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The stimulatory effect of the octadecaneuropeptide (ODN) on cytosolic Ca^{2+} in rat astrocytes is not mediated through classical benzodiazepine receptors

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Abstract

Diazepam-binding inhibitor has been initially isolated from the rat brain from its ability to compete with benzodiazepines for their receptors. We have recently shown that the octadecaneuropeptide (diazepam-binding inhibitor-(33–50) or ODN) induces an increase in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) in astroglial cells. The purpose of the present study was to determine whether central-type benzodiazepine receptors or peripheral-type benzodiazepine receptors are involved in the response of cultured rat astrocytes to ODN. The mixed central-/peripheral-type benzodiazepine receptor ligand flunitrazepam (10^{-10} to 10^{-6} M), the specific peripheral-type benzodiazepine receptor agonist Ro5-4864 (10^{-10} to 10^{-6} M) and the peripheral-type benzodiazepine receptor 'antagonist' PK 11195 (10^{-9} to 10^{-6} M) all induced a dose-dependent increase in $[Ca^{2+}]_i$. At high doses (10^{-7} to 10^{-5} M), the central-type benzodiazepine receptor agonist clonazepam also mimicked the stimulatory effect of ODN on $[Ca^{2+}]_i$. However, the $[Ca^{2+}]_i$ rise induced by ODN was blocked neither by PK 11195 nor by the central-type benzodiazepine receptor antagonist flumazenil (10^{-6} M each). Binding of [^{3}H]flunitrazepam to intact astrocytes was displaced by low concentrations of the peripheral-type benzodiazepine receptor ligands flunitrazepam, Ro5-4864 and PK 11195, and by high concentrations of clonazepam. In contrast, ODN did not compete for [^{3}H]flunitrazepam binding in intact cells. These data indicate that the effect of ODN on Ca^{2+} mobilization in rat astrocytes is mediated by high affinity receptors which are not related to classical benzodiazepine receptors.

Keywords: Endozepine; Octadecaneuropeptide (ODN); Diazepam-binding inhibitor; Benzodiazepine receptor; Glial cell; Ca^{2+} mobilization

1. Introduction

The term endozepines refers to a novel family of regulatory peptides that have been initially isolated from the rat brain on the basis of their ability to displace diazepam from its binding sites (Guidotti et al., 1983). All endozepines derive from an 86 amino acid polypeptide called diazepam-binding inhibitor which has been characterized in several vertebrate species, i.e., human, bovine (Marquardt et al., 1986), pig (Chen et al., 1988), rat (Knudsen et al., 1989), duck (Rose et al., 1994) and frog (Lihrmann et al., 1994). Proteolytic cleavage of diazepam-binding inhibitor generates several biologically active fragments including the triakontatetraneuropeptide (diazepam-binding inhibitor-(17–50)) (Slobodyansky et al., 1989) and the octadecaneuropeptide (diazepam-binding inhibitor-(33–50) or ODN) (Ferrero et al., 1986).

Immunohistochemistry and in situ hybridization techniques have revealed that endozepines are widely distributed in the central nervous system and in peripheral organs (Alho et al., 1988; Bovolin et al., 1990; Rhéaume et al., 1990; Tonon et al., 1990; Johansson et al., 1991; Steyaert et al., 1991; Tong et al., 1991) suggesting that endozepines could be involved in the regulation of multiple biological processes. In support of this hypothesis, it has been shown that endozepines stimulate steroidogenesis in adrenocortical and Leydig cells (Papadopoulos et al., 1991; Garnier et al., 1993), inhibit glucose-induced insulin secretion from pancreatic islets (Ostenson et al., 1994) and reverse the inhibitory effect of γ-aminobutyric acid

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(GABA) on pituitary melanotrope cells (Tonon et al., 1989).

Benzodiazepines interact with two families of receptors which differ in their molecular structure. The central-type benzodiazepine receptor is associated with the $GABA_A$ receptor/chloride channel complex (Knoflach et al., 1992), and activation of central-type benzodiazepine receptors allosterically modulates the effect of GABA (Sigel and Baur, 1988; Puia et al., 1989). Although central-type benzodiazepine receptors are predominantly expressed in neurons, several studies have shown the occurrence of $GABA_A$/central-type benzodiazepine receptors in glial cells (Bormann and Kettenmann, 1988; Ventimiglia et al., 1990; Bureau et al., 1995). The so-called peripheral-type benzodiazepine receptor is composed of three different subunits, i.e., an isoquinoline carboxamide-binding protein, a voltage-dependent anion channel and an adenine nucleotide carrier (McEnery et al., 1992). Peripheral-type benzodiazepine receptors are primarily located in the outer mitochondrial membrane (Anholt et al., 1986) but are also present in the plasma membrane (Oke et al., 1992; Garnier et al., 1993; Alho et al., 1994). Despite their name, peripheral-type benzodiazepine receptors are found both in peripheral organs (De Souza et al., 1985; Garnier et al., 1993; Lesouhaitier et al., 1996) and in the central nervous system (Benavides et al., 1983; Gavish et al., 1992; Lin et al., 1993). In particular, high concentrations of peripheral-type benzodiazepine receptors are present in glial cells (Itzhak et al., 1993; Alho et al., 1994; Moynagh et al., 1994).

Intracerebroventricular administration of diazepam-binding inhibitor-related peptides causes proconflict behavior (Ferrero et al., 1986; Slobodyansky et al., 1989) but the mode of action of endozepines remains unknown. Endozepines are synthesized in glial cells (Tonon et al., 1990; Malagon et al., 1993; Vidnyanszky et al., 1994) and released by astrocytes in primary culture (Lamacz et al., 1996). In addition, diazepam-binding inhibitor and the triakontatetraneuropeptide stimulate steroid biosynthesis in glioma cells (Guarneri et al., 1992; Papadopoulos et al., 1992) and ODN produces an increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in rat astrocytes (Lamacz et al., 1996). These data suggest that endozepines may act as intracrine and/or autocrine regulators of glial cell activity.

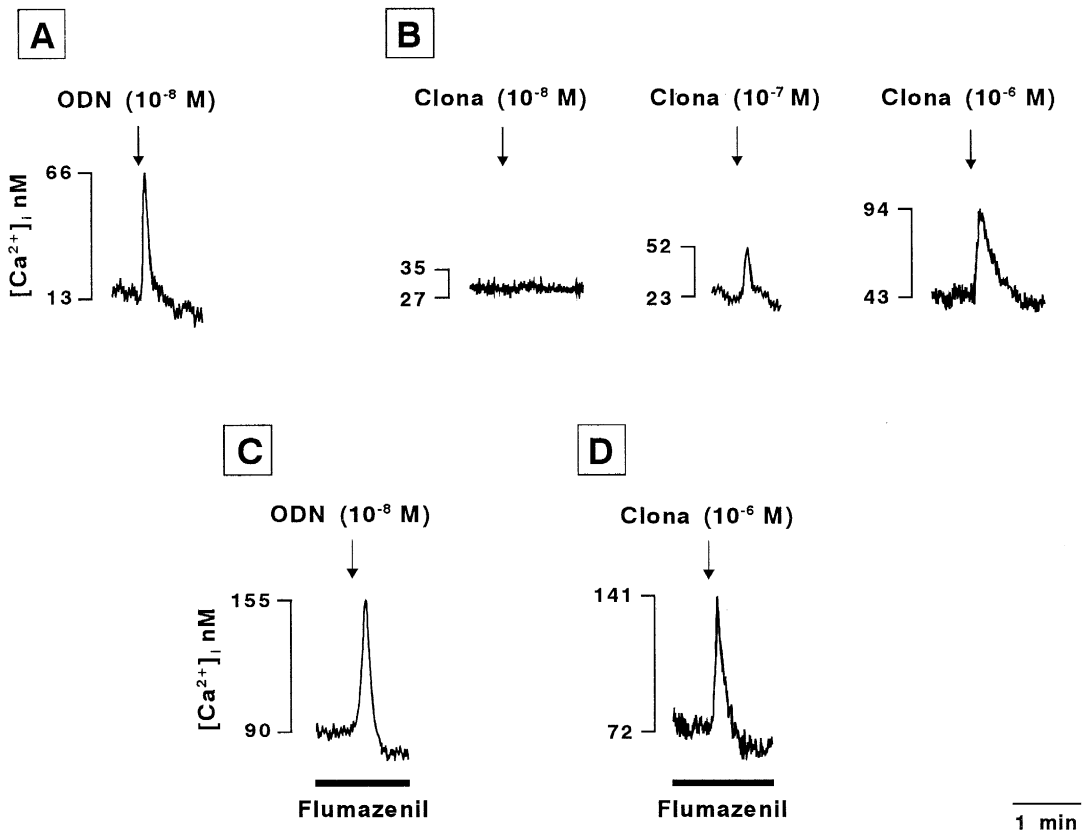

Fig. 1. Effects of ODN and central-type benzodiazepine receptor ligands on $[Ca^{2+}]_i$ in cultured rat astrocytes. (A,B) A 2-s pulse (arrow) of ODN (10^{-8} M) or clonazepam (Clona; 10^{-8} to 10^{-6} M) was administered in the vicinity of cells incubated in normal culture medium. (C,D) After a 10-min incubation with flumazenil (10^{-6} M), a 2-s pulse (arrow) of ODN (10^{-8} M) or clonazepam (10^{-6} M) was applied to other cells in the same dishes. The number of cells studied was (A) 60, (B) 41, (C) 28 and (D) 12.

The aim of the present study was to determine the pharmacological profile of the receptors mediating the ODN-induced $[Ca^{2+}]_i$ rise in cultured rat astrocytes.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM) and the antibiotic-antimycotic solution were purchased from Gibco-BRL (Eragny, France). Glutamine, Hepes and F12 culture medium were from Biowhittaker (Gagny, France). Fetal calf serum was from Biosys (Compiègne, France). Insulin, glucose, PK 11195 and bacitracin were from Sigma (St. Louis, MO, USA). Ro5-4864 was from Fluka (Mulhouse, France). Clonazepam, flumazenil and flunitrazepam were generous gifts from Hoffmann-La Roche (Basle, Switzerland). Indo-1 acetoxymethyl ester was from Molecular Probes (Eugene, OR, USA). Rat ODN was synthetized by the solid phase methodology on a 433A Applied Biosystems peptide synthesizer (St. Quentin en Yvelines, France) using the standard Fmoc procedure. [^{3}H]Flunitrazepam was from N.E.N. (Les Ulis, France).

2.2. Cell culture

Primary cultures of astrocytes were prepared from the brain of Wistar rats as previously described (Patte et al., 1995). 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, 0.4% glucose and 1% of the antibiotic-antimycotic solution. The tissues were disaggregated mechanically and filtered through a 82-μm nylon sieve (Tripette and Renaud, Combles, France). Dissociated cells were resuspended in culture medium supplemented with 10% fetal calf serum and seeded on coverslips in 35-mm dishes (Dutscher, Brumath, France) 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. After 4 days in culture, approximately 90% of the cells exhibited the morphological characteristics of type I astrocytes and were labeled with antibodies against glial fibrillary acidic protein (Lamacz et al., 1996).

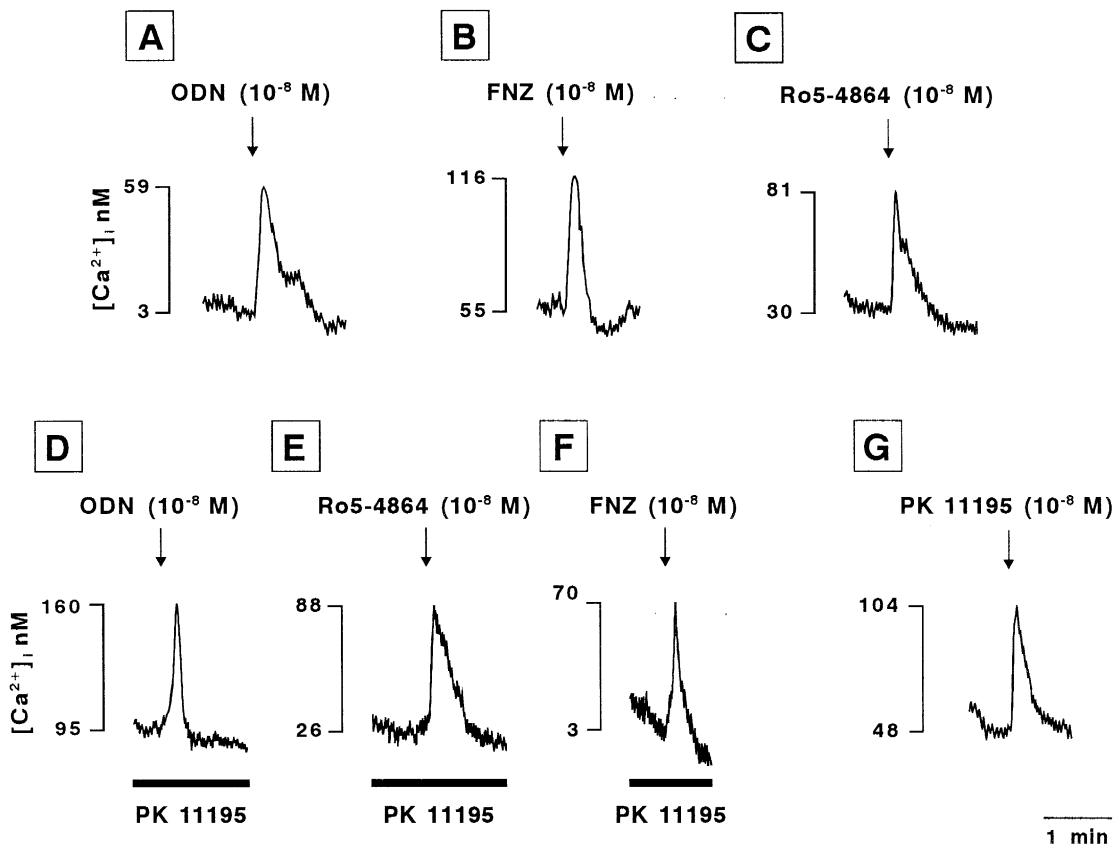

Fig. 2. Effects of ODN and peripheral-type benzodiazepine receptor ligands on $[Ca^{2+}]_i$ in cultured rat astrocytes. (A–C) A 2-s pulse (arrow) of ODN, flunitrazepam (FNZ) or Ro5-4864 (10^{-8} M, each) was administered in the vicinity of cells incubated in normal culture medium. (D–F) After a 10-min incubation with PK 11195 (10^{-6} M), a 2-s pulse (arrow) of ODN, FNZ or Ro5-4864 (10^{-8} M, each) was applied to other cells in the same dishes. (G) A 2-s pulse (arrow) of PK 11195 (10^{-8} M) was administered in the vicinity of cells incubated in normal culture medium. The number of cells studied was (A) 60, (B) 30, (C) 34, (D) 17, (E) 15, (F) 13 and (G) 10.

2.3. Measurement of cytosolic Ca^{2+} concentrations

The cells (5–7 days) were incubated at 37°C for 45 min in the dark with 5 μM indo-1 acetoxymethylester diluted in culture medium. The cells were then washed twice with 2 ml of fresh medium. The $[Ca^{2+}]_i$ was monitored by a dual-emission microfluorimeter system constructed from a Nikon Diaphot inverted microscope, as previously described (Gracia-Navarro et al., 1992). The fluorescence emission of indo-1, induced by excitation at 355 nm, was recorded at two wavelenghts (405 nm and 480 nm) by separate photometers (Nikon). The 405/480 signal ratio was determined using an analogic divider (constructed by Dr B. Dufy, Bordeaux, France) after conversion of single photon currents to voltage signals. All three signals, corresponding to absorbances at 405 nm and 480 nm, and the 405/480 ratio, were continuously recorded with a three-channel voltage recorder (BD 100/101; Kipp and Zonen, Delft, Netherlands). The actual values of $[Ca^{2+}]_i$ were calculated as previously described (Larcher et al., 1992). All secretagogues were ejected for 2 s in the vicinity of individual cells by a pressure ejection system. The doses of secretagogues indicated (10^{-11} to 10^{-5} M) correspond to the concentration contained in the ejection pipette.

2.4. Binding studies

Cells cultured for 9–12 days were washed twice with Tris buffer (pH 7.4) containing 0.1% bovine serum albumin, 5 mM $MgCl_2$ and 0.5 μg bacitracin/ml. The cells were incubated for 60 min at 4°C in the same buffer with 2 nM [^{3}H]flunitrazepam and graded concentrations of unlabeled flunitrazepam, Ro5-4864, PK 11195, clonazepam or ODN (10^{-10} to 10^{-5} M). Non-specific binding was determined by adding 10^{-5} M flunitrazepam. At the end of the incubation, the coverslips were removed from the dishes and washed six times with cold Tris buffer, and the radioactivity was counted.

3. Results

3.1. Microfluorimetric studies

The effects of synthetic rat ODN and benzodiazepine receptor ligands on $[Ca^{2+}]_i$ were studied by monitoring the fluorescence signal in cultured astrocytes. Ejection of 10^{-8} M ODN in the vicinity of the cells caused a rapid and transient increase in $[Ca^{2+}]_i$ (Fig. 1A). Administration of the specific central-type benzodiazepine receptor agonist clonazepam (10^{-8} M) did not modify $[Ca^{2+}]_i$ (Fig. 1B). At higher concentrations (10^{-7} and 10^{-6} M), clonazepam induced a weak stimulatory effect on $[Ca^{2+}]_i$. The central-type benzodiazepine receptor antagonist flumazenil (10^{-6} M) did not affect the increase in $[Ca^{2+}]_i$ induced by 10^{-8} M ODN (Fig. 1C). Similarly, flumazenil had no effect on clonazepam-evoked $[Ca^{2+}]_i$ rise (Fig. 1D).

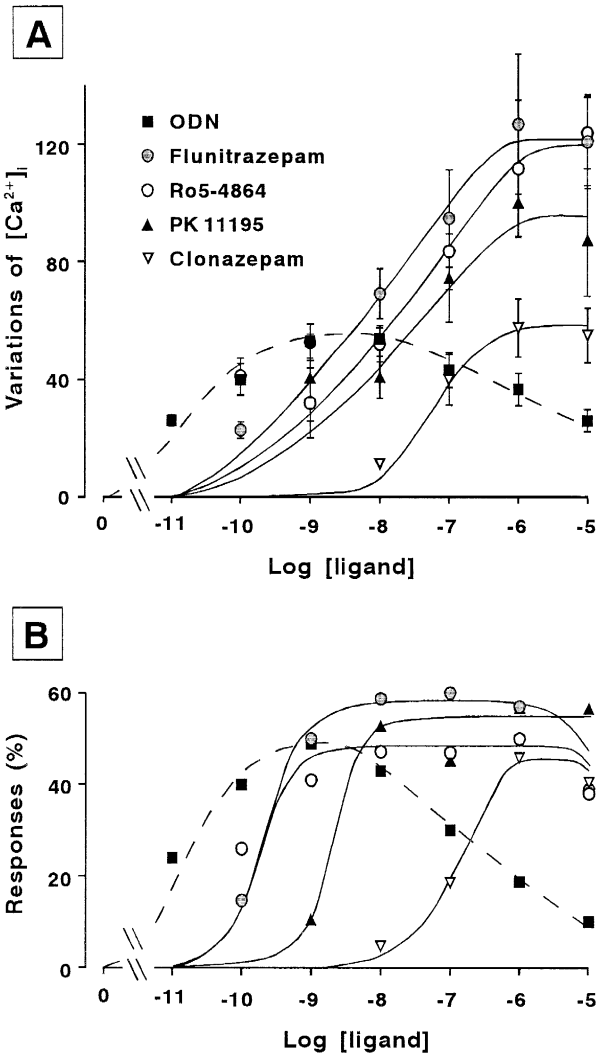

Fig. 3. Effects of graded concentrations of ODN and benzodiazepine receptor ligands on the amplitude of the $[Ca^{2+}]_i$ response (A) and the percentage of responding cultured rat astrocytes (B). Each point represents the mean response ± S.E.M. calculated from at least 10 cells.

The mixed central-/peripheral-type benzodiazepine receptor ligand flunitrazepam (10^{-8} M) and the specific peripheral-type benzodiazepine receptor agonist Ro5-4864 (10^{-8} M) both mimicked the effect of ODN on $[Ca^{2+}]_i$ (Fig. 2A–C). Preincubation of the cells with the supposed peripheral-type benzodiazepine receptor antagonist PK 11195 (10^{-6} M) did not inhibit the ODN-evoked $[Ca^{2+}]_i$ increase (Fig. 2D). However, PK 11195 also failed to block the $[Ca^{2+}]_i$ rise induced by flunitrazepam (Fig. 2E) or Ro5-4864 (Fig. 2F). In addition, a short pulse of PK 11195 (10^{-8} M) in the vicinity of the cells induced a robust increase in $[Ca^{2+}]_i$ (Fig. 2G).

The effects of graded doses of ODN and various benzodiazepine receptor ligands on $[Ca^{2+}]_i$ are compared in Fig. 3. At low concentrations (10^{-11} to 10^{-9} M), ODN in-

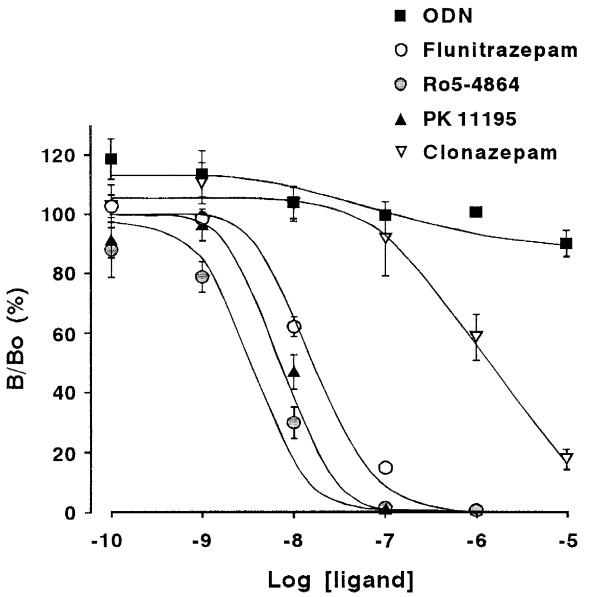

Fig. 4. Dose-displacement curves for inhibition of [^{3}H]flunitrazepam binding to cultured rat astrocytes by various benzodiazepine receptor ligands and ODN. Cultured astrocytes were incubated for 60 min at 4°C with 2 nM [^{3}H]flunitrazepam. The data are plotted as the percentage of specific [^{3}H]flunitrazepam bound vs. the concentration of competing drug. Each curve represents the mean ± S.E.M. of four independent experiments.

duced a dose-related increase in the amplitude of the Ca^{2+} wave (Fig. 3A) and in the proportion of responding cells (Fig. 3B). At higher concentrations (10^{-8} to 10^{-5} M), the effects of ODN on the amplitude of the response and percentage of responding cells gradually declined. In contrast, the dose-response curves obtained with benzodiazepine receptor ligands were strictly monophasic. The range of potency of these compounds on $[Ca^{2+}]_i$ increase was ODN ≫ flunitrazepam ≥ Ro5-4864 > PK 11195 ≫ clonazepam. In contrast, the efficacy of the peripheral-type benzodiazepine receptor ligands in raising $[Ca^{2+}]_i$ was 2-fold higher than that of ODN (Fig. 3A).

3.2. Binding studies

Benzodiazepine receptor ligands and ODN were examined for their ability to compete with [^{3}H]flunitrazepam binding sites in intact cultured astrocytes. Flunitrazepam, Ro5-4864, and PK 11195 were very potent in displacing the radioligand binding (Fig. 4). Clonazepam, at high concentrations, also induced a weak competition. In contrast, ODN, even at concentrations up to 10^{-5} M, failed to inhibit [^{3}H]flunitrazepam binding.

4. Discussion

Astroglial cells synthesize and release neuropeptides related to the endozepine ODN (Tonon et al., 1990; Vidnyanszky et al., 1994; Alho et al., 1994). We have recently observed that administration of nanomolar concentrations of ODN causes a robust increase of $[Ca^{2+}]_i$ in cultured astrocytes (Lamacz et al., 1996) suggesting that ODN interacts with a high affinity plasma membrane receptor. In order to determine whether the action of ODN was mediated through central- or peripheral-type benzodiazepine receptors, we have compared the effect of ODN and various benzodiazepine receptor agonists on the Ca^{2+} response in rat astrocytes.

We initially found that the ODN-evoked increase in $[Ca^{2+}]_i$ was mimicked by nanomolar concentrations of the non-selective benzodiazepine receptor agonist flunitrazepam and of the specific peripheral-type benzodiazepine receptor agonist Ro5-4864. Conversely, the central-type benzodiazepine receptor agonist clonazepam stimulated Ca^{2+} mobilization only at concentrations high enough to activate both central- and peripheral-type benzodiazepine receptors. In addition, the central-type benzodiazepine receptor antagonist flumazenil did not block the $[Ca^{2+}]_i$ increase induced by ODN and clonazepam. These data suggested that the ODN-evoked $[Ca^{2+}]_i$ rise could be mediated through activation of peripheral-type benzodiazepine receptors. Consistent with this hypothesis, it has been previously reported that the potentiating effect of ODN on lipopolysaccharide-induced production of cytokines in human monocytes can be accounted for by activation of peripheral-type benzodiazepine receptors (Taupin et al., 1991). The recent finding that peripheral-type benzodiazepine receptors can be located at the plasma membrane level (Oke et al., 1992; Garnier et al., 1993) provided further support for the hypothesis that the stimulatory effect of ODN on $[Ca^{2+}]_i$ in astrocytes could be mediated through peripheral-type benzodiazepine receptors.

Paradoxically, the isoquinoline carboxamide derivative PK 11195, which is generally considered as a peripheral-type benzodiazepine receptor antagonist (Zavala and Lenfant, 1987; Garnier et al., 1993; Costa et al., 1994), did not block the Ca^{2+} mobilization induced by ODN, Ro5-4864 and flunitrazepam. In fact, PK 11195 mimicked the stimulatory effect of ODN and peripheral-type benzodiazepine receptor agonists on $[Ca^{2+}]_i$ in cultured astrocytes. This observation was the first clue suggesting that the effect of ODN might not be ascribed to activation of authentic peripheral-type benzodiazepine receptors. Therefore, dose-response curves were established in order to compare the effects of ODN and benzodiazepine receptor ligands on $[Ca^{2+}]_i$ in astrocytes. For concentrations ranging from 10^{-11} to 10^{-9} M, ODN induced a dose-dependent increase in $[Ca^{2+}]_i$ while, at higher concentrations of ODN (10^{-8} to 10^{-5} M), the Ca^{2+} response gradually declined. The proportion of astroglial cells which responded to graded doses of ODN also exhibited a bell-shaped pattern. In contrast, all the benzodiazepines tested caused a monophasic, dose-dependent increase in $[Ca^{2+}]_i$

suggesting that ODN did not act through the same mechanism as peripheral-type benzodiazepine receptor ligands.

Binding experiments were next performed on intact astrocytes in order to examine whether ODN could interact with benzodiazepine receptors on the plasma membrane. The presence of peripheral-type benzodiazepine receptors in homogenate preparations from cultured astroglial cells has recently been reported using the selective peripheral-type benzodiazepine receptor radioligands [^{3}H]Ro5-4864 and [^{3}H]PK 11195 (Itzhak et al., 1993). The present data, obtained with the non-selective ligand [^{3}H]flunitrazepam, confirmed the existence of high affinity peripheral-type benzodiazepine receptors on rat astrocytes and revealed that ODN does not displace benzodiazepines from their binding sites. These data demonstrate that the effect of ODN on Ca^{2+} mobilization cannot be accounted for by interaction with peripheral-type benzodiazepine receptors.

The type of receptor responsible for the effect of ODN on astroglial cells is currently unknown. Recent studies have shown that ODN activates polyphosphoinositide metabolism in rat astrocytes through a pertussis toxin-sensitive G protein (Patte et al., 1995). In addition, the effect of ODN on Ca^{2+} mobilization is blocked by thapsigargin, a Ca^{2+}-ATPase inhibitor which causes depletion of intracellular Ca^{2+} stores (Lamacz et al., 1996). These observations, together with the present findings strongly suggest that, in rat astroglial cells, the neuropeptide ODN does not act on benzodiazepine binding sites but activates a novel type of high affinity receptors positively coupled to phospholipase C.

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