Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 8016–8019, September 1991
Biochemistry

Glycosyl-phosphatidylinositol/inositol phosphoglycan: A signaling system for the low-affinity nerve growth factor receptor

(development/inner ear/cochleovestibular ganglion/anti-inositol phosphoglycan antibody)

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Communicated by Sidney Udenfriend, June 7, 1991 (received for review April 15, 1991)

ABSTRACT **Nerve growth factor (NGF) exerts a variety of actions during embryonic development. At the early stages of inner ear development, NGF stimulates cell proliferation, an effect mediated through low-affinity receptors. We have studied the possibility that the glycosyl-phosphatidylinositol/inositol phosphoglycan (glycosyl-PtdIns/IPG) system is involved in transmitting this NGF signal. Endogenous glycosyl-PtdIns was characterized in extracts of cochleovestibular ganglia (CVGs) that incorporated [^{3}H]glucosamine, [^{3}H]galactose, [^{3}H]myristic acid, and [^{3}H]palmitic acid. Incubation of CVG with NGF produced a rapid and transient hydrolysis of glycosyl-PtdIns. Hydrolysis was complete at 100 ng/ml, and the half-maximal effect occurred at 25 ng/ml, overlapping with the concentration dependence of the mitogenic effect of NGF. An IPG was isolated from embryonic extracts. It had biological effects similar to those reported for the insulin-induced IPG in other tissues. It exerted a powerful mitogenic effect on CVG, comparable to that of NGF. Both the IPG- and NGF-induced cell proliferation were blocked by anti-IPG antibodies that recognized the endogenous IPG on a silica plate immunoassay. These results show that CVG possesses a fully active glycosyl-PtdIns/IPG signal transduction system and that the proliferative effects associated with NGF binding to low-affinity receptors require IPG generation.**

Cell proliferation during embryonic development is strictly regulated and this control is believed to be exerted by the local expression of growth factors and their receptors. Insulin and nerve growth factor (NGF) emerge among the molecules that are able to trigger and support cell division in distinct embryonic neuronal and nonneuronal populations (1–3). In a variety of cells, insulin stimulates the hydrolysis of a membrane glycophospholipid containing inositol, sugars, and saturated fatty acids (4–6). This glycosyl-phosphatidylinositol (glycosyl-PtdIns) has been found in several cell membranes and bears a remarkable resemblance to the glycosyl-PtdIns anchor of membrane proteins (7). NGF also promotes the hydrolysis of a membrane glycosyl-PtdIns in cells where NGF is known to have profound biological effects (8). The hydrolysis of glycosyl-PtdIns produces a rapid intracellular accumulation of its polar head group, an inositol phosphoglycan (IPG) that has been shown to mimic a variety of the biological effects of insulin (refs. 9–12; for a review, see ref. 13). Recent experiments have demonstrated that a rat liver-derived IPG was able to copy the effects of both insulin and NGF on the early developing inner ear of the chicken embryo (14). The two growth factors differentially regulate cell division in the otic vesicle and the associated cochleovestibular ganglion (CVG) (1, 2). An interesting possibility is, therefore, that IPG would be conserved for some of the developmental actions of insulin and NGF, which could use a common signaling pathway, shared perhaps with other related growth factors, to regulate cell growth. The present work provides further support for the involvement of this glycosyl-PtdIns/IPG pathway in transducing the mitogenic effects of NGF on the early developing inner ear by showing the following results: (*i*) the presence of endogenous glycosyl-PtdIns and IPG, the latter with strong mitogenic activity; (*ii*) the ability of NGF to stimulate glycosyl-PtdIns hydrolysis in parallel with its biological activity; and (*iii*) the ability of anti-IPG antibodies to block the biological effects of NGF.

MATERIALS AND METHODS

Preparation of Explant Cultures. CVGs (statoacoustic ganglia) were aseptically isolated from 72-h chicken embryos, as described (2), and staged according to ref. 15. The standard culture medium consisted of serum-free medium 199 with Hanks' salts and 2 mM glutamine (Flow Laboratories), supplemented with 15 mM $NaHCO_3$. Incubations were carried out at 37°C in a water-saturated atmosphere containing 5% CO_2/95% air.

Histology. CVGs were fixed in 4% (wt/vol) paraformaldehyde and processed for histology. Determinations of tissue volume were made using morphometrical methods on serial sections as described in detail in ref. 16.

CVG Labeling and Glycosyl-PtdIns Purification. CVGs were radioactively labeled for 24 h in the presence of [^{3}H]glucosamine, [^{3}H]galactose, [^{3}H]myristic acid, or [^{3}H]palmitic acid at 100 μCi/ml (New England Nuclear; 1 Ci = 37 GBq). At the end of the incubation period, CVGs were collected, placed in 0.2 ml of phosphate-saline buffer, and 0.2 ml of ice-cold 10% (wt/vol) trichloroacetic acid was added to each sample. After standing for 15 min at 4°C, cellular lipids were extracted and glycosyl-PtdIns was purified as indicated (5).

Purification of IPG. IPG was prepared by treating glycosyl-PtdIns purified from either rat liver or 3-day chicken embryos with PtdIns-specific bacterial phospholipase C, as described (5, 14). PtdIns-specific phospholipase C from *Bacillus thuringiensis* was a generous gift of S. Udenfriend (Roche Institute of Molecular Biology, Nutley, NJ). The concentration of IPG was calculated by measuring free amino groups, considering that each molecule of IPG contains one amino group. The biological activity of IPG from both sources was assessed *in vitro* by testing its capacity to inhibit the phosphorylation of histone IIA by the cAMP-dependent protein kinase (17).

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Abbreviations: CVG, cochleovestibular ganglion; IPG, inositol phosphoglycan; glycosyl-PtdIns, glycosyl-phosphatidylinositol; NGF, nerve growth factor.

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Immunodetection of Chicken Embryo Purified IPG by Anti-IPG Antibodies. Polyclonal rabbit anti-IPG antibodies were raised and tested as described (11). IgG was purified from immune sera by ammonium sulfate fractionation followed by affinity chromatography with protein A-agarose. IPG purified from either rat liver or chicken embryos and several simple sugars and inositols were spotted on a silica G60 TLC plate (Merck). Antigens were detected by autoradiography as described (18).

RESULTS AND DISCUSSION

Hydrolysis of Glycosyl-PtdIns by NGF. The glycolipid precursor of IPG was characterized by incubating isolated CVGs overnight with the following radiolabeled precursors of glycosyl-PtdIns: [^{3}H]glucosamine, [^{3}H]galactose, [^{3}H]myristic acid, or [^{3}H]palmitic acid. Polar lipids were then extracted and resolved by sequential acid–base TLC (5). After these treatments, a single labeled fraction with an R_f of 0.5 was recovered, which was further purified by two-dimensional TLC. As shown in Fig. 1*A*, a single spot was observed by fluorography of the plate. The chromatographic profile obtained for CVG-isolated glycosyl-PtdIns was identical to that observed for rat liver glycosyl-PtdIns. Further studies were performed with the single fraction obtained after TLC in a basic solvent system. CVG-isolated glycosyl-PtdIns incorporated [^{3}H]glucosamine, [^{3}H]galactose, [^{3}H]myristic acid, and [^{3}H]palmitic acid. This pattern is similar to that reported for the insulin-modulated glycosyl-PtdIns (5, 6). The presence of glycosyl-PtdIns was confirmed by demonstrating that the glucosamine molecule was covalently linked to PtdIns. The glycolipid fraction, with an R_f of 0.5, from ganglia metabolically labeled with [^{3}H]glucosamine was treated with either sodium nitrite (pH 3.75) or PtdIns-specific phospholipase C from *B. thuringiensis*. Both treatments produced the hydrolysis of the CVG-isolated glycosyl-PtdIns, measured as radioactivity released in the aqueous phase. The percentage of glycosyl-PtdIns hydrolysis was 61.1% for the deamination treatment and 34.65% for the phosphodiesteric cleavage (data determined in two experiments performed in duplicate). These results indicate that CVG cells contain a glycosyl-PtdIns molecule that could serve as the endogenous source of IPG.

The ability of NGF to stimulate hydrolysis of glycosyl-PtdIns was determined by incubating CVG, prelabeled with [^{3}H]glucosamine, with NGF (25 ng/ml). Glycosyl-PtdIns hydrolysis was measured at the times indicated and analyzed by TLC (Fig. 1*B*). NGF induced a loss of glycosyl-PtdIns of about 30% within 0.5 min and 50% within 1 min. Recovery started immediately thereafter and the new steady-state value was reached about 10 min after the addition of the growth factor. The dose dependency of glycosyl-PtdIns hydrolysis was studied following a similar experimental approach (Fig. 1*C*). Hydrolysis was almost complete at NGF concentrations >100 ng/ml, and the half-maximal effect was observed with NGF at 25 ng/ml. These results indicate that the binding of NGF to low-affinity receptors regulates the rate of glycosyl-PtdIns hydrolysis. Hence, glycosyl-PtdIns hydrolysis may represent an early event in the transduction of the NGF signal in CVG.

It is interesting to note that the dose–response profile of glycosyl-PtdIns hydrolysis overlaps with that of cell proliferation induced by NGF (Fig. 1*C Inset*, data from ref. 2). This would indicate that the mitogenic effect of NGF could be linked to the hydrolysis of glycosyl-PtdIns.

CVG from 72-h chicken embryos shows only low-affinity NGF receptors (3), thus the activation of the glycosyl-PtdIns/IPG system would be linked to specific binding of NGF to the low-affinity receptor. This fact leads to the question of whether NGF is sharing low-affinity receptors

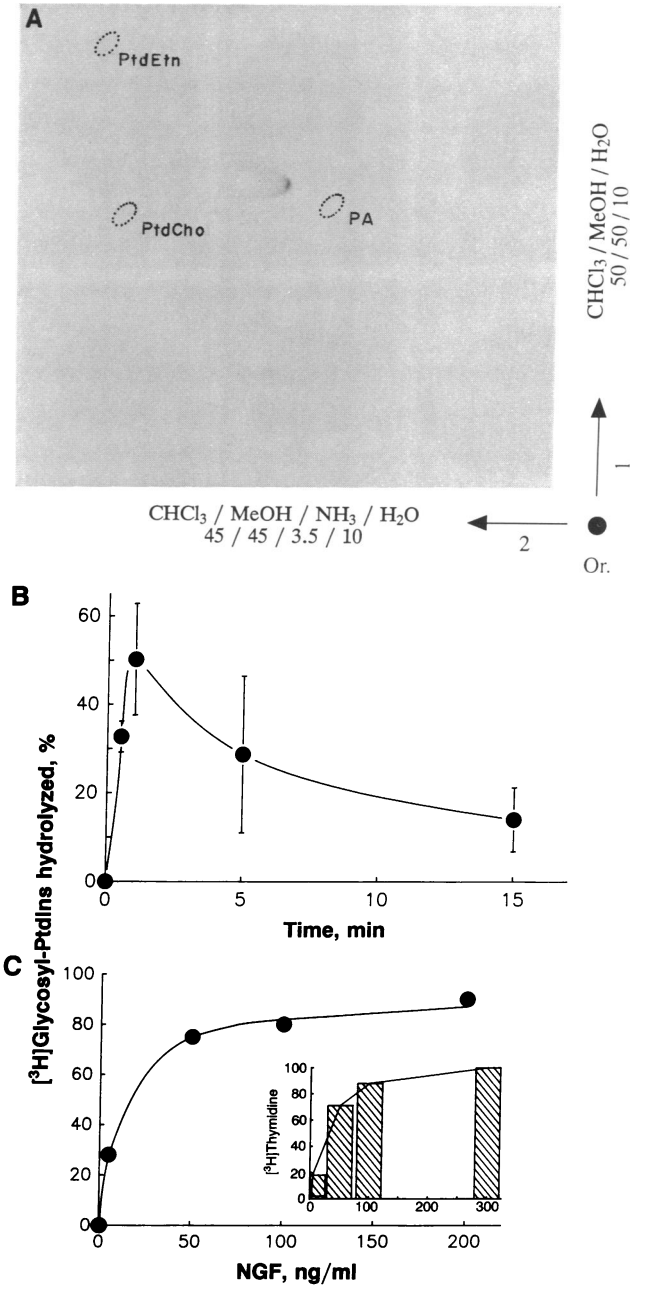

FIG. 1. Hydrolysis of glycosyl-PtdIns by NGF. (*A*) Two-dimensional TLC of [^{3}H]glycosyl-PtdIns isolated from chicken embryos. [^{3}H]Glycosyl-PtdIns was spotted at one corner of the plate (Or.) and the chromatogram was developed first in chloroform/methanol/water, 50:50:10 (vol/vol), dried briefly, turned 90°, and further developed in chloroform/methanol/ammonia/water, 45:45:3.5:10 (vol/vol). Finally, the plate was dried, and labeled glycosyl-PtdIns was detected by fluorography of the plate. Phospholipid standards were visualized with I_2 and are indicated by dotted lines. PtdEtn, phosphatidylethanolamine; PtdCho, phosphatidylcholine; PA, phosphatidic acid. (*B*) Time course of hydrolysis of glycosyl-PtdIns. Cochlear ganglia were incubated with [^{3}H]glucosamine and exposed to NGF (25 ng/ml). The fraction of glycosyl-PtdIns hydrolyzed was plotted against the time of incubation. [^{3}H]Glucosamine incorporated per ganglion at zero time was 981 ± 155 dpm. Results are mean ± SEM of four experiments. (*C*) Dose–response effect of NGF on glycosyl-PtdIns hydrolysis. Experiments were done as described in *B*. Glycosyl-PtdIns hydrolyzed was measured after incubation for 1 min with NGF as indicated. Results are from one representative experiment out of three. (*Inset*) Cell proliferation rate of CVG as a function of NGF in the incubation medium [data from Represa and Bernd (2)]. Data for [^{3}H]thymidine are percent of maximal [^{3}H]thymidine incorporation.

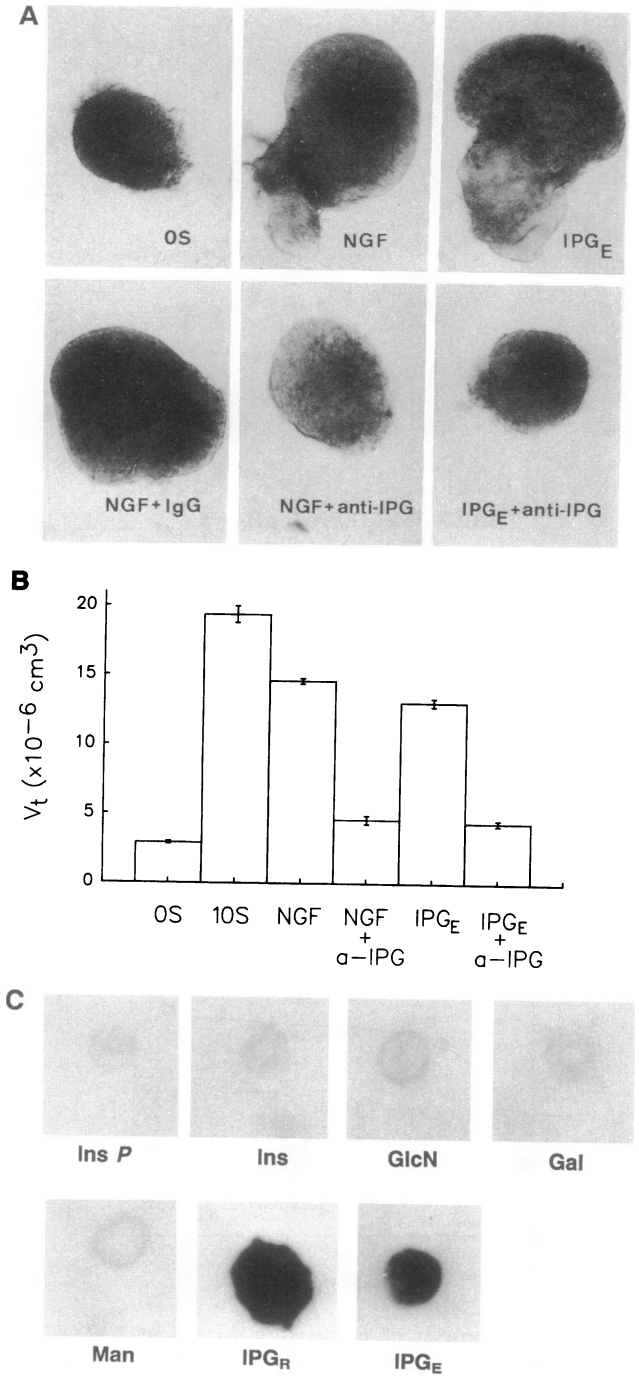

FIG. 2. Blockade of the mitogenic effect of NGF by anti-IPG antibodies. (*A*) Isolated CVGs were grown in culture for 24 h in medium 199 alone (OS) or containing NGF (NGF), embryon-derived IPG (IPG$_E$), NGF plus IgG from preimmune rabbit (NGF + IgG), NGF plus anti-IPG antibody (NGF + anti-IPG), or embryon-derived IPG plus anti-IPG antibody (IPG$_E$ + anti-IPG). Concentrations used were as follows: NGF, 50 ng/ml; IPG, 5 μg/ml; anti-IPG antibody, 20 μg/ml; preimmune IgG, 20 μg/ml. (*B*) Effect of anti-IPG antibody on tissue volume. Measurements of tissue volume (V_t) were done on ganglia from experiments performed as in *A* that were processed for histology. Labels are as in *A*, except that α-IPG is anti-IPG antibody. 10S indicates ganglia grown in medium 199 plus 10% fetal calf serum. Results are mean ± SEM of three experiments. (*C*) Recognition of chicken embryo IPG by anti-IPG antibody. IPG extracted from either rat liver (IPG$_R$) or from chicken embryos (IPG$_E$) was spotted on a silica G60 TLC plate and incubated with anti-IPG antibody at 4 μg/ml, and antigens were detected by autoradiography. Other sugars and inositols were assayed in parallel and the names are indicated. Ins*P*, inositol phosphate. Concentrations were as follows: IPG$_R$, 50 μM; chicken embryo IPG (IPG$_E$), 5 μM; all other substances, 1 mM.

with other neurotrophic factors and, hence, whether the glycosyl-PtdIns hydrolysis could be a candidate system for neurotrophic factor signaling (19).

Blockade of NGF-Induced Proliferation by Anti-IPG Antibodies. The presence of an endogenous glycosyl-PtdIns plus the fact that rat liver-derived IPG has proliferative effects in CVG (14) raise the question of the occurrence of an endogenous active glycosyl-PtdIns-derived product with biological activity in the chicken embryo. A molecule purified by PtdIns-phospholipase C hydrolysis of a glycosyl-PtdIns purified from chicken embryos was assayed for biological effects. The activity of chicken-derived IPG was evaluated *in vitro* by determining its ability to inhibit the catalytic subunit of cAMP-dependent protein kinase (17). Chicken-derived IPG, NGF, or liver-derived IPG (14) induced cell proliferation of isolated CVG to the same extent (Fig. 2*A*).

An anti-IPG antibody has been reported to selectively block a variety of insulin actions (11). This antibody (20 μg/ml) was able to abolish both IPG and NGF stimulation of CVG growth, as estimated from the size of the CVGs (Fig. 2*A*) and morphometrical measurements of tissue volume (Fig. 2*B*). A similar result was observed with IPG that was purified from either chicken embryo or rat liver. Anti-IPG antibodies, on the other hand, had no effect on the proliferation of either control CVG kept in culture without additions (bar OS) or CVG stimulated to grow with 10% fetal calf serum (bar 10S). The IgG fraction purified from serum of a nonimmunized rabbit was unable to block the effects of IPG or NGF. At this stage it seemed important to assess the specificity of the recognition of the chicken-derived IPG by the anti-IPG antibody. Fig. 2*C* shows the ability of anti-IPG antibodies to recognize both rat liver- and chicken embryo-purified IPGs in a immunodection assay performed on a TLC plate (IPG$_R$ and IPG$_E$, respectively, in Fig. 2*C*). Simple sugars (galactose, glucosamine, or mannose) and inositol and inositol monophosphate did not show cross-reaction with the antibody.

It has been known for some time that there are two classes of NGF receptors on most responsive cells, low-affinity and high-affinity classes. The low-affinity NGF receptor is a protein of 75 kDa (20) and the *trk* protooncogene has been identified as the gene for the high-affinity receptor (21, 22). It has been proposed that these receptors are two independent entities and that only the high-affinity receptor is the biologically active form (23). However, CVG from 72-h chicken embryos express only low-affinity NGF receptors and NGF exerts a strong proliferative effect on CVG (3). The present results show that CVG possesses a fully active glycosyl-PtdIns/IPG signaling system that operates as a transducing pathway for the low-affinity NGF receptor and perhaps for receptors of other related neurotrophic factors that regulate cell proliferation during embryonic development.

We thank Dr. S. Udenfriend for the PtdIns-specific phospholipase C. I.V.-N. was supported by a short-term fellowship from the Comunidad de Madrid while working at the University of Valladolid. M.A.A. is fellow of Ministerio de Educación y Ciencia, Spain, and R.C. is fellow of Fundación Conchita Rabago. This work was supported in part by grants from Dirección General de Investigación, Ciencia y Tecnología (PM90-0001) Comunidad de Madrid and Europharma to J.M.M., Dirección General de Investigación, Ciencia y Tecnología (PB89-0357) to F.G., and Fondo de Investigaciones Sanitarias (91/0361) to J.R., and National Institutes of Health NIDDK 40753 to G.R.

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