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N,*N*,*N*′,*N*′-Tetrakis(2-pyridylmethyl)ethylenediamine inhibits ligand binding to certain G protein-coupled receptors

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Abstract

N,*N*,*N*′,*N*′-Tetrakis(2-pyridylmethyl)ethylenediamine (TPEN) is used widely in biological systems to chelate certain heavy metals, particularly Zn^{2+}. Here we show that TPEN inhibits ligand binding to certain G protein-coupled receptors and is an antagonist at muscarinic receptors. In intact human neuroblastoma SH-SY5Y cells, the binding of the muscarinic receptor ligand [*N*-methyl-^{3}H]scopolamine methyl chloride was inhibited by TPEN ($K_i \sim 26$ μM), as was muscarinic receptor agonist-induced inositol 1,4,5-trisphosphate formation ($K_i \sim 26$ μM). This antagonism was not due to metal ion chelation, indicating that it resulted from a direct interaction of TPEN with muscarinic receptors. Examination of the effects of TPEN on other receptors in SH-SY5Y cell membrane preparations showed that the binding of the nonpeptide opioid receptor ligand [15,16-^{3}H]diprenorphine was strongly inhibited, whereas binding of [^{125}I]vasoactive intestinal polypeptide was not. This pattern of selectivity was also seen in AR4-2J rat pancreatoma cell membranes, in which TPEN inhibited ligand binding to muscarinic receptors, but not that to cholecystokinin receptors. In conclusion, these data show that TPEN inhibits ligand binding to certain G protein-coupled receptors and exhibits selectivity towards those receptors whose transmembrane helices form the predominant site for ligand interaction. TPEN may have widespread antagonistic activity towards G protein-coupled receptors of this kind.

Keywords: TPEN; Ligand binding; G protein-coupled receptor

1. Introduction

N,*N*,*N*′,*N*′-Tetrakis(2-pyridylmethyl)ethylenediamine (TPEN) is a membrane-permeable metal ion chelator with structural similarity to EDTA (Arslan et al., 1985). TPEN has particularly high affinity for several heavy metals [e.g., stability constant (log K_i) values for Cu^{2+}, Zn^{2+}, and Fe^{2+} are 20.4, 15.6, and 14.6, respectively] (Smith and Martell, 1975; Arslan et al., 1985; Cherny et al., 1999). This property has led to the use of TPEN in a variety of settings, often as a tool to probe the functions of Zn^{2+}. For example, depletion of intracellular Zn^{2+} with TPEN causes HeLa and airway epithelial cell apoptosis in vitro (Chimienti et al., 2001; Carter et al., 2002), and TPEN inhibits the neurotoxic effects of Zn^{2+} in vivo (Armstrong et al., 2001). Intriguingly, due to its chelating powers, TPEN has also been shown to solubilize β-amyloid deposits in vitro (Cherny et al., 1999), and clioquinol, which also selectively chelates Cu^{2+} and Zn^{2+}, has been shown to reduce β-amyloid deposition in vivo (Cherny et al, 2001), leading to suggestions that these compounds might be useful in the prevention and treatment of Alzheimer's disease. Most recently, TPEN has been used in cell-free systems (Lorick et al., 1999; Bays et al., 2001) to inhibit Zn^{2+}-dependent ubiquitin–protein ligases.

Our preliminary studies with TPEN on ubiquitin–protein ligases (Wojcikiewicz et al., 2003) alerted us to the possibility that it might also interfere with signaling via G protein-coupled receptors. In the present study, we have investigated this possibility and have shown that TPEN inhibits ligand binding to and acts as an antagonist at certain G protein-coupled receptors.

2. Experimental

2.1. Materials

SH-SY5Y and AR4-2J cells were cultured as described (Wojcikiewicz, 1995). TPEN and receptor agonists and

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antagonists were obtained from Sigma (St. Louis, MO). TPEN stock solutions were made up of dimethyl sulphoxide (DMSO) at 30–50 mM and were stored at −20 °C. [*N*-Methyl-^{3}H]scopolamine methyl chloride ([^{3}H]NMS; 83.5 Ci/mmol), [15,16-^{3}H]diprenorphine ([^{3}H]DPN; 50 Ci/mmol), [^{125}I]vasoactive intestinal polypeptide ([^{125}I]VIP; 2200 Ci/mmol), and [^{125}I]cholecystokinin-8 ([^{125}I]CCK; 2200 Ci/mmol) were obtained from Perkin-Elmer Life Sciences.

2.2. Measurement of radioligand binding

Cells were detached from plates with 155 mM NaCl, 10 mM HEPES, 1 mM EDTA, pH 7.4, and were centrifuged at 500 × *g* for 2 min at 25 °C. For experiments with intact cells, pellets were resuspended in Krebs–HEPES buffer (118 mM NaCl, 4.7 mM KCl, 25 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.3 mM $CaCl_2$, 10 mM glucose, 10 mM HEPES, pH 7.4) and cell suspensions (30 μg of proteins in 1 ml) were incubated with 0.2 nM [^{3}H]NMS and other agents for 20 min at 25 °C. To prepare cell membranes, pellets were resuspended in ice-cold 10 mM HEPES and 10 mM EDTA (pH 7.4), disrupted with a Polytron homogenizer (3 × 10 s), centrifuged (27,000 × *g* for 15 min at 4 °C), and resuspended in ice-cold 10 mM HEPES and 1 mM $MgCl_2$, pH 7.4. These preparations (20–400 μg of membrane proteins in 0.5–1 ml) were then incubated at 25 °C with either 0.2 nM [^{3}H]NMS or 0.1 nM [^{3}H]DPN for 20 min, or with 0.36 nM [^{125}I]CCK or 45 pM [^{125}I]VIP for 60 min. Vehicle for TPEN (DMSO) was present at appropriate concentrations in all incubations and nonspecific binding was defined with 10 μM atropine, 10 μM naloxone, 1 μM CCK, or 1 μM VIP. Bound and free ligands were separated by filtration through Whatman GF/B filters and two washes with 4 ml of incubation buffer at 4 °C, and radioactivity associated with the filters was assessed in 4 ml of scintillation fluid after 24 h of extraction. To minimize nonspecific binding in incubations with [^{125}I]VIP, four washes were performed, 1% bovine serum albumin was included in incubations and washes, and filters were presoaked in 0.01% polyethylenimine (Sigma).

Specific binding was fitted to sigmoid curves using Prism 2 software (GraphPad Software) to obtain IC_{50} (half-maximal inhibition) values and Hill slopes for TPEN inhibition. Since [^{3}H]NMS and [^{3}H]DPN were used at concentrations equal to their dissociation constants (K_d) in SH-SY5Y cells (Lambert et al., 1989; Campbell et al., 1995), inhibition constant (K_i) values for TPEN were calculated from IC_{50} values using the relationship, $K_i = 0.5 \times IC_{50}$ (Cheng and Prusoff, 1973).

2.3. Measurement of inositol 1,4,5-trisphosphate ($InsP_3$) concentration

Inositol 1,4,5-trisphosphate concentration in SH-SY5Y cell suspensions was measured with a radioreceptor assay exactly as described (Oberdorf et al., 1999). Present in all incubations was 0.2% DMSO. As carbachol was used at a concentration equal to that causing half-maximal response (EC_{50}), K_i values for TPEN were calculated from IC_{50} values using the relationship, $K_i = 0.5 \times IC_{50}$ (Cheng and Prusoff, 1973).

2.4. Miscellaneous

Data shown or quoted are mean ± S.E.M. (where $n \geq 3$) or range (where $n = 2$) of results from n independent experiments, or are mean ± S.E.M. of triplicate determinations from individual experiments representative of at least two with similar results. Statistical analysis was by t test and significance was ascribed when $P < 0.05$.

3. Results

The initial experiments that led us to investigate whether TPEN might bind to and inhibit G protein-coupled receptors were performed on intact SH-SY5Y cells. These cells express muscarinic receptors, predominately the m3 subtype (Lambert et al., 1989), and the muscarinic receptor agonist, carbachol, stimulates phospholipase C activity and leads to $InsP_3$ formation with $EC_{50} \sim 10$ μM under the experimental conditions employed in this study (Lambert and Nahorski, 1990; Wojcikiewicz et al., 1994; data not shown). Fig. 1A

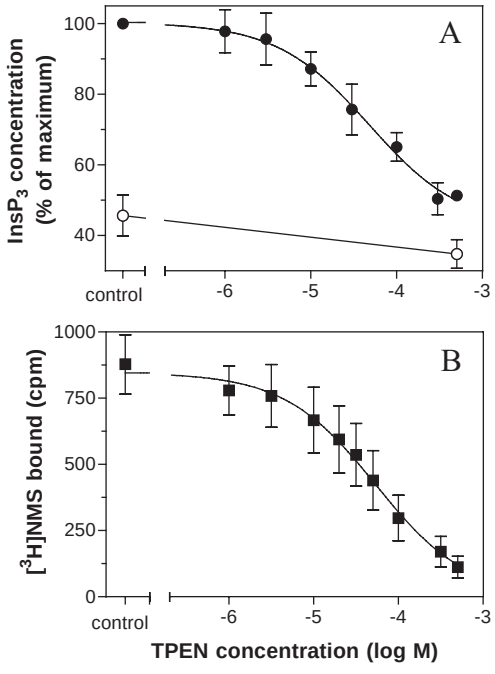

Fig. 1. TPEN inhibits $InsP_3$ formation and [^{3}H]NMS binding in intact SH-SY5Y cells. (A) Cells were incubated without carbachol (○) or with 10 μM carbachol (●) in the presence of the indicated concentrations of TPEN, and $InsP_3$ concentration was measured ($n = 3$). (B) Cells were incubated with 0.2 nM [^{3}H]NMS in the presence of the indicated concentrations of TPEN, and specific [^{3}H]NMS binding was determined ($n = 6$).

shows that 10 μM carbachol-stimulated $InsP_3$ formation was inhibited by TPEN with $IC_{50} = 51 \pm 14$ μM ($n = 3$), yielding a K_i for TPEN of ~ 26 μM. To explain these findings, we examined whether TPEN blocks binding to muscarinic receptors. Fig. 2B shows that TPEN inhibits the binding of the muscarinic receptor ligand [^{3}H]NMS to intact SH-SY5Y cells with $IC_{50} = 52 \pm 22$ μM ($n = 6$), yielding a K_i for TPEN of ~ 26 μM. Thus, the inhibitory effect of TPEN on carbachol-stimulated $InsP_3$ formation appears to result from blockade of ligand binding to muscarinic receptors and indicates that TPEN is a muscarinic receptor antagonist.

To show that these effects of TPEN were not indirect (e.g., a consequence of chelation of intracellular ions), we also examined its effects on ligand binding to membranes from disrupted cells. Fig. 2A shows that TPEN also inhibited [^{3}H]NMS binding to SH-SY5Y cell membrane preparations, with $IC_{50} = 28 \pm 9$ μM ($n = 6$) and a K_i ~ 14 μM. Further, the effects of TPEN were not inhibited by coincubation with 500 μM Zn^{2+} (in fact, they were significantly enhanced to a new $IC_{50} = 8 \pm 2$ μM; Fig. 2A) and were not mimicked by equimolar amounts of EDTA (data not shown), which has heavy metal affinities similar to those for TPEN. Thus, the effects of TPEN are not simply due to chelation of metal ions and, in total, these data indicate that TPEN interacts directly with muscarinic receptors to inhibit ligand binding.

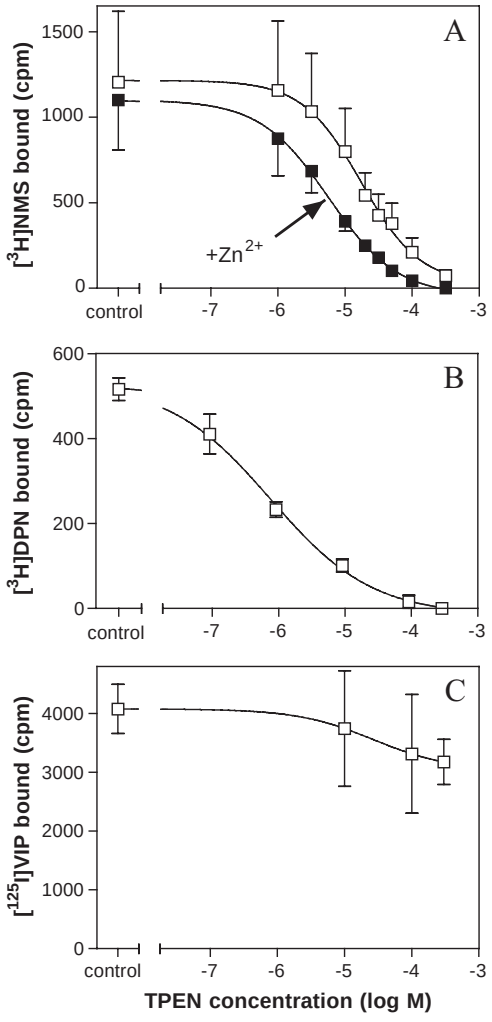

Fig. 2. Effects of TPEN on ligand binding to SH-SY5Y cell membranes. (A) Specific [^{3}H]NMS binding in the absence (□) or presence of 500 μM $ZnCl_2$ (■); 500 μM $MgCl_2$ was added to incubations as a control when $ZnCl_2$ was absent ($n = 6$). (B) Specific [^{3}H]DPN binding. (C) Specific [^{125}I]VIP binding ($n = 2$).

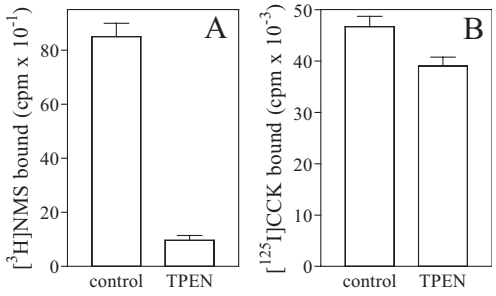

Fig. 3. Effects of TPEN on ligand binding to AR4-2J cell membranes. Specific [^{3}H]NMS binding (A) and [^{125}I]CCK binding (B) was determined in the absence or presence of 100 μM TPEN.

In order to determine whether the effects of TPEN were specific to muscarinic receptors, we examined whether binding to μ-opioid and VIP receptors was affected by TPEN. Fig. 2B shows that binding of the nonpeptide μ-opioid receptor antagonist [^{3}H]DPN was potently inhibited by TPEN with $IC_{50} = 0.8$ μM and a K_i ~ 0.4 μM. In contrast, [^{125}I]VIP binding was not significantly inhibited (Fig. 2C). Further, we examined the effects of TPEN on ligand binding to AR4-2J cell membranes, since these cells also express multiple G protein-coupled receptors (Simeone et al, 1995; Oberdorf et al., 1999). Fig. 3A shows that [^{3}H]NMS binding to AR4-2J cell membranes was inhibited ~ 90% by 100 μM TPEN, similar to that seen in SH-SY5Y cells. In contrast, 100 μM TPEN only slightly inhibited [^{125}I]CCK binding (by ~ 15%), and this change was not significant (Fig. 3B).

4. Discussion

The data presented indicate that TPEN interacts with the ligand-binding site of some, but not all, G protein-coupled receptors. In the case of muscarinic receptors, this interaction blocks [^{3}H]NMS binding and inhibits carbachol-induced receptor activation, showing that TPEN is a muscarinic receptor antagonist. This antagonism appears to reflect a direct interaction of TPEN with muscarinic receptors, since it was observed in membrane preparations as well as in intact cells, and was not a consequence of metal ion chelation.

Of the G protein-coupled receptors examined in this study, TPEN inhibited binding to muscarinic and opioid receptors, but was inactive at VIP and CCK receptors. The explanation of this selectivity may rest with the fact that the

different ligands for these receptors differ in the manner in which they bind. On one hand, for muscarinic and non-peptide opioid receptor ligands (which are characteristic of ‘‘small molecule’’ ligands), the predominant determinants of binding affinity are located deep within a ligand-binding crevice composed of transmembrane helices 3, 5, 6, and 7 of their G protein-coupled receptors (Wess, 1994; Law et al., 1999; Gether, 2000). In contrast, for the polypeptides VIP and CCK, these determinants are primarily in their receptor’s extracellular N-termini and loops, and in outer regions of transmembrane helices 1–3 (Holtmann et al., 1995; Silvente-Poirot et al., 1998; Solano et al., 2001). Thus, we suggest that TPEN either interacts selectively with the ligand-binding crevice of muscarinic and opioid receptors, or that it interacts with this region in all of the G protein-coupled receptors, but only inhibits binding of the ligands that interact predominantly with this region (namely, the small-molecule ligands, but not the peptides). In this regard, it is well known that a conserved aspartic acid residue in transmembrane helix 3 of many G protein-coupled receptors interacts strongly with the positively charged amine head group of numerous small-molecule ligands, such as catecholamines and acetylcholine (Gether, 2000). As TPEN contains protonated amines at physiological pH (Arslan et al, 1985; Smith and Martell, 1975), it is tempting to speculate that it may also interact with this aspartic acid. Ionic interactions of this kind may also provide an explanation for the intriguing observation that inclusion of Zn^{2+} in incubations with TPEN enhanced its potency at muscarinic receptors.

In conclusion, our data indicate that TPEN interacts with and inhibits certain G protein-coupled receptors. Clearly, this information should be taken into account when interpreting studies in which TPEN is used in biological systems and may limit its potential as a therapeutic agent. The ability of TPEN to inhibit ligand binding to both muscarinic and opioid receptors suggests that it may have widespread antagonistic activity against G protein-coupled receptors that bind small molecules, and that modifications to the structure of TPEN may lead to the development of more specific agents.

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