviolet inspection of the two-dimensional chromatograms revealed the cyclic AMP and adenine spots, the latter containing some 100-fold higher ^{14}C activity than did the former but lacking ^{3}H activity, to be separated from each other but to lay very close together. Thus there was a real possibility that [^{14}C]adenine formed from [8-^{14}C]ATP could cover a larger area than that delineated by ultraviolet as adenine, and that some of this would be cut out together with the cyclic AMP spot. Therefore, a third chromatographic run was carried out in direction opposite to the second, using *n*-butanol saturated with water as advocated by DOUŠA AND RYCHLÍK[10]. As a result the adenine spot and its ^{14}C activity (unlabeled adenine had been added) did move away from cyclic AMP spot, whereas the latter did not essentially change in position. The ^{3}H content of the cyclic AMP spot was not, but its ^{14}C activity was much decreased by the latter procedure as illustrated by the 15-fold lower basal enzyme activity now obtained as compared with that after only two chromatographic runs. The adenine spot contained 0.3% of the added radioactivity in experiments carried out in the absence of membranes (blanks), and 1–2.5% in the presence of

membranes. The hypoxanthine spot, lying directly below adenine, contained only 0.03 % and 0.06%, respectively. The origin of adenine has not been studied; some could have been present in [8-^{14}C]ATP or formed by thermal degradation on stopping the reaction. Since the amount of adenine was markedly increased when membranes had been present, it follows that either thermal or enzymic degradation of membrane-catalyzed breakdown products of ATP to adenine had occurred.

These results showed that after the first two chromatographic runs the cyclic AMP spot, as cut out from the paper, still contained traces of a highly ^{14}C-labeled compound, very presumably adenine. For this reason the three chromatographic runs were applied in all subsequent experiments.

Rat-liver plasma membranes

As shown in Table I rat-liver membranes now showed a very low basal adenyl cyclase activity ranging form 1.3 to 4.0 nmoles cyclic AMP formed per mg protein per h. This activity was some 20-fold enhanced by 10^{-5} M glucagon (range: 28–53 nmoles cyclic AMP formed), 5 fold by 10^{-4} M epinephrine (range, 10–15 nmoles cyclic AMP in four experiments; in a fifth experiment only 5.3 nmoles were formed), and 10 fold by 10^{-2} M NaF (range: 20–30 nmoles). That fluoride stimulated the adenyl cyclase activity and did not act indirectly by inhibiting ATP breakdown by membrane ATPase follows from the condition that the fluoride stimulation occurred in the presence of the ATP regenerating system.

Bovine growth hormone (25 μg) had no effect on the adenyl cyclase of rat liver plasma membranes. However, in all experiments (some typical ones in Table II) a small enhancing effect (1.4–2.3-fold) of insulin ($2 \cdot 10^{-5}$ M) was noted either on fresh membranes or following their preincubation with insulin for 5–10 min at 0° prior to adding ATP. Since the insulin supplied might have been contaminated by small amounts of glucagon, no definite conclusion as to the effect of insulin on adenyl cyclase could be drawn. By a similar preincubation the stimulatory effect of glucagon was not affected, but that of epinephrine was abolished.

TABLE I

ADENYL CYCLASE ACTIVITY OF ISOLATED PLASMA MEMBRANES FROM RAT LIVER AND RAT HEPATOMA-484A

Rat liver was homogenized in 1 mM $NaHCO_3$ of pH 7.5 without or with 2 mM $CaCl_2$ (abbreviated as b and ca, respectively); rat hepatoma was homogenized in the latter medium (ca). As indicated in the text, plasma membranes were washed with EDTA prior to assay in some of the experiments.

Membrane source (homogenization medium)	*nmoles cyclic AMP formed per mg protein per h*			
	Basal activity	*Glucagon (10^{-5} M)*	*Epinephrine (10^{-4} M)*	*NaF (10^{-2} M)*
Rat liver (b)	2.3 ± 1.0 (6)	44.2 ± 8.3 (6)	11.1 ± 2.8 (5)	25.3 ± 4.2 (4)
Rat liver (ca)	1.1 ± 0.4 (2)	27.5 ± 1.5 (2)	4.5 ± 2.8 (2)	20.0 ± 1.1 (2)
Rat liver (ca), EDTA washing	2.2 ± 0.3 (2)	35.5 ± 2.1 (2)	11.1 ± 1.1 (2)	29.1 ± 1.1 (2)
Rat hepatoma (ca)	0.8 ± 0.2 (3)	7.4 ± 1.8 (3)	1.1 ± 0.3 (3)	9.2 ± 1.2 (3)
Rat hepatoma (ca), EDTA washing	0.9 ± 0.5 (2)	6.7 ± 3.1 (2)	1.5 ± 0.4 (2)	7.6 ± 1.6 (2)

TABLE II

EFFECT OF PREINCUBATING RAT-LIVER PLASMA MEMBRANES WITH HORMONES FOR 5–10 min AT 0° ON THE ADENYL CYCLASE ACTIVITY

Some typical experiments are listed.

Preincubation	*nmoles cyclic AMP formed per mg protein per h*					
	Without hormone	*Glucagon*	*Epinephrine*	*Growth hormone*	*Insulin*	
					10^{-5} *M*	$2 \cdot 10^{-5}$ *M*
−	2.1	48	15.0	2.6	2.4	2.5
+	2.9	52	2.8	3.3	2.5	6.6
−	3.8	51	13.1			
+	4.5	50	4.7			
−	3.0			3.5	4.0	4.7
+	4.0			4.2	5.1	5.6

Rat hepatoma-484A plasma membranes

Plasma membranes in the above experiments were isolated from rat-liver homogenates prepared in 1 mM $NaHCO_3$. As reported previously[9], plasma membranes cannot be isolated from rat hepatoma-484(A) homogenized in this medium, unless it is fortified with 2 mM $CaCl_2$. When plasma membranes were isolated from rat-liver homogenates prepared in 1 mM $NaHCO_3$ containing 2 mM $CaCl_2$, the basal and hormonestimulated adenyl cyclase activities tended to be lower (Table I) than those recorded in the aforementioned experiments. This effect was counteracted by washing the liver plasma membranes, thus isolated, with 1 mM $NaHCO_3$ containing 1 mM EDTA of pH 7.4 (10 min at 0°, followed by reisolation through centrifugation for 10 min at 1500 × *g*) prior to enzyme assay. These results were obtained in comparative experiments which were carried out in order to explore whether there was any gross difference in enzyme activity and hormone response induced by the use of Ca^{2+} in the homogenization medium, a condition imparative for isolating the hepatoma membranes. The results obtained with the rat hepatoma membranes demonstrated, however, that irrespective of whether these membranes were washed with EDTA or not, the results were similar (Table I). The hepatoma membranes exhibited very low basal adenyl cyclase activity, since on the average less than 1 nmole cyclic AMP was formed; this activity was less than one-half the activity of the liver membranes. Glucagon stimulated less, *i.e.* some 9-fold, so that the hepatoma membrane enzyme activity in the presence of this hormone amounted to only 16 % that of the liver membranes. Epinephrine was hardly if at all active, but fluoride showed about the same relative stimulation as it did with the liver membranes, though the specific activity of the hepatoma membrane enzyme was some 35 % that of the liver membranes in the presence of fluoride.

Mouse liver and hepatoma-147042 plasma membranes

The plasma membranes from these mouse tissues were isolated from plain bicarbonate homogenates[8], and the enzymic data can be directly compared. As shown in Table III, the basal, glucagon- and fluoride-stimulated adenyl cyclase activities of the mouse-liver plasma membranes were similar to, whereas the epinephrine-

TABLE III

ADENYL CYCLASE ACTIVITY OF ISOLATED PLASMA MEMBRANE FROM MOUSE LIVER AND MOUSE HEPATOMA-147042

Tissues homogenized in 1 mM $NaHCO_3$. Typical experiments to illustrate the variations encountered in epinephrine stimulation; otherwise the results were identical. Note that epinephrine stimulation varied independently from glucagon stimulation.

Membrane source	*nmoles cyclic AMP formed per mg protein per h*			
	Basal activity	*Glucagon*	*Epinephrine*	*NaF*
Liver	1.8	46.1	8.1	28.1
	1.4	46.0	3.1	39.8
Hepatoma	1.6	32.1	5.2	21.0
	1.5	34.3	2.3	27.5

stimulated activity was definitely lower than the corresponding values obtained for the rat-liver membranes. The basal adenyl cyclase of the plasma membranes isolated from mouse hepatoma-147042 was similar in activity to that of the mouse-liver membranes, but the hormone- and fluoride-stimulated activities displayed by the former membranes were some 30% lower. This decrease was relatively small in comparison with the much greater decreases observed in the rat hepatoma *versus* liver system.

DISCUSSION

Using isolated rat-liver plasma membranes, MARINETTI *et al.*[4,5] found high basal adenyl cyclase activity ($\geqslant$ 50 nmoles cyclic AMP per mg protein per h) and less than 2-fold stimulation by glucagon and epinephrine. From the findings reported in the present paper it is concluded that these results are influenced by the incomplete separation of the ATP reaction and breakdown products. Contrary to what has been found[1] for the adenyl cyclase of other cells and their plasma membranes, fluoride inhibited the liver-membrane adenyl cyclase in the experiments of RAY *et al.*[5]. In the present experiments fluoride activated the adenyl cyclase of rat- and mouse-liver and hepatoma plasma membranes by acting directly on the enzyme rather than indirectly on membrane ATPase.

The basal, fluoride- and glucagon-stimulated adenyl cyclase activities of our rat-liver plasma membranes closely resemble those reported by POHL *et al.*[6,7]. These authors followed the procedure of KRISHNA *et al.*[12] using [α-^{32}P]ATP as substrate, Dowex and differential absorption on $BaSO_4$ to obtain cyclic AMP. Although this method gave a rather poor cyclic AMP recovery (35–50%; our recoveries amounted to 65–75%), and has been subject to criticism[13], it is significant and convincing as regards results, that POHL's *et al.*[6,7] and our data are so strikingly similar. POHL *et al.* however, found no[6] or slight[7] activation of the adenyl cyclase of isolated rat-liver plasma membranes by epinephrine, in contrast to our results, and those of BITENSKY *et al.*[14,15] using rat-liver homogenate and a particulate fraction derived therefrom. Since in liver there appear to be two adenyl cyclases or at least two different receptors, one responding to glucagon and one to epinephrine[14,15], the result of POHL *et al.*[6,7]

(and the cases in which we obtained low activation by epinephrine) may be accounted for by (i) a greater lability of the epinephrine receptor or activation process (see preincubation experiments of Table II; *cf.* also ref. 15), or (ii) membrane fractionation leading to loss of one enzyme during preparation if the two enzymes are located differentially[16]. For routine use, POHL *et al.*[7] used stored membranes, whereas in our experiments fresh membranes were always incubated on the same day of their preparation. POHL's *et al.*[7] membrane isolation method was somewhat different from ours, but both are based on the original Neville procedure from which RAY[17] has devised a more divergent modification. To what extent membrane preparation *per se* has influenced the enzymic results of RAY *et al.*[4,5] cannot be judged at the moment.

The hormonal control of the plasma-membrane adenyl cyclase affords an unique situation in that a humoral signal by interacting with the cell surface, transmits its message, *via* production of the cyclic AMP messenger, to the cell interior. Most striking among the various cellular responses recorded is that involving immediate or delayed cell proliferation in a number of normal tissues, *i.e.* mouse salivary gland[18], and rat thymic[19] and peripheral[20] lymphocytes; *cf.* ref. 21 for gastric epithelium. The study of the mechanism by which cyclic AMP appears to control the initiation of DNA synthesis and cell proliferation could lead to a better understanding of the factors and processes involved in the growth control of these cells, and its loss in corresponding neoplastic cells. Moreover, the adenyl cyclase system may allow to formulate how, in biochemical terms, the cell surface is involved in growth control. This involvement has been indicated by the phenomenon of contact inhibition of growth (replication) and the latter's loss in transformed cells[22], and is currently receiving much attention in view of the concurrent changes in tumor-cell surface agglutinin receptors[23–26].

Theoretically, the neoplastic state could be distinct from normal by a sustained high concentration (activity) of a critical component (enzyme) released from control, *versus* a low but signalcontrolled (activated) normal level. That this situation does not generally apply to hepatoma in respect of the adenyl cyclase system, follows from our results. Therefore the contrary finding of BROWN *et al.*[27] on other rat hepatomas cannot support the hypothesis of a "derepressed", hormone-insensitive adenyl cyclase activity being involved in neoplastic growths, as suggested by MACMANUS *et al.*[20] Moreover, the experiments of BROWN *et al.*[27] have been carried out with crude preparations (20000 × *g* pellet prepared from rat liver or hepatomas) and a rather insensitive assay method. These conditions, together with the circumstance that hormone stimulation was measured in the presence of fluoride, whereas the latter may inhibit the former[1], and the small enzyme differences actually reported by BROWN *et al.*[27] do not allow to draw firm conclusions from their data. (In other experiments of the latter authors[28], epinephrine in the absence of fluoride stimulated the adenyl cyclase of a hepatoma preparation to a greater extent than it did the liver activity.)

Our results show a decreased adenyl cyclase activity in the plasma membranes of the rather anaplastic rat hepatoma-484A, and smaller enzyme activation by glucagon as compared with liver plasma membranes. The virtual lack of effect of epinephrine on the hepatoma-membrane enzyme supports the view of the presence in hepatic tissue of two enzymes, one activatable by glucagon and one by epinephrine (*cf.* also Table III), but does not necessarily imply the absence of the epinephrine responding receptor or enzyme from the hepatoma cell surface *in situ*, for the reasons mentioned above. It has also been shown that relatively little difference in the adenyl

cyclase system of plasma membranes from mouse liver and a well differentiated mouse hepatoma existed. Thus, the present results on two representatives of the liver-hepatoma model demonstrate the not uncommon finding of differences in metabolic characters according to differentiation status and growth rate[9].

Decreased adenyl cyclase has also been briefly reported by BÜRK[29] in a polyoma transformed hamster cell line. Of much interest in this connection is the recent finding of JOHNSON *et al.*[30] that cyclic AMP and its derivates restored several morphological characteristics of normal fibroblasts (contact inhibition) in transformed cells. Furthermore some types of tumors seem to have their metabolism adapted to low levels of cyclic AMP, higher concentrations being inhibitory to the tumor but not to the corresponding normal cells[31]. According to the same principle the inhibitory effect of epinephrine on mitosis, reported[32] for some epithalia, would be circumvented by the corresponding tumors following a decrease in amount (or affinity) of their epinephrine receptors. However, in view of the differences and uncertainties it remains to be further established whether and how the adenyl cyclase system is related to neoplastic disease, either specific or general.

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