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EFFECTS OF CHRONIC LOW-LEVEL LEAD EXPOSURE ON mRNA EXPRESSION, ADP-RIBOSYLATION AND PHOTOAFFINITY LABELING WITH [α-^{32}P]GUANINE TRIPHOSPHATE-γ-AZIDOANILIDE OF GTP-BINDING PROTEINS IN NEURONS ISOLATED FROM THE BRAIN OF NEONATAL AND ADULT RATS

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Abstract—The effects of chronic lead exposure on mRNA expression, ADP-ribosylation and photoaffinity labeling with [α-^{32}P]guanine triphosphate-γ-azidoanilide ([^{32}P]GTP-A) of α_i or α_s subunit of G protein were investigated in neurons isolated from the brain of neonatal and adult rats exposed to lead acetate or sodium acetate (for control). Rats were exposed by oral feeding for 10 days or 20 weeks to a low level of lead acetate or sodium acetate. The exposure started either prenatally or at an adult age. The expression of α_i-mRNA in neurons obtained from the brain of control neonatal rats was significantly higher than that of the expression in samples obtained from the brain of control adult rats or the brain of rats exposed to lead at an adult age. The expression of α_i-mRNA in neurons obtained from the brain of control neonatal rats, lead-exposed neonatal rats and adult rats prenatally exposed to lead did not differ significantly. Chronic lead exposure did not affect the expression of α_s-mRNA in neurons obtained from the brain of neonatal and adult rats. The ADP-ribosylation or the photoaffinity labeling with [^{32}P]GTP-A of α_i or α_s subunits reflected the developmental pattern of the expression of α_i or α_s-mRNA. The incorporation of radioactivity in α_i-subunit obtained from the brain of control neonatal rats, lead-exposed neonatal rats and rats prenatally exposed to lead was greater than the incorporation in α_i-subunit obtained from the brain of control adult rats or rats exposed to lead at an adult age. The incorporation of radioactivity did not differ significantly in α_s-subunits obtained from control or lead-exposed neonatal and adult rats. These observations indicate that (1) the mRNA expression, ADP-ribosylation and photoaffinity labeling with [^{32}P]GTP-A of α_i-subunit decrease, whereas the mRNA expression, ADP-ribosylation and photoaffinity labeling with [^{32}P]GTP-A of α_s-subunit do not change as animals age after postnatal day 10, (2) chronic prenatal lead exposure delays the age-dependent decrease in mRNA expression, ADP-ribosylation and photoaffinity labeling of α_i subunit, and (3) chronic adult exposure does not cause these changes.

Although chronic lead exposure is known to impair the functional capacity of cholinergic, adrenergic, GABAergic, glutaminergic and opiate receptors in the central nervous system (CNS) [1–7], the mechanism by which the receptors are affected by lead exposure is not fully understood. Previous studies have shown that the mammalian brain contains a family of structurally homologous GTP-binding proteins that couple the cholinergic [8–12], adrenergic [13–16], glutaminergic [17] and opiate [18–21] receptors to a variety of effector mechanisms including the hydrolysis of polyphosphoinositide in brain and other cells. This investigation is based on the hypothesis that chronic lead exposure affects the functional capacity of various central receptors by affecting G proteins present in the brain of the developing rats. There are at least two types of G proteins that regulate the receptor function by stimulating or inhibiting adenylate cyclase activity in the mammalian CNS [22]. The stimulatory G proteins (G_s) bind with guanine nucleotides (GTP, GDP or Gpp(NH)p†) and serve as a substrate for ADP-ribosylation catalyzed by cholera toxin [23]. The inhibitory G protein (G_i) also binds with guanine nucleotide but serves as a substrate for ADP-ribosylation catalyzed by a pertussis toxin [22]. Both G_s and G_i consist of α (GTP binding site and ribosylation substrate), β (guanine triphosphatase), and γ (regulatory) subunits. Although the β and γ subunits of both G proteins are structurally identical, the α subunits of the two proteins are structurally different [22]. The regulatory properties of G proteins are modified by GTP, GDP, Gpp(NH)p, *N*-ethylmaleimide (NEM), ADP-ribosylation, and cations such as Na^+ or Mn^+ [13, 24, 25]. The observations of a preliminary study that (1) NEM and Gpp(NH)p modified the effects of chronic lead exposure on cholinergic receptors in the CNS, and (2) chronic lead exposure altered the ADP-

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† Abbreviations: Gpp(NH)p, guanosine 5′-($\beta\gamma$-imido)triphosphate; NEM, *N*-ethylmaleimide; SDS, sodium dodecyl sulfate; and [^{32}P]GTP-A [α-^{32}P]guanine triphosphate-γ-azidoanilide.

ribosylation of G proteins in rat brain* support the proposed hypothesis that G proteins may be involved in the development of toxicity following chronic lead exposure. In this investigation the effects of chronic lead exposure on mRNA expression, photoaffinity labeling with [α-^{32}P]guanine triphosphate-γ-azidoanilide ([^{32}P]GTP-A) and ADP-ribosylation of α_s and α_i subunits of G proteins were studied in neurons isolated from the brain of neonatal and adult rats exposed to lead acetate or sodium acetate.

MATERIALS AND METHODS

Materials

The radioactive chemicals were purchased from NEN Radiochemicals Corp., Wilmington, DE. Other chemicals were obtained from the Sigma Chemical Co., St. Louis, MO. The organic solvents were obtained from Fisher Scientific, Minneapolis, MN. The gel electrophoresis apparatus consisted of an Ephortic power supply and an Ephortic vertical slab. A Beckman LS 3801 scintillation counter was used.

Structure of the probes

The expression of α_i-subunit mRNA was studied by using a synthetic ^{32}P-labeled oligonucleotide

3′-AAACTACGGCAGTGACTGCAGT-
AGTAGTTCTTGTTGGACTTCCTG-
ACACCGGAGAAG-5′

that was complementary to the nucleotide sequence encoding amino acids 337–355 of α_i [26, 27]. The expression of α_s-subunit mRNA was studied by using a synthetic oligonucleotide

5′-CATTTGCTTCACAATGGTGCTTTTACC-3′

that was complementary to the sequence encoding amino acids 52–60 of α_s [28]. The probes were diluted with hybridization buffer to a specific activity of 2×10^6 cpm/fmol. The specificity of the probes was investigated by hybridization of RNA obtained from neurons.

Methods

Treatment of rats with lead acetate. Rats were exposed prenatally or at an adult age to lead acetate or sodium acetate (for control) for 20 weeks at a dose of 1.0 mg/kg/day as described by Singh and Ashraf [29].

For prenatal exposure, pregnant rats (obtained from the Animal Services, University of Minnesota, 14- to 16-weeks-old at 1 week of gestation) were exposed to a 1.0 mg/kg/day dose of lead acetate (N = 50) or sodium acetate for control (N = 50) by gastric intubation as described previously [29]. During week 3 of gestation, each rat was placed in individual cages and allowed to litter. After birth, the pups were further exposed for 20 weeks to lead, initially (for up to 2 weeks) by allowing them to receive milk from an exposed mother and then by feeding them lead acetate via gastric intubation (1.0 mg/kg/day). After 10 days or 20 weeks of the treatment, each animal was killed by thoracic stun and decapitation in the following groups: group 1, prenatally exposed to lead and killed on day 10 of the treatment; group 2, prenatally exposed to lead and killed at week 20 of the treatment; group 3, prenatally exposed to sodium acetate and killed at day 10 of the treatment; and group 4, prenatally exposed to sodium acetate and killed at week 20 of the treatment. Each skull was dropped into an ice-cold sucrose solution (0.32 M) and blood was drained into a centrifuge tube immediately after decapitation. The skull was dissected, the brain was removed, and the cerebral cortexes were placed in ice-cold 0.32 M sucrose and used for the isolation of neurons. All animals were kept under constant temperature with a 12-hr light and 12-hr dark cycle and received laboratory chow and water *ad lib.* throughout the experiment.

For adult exposure, 10-week-old rats were exposed for 20 weeks to lead acetate (group 5) or sodium acetate (group 6) by gastric intubation as described previously [29]. Animals were killed and tissue samples were processed as described above.

Isolation of neurons. Brain samples obtained from control or lead-exposed rats were processed for the isolation of neurons as described by Norton and Poduslo [30]. After sucrose gradient centrifugation, the four layers were identified and labeled from the top as layers A to D. Layer C which contained a mixture of neuronal and glial cells was processed further by sucrose gradient centrifugation to separate neurons from the glial cells [30]. The neuronal fraction was pooled with layer D which contained predominantly neurons. The pooled samples were concentrated to 500 μL under reduced pressure. Brain samples from 50 neonatal or 20 adult rats produced approximately 100 mg of neurons.

Expression of α_s and α_i mRNAs. Total RNA was extracted from the neurons according to Chirgwin *et al.* [31]. Total RNA was fractionated for Northern analysis by electrophoresis in 1.0% agarose gel containing 6% formaldehyde [32] and transferred onto a nitrocellulose filter. Northern blots were hybridized overnight at 40° in the presence of hybridization buffer [saline citrate buffer (0.6 M NaCl and 0.06 M sodium citrate, pH 7.4), 50% formamide, Denhardt's solution (0.02% each of polyvinylpyrrolidone, bovine serum albumin and Ficoll), 1.0% *N*-lauroylsarcosine, 0.02 M sodium phosphate (pH 7.4), 10% dextran sulfate, yeast tRNA (500 μg/mL), salmon sperm DNA (100 μg/mL) and 60 mM dithiothreitol] and the radiolabeled α_i probe ($1–2 \times 10^6$ cpm/fmol). Then the sample was removed, washed with the hybridization buffer, and placed in another dish containing the hybridization buffer and the α_s-probe. Hybridization was continued for another 24 hr. After hybridization, the samples were subjected to autoradiography at −80° for 5 days. The hybridized bands were scanned by using a densitometer and the optical density (O.D.) was recorded. In some preliminary experiments, the extracted RNA was hybridized with individual probes to identify the individual mRNAs.

Isolation of G_i and G_s. A 100-μL aliquot of each sample was processed by a modification of the procedure described by Neer *et al.* [24]. Individual samples (100 mg neurons) were mixed with 5 mL of

* Singh AK, unpublished data.

Table 1. Blood, brain and neuronal lead levels

Groups*	Blood lead (μg/dL)	Brain lead (ng/g)	Neuron lead (ng/mg protein)	Body wt (g)
N_C	1.6 ± 0.5	1.7 ± 1	<0.5	15 ± 3
N_L	33 ± 5	150 ± 30	20 ± 2	17 ± 5
A_C	3 ± 1	2 ± 0.5	<0.5	273 ± 25
A_{L1}	15 ± 5†	80 ± 10†	11 ± 2†	280 ± 30
A_{L2}	21 ± 3†	100 ± 20†	13 ± 2†	275 ± 20

Values are means ± SD, N = 5.

* N_c, neonatal control; N_L, neonatal rats prenatally exposed to lead; A_c, control adult; A_{L1}, adult rats prenatally exposed to lead; and A_{L2}, rats exposed to lead at an adult age.

† Significantly different ($P < 0.05$) when compared to the N_L values.

a solution containing Tris·Cl (50 mM, pH 8.1), sucrose (5%), $MgCl_2$ (6 mM), EDTA (1 mM), benzamidine (3 mM), dithiothreitol (1 mM) and trypsin inhibitors (1.0 μg/mL), and the mixture was homogenized. The homogenate was centrifuged at 100,000 *g* for 60 min and the pellets were collected. The pellets were further processed and subjected to DEAE-Sephacel chromatography as described by Neer *et al.* [24]. To accommodate a smaller amount of sample, a 10-mL column was used and the protein was eluted in 0.5-mL fractions. Each fraction was analyzed for the presence of G_s as described by Bender and Neer [33]. The fractions containing G_s and G_i proteins were collected and concentrated to 100 μL under reduced pressure.

Photoaffinity labeling with [^{32}P]GTP-A of G proteins. A 50-μL portion of the concentrated samples was incubated for 2 hr at 25° with [^{32}P]GTP-A (10^6 dpm, 10^{-13} mmol) as described by Schafer *et al.* [34]. After incubation, each sample was irradiated at 4° with UV light for 2 hr. After irradiation, each sample was mixed with 200 μL of $HClO_4$ (50 mM), the mixture was centrifuged (10,000 *g* for 1 min), and the pellets were collected and redissolved in 100 μL of Tris base (100 mM). The mixture was sonicated and subjected to sodium dodecyl sulfate (SDS) electrophoresis as described by Nelson *et al.* [35]. The gel slab was cut longitudinally into two equal halves. One portion of the gel was stained for proteins as described by Singh [36]. The other portion of the gel was cut into 5-mm pieces (such that each protein band was in one fraction), and radioactivity was determined in the presence of an aqueous scintillant. G_i and G_s were identified by using protein standards of molecular weight 41,000 and 45,000, respectively. Different protein bands were also identified by subjecting the gel plates to autoradiography performed at 4° for 5 days.

ADP-ribosylation of α_i and α_s (in the presence or absence of added β proteins) for samples obtained from control and lead-exposed rats. The concentrated samples were mixed with NAD (5 μM) containing [^{32}P]NAD (0.5 μCi), ATP (3 mM), GTP (2 mM), isoniazide (10 mM), thymidine (10 mM) and pertussis toxin (60 ng) or cholera toxin (100 ng) in a total volume of 50 μL. In some samples exogenous β protein was added before ADP-ribosylation. Each sample was subjected to SDS-polyacrylamide gel electrophoresis as described by Neer *et al.* [24] for α_i and Cassel and Slinger [23] for α_s. The gel was cut into 0.5-cm pieces and counted in the presence of an aqueous scintillant in a Beckman scintillation counter. The radioactivity level in the G_i or G_s protein fraction represented ADP-ribosylated protein. Different protein bands were also identified by subjecting the gel plates to autoradiography performed at 4° for 5 days.

Lead analysis. Blood, whole brain and neuronal samples were analyzed for the presence of lead by atomic absorption spectrophotometry as described by Singh and Ashraf [29]. Brain and neuronal samples were digested in 0.5 mL nitric acid and the solution was dried by heating at 250°. The dried residue was redissolved in 0.5 mL of sodium acetate (1.0 M in 0.2 M sodium chloride) and the mixture was analyzed by atomic absorption spectrophotometry. Blood samples were diluted with 0.1% Triton X-100 solution and homogenized for 10 sec. The homogenate was processed for the analysis of lead as described previously. A standard curve was prepared by analyzing samples containing 10–100 ng lead.

RESULTS

Body weight, food and water intake and the levels of lead

The weight gain in control rats and in lead-exposed rats was similar (Table 1). The food and water intake by control and lead-exposed rats also did not differ significantly. Blood-lead levels in lead-exposed rats ranged from approximately 30 μg/dL in neonatal rats to <20 μg/dL in adult rats. Since the Center for Disease Control has established that blood-lead levels ranging from 30 to 80 μg/dL caused "subclinical lead poisoning" in lead-exposed children, the exposure level used in this investigation is in the range of levels of lead present in the environment that may cause low-level toxicity in children. The relationship between the brain or neuronal lead levels and the development of toxicity is not yet established.

RNA hybridization

Sample from control rats. This study indicated that the neuronal RNA hybridized with both G_i and G_s

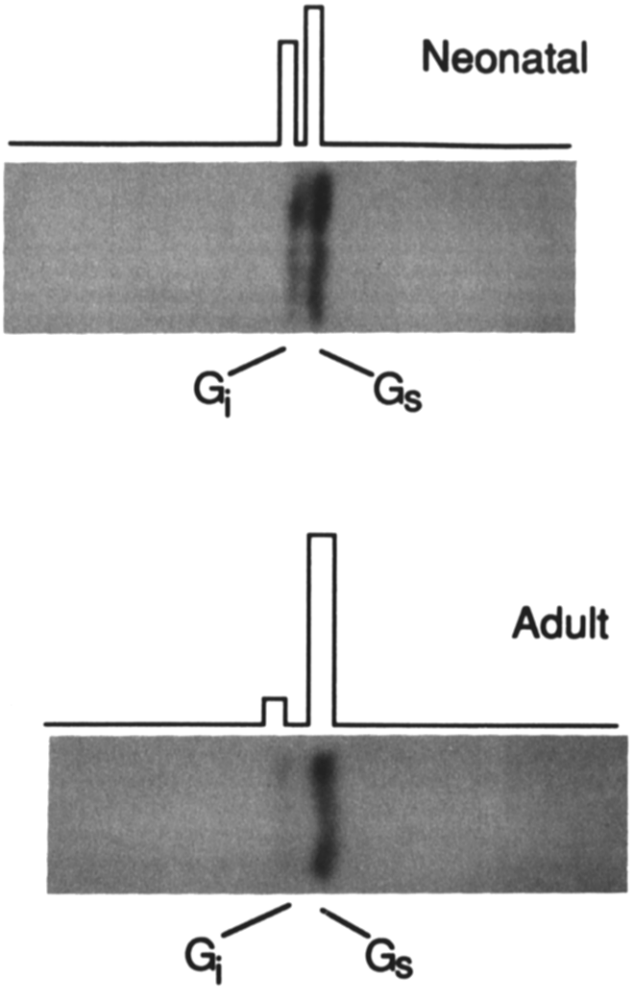

Fig. 1. mRNA expression of α_s and α_i subunits of G proteins in neurons isolated from the brain of neonatal and adult rats (G_s: cholera toxin sensitive G proteins and G_i: pertussis toxin sensitive G proteins). Total RNA was extracted from control or lead-exposed neurons, and individual RNA was separated by electrophoresis. The expressions of α_s and α_i mRNAs were studied by hybridizing the samples with an α_i probe followed by an α_s probe.

probes at kb 2.6 and 1.7, respectively (Fig. 1). While the G_s probe exhibited comparable hybridization with neonatal-RNA and adult-RNA (Fig. 2), the G_i probe exhibited significantly greater hybridization with neonatal-RNA than with adult-RNA (Fig. 2). The [mRNA:G_s probe hybridization/mRNA:G_i probe hybridization] ratio for adult samples was significantly greater than the ratio for neonatal samples.

Samples from lead-exposed rats. This study indicated that chronic prenatal lead exposure (1) did not affect the hybridization of the G_s or G_i probe with respective mRNAs in samples obtained from neonatal rats (group 1), (2) significantly increased the hybridization of the G_i probe with G_i-mRNA in samples obtained from adult rats (group 2), and (3) did not affect the hybridization of the G_s probe with G_s-mRNA in samples obtained from adult rats (groups 1–6). Chronic exposure of adult rats to lead did not affect the mRNA expression of G proteins. The [mRNA:G_s probe hybridization/mRNA:G_i probe hybridization] ratios were comparable for samples obtained from the brain of neonatal (group 1) and adult (group 2) rats prenatally exposed to lead. However, the [mRNA:G_s probe hybridization/mRNA:G_i probe hybridization] ratio for samples obtained from the brain of rats exposed to lead at an adult age (group 5) was greater than the ratio for samples obtained from the brain of control neonatal rats (group 3), lead-exposed neonatal rats (group 1)

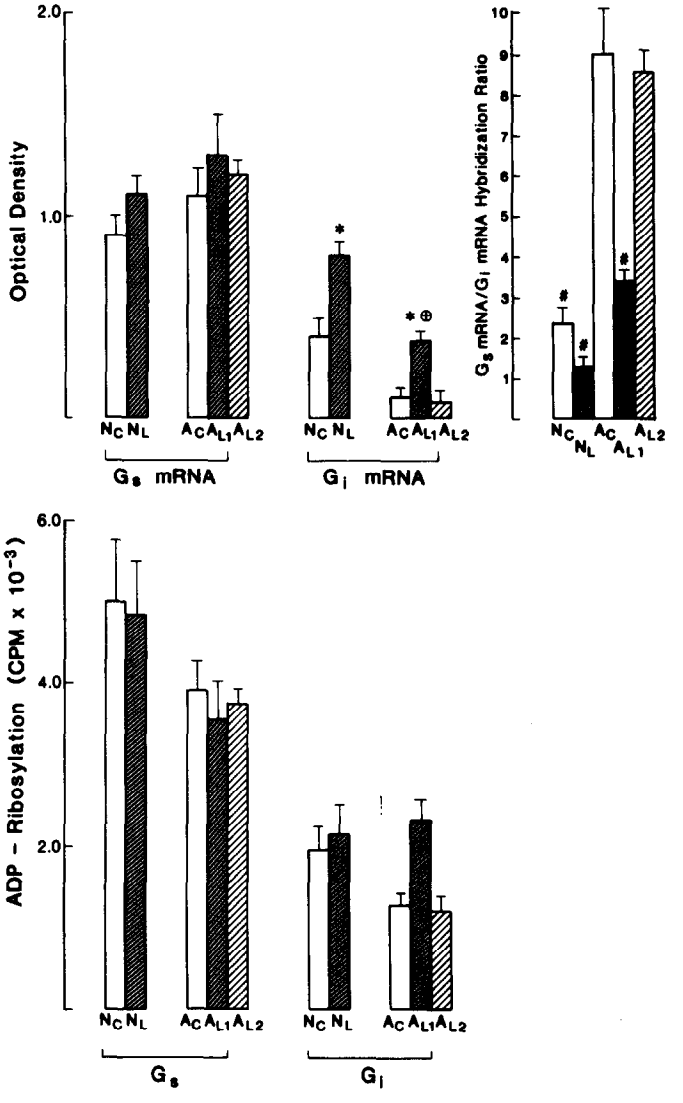

Fig. 2. Optical density of the autoradiographic plates for the hybridization of α_s and α_i probes with mRNA, and the radiolabeling due to ADP-ribosylation of α subunits in neurons obtained from the brain of control or lead-exposed neonatal and adult rats. The G_s:mRNA/G_i:mRNA hybridization ratio was calculated from the optical density values. Abbreviations: N_c, control neonatal rats; A_C, control adult rats, N_L, neonatal rats prenatally exposed to lead; A_{L1}, adult rats prenatally exposed to lead; and A_{L2}, rats exposed to lead at an adult age. Key: (*) $P < 0.05$ (significant) when N_L values were compared with N_C values or A_{L1} values were compared with the A_C or A_{L2} values; (⊕) $P < 0.05$ when A_{L1} values were compared with N_L values; and (#) $P < 0.05$ when the hybridization ratios for N_C, N_L and A_{L1} were compared with the A_C or A_{L2} values. Values are means ± SD, N = 5.

and prenatally lead-exposed adult rats (group 2) (Fig. 2).

Photoaffinity labeling with [^{32}P]GTP-A of G_i and G_s proteins

Typical autoradiographs for the photoaffinity labeling of G_i protein obtained from control and lead-exposed rats are shown in Fig. 3. The photoaffinity labeling of G_i protein obtained from neonatal samples (group 3) was significantly greater than the labeling of G_i protein obtained from adult samples (group 6). However, the labeling of G_s protein obtained from neonatal (group 3) and adult (group 6) samples did not differ significantly (Fig. 4). Chronic prenatal lead exposure significantly increased the labeling of G_i protein without affecting the labeling of G_s protein in neurons (Fig. 4). Chronic exposure of adult rats to lead did not cause these changes (Fig. 4).

ADP-ribosylation of G_i and G_s protein

Typical autoradiographs for the ADP-ribosylation of α_i subunit of G_i protein obtained from control and lead-exposed rats are shown in Fig. 5. This

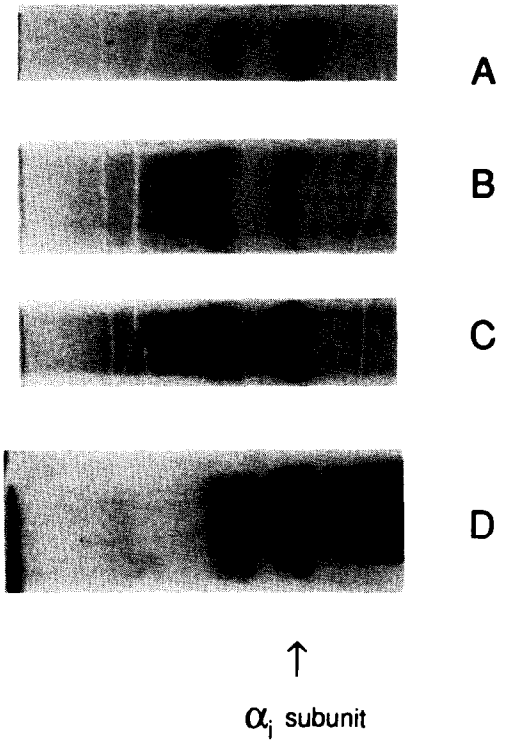

Fig. 3. SDS-polyacrylamide gel electrophoresis of G_i protein labeled with [α-^{32}P]guanine triphosphate-γ-azidoanilide ([^{32}P]GTP-A). G_i proteins were obtained from control neonatal rats (C), control adult rats (B), adult rats prenatally exposed to lead (D) and rats exposed to lead at an adult age (A). Each sample was incubated with [^{32}P]-GTP-A and irradiated at 4° with UV light. The radiolabeled protein was subjected to SDS electrophoresis as described in Materials and Methods.

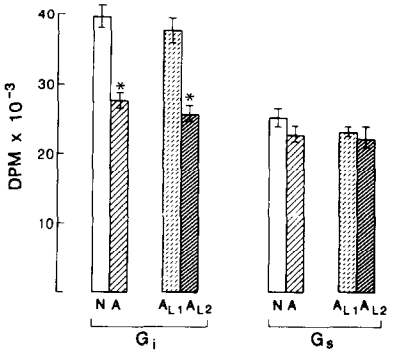

Fig. 4. Photoaffinity labeling with [^{32}P]GTP-A of pertussis toxin sensitive G proteins (G_i) and cholera toxin sensitive G proteins (G_s). Values are means ± SD, N = 5. Abbreviations: N, control neonatal; A, control adult; A_{L1}, adult rat prenatally exposed to lead; and A_{L2}, rats exposed to lead at an adult age. Key: (*) $P < 0.05$ (significant) when N_C values were compared with A_C values or A_{L1} values were compared with A_{L2} values for G_i.

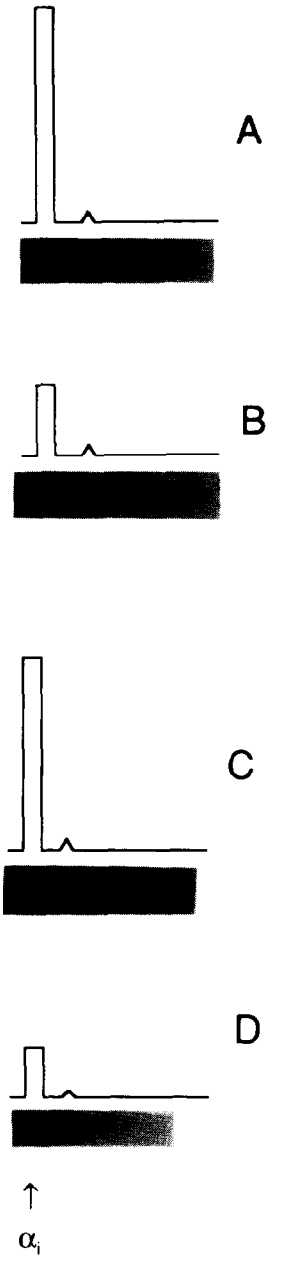

Fig. 5. SDS-polyacrylamide gel electrophoresis of the α_i protein [^{32}P]ADP-ribosylated by pertussis toxin. α_i Subunit was obtained from control neonatal rats (A), control adult rats (B), adult rats neonatally exposed to lead (C) and rats exposed to lead at an adult age (D). Each sample was incubated with the toxin (60 ng) and the ribosylated sample was subjected to electrophoresis as described in Materials and Methods.

experiment indicated that the α_i protein obtained from neonatal rats (groups 1 and 3) exhibited significantly greater incorporation of radioactivity than the α_i protein obtained from adult rats (groups 4 or 6). The α_i protein obtained from adult rats that

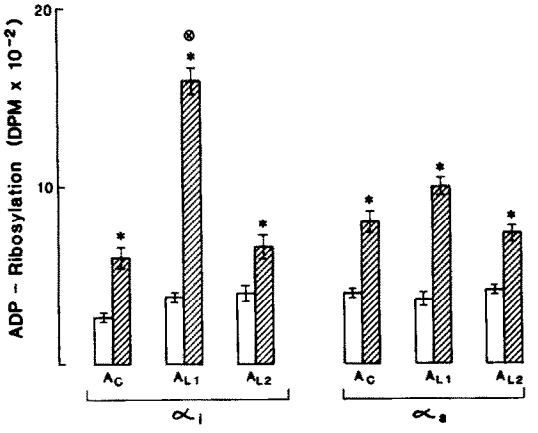

Fig. 6. ADP-ribosylation of α subunits in the presence (▨) or absence (□) of exogenously added β subunit. Abbreviations: A_c, control adult; A_{L1}, adult rat prenatally exposed to lead; and A_{L2}, rats exposed to lead at an adult age. Key: (*) $P < 0.05$ (significant) when values in the presence of the added β subunit were compared with values in the absence of the added β subunit; (⊗) $P < 0.05$ (significant) when the values in the presence of β subunit from A_{L1} were compared with other samples in the presence of β subunit. Values are means ± SD, N = 5.

were prenatally exposed to lead (group 2) exhibited greater incorporation of radioactivity than the α_i protein obtained from control rats (group 6) or from rats that were exposed to lead at an adult age (group 5) (Fig. 6). Addition of β protein significantly increased ADP-ribosylation of α_i protein in control and lead-exposed samples; however, the incorporation of radioactivity in samples obtained from rats that were prenatally exposed to lead (group 2) was significantly greater than the incorporation in samples obtained from control rats (group 6) or the rats that were exposed to lead at an adult age (group 5). Unlike the ADP-ribosylation of α_i protein, the ADP-ribosylation of α_s protein was not affected by chronic lead exposure (Fig. 6).

DISCUSSION

Using the mRNA-hybridization technique, the expression of α_i and α_s mRNAs has been studied in brain, liver, ovary and other tissues [26–28]. The present study indicated that although isolated neurons expressed both α_i and α_s mRNAs, the expression of α_i mRNA decreased as the animals aged whereas the expression of α_s mRNA remained unchanged. The size of the transcripts in isolated neurons was similar to that reported by Suki *et al.* [27] in liver and ovary. The observations that the hybridization of the α_i or α_s probe with RNA exhibited only one band for each probe indicates the presence of one active gene for each protein. The developmental patterns of α_s and α_i mRNAs are also reflected in the photoaffinity labeling with [^{32}P]GTP-A and the ADP-ribosylation of G proteins. While the synthesis of α_i exhibited an age-dependent decrease, the synthesis of α_s protein did not. Unlike the observations of this study, previous studies have shown that (1) the α_i or α_s probes encode more than one mRNA in various tissues, indicating the presence of multiple active genes expressing different α proteins in brain and liver [27, 28] and (2) the ADP-ribosylation of α_i subunit obtained from brain either does not change [15, 19] or decreases with age [19]. Although the cause for the observed differences is not fully understood, the tissue samples and the method used for sample preparation in this study were different from the methods used in previous studies.

This study indicated that chronic prenatal lead exposure delayed the age-dependent decrease in mRNA expression, ADP-ribosylation and photoaffinity labeling of α_i subunit, and that chronic adult lead exposure did not cause these changes in neurons. It is therefore suggested that chronic lead exposure alters the normal developmental pattern of G_i in neurons in developing young. Previous studies have shown that (1) the coupling activity of receptors with G proteins develops parallel to the development of G_i protein [19] and (2) the neonatal receptors are functionally associated with G_i proteins, whereas the adult receptors are not. Therefore, chronic prenatal lead exposure may alter the functional capacity of the CNS receptors by altering the ontogenesis of G_i proteins and/or the G protein–receptor interaction. It is also possible that alterations in the [mRNA : α_s-probe hybridization/mRNA : α_i-probe hybridization] ratio may indicate chronic exposure to lead in developing animals.

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