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Transfer of functional opiate receptors from membranes to recipient cells by polyethylene glycol-induced fusion

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Opiate receptor-mediated inhibition of adenylate cyclase activity was elicited in membranes of C6BUI glioma cells and S49 cyc$^-$ lymphoma cells after fusion with opiate receptor-containing membranes derived from NG108-15 neuroblastoma × glioma hybrid cells. The fusion was induced by polyethylene glycol using procedures developed by Orly and Schramm [(1976) Proc. Natl. Acad. Sci. USA 73, 4410–4414]. Prior to fusion, the adenylate cyclase activity of the donor, NG108-15 cell membrane, was inactivated by *N*-ethylmaleimide treatment. Prostaglandin E_1 receptors and the stimulatory GTP-binding protein N_s were transferred to the recipient cells along with opiate receptors. Thus, inhibitory receptors can be tranferred to foreign adenylate cyclase systems just as stimulatory receptors had earlier been found to do. Furthermore, opiate receptors have been shown to function in non-neuronal cells.

Opiate receptor; Reconstitution; Membrane fusion; Adenylate cyclase

1. INTRODUCTION

The adenylate cyclase system is composed, minimally, of a catalytic unit, GTP-binding transducing proteins (the N or G proteins) and receptors [2]. Membrane fusion has been a useful tool for the analysis of β-adrenergic and glucagon receptor-coupled adenylate cyclase [1,3–5]. It has also been shown that purified β-adrenergic receptor incorporated into liposomes can be coupled to adenylate cyclase by fusion with *Xenopus* erythrocytes [6]. These fusion experiments were designed to test the ability of such purified receptors to activate fully the adenylate cyclase system. Here, we describe the generation of opiate inhibition of adenylate cyclase in C_6 glioma and S49 cyc$^-$ cells as a result of fusion with membranes of NG108-15 neuroblastoma × glioma hybrid cells (treated so as to inactivate adenylate cyclase). The experiments show that opiate receptors can be coupled to adenylate cyclase in membranes of neuronal and non-neuronal origin.

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Abbreviations: NEM, *N*-ethylmaleimide; PEG, polyethylene glycol; DALAMID, (D-Ala^2)enkephalin(5-L-methioninamide); Mops, 4-morpholinepropanesulfonic acid

2. MATERIALS AND METHODS

2.1. *Cell culture*

Neuroblastoma × glioma, NG108-15, hybrid cells were cultured as in [7] except that they were grown in 850-cm^2 roller bottles. Cell pastes were stored at −70°C. C_6 BU1 glioma and S49 lymphoma cyc$^-$ cells were grown in Dulbecco's

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modified Eagle medium supplemented with 10% fetal bovine serum also in 850-cm^2 roller bottles.

2.2. *Preparation of membranes for fusion*

All operations described below were carried out at 4°C. NG108-15 cell pastes were thawed and homogenized in 0.32 M sucrose, 5 mM $MgCl_2$, 0.01 M Tris-HCl, pH 7.5. After centrifugation for 30 min at 40000 rpm in a Beckman 60 Ti fixed-angle rotor, the crude membranes were resuspended in 10 mM Mops, 1 mM mercaptoethanol (pH 7.4) at a final concentration of 0.5 mg/ml protein. Resuspended membranes were incubated for 25 min with 5 mM NEM in the presence of 100 mM NaCl (final concentration). Then 1.66 vols of 10 mM Mops, 5 mM mercaptoethanol (pH 7.4) were added to inactivate the NEM. When the binding sites were to be protected with opiates, membranes were incubated for 15 min before treatment with NEM. The membranes were then centrifuged for 30 min at 40000 rpm as before and the protein concentration was adjusted to 3 mg/ml in 10 mM Mops, 1 mM mercaptoethanol (pH 7.4). Liposomes prepared by sonication (using a bath sonicator until the suspension became homogeneous) from crude soybean phospholipids (asolectin) (10 mg/ml) in 10 mM Tris-HCl (pH 7.5), 1 mM EDTA were added (0.25 mg/ml) and after 10 min, $MgCl_2$ was added to 10 mM for a further 15 min incubation. Membranes were diluted with 10 mM Tris-HCl (pH 7.5) to 1.5 mg/ml and divided into aliquots which were placed in small polycarbonate tubes in multiples of 1.5 ml. After centrifugation at 15000 rpm for 30 min in a Beckman 50 Ti fixed-angle rotor, the supernatant was removed and pellets were stored at −70°C until use.

2.3. *Fusion procedure*

Freshly harvested S49 cyc$^-$ or C_6 BU_1 cells were suspended in 5 mM KCl, 135 mM NaCl, 0.8 mM $MgCl_2$, 20 mM Tris-HCl (pH 7.4) (solution A) to 2×10^7 cells/ml. 1 ml cells was added to each tube of NEM-treated membranes. Cells were centrifuged on top of the pellet at 5000 rpm for 5 min. The supernatant was removed, the walls of the tubes were dried with absorbent paper and the pellets were gently mixed with a glass rod. The tubes were transferred to a 37°C bath and 500 μl PEG (at 37°C) were added (4.24 g PEG + 5.76 ml solution A + 20 μl of 1 N NaOH) and the tubes were vortex-mixed for 2 s. 100 s later, 1 ml solution A supplemented with 2 mM ATP and 0.1 mM EDTA was added followed by a second vortex-mixing. Exactly 2 min later a further 9 ml supplemented solution A was added. After mixing by inversion of the parafilm-covered tubes, centrifugation was carried out at 10000 rpm for 10 min in a Beckman 50 Ti rotor at 4°C. To the pellet was added 3 ml hypotonic medium (5 mM $MgCl_2$, 0.1 mM EGTA, 1 mM mercaptoethanol, 10 mM Tris-HCl, pH 7.5) and the resulting suspension was centrifuged at 20000 rpm for 10 min in a Beckman 50 Ti rotor. The pellets were resuspended in 1 ml hypotonic medium. Adenylate cyclase assays were performed by the method of Salomon et al. [8] as modified by Sharma et al. [9]. Assay mixtures (final volume 100 μl) contained 0.2 mM [α-^{32}P]ATP (2 μCi), 45 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 10 mM creatine phosphate, 10 U creatine phosphokinase, 1 mM [^{3}H]cAMP (20000 cpm), 50 mM NaCl and 10 μM GTP. GTPase assays were performed as in [10] except that the total membrane preparation was used and opiate binding performed as described [11].

2.4. *Reagents*

[G-^{3}H]cAMP (22.1 Ci/mmol), [^{3}H]DALAMID (43.6 Ci/mmol), [γ-^{32}P]GTP (tetraethylammonium salt) (10–50 Ci/mmol) and [^{3}H]etorphine were purchased from New England Nuclear. [α-^{32}P]ATP (6–10 Ci/mmol) was from ICN. Asolectin was from Associated Concentrates; PEG 6000, Mops, NEM, EDTA and isoproterenol were from Sigma; other chemicals were of the highest grade commercially available.

3. RESULTS

3.1. *Preparation of NEM membranes*

The general procedure used, as in the work of Schramm and co-workers [1,3,4], was to inactivate the adenylate cyclase of donor NG108-15 membranes without markedly affecting receptor-binding characteristics. As shown in fig.1, NEM treatment of NG108-15 membranes almost completely abolishes adenylate cyclase activity and, under the conditions used, has little effect on opiate binding to receptors. As shown by others [12,13], NaCl protects opiate-binding sites from

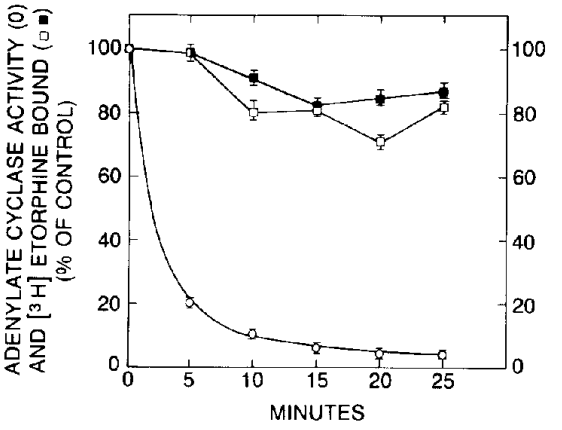

Fig.1. Susceptibility of opiate agonist binding and adenylate cyclase activity in NG108-15 membranes to 5 mM NEM treatment. Experiments were as described in section 2 except that at each time indicated, the reaction was stopped with mercaptoethanol. Opiate binding and adenylate cyclase activities were measured after three washes with 10 mM Mops, 1 mM mercaptoethanol (pH 7.4). (■—■) Binding data for membranes preincubated with 10^{-8} M naloxone before NEM treatment.

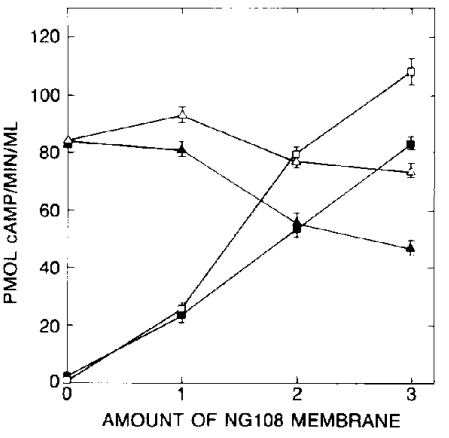

Fig.2. Hormone-stimulated adenylate cyclase activity after fusion of increasing amounts of NEM-treated NG108-15 membranes with C_6BU_1 glioma cells (2×10^7 cells/fusion). Isoproterenol (5×10^{-5} M) (△, ▲) and PGE_1 (10^{-5} M) (□, ■) stimulation of adenylate cyclase with (▲, ■) and without (△, □) 10^{-5} M etorphine. Bars indicate SE of quadruplicate determinations. Basal activity of approx. 100 pmol/min per ml was subtracted. One part of NEM-treated NG108-15 membranes was 0.5 mg proteins.

inactivation with NEM and in our experiments was found to be necessary to ensure selective impairment of adenylate cyclase only. Inclusion of opiate ligands (either agonists or antagonists) gave only a slight further protection of binding sites and was not generally deemed necessary in our experiments.

3.2. *Fusion with C_6 glioma cells*

C_6 glioma cells have long been known to be completely devoid of opiate receptors [7] but do have a well-characterized adenylate cyclase which is stimulated by β-adrenergic receptors [14]. These cells are therefore useful recipients for fusion with opiate receptor-containing membranes. The results of fusion of a constant amount of C_6 cells with varying amounts of NEM treated NG108-15 membranes are described by the data of fig.2. C_6 cells alone, carried through the fusion procedure, have adenylate cyclase activity which is stimulated by isoproterenol but unaffected by the opiate agonist, etorphine. C_6 cells are also unaffected by PGE_1 since they do not carry these receptors. When fused with NEM-treated NG108-15 membranes, however, opiate receptor activity becomes apparent as a decrease in adenylate cyclase activity. Interestingly, there is the simultaneous appearance of stimulation of adenylate cyclase by PGE_1 as well, because the membranes were derived from cells containing such receptors. Not shown in the figure are data from the control with NEM-treated NG108-15 membranes alone. These controls were a part of every experiment and always showed negligible amounts of adenylate cyclase activity, almost indistinguishable from blanks, without enzyme.

That the opiate inhibition of adenylate cyclase, after fusion of membranes with C_6 cells, is due to a receptor-mediated process is demonstrated by its reversal by naloxone (fig.3). This figure shows that C_6 adenylate cyclase is unaffected by either etorphine or naloxone when fused alone. After fusion with NEM-treated NG108-15 membranes both basal and isoproterenol-stimulated adenylate cyclase are inhibited by etorphine and this inhibition is reversed by naloxone. Again, NEM-treated NG108-15 membranes alone and carried through the fusion procedure had no measurable adenylate cyclase activity.

Because opiate inhibition of adenylate cyclase is correlated with stimulation of an associated GTPase activity [10], we measured low-K_m

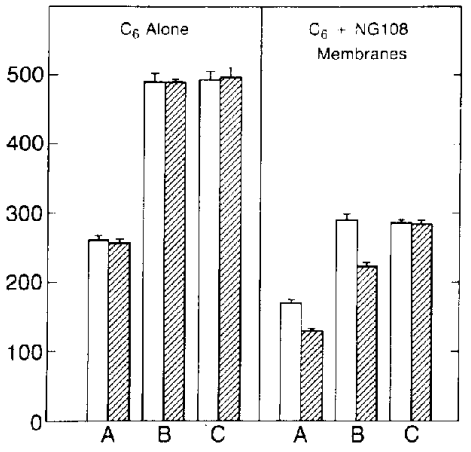

Fig.3. Coupling of δ opiate receptors to adenylate cyclase of C_6 BU_1 glioma cells by fusion with NEM-treated NG108-15 cells. (Left) Results of C_6 BU_1 cells fused alone; (right) glioma cells fused with NEM-treated NG108 membranes. (A) Basal activity; (B,C) isoproterenol (5×10^{-5} M)-stimulated adenylate cyclase activities. Effects of the opiates are shown in the hatched columns. 10^{-5} M etorphine was added in every case and naloxone (5×10^{-4} M) was also included in columns C. Bars at the top of the columns show the SE of quadruplicate determinations. One part of NEM-treated NG108-15 membranes was 0.5 mg total proteins.

GTPase activity after fusion of NEM-treated NG108-15 membranes with C_6 cells. In a typical experiment (table 1), the low-K_m GTPase present in C_6 cells or NEM-treated NG108-15 membranes fused alone is not stimulated by opiates but when the cells and membranes are fused together, small but reproducible stimulation by DALAMID is observed.

3.3. *Fusion with S49 cyc⁻ lymphoma cells*

The cyc⁻ variant of S49 lymphoma cells is defective in the guanine nucleotide regulatory protein, N_s, which is required for hormone-stimulated adenylate activity [15]. It has been shown that this missing component is easily reinserted into the cells using several methods, including membrane fusion [5]. Thus, hormone-stimulated adenylate cyclase measured with these cells after fusion with NEM-treated NG108-15 membranes is a useful indication of successful fusion. An experiment describing the results of such a fusion is shown in fig.4. This figure shows that both basal (GTP-stimulated) and

Table 1

Reconstitution of opiate-stimulated GTPase activity by fusion of NEM-treated NG108-15 membranes with C_6 cells

Fused components	pmol P_i/min per ml	
	Basal	DALAMID (10^{-5} M)
C_6 alone	7.4 ± 0.5	6.9 ± 0.4
NEM-treated NG108-15 membranes alone	1.2 ± 0.2	1 ± 0.4
C_6 + NEM-treated NG108-15 membranes	11.4 ± 0.3	13.8 ± 0.3

Data are means ± SE of quadruplicate determinations. The experiment was performed twice

hormone (PGE_1)-stimulated adenylate cyclase activity is markedly increased in cyc⁻ membranes after fusion with NEM-treated NG108-15 membranes and that opiate inhibition of this activity is observable. Similar data have been obtained with isoproterenol stimulated adenylate cyclase (not shown). Thus, opiate receptor function has been

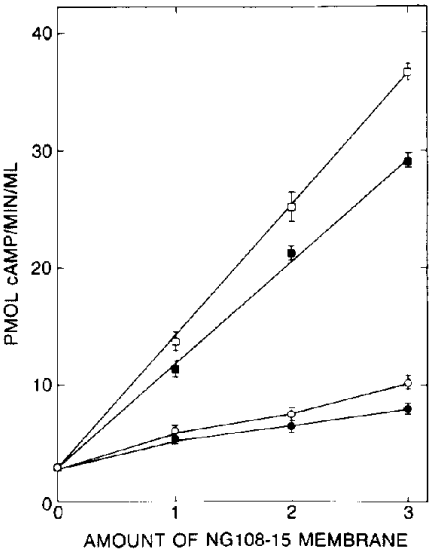

Fig.4. Fusion of increasing amounts of NEM treated NG108 membranes with S_{49} cyc⁻ cells (3×10^7 cells/fusion). Basal (○,●) and PGE_1 (10^{-5} M (□, ■)-stimulated adenylate cyclase activity in the presence (●, ■) or absence (○, □) of 10^{-5} M etorphine. Bars are SE of quadruplicate determinations.

transferred to cyc⁻ membranes along with N_s by the fusion procedure.

4. DISCUSSION

The present results show that opiate receptors can be coupled to adenylate cyclase in cell membranes originally devoid of such receptors using the membrane-cell fusion procedures developed by Schramm and colleagues. Thus, inhibitory receptors share with stimulatory receptors the ability to migrate between and presumably within membranes and to interact with adenylate cyclase from extraneous sources. One or all of the components of the adenylate cyclase inhibitory as well as stimulatory complexes must diffuse readily within the membrane.

Successful reconstitution of opiate receptor function by membrane-cell fusion is dependent upon the membrane and, therefore, receptor concentration. In our hands, approx. 1 pmol receptor is required per fusion in order to observe reliably coupling to adenylate cyclase. The assays are performed on aliquots corresponding to 5% or less of each fusion reaction and the number of receptors present/assay is 2–5-times that used normally when assaying opiate inhibition of NG108-15 membranes. Thus, the efficiency of the fusion reaction is high.

Although opiate receptors are naturally associated with neuronal tissue we have shown here that they can be incorporated into lymphoid cells and function in such cells. Thus, the adenylate cyclase machinery of diverse cell types contains freely exchangeable components, for inhibition as well as for stimulation. Interestingly, C_6 glioma cells, which are richly endowed with N_i (the inhibitory GTP-binding protein) have not yet been shown to contain inhibitory receptors. The insertion of opiate receptors into these cells has thus allowed the first demonstration of inhibitory hormone function in such cells.

Demonstration of the insertion of opiate receptors from membranes into recipient cells is a step towards the development of a reconstitution assay for receptor function. Experiments are in progress which are aimed at incorporation of purified opiate receptors from solution with membranes in ways analogous to those already used for β-adrenergic receptors [6].

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