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The endozepine ODN stimulates polyphosphoinositide metabolism in rat astrocytes

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Abstract Astrocytes synthesize a series of peptides called endozepines which act as endogenous ligands of benzodiazepine receptors. The present study demonstrates that the endozepine ODN causes a dose-dependent increase in inositol trisphosphate and a parallel decrease in phosphatidylinositol bisphosphate in cultured rat astrocytes. Pre-incubation of astrocytes with the phospholipase C inhibitor U 73122 or with pertussis toxin totally blocked polyphosphoinositide metabolism. These data show that, in rat astrocytes, ODN stimulates a phospholipase C coupled to a pertussis toxin-sensitive G protein.

Key words: Endozepine; Octadecaneuropeptide; Polyphosphoinositide metabolism; Glial cell

1. Introduction

The term endozepines designates a novel family of regulatory peptides that have been isolated as a result of their ability to displace benzodiazepine (BZD) binding from their receptors [1–3]. All endozepines derive from an 86 amino acid precursor polypeptide called diazepam-binding inhibitor (DBI) which generates, through proteolytic cleavage, several biologically active fragments, including the triakontatetraneuropeptide DBI[17–50] (TTN), the octadecaneuropeptide DBI[33–50] (ODN) and the triakontaseptaneuropeptide DBI[39–75] [4–6].

Endozepines are widely distributed in the central nervous system and in peripheral organs [7,8]. In the rat brain, endozepines are primarily synthesized in glial cells [9,10] and ODN has been shown to inhibit [^{3}H]diazepam binding in cultured rat astrocytes [11], suggesting that endozepines may act as paracrine and/or autocrine factors in astroglial cells.

There is now clear evidence that glial cells express a large variety of receptors for neuropeptides, which are functionally coupled to second messenger systems [12,13]. We have recently found that ODN stimulates calcium mobilization in rat astrocytes [14]. This observation led us to investigate the possible effect of endozepines on polyphosphoinositide metabolism.

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Abbreviations: DBI, diazepam-binding inhibitor; ODN, octadecaneuropeptide; PTX, pertussis toxin; TTN, triakontatetraneuropeptide.

2. Materials and methods

2.1. Chemicals

Dulbecco's modified Eagle's medium (DMEM) and the antibiotic–antimytotic solution were purchased from Gibco. F12 culture medium was from Biowhittaker. Fetal calf serum was obtained from Biosys. Synthetic ODN was from Bachem. Myo-[^{3}H]inositol was provided by Amersham. The phospholipase C inhibitor U 73122 was from Tebu. Pertussis toxin (PTX) and all other reagents were purchased from Sigma.

2.2. Cell culture

Primary cultures of astrocytes were prepared from the brain of Wistar rats as previously described [15]. Briefly, cerebral hemispheres from newborn rats were collected in DMEM/F12 (2:1; v/v) culture medium supplemented with 2 mM glutamine, 1% insulin, 5 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid), 0.4% glucose and 1% of the antibiotic–antimytotic solution. The tissues were disaggregated mechanically and filtered through a 82-μm nylon sieve (Tripette and Renaud, France). Dissociated cells were resuspended in culture medium supplemented with 10% fetal calf serum and seeded in 35-mm dishes (Dutscher) at a density of 10^6 cells/dish. The cells were incubated at 37°C in a humid atmosphere (5% CO_2) and the medium was changed twice a week.

2.3. Reverse-phase HPLC analysis

Cultured astrocytes were incubated with 10^{-8} M synthetic ODN for 30 min. The culture medium was collected and analyzed by high-performance liquid chromatography (HPLC) on a (250 × 4.6 mm) Lichrosorb C18 column (Alltech) equilibrated with acetonitrile/water/trifluoroacetic acid (20:79.9:0.1; v/v/v; pH 2.3) at a flow rate of 1 ml/min. The concentration of acetonitrile in the eluting solvent was held for 10 min at 20% and raised to 60% over 60 min using a linear gradient. One-ml fractions were collected and evaporated in a Speed-Vac concentrator (Savant). The concentration of ODN was measured in each fraction by radioimmunoassay as previously described [16].

2.4. Measurement of polyphosphoinositide metabolism

Astrocytes were incubated with 10 μCi/ml myo-[^{3}H]inositol (100 Ci/mmol) at 37°C in glucose-free culture medium. For incorporation studies, ODN (10^{-8} M) was added to the incubation medium for 0–30 min and ^{3}H-labeled inositol phosphates were analyzed as previously described [17]. For equilibrium studies, astrocytes were prelabeled with [^{3}H]inositol at 37°C for 24 h. Then the cells were washed five times with DMEM supplemented with 1 mmol inositol/l and preincubated with 10 mM LiCl for 10 min. Astrocytes were incubated either with a single dose of ODN (10^{-8} M) for various durations (30 s–2 min) or with various doses of ODN (10^{-10}–10^{-7} M) for 1 min. In one set of experiments, astrocytes were preincubated with U 73122 (10^{-5} M) alone for 10 min and then with U 73122 plus ODN (10^{-8} M) for 1 min. In another set of experiments, astrocytes were preincubated with PTX (0.2 μg/ml) for 4 h and then with PTX plus ODN (10^{-8} M) for 1 min. In all experiments the incubations were stopped by removing the medium and adding ice-cold 10% trichloroacetic acid for 30 min at 4°C. The cells were then homogenized and the homogenate was centrifuged. Inositol phosphates contained in the supernatant were analyzed by ion-exchange chromatography, and polyphosphoinositides contained in the

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pellet were separated by thin layer chromatography as previously described [17].

3. Results

3.1. Effect of ODN on [^{3}H]inositol incorporation into phospholipids

Exposure of astrocytes to ODN (10^{-8} M) increased the rate of incorporation of [^{3}H]inositol into the phospholipid fraction (Fig. 1). The effect of ODN was maximum after 15 min and vanished within 30 min. HPLC analysis of ODN after a 30-min incubation with astrocytes revealed that the peptide was not degraded (Fig. 2) and that 90% of authentic ODN was still present in the culture medium at the end of the incubation period.

3.2. Effect of ODN on polyphosphoinositide metabolism

The time–course of the effect of ODN on the formation of inositol phosphates and the concentration of phosphoinositides was studied in [^{3}H]inositol-labeled astrocytes. Administration of ODN (10^{-8} M) caused a rapid and transient increase in IP_2 and IP_3 formation (Fig. 3A,B) and a concomitant decrease in PIP and PIP_2 concentrations (Fig. 3C,D). The response to ODN reached a maximum after 1 min and the concentrations of inositolphosphates and phosphoinositides returned to basal levels within 2 min.

The effects of graded doses of ODN on polyphosphoinositide metabolism are shown in Fig. 4. For concentrations ranging from 10^{-9} to 10^{-8} M, ODN induced a dose-related increase in IP_2 and IP_3 formation (Fig. 4A,B) and a symmetrical decrease of PIP and PIP_2 concentrations (Fig. 4C,D). At higher concentrations (3×10^{-8} and 10^{-7} M), the effect of ODN on IP_2 and IP_3 formation gradually disappeared (Fig. 4A,B).

3.3. Involvement of phospholipase C in the mechanism of action of ODN

Incubation of astrocytes for 10 min with the phospholipase

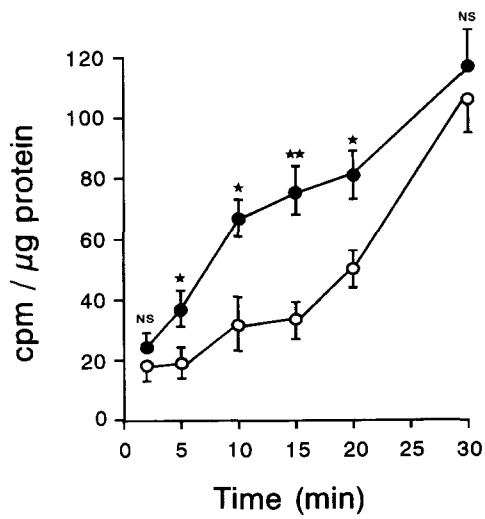

Fig. 1. Time–course of the effect of ODN (10^{-8} M) on [^{3}H]inositol incorporation into phosphoinositols in astrocytes. Cells were incubated with [^{3}H]inositol in the absence (○) or presence (●) of ODN for the times indicated. Each point represents the mean ± S.E.M. of four independent determinations. $*P < 0.05$, $**P < 0.01$, compared with the respective control.

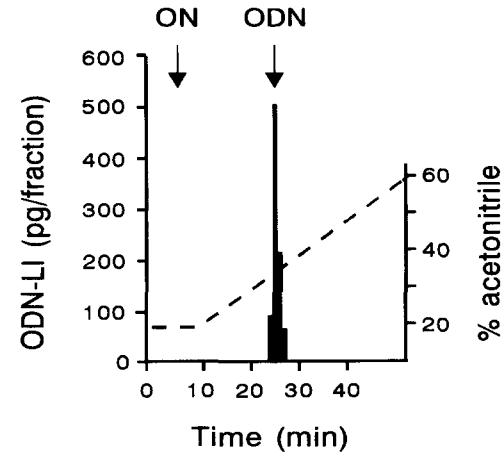

Fig. 2. Reverse-phase HPLC analysis of synthetic ODN after a 30-min incubation with cultured astrocytes at 37°C. All fractions collected (1 ml each) were dried and assayed for ODN-like immunoreactivity (ODN-LI). The arrows indicate the elution position of synthetic ODN and its C-terminal octapeptide (ON). The broken line shows the concentration of acetonitrile in the eluting solvent.

C inhibitor U 73122 (10^{-5} M) did not affect the basal level of IP_3 and PIP_2, but totally blocked the effect of ODN (10^{-8} M) on IP_3 formation (Fig. 5A) and PIP_2 breakdown (Fig. 5B). Similarly, incubation of the cells with PTX (0.2 µg/ml) for 4 h had no effect on the basal concentration of IP_3 and PIP_2, but totally suppressed the action of ODN on IP_3 formation (Fig. 5A) and PIP_2 breakdown (Fig. 5B).

4. Discussion

Although endozepines are extremely abundant and widely distributed in the central nervous system [7,8], the effect of these neuropeptides on second messenger systems have never been investigated in glial cells. The present study demonstrates that nanomolar concentrations of ODN stimulate polyphosphoinositide metabolism in cultured rat astrocytes. First, ODN increased the rate of incorporation of [^{3}H]inositol into the membrane lipid fraction. Second, ODN stimulated inositol phosphate formation and phosphatidylinositol metabolism in glial cells. The effect of ODN on [^{3}H]inositol incorporation only occurred during the first 20 min and disappeared after longer periods of incubation. HPLC analysis showed that the ODN molecule was still intact after a 30-min incubation with astrocytes, indicating that the disappearance of the effect of ODN could not be ascribed to degradation of the neuropeptide by astroglial cells. These data suggest that the action of ODN on astrocytes is associated with a rapid desensitization process. Consistent with this hypothesis, we have recently observed that repeated pulses of ODN induce an increase in cytosolic calcium concentrations in rat astrocytes, with gradual attenuation of the response (Lamacz et al., unpublished data).

It is now established that glial cells possess a large array of receptors for classical neurotransmitters and neuropeptides (see [18] for review), most of which are coupled to phospholipase C. The effect of ODN on inositol phosphate formation and phosphatidylinositol breakdown suggests that the action of endozepines on astroglial cells is mediated by a membrane receptor coupled to a phospholipase C. In support of this notion, the

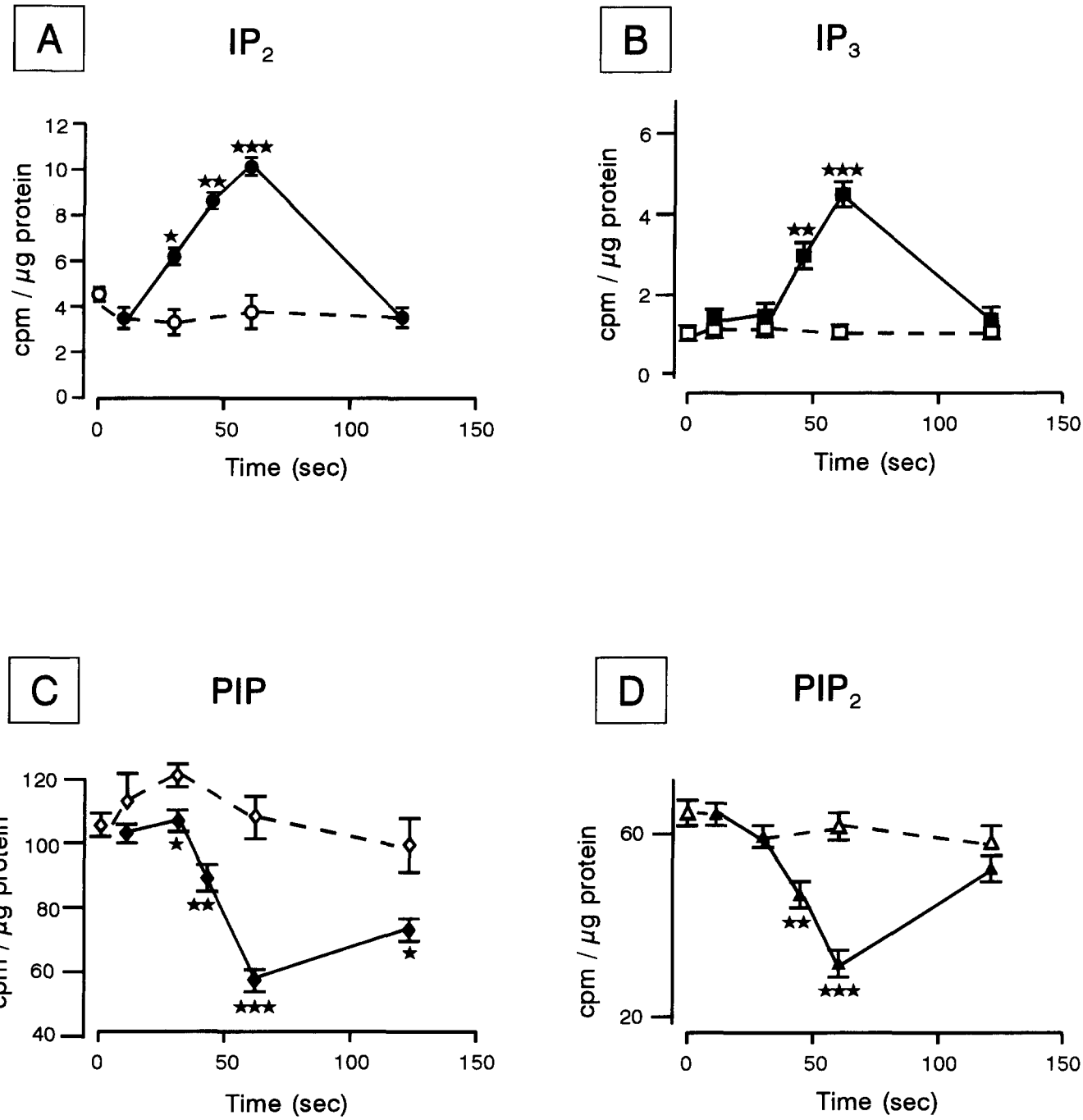

Fig. 3. Time-course of the effect of ODN (10^{-8} M) on polyphosphoinositide metabolism in cultured astrocytes. (A) Inositol bisphosphate (IP_2); (B) inositol trisphosphate (IP_3); (C) phosphatidylinositol monophosphate (PIP); (D) phosphatidylinositol bisphosphate (PIP_2). Control values are represented by open symbols. Each point represents the mean (±S.E.M.) of four independent determinations. ***$P < 0.001$, **$P < 0.01$ and *$P < 0.05$, compared with the respective control.

present data show that the ODN-evoked stimulation of polyphosphoinositide metabolism was totally blocked by U 73122, a drug which selectively inhibits phospholipase C-dependent processes [19]. We also observed that the effect of ODN on phosphatidylinositol turnover was abolished when astrocytes were incubated with pertussis toxin, indicating that a PTX-sensitive G protein is involved in the mechanism of action of endozepines on glial cells.

The administration of graded concentrations of ODN on astrocytes revealed that the dose–response curves exhibited a bell shape with a maximum effect at a concentration of 10^{-8} M. In agreement with this finding, we have recently observed that nanomolar doses of ODN increase cytosolic calcium while micromolar doses do not stimulate calcium mobilization and may even reduce intracellular calcium levels (Lamacz et al., unpublished data). These data suggest that ODN acts through two

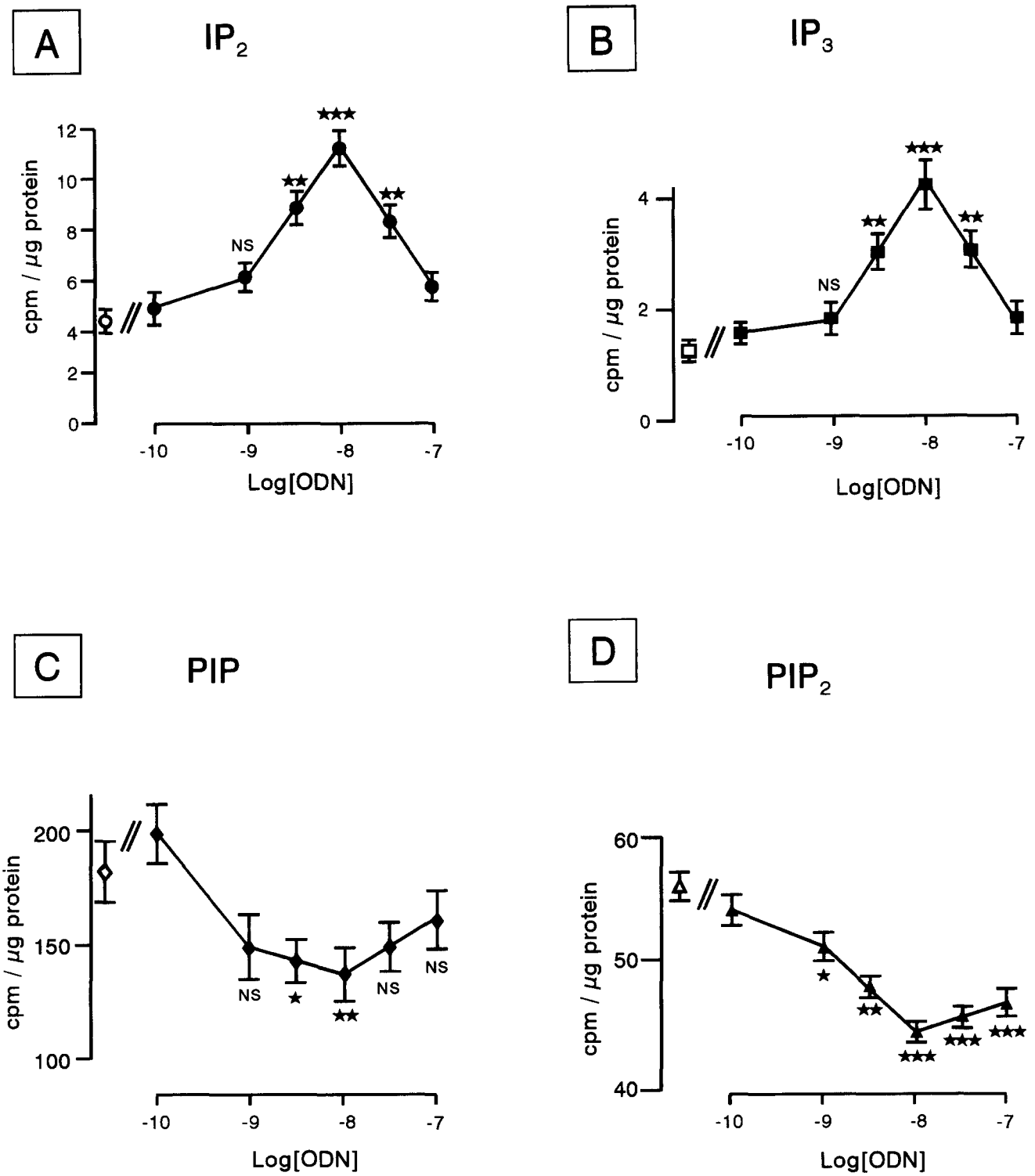

Fig. 4. Effect of increasing concentrations of ODN (10^{-10} to 10^{-7} M) on polyphosphoinositide metabolism in cultured astrocytes. Each point represents the mean (±S.E.M.) of four independent determinations. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, compared with the respective control.

types of receptors on rat astrocytes: high affinity receptors positively coupled to phospholipase C and low affinity receptors negatively coupled to phospholipase C.

In conclusion, the present study has demonstrated that nanomolar concentrations of ODN stimulate polyphosphoinositide metabolism through a PTX-sensitive G protein. Since rat astrocytes synthesize and release substantial amounts of ODN-related peptides [8–10], these data support the view that endozepines may be involved in paracrine and/or autocrine communication in astroglial cells.

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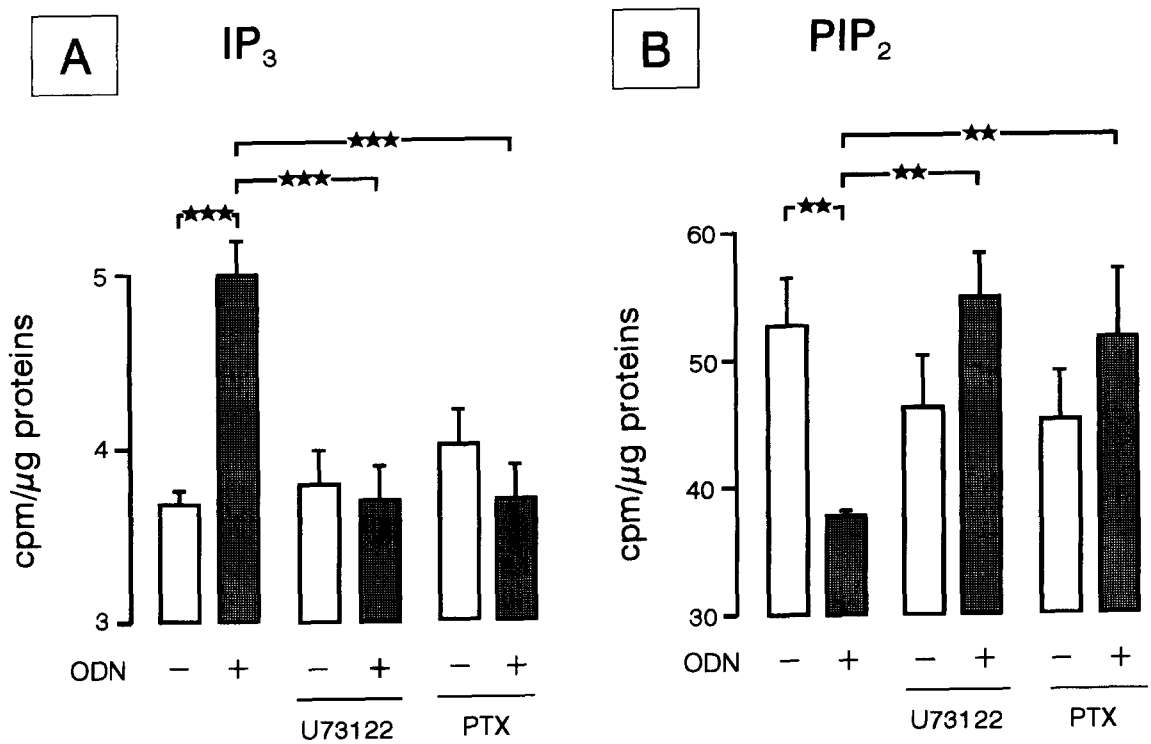

Fig. 5. Effect of U 73122 and pertussis toxin (PTX) on inositol trisphosphate (A) and phosphatidylinositol bisphosphate (B) levels in cultured astrocytes. The cells were preincubated in the absence or presence of U 73122 (10^{-5} M) for 10 min or PTX (0.2 μg/ml) for 4 h and then incubated for 1 min with culture medium alone (open bars) or 10^{-8} M ODN (shaded bars). Values are means (± S.E.M.) of four independent determinations. $^{**}P < 0.01$, $^{***}P < 0.001$.

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