The glucagon receptor from liver can be functionally fused to caudate nucleus adenylate cyclase

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1. INTRODUCTION

The caudate nucleus region of mammalian brain which is innervated by dopaminergic neurons contains dopamine-stimulated adenylate cyclase [1–5]. This observation together with other evidence [6–8] led to the suggestion that the effects of dopamine in the central nervous system are mediated through the activation of adenylate cyclase. However, it has been established that there are two classes of dopamine receptor D_1 and D_2 and that only D_1 receptors are coupled to adenylate cyclase [9–11]. D_1 receptors may also be restricted to a minor component of the caudate nucleus [12,13]. It may not be surprising, therefore, that in isolated plasma membranes from bovine caudate nucleus we find that adenylate cyclase is not activated by dopamine. However, the process of membrane purification may impair the function of adenylate cyclase. The aim of this study was to test whether bovine caudate nucleus adenylate cyclase can respond to any hormone receptor. To this end we fused rat liver plasma membranes containing a functional glucagon receptor with brain membranes, using the polyethylene glycol method in [14,15]. After fusion, glucagon stimulated caudate nucleus adenylate cyclase showing that the enzyme is able to functionally couple to a foreign hormone receptor. We conclude that it is unlikely that these membranes contain dopamine receptors which stimulate adenylate cyclase.

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2. EXPERIMENTAL

2.1. *Preparation of membranes*

Rat liver plasma membranes were prepared as in [16] and bovine caudate nucleus plasma membranes as in [17]. Protein concentrations were determined by a scaled down modification of the method in [18].

2.2. *Determination of adenylate cyclase activity*

Adenylate cyclase activity was assayed at 30°C in a mixture containing 50 mM Tris–HCl (pH 7.4) 10 mM $MgCl_2$, 1 mM cyclic 3′,5′-AMP, 1 mM dithiothreitol, 5 units of creating kinase, 20 mM creatine phosphate, 1% bovine serum albumen, 0.5 mM ATP and [α-^{32}P]ATP to spec. act. 12 cpm/pmol. The incubation was stopped and cyclic 3′,5′-AMP separated from other nucleotides as in [19].

2.3. *Fusion of rat liver plasma membranes with nucleus plasma membranes*

The fusion procedure follows that in [14,15]. Rat liver plasma membranes were suspended in 20 mM Tris–HCl (pH 7.4) at 2 mg membrane protein/ml at 0°C. Freshly prepared ice-cold solution of *N*-ethylmaleimide (44 μl 50 mM) was added

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dropwise with continuous mixing to 400 μl membrane suspension to give 5 mM final conc. The mixture was incubated for 5 min at 0°C and the reaction was then stopped by the addition of 10 μl 250 mM dithiothreitol. The mixture was spun for 2 min at 4°C in a microfuge. The pellet was gently blotted with a tissue and resuspended in 1.5 ml ice-cold buffer containing 20 mM Tris–HCl (pH 7.4), 2 mM $MgCl_2$ and 1 mM EDTA (TME buffer). Sonicated suspension (40 ml) of crude asolectin (1 mg/ml) and 15 μl 1 M $MgCl_2$ were added one at a time and mixed and the mixture was incubated at 0°C for 10 min. A sample (500 μl) was removed to determine the adenylate cyclase activity of the liver membranes prior to fusion and the remaining suspension centrifuged for 2 min at 0°C in a microfuge. The pellet was blotted as before and 100 μl of a 2 mg/ml suspension in TME buffer of bovine caudate nucleus plasma membranes was layered over the liver membrane pellet. The tube was centrifuged as before and the supernatant discarded. The combined pellet was incubated at 30°C for 5 min and 0.5 ml of a freshly prepared solution of polyethylene glycol 6000 (PEG) (52%, w/w) were added. The pellet was resuspended this requiring vigorous mixing and taking ~40 s and the tube returned to a 30°C water bath. At 2 min after the addition of the polyethylene glycol solution and at 2 min intervals thereafter the following volumes of TME buffer at 30°C were added with mixing: 0.2, 0.3, 0.5, 1.5, 3.0, 5.0, 7.0 ml. The mixture was then centrifuged at $20000 \times g$ for 10 min at 0°C in an angle rotor. The supernatant was carefully decanted and the pellet was resuspended in 400 μl of ice-cold TME buffer giving ~650 μl suspension total volume.

3. RESULTS AND DISCUSSION

The response of rat liver plasma membrane adenylate cyclase to glucagon and guanine nucleotides is shown in fig. 1A. GTP stimulated basal activity by 1.7-fold and p(NH)ppG by 8-fold over 20 min at 30°C. Glucagon increased the basal activity by 1.7-fold, the activity in the presence of GTP by 3-fold and the activity in the presence of p(NH)ppG by 1.7-fold. Preincubation of liver plasma membranes with 5 mM *N*-ethylmaleimide for 10 min at 0°C almost abolished adenylate cyclase activity under any experimental condition (fig. 1A). Treatment of liver plasma membranes with polyethylene glycol (PEG) following the same protocol used in the fusion process caused a 3–4-fold reduction in activity under all experimental conditions, probably reflecting inactivation of the catalytic unit. In contrast the PEG treatment had little effect on the adenylate cyclase activity of brain plasma membranes (fig. 1B). GTP stimulated the basal activity of untreated membranes or of PEG treated membranes by ~1.7-fold and p(NH)ppG by ~3–4-fold, respectively. Glucagon had no effect on the adenylate cyclase activity of either native brain membranes or brain membranes which had been treated with PEG. We also tested the effect of addition of the crude phospholipid preparation asolectin since in [14] the presence of asolectin improved the fusion of liver plasma membranes with Friend erythroleukemia cell plasma membranes. Here, we found that asolectin over 60–240 μg/48 μg plasma membrane protein had no effect on the activity of adenylate cyclase in either the liver or brain plasma membranes and no effect on the fusion procedure.

The procedure for the fusion of liver plasma membranes with brain plasma membranes is detailed in section 2. After fusion of *N*-ethylmaleimide treated liver plasma membranes with brain plasma membranes, the brain adenylate cyclase in the fused membranes was activated by glucagon (fig. 1C, fig. 2). The stimulation of basal activity was 1.9-fold which is comparable with the stimulation in native liver membranes. In the presence of GTP the stimulation by glucagon was 1.3-fold and in the presence of p(NH)ppG 1.2-fold. This is considerably less than the stimulation in native liver membranes. In the absence of glucagon the activity was comparable with the activity in brain plasma membranes which had been treated with PEG. The experiment shown in fig. 1C is representative of 8 successful fusion expt. In only 1 expt was there no response to glucagon. The data from 7 expt are summarised in table 1. In the last column, the ratio of the (p(NH)ppG + glucagon)-stimulated activity to the glucagon-stimulated activity is given. This ratio may be taken as a qualitative assessment of the effectiveness of the coupling after fusion. It is clear that the activation is less effective in the fused membranes than in native liver membranes. However, the basal specific activity of brain membranes was much higher than the basal specific activity of

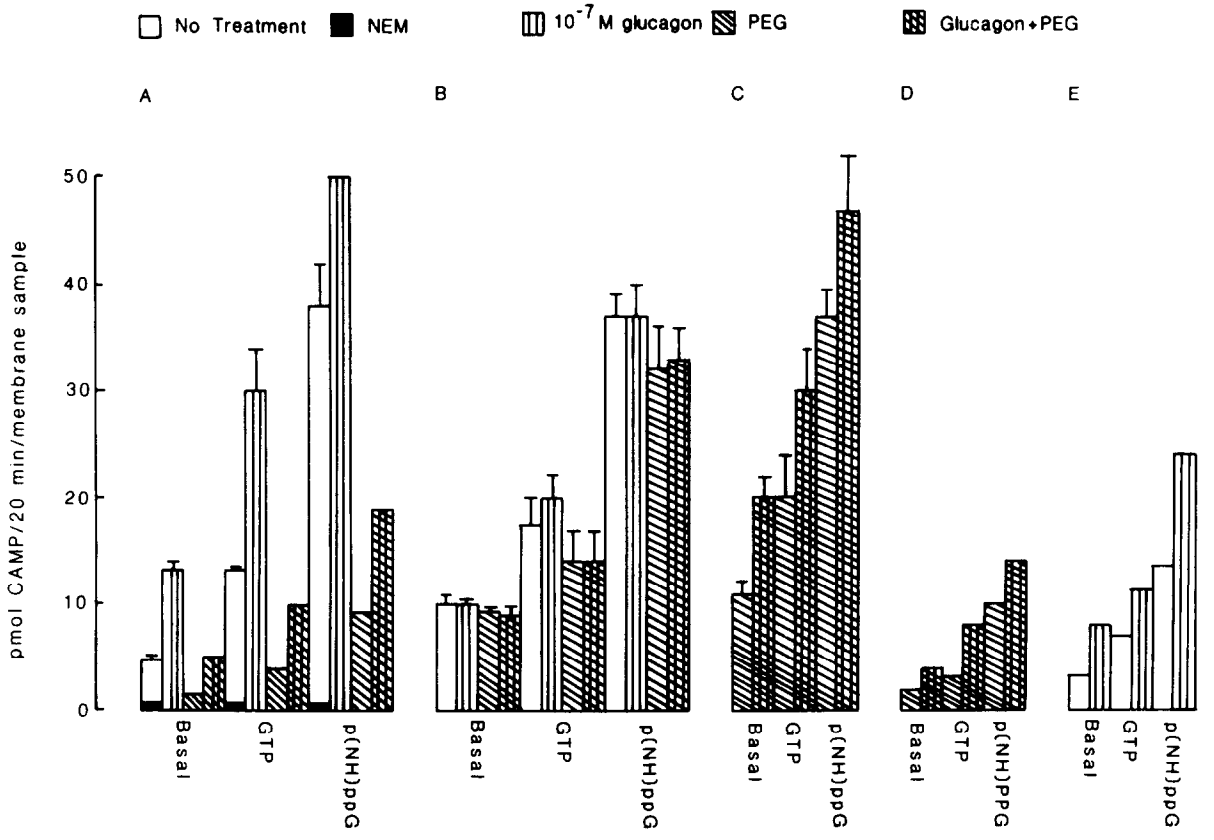

Fig. 1. Rat liver and bovine caudate nucleus plasma membranes were fused as in section 2. Adenylate cyclase assay incubations were for 20 min at 30°C. The following preparations were assayed: (A) liver plasma membranes (10 μg/assay) before and after treatment with PEG and *N*-ethylmaleimide; (B) brain plasma membranes (6 μg/assay) before and after treatment with PEG; (C) liver membranes pretreated with *N*-ethylmaleimide (16 μg/assay) fused with brain plasma membranes (6 μg/assay); (D) brain plasma membranes pretreated with NEM (22 μg/assay) fused with liver plasma membranes (6 μg/assay). This experiment followed the above protocol except that brain adenylate cyclase activity was destroyed by *N*-ethylmaleimide and replaced the liver as donor; (E) a mixture of liver plasma membranes (5 μg/assay) and brain plasma membranes (1.5 μg/assay) not treated with PEG but incubated at 30°C for the same time as the PEG treatment (14 min).

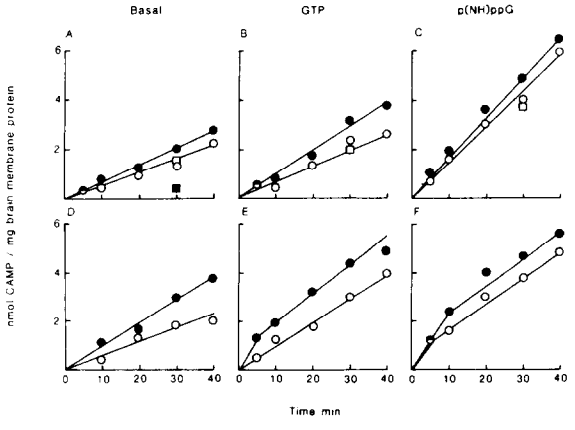

Fig. 2. Time course of cyclic AMP production generated by brain adenylate cyclase fused with liver membranes which were treated with *N*-ethylmaleimide. Fusion was performed as in section 2. The concentration of NEM-treated liver membrane was varied from 16.4 μg (A,B,C)–32.8 μg (D,E,F)/6 μg of brain membranes. Adenylate cyclase activity was determined as in section 2. Control incubations are shown by open symbols, incubations in the presence of 10^{-7} M glucagon by closed symbols. The control incubations for the fusion were assayed in duplicate at 30 min: (■) NEM-treated liver membranes; (□) PEG-treated brain membranes in the presence or the absence of glucagon. There was no effect of glucagon.

Table 1

Activation ratios elicited by glucagon, GTP and GppNHp in the various membrane preparations

Membrane preparation	Basal + glucagon/ − glucagon	GTP + glucagon/ − glucagon	p(NH)ppG + glucagon/ − glucagon	GTP / basal	p(NH)ppG + glucagon / basal + glucagon
Liver $n = 3$	2.5 ± 0.2	3.0 ± 1.0	1.7 ± 0.4	1.7 ± 0.1	3.8 ± 0.32
Liver PEG $n = 1$	3.5 ± 0.1	2.5 ± 0.27	2.0 ± 0.2	2.5 ± 0.28	4.0 ± 0.4
Brain $n = 3$	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	1.6 ± 0.2	3.0 ± 0.3
Brain PEG $n = 3$	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	1.7 ± 0.4	4.2 ± 0.2
Liver + Brain $n = 1$	1.8 ± 0.08	1.8 ± 0.02	1.9 ± 0.02	2.0 ± 0.08	4.0 ± 0.2
Liver NEM + brain fused $n = 7$	1.57 ± 0.24	1.32 ± 0.2	1.175 ± 0.17	1.7 ± 0.3 ($n = 5$)	2.2 ± 0.15

The fusion, treatment of membranes with NEM and PEG and the adenylate cyclase determinations was as in section 2. Glucagon was added at 10^{-7} M, GTP and p(NH)ppG at 10^{-4} M. The standard deviations are presented in each case, where $n > 1$; otherwise the average and limits are given. Each experimental condition was assayed in duplicate. n = no. individual expt.

liver membranes and the stimulation by p(NH)ppG was only 3–4-fold in the brain compared to 8-fold in the liver. The p(NH)ppG-stimulated state in these systems is probably close to the maximum possible adenylate cyclase activity. Thus the potential range of glucagon activation working on brain adenylate cyclase is less and it is not therefore surprising that the actual activation is also less.

When brain plasma membranes were treated with *N*-ethylmaleimide to inhibit adenylate cyclase and then fused to untreated liver plasma membranes, the liver plasma membrane adenylate cyclase was still activated by glucagon (fig. 1D). However, the activation was slightly less than in native membranes. The adenylate cyclase activity of a mixture of liver plasma membranes and brain plasma membranes which had not been treated with NEM or fused corresponded for the most part to the sum of the activities of the individual membrane preparations. It appears therefore that fusion is required for glucagon to activate brain adenylate cyclase and no loosely bound factor is able to transmit the message from the hormone receptor in one plasma membrane to adenylate cyclase in the other.

The time course of adenylate cyclase activity in brain plasma membrane fused with *N*-ethylmaleimide-treated liver plasma membrane is shown in fig. 2. The stimulation by glucagon in the fused membranes was dependent upon the ratio of liver plasma membranes to brain plasma membranes used in the fusion. Increasing the liver plasma membrane protein from a protein ratio of 2.5 : 1 compared to the brain membrane to 5 : 1 increased the response to glucagon. Further increases in the ratio of liver membrane protein to brain membrane protein did not increase the response to glucagon. Dopamine did not activate adenylate cyclase in the brain membranes either before or after fusion to liver plasma membranes which had been treated with NEM.

The stimulation of brain adenylate cyclase by glucagon is a convincing demonstration of functional coupling of the glucagon receptor to adenylate cyclase. This implies that the liver plasma membrane and brain plasma membranes have fused. It was also possible to separate the fused membranes from unfused liver and brain plasma membranes on a sucrose gradient. The buoyant density of liver plasma membranes is greater than that of brain plasma membranes. It seemed likely therefore that the fused membranes would have a

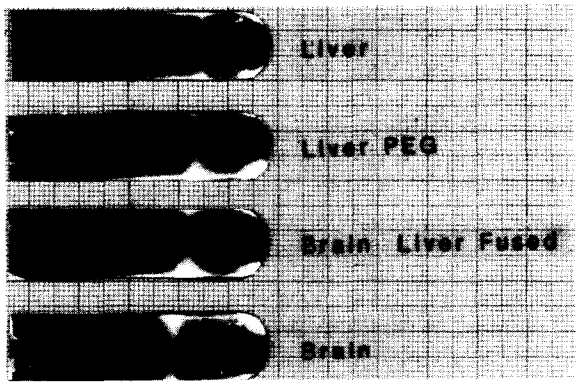

Fig. 3. Separation of liver, brain and fused plasma membranes on a sucrose gradient. Liver and brain membranes were fused as in section 2. Sucrose, 2.5 ml 50% (w/v) and 2.5 ml 20% (w/v), was used to form a 20–50% linear gradient of 5 ml total vol. Between 300–400 μl of membrane suspension (0.8 mg total membrane protein) were applied to the top of the gradient which was then centrifuged at 100000 × g for 90 min in a swingout rotor (Beckman). The tubes were photographed against a background with lighting from below and behind at a slight angle.

buoyant density intermediate between the two and that this could be used as a method of separation. The results of centrifugation on a 20–50% (w/v) sucrose gradient are shown in fig. 3. Liver membranes were clearly separated from brain membranes and this was not affected when the membranes were mixed. Treatment with polyethylene glycol slightly reduced the density of the brain membranes and caused a more marked reduction in the density of the liver membranes. This reduction in density probably reflects the fact that polyethylene glycol remained associated with the membranes even after washing. However, there was still a clear separation. The fused membranes formed a somewhat more diffuse band between the polyethylene glycol-treated liver and brain membranes. This confirms the conclusion that the membranes have fused, and also provides a means of separation of fused membranes from residual unfused material which will be of great value in further studies.

These experiments show that brain adenylate cyclase will couple to a hormone receptor leading to activation. The lack of response to the dopamine receptor in the native membranes cannot therefore be explained by a defect in the catalytic unit of the brain enzyme. This leaves the possibility of a lack of function in either the GTP binding protein (G/F) which is thought to mediate the coupling between receptors and adenylate cyclase [20] or in the dopamine receptor. In one strain of S49 lymphoma cells there is a defect in the G/F subunit which allows the activation of adenylate cyclase by GTP and p(NH)ppG while not allowing activation by hormone receptors [21]. This could be analogous to the situation in the native brain membranes. Nevertheless, a lack of function of native brain G/F cannot account for the lack of dopamine response in the fused membranes. A G/F protein which mediates the activation of adenylate cyclase by one hormone receptor seems to be always able to mediate activation by any other receptor present [22–24]. Thus, the activation of brain adenylate cyclase in the fused membranes by glucagon points to the presence of a functional G/F protein. The lack of response to dopamine in the fused membranes therefore implies that the dopamine receptors cannot functionally couple with the other components of the system. Whether the G/F subunit in the fused membranes is derived from the liver plasma membranes or the brain plasma membranes is not clear at present.

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