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Inhibition of opiate receptor-mediated signal transmission by rabies virus in persistently infected NG-108-15 mouse neuroblastoma–rat glioma hybrid cells

(opiate receptor coupling/regulatory G/F protein/GTPase/adenylate cyclase/persistent rabies virus infection)

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ABSTRACT **Acute and persistent rabies virus infection of mouse neuroblastoma–rat glioma hybrid cells (NG-108-15) results in a loss of the normal inhibiting function of opiates via the opiate receptor on hormone-stimulated adenylate cyclase activity. Previous studies of these persistently infected cells have shown a decrease in the affinity of the opiate receptors for agonists without any change in the number of these receptors. We now demonstrate that persistently infected cells are unable to couple the opiate receptors to the inhibitory regulatory protein N_i of the adenylate cyclase, as measured by the loss of stimulation of the GTPase activity of this protein. However, the unstimulated basal GTPase activities of the regulatory components N_i and N_s are unchanged in the persistently infected cells. These studies also reveal a disorder of the stimulation of the adenylate cyclase by GTP or fluoride via the stimulating regulatory G/F protein (N_s) in persistently infected cells, whereas direct stimulation of the catalytic subunit of the adenylate cyclase by forskolin remains unchanged. Therefore, there are different points of dysfunction caused by the persistent rabies infection in the signal pathway from the opiate receptor to the adenylate cyclase and from the stimulating N_s protein to the enzyme: (*i*) opiate receptor binding is reduced by a decrease of agonist affinity (previously published data), (*ii*) the stimulation of GTPase activity of the inhibiting regulatory component N_i of the adenylate cyclase system is inhibited, and (*iii*) the signal pathway from the stimulating regulatory component of the adenylate cyclase system to the unchanged activity of the catalytic subunit is defective.**

Cells of the mouse neuroblastoma–rat glioma hybrid line NG-108-15 (108CC-15) have a variety of membrane receptors on their surfaces, including opiate receptors. Many fundamental studies of opiate receptor functions have been carried out with these cells and have enriched our knowledge of the biochemical basis of opiate receptor function (1–7). These cells are very suitable for studying the effect of persistent infections by neurotropic viruses on receptor specific functions in cells derived from the central nervous system. Unlike acute infections, these persistent infections cause no destruction of the cells and cause only transient changes, if any, in the growth parameters of these cells. In the case of rabies virus pathogenesis of animals and man it is supposed that the observed central nervous deficiencies are mainly caused by the interaction of the virus with its neuronal host cells, because pathological examination shows little immune-mediated cell destruction in the infected region of the brain (8). It appears that the limbic system plays an important role during rabies virus pathogenesis, thus giving rise to the term "limbic tropism" (8). It is known that the limbic system contains large numbers of encephalinergic neurons with a high concentration of opiate receptors (9, 10). To investigate the possible influence of rabies virus infection on opiate receptor mechanisms we established acute and persistent rabies virus infections in NG-108-15 cells. We have previously observed that the normal inhibitory effect of the agonist binding to opiate receptor on adenylate cyclase activity was lost when the infected cells were incubated simultaneously with prostaglandin E_1 as an adenylate cyclase stimulatory hormone and with opiates as agonists for the cAMP formation-inhibiting function of opiate receptors (11). Further studies have demonstrated a loss of 30–40% of affinity of the opiate receptors in NG-108-15 cells in acute infections. This was increased to 80–90% in long-time persistence. The number of the opiate receptors was unchanged, however, in both cases (12).

According to Cassel and Selinger (13), adenylate cyclase is activated by binding of GTP to the stimulating regulatory protein N_s. This complex formation is increased after the occupation of stimulating hormone receptors by their appropriate hormones. But after occupation of inhibiting receptors, like opiate receptors, by their agonists the GTPase activity of an inhibiting regulatory GTP-binding protein N_i of the adenylate cyclase system is stimulated (7). A model of adenylate cyclase regulation by inhibiting and stimulating regulatory coupling components (N_i and N_s) has been recently discussed by Jakobs and Schultz (14). Hamprecht and coworkers found in the opiate receptor system of NG-108-15 (108CC-15) cells such a GTP-binding regulatory component which inhibits the adenylate cyclase system (15, 16). The coupling of opiate receptors to the inhibiting regulatory protein N_i can best be studied by opiate-dependent stimulation of GTPase. This is inhibited by opiate antagonists, showing that it is a process specific for opiate receptors. We have carried out these studies to determine if rabies virus persistence results in dysfunctions in the signal pathway from opiate receptors to the regulatory N_i protein and to the adenylate cyclase unit in NG-108-15 cells. In this paper we demonstrate a disrupted coupling of the opiate receptor to the regulatory N_i protein and a lack of GTP or fluoride stimulation of the adenylate cyclase via the stimulating N_s protein in cells persistently infected by rabies virus. However, direct stimulation of adenylate cyclase by the plant diterpene forskolin shows that the catalytic subunit of the cyclase remained unchanged in the infected cells.

MATERIALS AND METHODS

Cells. NG-108-15 (108CC-15) mouse neuroblastoma–rat glioma hybrid cells were cultured as described (1).

Virus. Rabies virus (HEP Flury strain) was propagated in BHK 21 A cells at 37°C and isolated as described by Madore and England (17) for the ERA strain.

Establishment of the Persistent Infection. 108-CC-15 cells were persistently infected with the HEP Flury strain as de-

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Abbreviations: p[NH]ppA and p[NH]ppG, adenosine and guanosine [β,γ-imido]triphosphates.

scribed (12). The state of infection (presence of cytoplasmic and membrane-associated virus antigen) was monitored by indirect and direct immunofluorescence (18).

Materials. [γ-^{32}P]GTP (39 Ci/mmol; 1 Ci = 37 GBq) was obtained from Amersham Buchler (Amersham, England). GTP, ATP, creatine phosphate, adenosine 5'-[β,γ-imido]triphosphate (p[NH]ppA), and guanosine 5-[β,γ-imido]triphosphate (p[NH]ppG) were from Boehringer Mannheim (Mannheim, F.R.G.); ouabain and dithiothreitol were from Sigma (St. Louis, MO). Methadone was a generous gift from Hoechst (Frankfurt, F.R.G.), and naloxone was a gift from Goedecke (Freiburg, F.R.G.). Forskolin came from Calbiochem–Behring (Giessen, F.R.G.). All other organic and inorganic chemicals were pro analysi quality (p. A.) of Merck (Darmstadt, F.R.G.).

Preparation of Membranes. NG108-15 cells were grown in 800-ml plastic tissue culture flasks (Nunc, Roskilde, Denmark), harvested by shaking off in phosphate-buffered saline (19), centrifuged at 400 × g, and frozen at −70°C. Frozen cells were thawed and suspended in 9 vol of cold 0.01 M Tris·HCl buffer (pH 7.5) at room temperature containing 0.1 mM EDTA and were then homogenized with 25 strokes of a tight-fitting Dounce homogenizer in an ice bath. The homogenate was centrifuged at 500 × g for 10 min to remove nuclei and unbroken cells. Membranes were collected by centrifugation of the supernatant fluid at 48,000 × g for 10 min, washed in 10 vol of the same buffer, and, after a second centrifugation, were resuspended in the same buffer at a protein concentration of 2 mg/ml and then stored in aliquots at −70°C until used (7).

GTPase Assay. A modification of the method of Cassel and Selinger (7, 20) was used to measure release of radioactive inorganic phosphate from [γ-^{32}P]GTP. The assay mixture, held on ice, contained 0.5 μM [γ-^{32}P]GTP (50,000 cpm), 1 mM ATP, 1 mM ouabain, 1 mM p[NH]ppA, 10 mM creatine phosphate, 5 units of creatine kinase, 100 mM NaCl, 5 mM $MgCl_2$, 2 mM dithiothreitol, 0.1 mM EDTA, 12.5 mM Tris·HCl buffer, and *l*-methadone (concentrations shown in Fig. 1) in a final volume of 100 μl at pH 7.5. To 100-μl aliquots of the reaction mixture 5 μl of membrane protein (1–2 mg/ml) was added. Hydrolysis of GTP at 0°C was negligible. The reaction was initiated by transferring the tubes to a 37°C water bath and after 10 min the tubes were immersed in an ice bath and agitated for 30 sec. Aliquots (0.9 ml) of 20 mM phosphoric acid (pH 2.3) containing 5% (wt/vol) activated charcoal were then added. After centrifugation for 10 min at 1,800 × g, radioactivity was measured in 200-μl aliquots of the supernatant fluids. Analysis of charcoal revealed that only [^{32}P]GTP was absorbed, and radioactive inorganic phosphate remained in the supernatant fluid.

Determination of Protein. Protein concentration was determined according to Lowry *et al.* (21).

Adenylate Cyclase Assay. Adenylate cyclase activity was measured as described (22) and the forskolin concentration used in the assay was 100 μM.

cAMP Determination. cAMP concentrations were measured according to a method of Gilman (23) and Tovey *et al.* (24).

RESULTS

GTPase and Opiates. Membrane preparations from uninfected and persistently rabies virus-infected NG-108-15 cells showed drastic differences in their susceptibility to opiate stimulation of the GTPase activity by the regulatory components of adenylate cyclase. Fig. 1 shows the methadone concentration dependence of the GTPase reaction in membranes from both types of cells. It is clearly demonstrated that even at high concentrations of methadone (up to 10^{-1} M) the GTP hydrolysis cannot be increased in membranes of infected NG-108-15 cells. It can also be seen that the level of GTPase

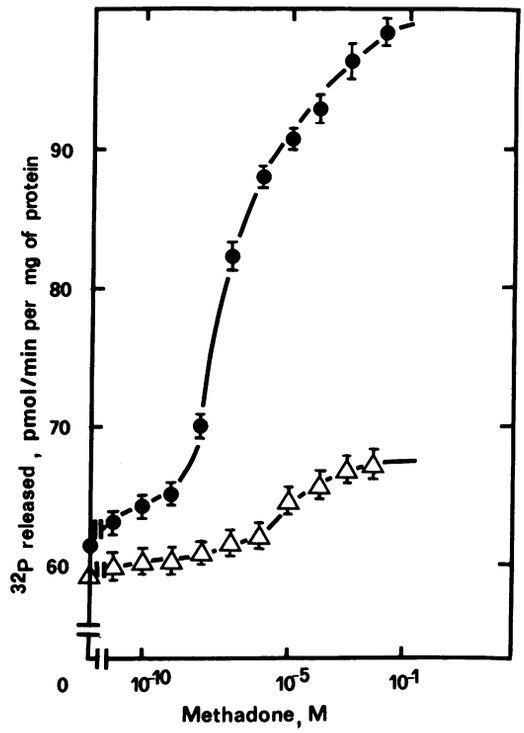

FIG. 1. Methadone concentration dependence of the GTPase reaction in membranes of uninfected (●) and persistently rabies virus-infected (△) NG-108-15 cells. Each vial contains 2.5 μg of membrane protein, which was incubated in the test mixture for 10 min. Standard deviation among four samples was 5%.

activity without methadone stimulation is equal in infected and uninfected cells. This is more clearly demonstrated in Fig. 2*C*, which shows the GTP concentration dependence of the GTPase reaction without opiate stimulation. A Lineweaver–Burk plot analysis of these data (Fig. 2*D*) resulted in a Michaelis–Menten constant K_m of 2×10^{-6} M and a maximal velocity $V_{max} = 1.7 \times 10^{-10}$ mol/min per mg of protein for the reaction. However, in the presence of opiate (100 μM methadone) a clear difference can be observed in the GTP hydrolysis between uninfected and persistently infected cells (Fig. 2*A*). Although the Michaelis–Menten constant ($K_m = 2 \times 10^{-6}$ M) for the reaction is equal with stimulated membranes from infected and uninfected cells, the V_{max} for uninfected cells (3.59×10^{-10} mol/min per mg of protein) is higher than that for infected cells (2.25×10^{-10} mol/min per mg of protein) (Fig. 2*B*). The examination of substrate dependence of GTP hydrolysis with or without methadone stimulation in membranes from infected and uninfected cells is illustrated in Fig. 3. Methadone causes little stimulation in the membranes of infected cells but causes a doubling of the reaction velocity in the membranes of the uninfected cells. This defective coupling of the opiate receptor to the inhibiting regulatory protein N_i of the adenylate cyclase in the case of the persistent rabies virus infection can be due either to a dysfunction of the receptor site or to a disorder of the regulatory protein. The question then arises: are the remaining functional components of the signal pathway from the opiate receptor to the regulatory protein and to the adenylate cyclase additionally affected in cells persistently infected by rabies virus?

Functional Ability of the Regulatory G/F Protein. As can be seen from the above data (Fig. 2), the basal GTPase activity of the regulatory GTP-binding proteins was not affected by rabies virus infection. The function of the stimulating protein N_s can be tested by activation of the catalytic subunit via the regulatory G/F protein with GTP, guanosine [β,γ-

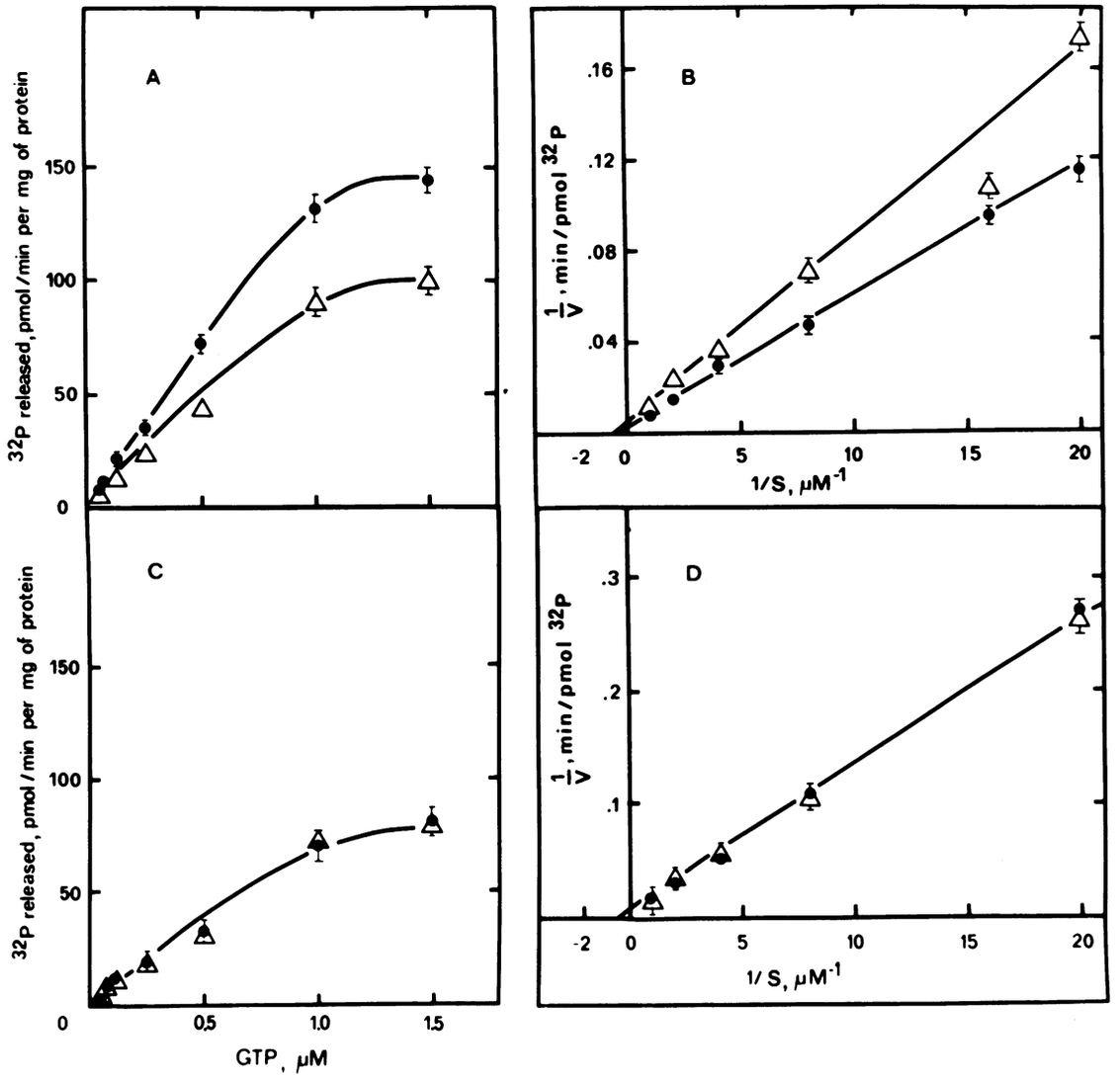

FIG. 2. GTP concentration dependence of the GTP hydrolysis in membranes (2.5 μg) of uninfected (●) and persistently rabies virus-infected (△) NG-108-15 cells. (*A*) Kinetics of the GTP hydrolysis in the presence of methadone (100 μM) for the membrane preparations of infected and uninfected cells. (*B*) Lineweaver–Burk analysis of the enzyme kinetics from *A*. (*C*) Enzyme kinetics of the GTP hydrolysis in membrane preparations (2.5 μg) of uninfected and persistently infected cells in the absence of opiate. (*D*) Lineweaver–Burk analysis of the enzyme kinetics from *C*. Each vial was incubated for 10 min. Standard deviation among four samples was 5%.

imido]triphosphate (p[NH]ppG), or fluoride. However, in persistently infected cells we see a total suppression of the activation of the adenylate cyclase after GTP or fluoride stimulation (Fig. 4). In contrast, we have previously reported that there is no change in the fluoride activation of adenylate cyclase in acutely rabies infected NG-108-15 cells (17). To localize the defect in adenylate cyclase activation we attempted to directly stimulate the catalytic subunit of the adenylate cyclase and bypass the receptors and the regulatory N_s protein. This is feasible with the diterpene forskolin, which directly activates the catalytic subunit of the adenylate cyclase system (25, 26).

Activity of the Catalytic Subunit of the Adenylate Cyclase. Forskolin stimulates the catalytic subunit of the adenylate cyclase in the concentration range of 10–200 μM without requiring the regulatory G/F protein. This compound was used at 200 μM to determine if the catalytic subunit of the adenylate cyclase was changed functionally by persistent rabies virus infection. The kinetics of forskolin stimulation with membrane preparations from uninfected and infected cells were compared (Fig. 5). The activation of both membrane preparations was very similar during the 25-min period of incubation. Therefore, the activity of the catalytic subunit was not affected by the virus infection.

DISCUSSION

This study shows a strongly reduced opiate receptor coupling to the inhibiting regulatory protein N_i of adenylate cyclase in membranes of persistently rabies virus-infected NG-108-15 cells. The coupling was measured by stimulating the GTPase activity of the regulatory N_i protein. There are other GTPase activities present in these cells, but under our test conditions only the opiate receptor-dependent enzyme was measured. This has a K_m of 2×10^{-6} M compared to a K_m of 7×10^{-4} M for the other GTPase, which is insensitive to opiates (7, 20). This latter enzyme plays only a minor role under our test conditions because its maximal activity is achieved at GTP substrate concentrations 50-fold higher than those used in our tests. The activity of this enzyme is only 5% of that of the opiate-dependent GTPase and was corrected for in our calculations. The dysfunction of the stimulation capability of the GTPase activity of the regulatory N_i protein could be due to various causes. It could be

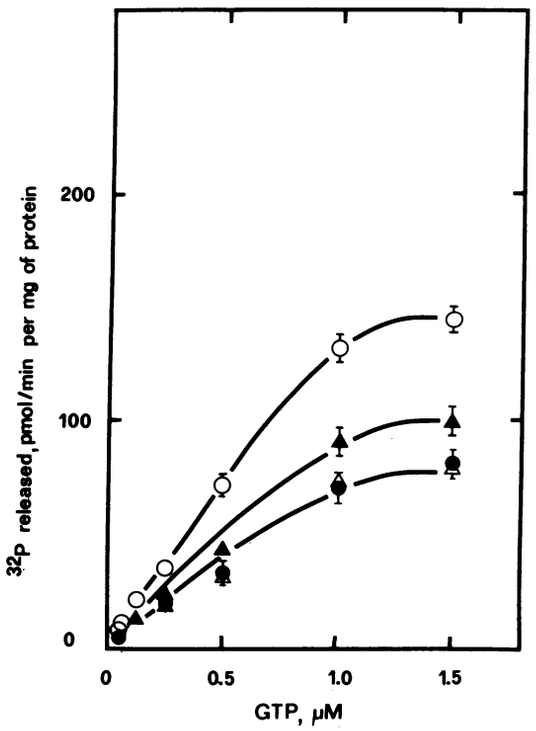

FIG. 3. Summary of the results in Fig. 2 for the enzyme kinetics with and without the opiate methadone for stimulated and unstimulated GTP hydrolysis by membranes of NG-108-15 cells. ●, Unstimulated membranes from uninfected cells; △, unstimulated membranes of persistently rabies virus-infected cells; ○, opiate-stimulated membranes of uninfected cells; ▲, opiate-stimulated membranes of persistently rabies virus-infected cells.

due to the reduced affinity of the receptor, which leads to decreased opiate binding. However, the number of opiate receptors is unchanged compared to uninfected cells and even very high concentrations of opiate cannot increase the stimulation of GTP hydrolysis when all receptors are occupied by opiate molecules (see Fig. 1). Reduction of the stimulation could also be due to a disorder of the regulatory N_i protein in its enzyme activity. However, the V_{max} and K_m values of the unstimulated basal GTP hydrolysis without opiates by membranes from infected and uninfected cells are identical. Therefore, it appears that the GTPase activities of the regulatory protein are functional. Because opiate receptors have been shown to be defective in their binding to opiates it is also possible that the receptor is defective at the coupling site to the inhibiting regulatory N_i protein. This we have demonstrated by measurement of GTP hydrolysis at methadone concentrations at which all the receptors are occupied. We have also shown that the interaction of these regulatory subunits with the adenylate cyclase is blocked after GTP or fluoride stimulation of the adenylate cyclase activity *in vitro*.

The catalytic subunit of the adenylate cyclase systems, however, is fully active, as shown by the tests done with forskolin. Taken together, these results indicate that the persistent rabies virus-infected NG-108-15 cells show a reduced affinity of the opiate receptors for opiate agonists, a disorder of the coupling of the opiate receptor to the regulatory inhibiting N_i protein, and a block of the regulation signal from the stimulating regulatory protein N_s to the adenylate cyclase, which has an unchanged enzyme activity.

It is still uncertain how the virus infection causes these observed dysfunctions of the receptor adenylate cyclase system. It is possible that there is direct influence of the viral proteins on cellular functions—e.g., a direct interaction between the viral membrane proteins and the cellular components of the receptor adenylate cyclase system. However, it is difficult to understand why in the case of acute rabies virus infection there is less change in agonist binding to receptors and no alteration in the fluoride- or p[NH]ppG stimulated cAMP generation *in vitro* compared to persistently infected cells, since in acute infections massive viral antigen accumulation could also be detected by immunofluorescence in the cytoplasm and cell membranes. Previous data with paramyxovirus-infected C6 rat glioma cells (22, 27) support the idea

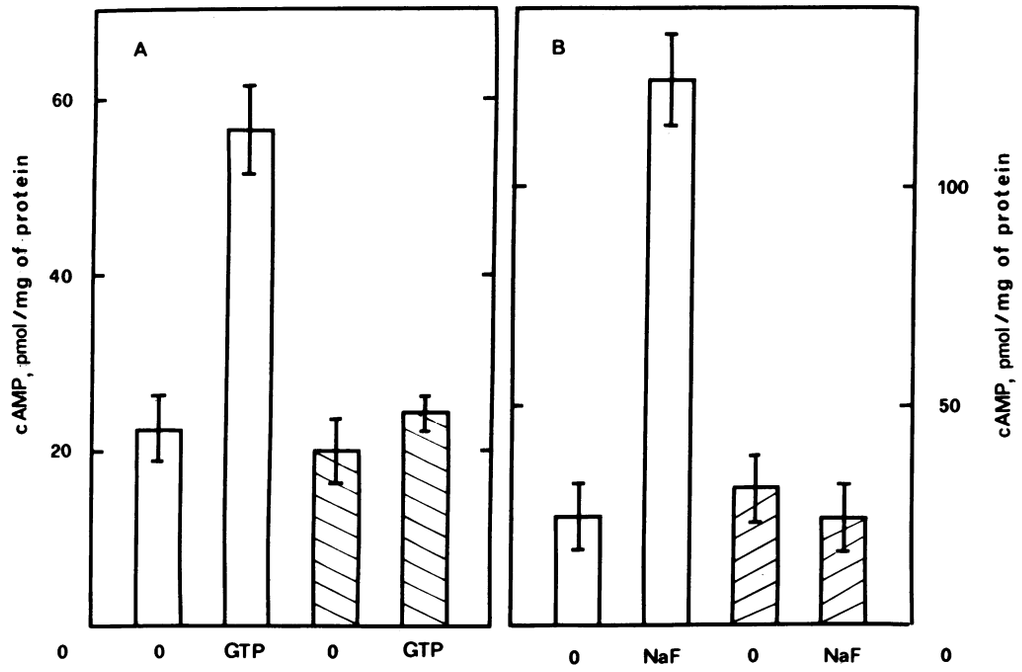

FIG. 4. Stimulation of the adenylate cyclase for cAMP generation by GTP (1.5 μM) (*A*) and NaF (10 mM) (*B*) in membranes (200 μg) from uninfected (empty bars) and persistently rabies virus-infected (hatched bars) NG-108-15 cells. Each sample was incubated for 10 min. Standard deviation among six samples was 10–12%.

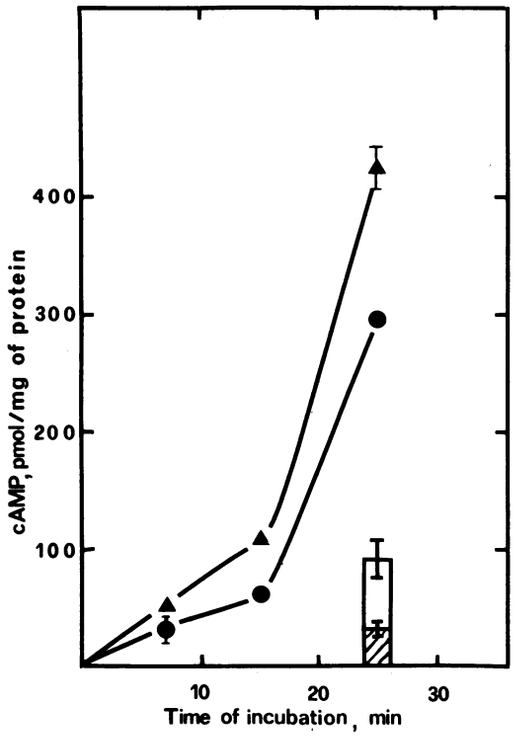

FIG. 5. Stimulation of the catalytic subunit of adenylate cyclase by forskolin (200 μM) in membrane preparations from uninfected (●) and persistently rabies virus-infected (▲) NG-108-15 cells. Each sample contained 200 μg of membrane protein and the measurements in the time course were done after 7, 15, and 25 min. The column shows the cAMP formation after a 25-min incubation with NaF (10 mM) (empty bar) and the basic level of cAMP after the same incubation without stimulation (hatched bar). The standard deviation among three samples was about 10%.

that there is more than a direct influence of viral proteins against such cell functions. It is possible that cytoplasmic virus factors closely related to membrane processes are involved. It is also possible that secondary cell regulatory events are involved that lead to alterations in the cell membrane and thus affect the receptor functions.

Investigations of [^{3}H]methyl group incorporation from the donor [*methyl*-^{3}H]methionine into membrane phospholipids exhibited no differences between uninfected and persistently rabies virus infected NG-108-15 cells as was observed in persistent infections of C6 cells with canine distemper virus and measles subacute sclerosing panencephalitis virus (28). The *in vitro* studies shown here together with our previously published data (12) show which sites in the complex cellular opiate receptor adenylate cyclase system are damaged after virus infection. These findings lead to the final conclusion that there is a disruption of the regulation of cell functions upon persistent infection with rabies virus.

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