5-(5-Chlorothien-2-yl)-1-(β-D-2-deoxyribofuranos-1-yl)-uracil (16d). 5-Thien-2-yl-1-(2-β-D-deoxyribofuranos-1-yl)uracil (385 mg) was acetylated as described for **16b**. This foam was dissolved in 10 mL of pyridine; 160 mg (1.18 mmol) of NCS was added and the mixture was heated for 4 h at 70 °C. Chromatographic purification (CH_2Cl_2/CH_3CN, 87/13) yielded 190 mg of a foam. The acetyl groups were removed by treatment with 20 mL of methanol saturated with ammonia. After evaporation and purification 120 mg (28% yield) of **13b** was obtained. The product was crystallized from acetone. Mp: 220–222 °C. UV (MeOH): λ_{max} = 274 nm (ϵ = 8800), λ_{max} = 327 nm (ϵ = 11 500). ^{1}H NMR (DMSO-d_6): δ 2.23 (t, 2 H, H-2′), 3.68 (br s, 2 H, H-5′), 3.87 (m, 1 H, H-4′), 4.31 (m, 1 H, H-3′), 5.29 (br s, 2 H, 3′-OH and 5′-OH), 6.19 (t, 1 H, H-1′), 7.03 and 7.23 [2 × (d, 1 H, J = 3.95 Hz, H-3″ and H-4″)], 8.64 (s, 1 H, H-6), 11.76 (s, 1 H, NH) ppm. ^{13}C NMR (DMSO-d_6): δ 40.5 (C-2′), 60.7 (C-5′), 69.8 (C-3′), 85.1 (C-1′), 87.7 (C-4′), 107.5 (C-5), 121.1 and 125.7 (C-3″ and C-5″), 127.4 (C-5″), 133.0 (C-2″), 135.7 (C-6), 149.1 (C-2), 161.3 (C-4) ppm. Anal. ($C_{13}H_{13}ClN_2O_5S$): C, H, N.

Antiviral Activity. The different molecules were evaluated for their antiviral in vitro activity according to well-established procedures.[17,18] The origin of the viruses [herpes simplex type 1 (HSV-1) (strains KOS, F and McIntyre), thymidine kinase deficient (TK$^-$) HSV-1 strains (B2006 and VMW 1837), herpes simplex virus type 2 (HSV-2) (strains G, 196 and Lyons), varicella-zoster virus (VZV) (strains Oka and YS), TK$^-$ VZV (strains 07-1 and YS-R), vaccinia virus (VV), vesicular stomatitis virus (VSV), and cytomegalovirus (CMV) (strains AD169 and Davis)] has been described previously.[18,19] Cytotoxicity measurements were based on either microscopically examination of detectable alteration, normal cell morphology, or inhibition of cell growth. Two different lines of human fibroblasts [HEL (human embryonic lung) and E_6SM (human embryonic skin–muscle)] were used for both the antiviral activity and cytotoxicity assays. All assays were done in 96-well microtiter plates.

Acknowledgment. This study was supported in part by the AIDS Basic Research Programme of the European Community and by grants from the Belgian Fonds voor Geneeskundig Wetenschappelijk Onderzoek (Projects no 3.0040.83 and 3.0097.97) and the Belgian Geconcerteerde Onderzoeksacties (Project no 85/90-79). Robert Snoeck is a senior research assistant from the Belgian National Fund for Scientific Research. We thank Dominique Brabants for her dedicated editorial assistance and Anita Van Lierde, Frieda De Meyer, and Anita Camps for their efficient technical assistance.

Registry No. **3a**, 133040-36-5; **3b**, 134333-64-5; **4a**, 31356-86-2; **4b**, 54-42-2; **5** (X = O), 51583-40-5; **5** (X = S), 37496-13-2; **6c**, 92233-50-6; **6d**, 89647-10-9; **7**, 84500-33-4; **8**, 54663-78-4; **8** (X = O), 118486-94-5; **9b**, 134333-65-6; **10**, 133787-21-0; **11**, 134333-66-7; **12b**, 134333-67-8; **13**, 4330-21-6; α-**14a**, 104267-95-0; β-**14a**, 31356-86-2; **14b**, 60110-67-0; α-**15a**, 127235-39-6; β-**15a**, 127235-40-9; α-**15b**, 134333-68-9; α-**15c**, 127235-62-5; α-**15d**, 127235-63-6; β-**15d** diacetyl derivative, 133787-21-0; **16a**, 134333-69-0; **16b**, 134333-70-3; **16c**, 134333-71-4; **16d**, 134333-72-5; *N*-methylimidazole, 616-47-7; 5-iodouracil, 696-07-1; 1,2,4-triazole, 288-88-0; thiazole, 288-47-1; 5-iodo-bis-*O*-(trimethylsilyl)uracil, 38953-72-9.

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Synthesis and Binding of [$^{125}I_2$]Philanthotoxin-343, [$^{125}I_2$]Philanthotoxin-343-lysine, and [$^{125}I_2$]Philanthotoxin-343-arginine to Rat Brain Membranes

R. A. Goodnow, Jr.,[†] R. Bukownik,[†] K. Nakanishi,*[,†] P. N. R. Usherwood,[§] A. T. Eldefrawi,[‡] N. A. Anis,[‡] and M. E. Eldefrawi*[,‡]

Department of Chemistry, Columbia University, New York, New York 10027, Department of Pharmacology and Experimental Therapeutics, School of Medicine, University of Maryland, Baltimore, Maryland 21201, and University of Nottingham, Nottingham NG7 2RD, U.K. *Received September 28, 1990*

$^{125}I_2$-iodinated philanthotoxin-343 (PhTX-343) (**10**), [$^{125}I_2$]PhTX-343-arginine (**11**), and [$^{125}I_2$]PhTX-343-lysine (**12**) were synthesized and evaluated as probes for glutamate receptors in rat brain synaptic membranes. It was found that these probes were not specific for the glutamate receptors but may be useful for investigating the polyamine binding site. Filtration assays with Whatman GF/B fiber glass filters were unsuitable because the iodinated PhTX-343 analogues exhibited high nonspecific binding to the filters, thus hindering detection of specific binding to membranes. When binding was measured by a centrifugal assay, [$^{125}I_2$]PhTX-343-lysine (**12**) bound with low affinity (K_D = 11.4 ± 2 μM) to a large number of sites (37.2 ± 9.1 nmol/mg of protein). The binding of [$^{125}I_2$]PhTX-343-lysine was sensitive only to the polyamines spermine and spermidine, which displaced [$^{125}I_2$]PhTX-343-lysine (**12**) with K_i values of (3.77 ± 1.4) × 10^{-5} M and (7.51 ± 0.77) × 10^{-5} M, respectively. The binding was insensitive to glutamate receptor agonists and antagonists. Binding results with [$^{125}I_2$]PhTX-343-arginine (**11**) were similar to those of [$^{125}I_2$]-PhTX-343-lysine. Considering the high number of toxin binding sites (10000-fold more than glutamate) in these membranes and the insensitivity of the binding to almost all drugs that bind to glutamate receptors, it is evident that most of the binding observed is not to glutamate receptors. On the other hand, PhTX analogues with photoaffinity labels may be useful in the isolation/purification of various glutamate and nicotinic acetylcholine receptors; they could also be useful in structural studies of receptors and their binding sites.

Introduction

Glutamate receptor pharmacology has attracted a great deal of attention in the last few years[1–4] because of the possible involvement of glutamate receptors in degenerative brain diseases,[5] mechanism of memory,[6] and ischemic damage.[7] Glutamate receptors are classified into three types according to their sensitivity to the exogenous excitatory amino acids quisqualate, kainate, and *N*-methyl-D-aspartate (NMDA). A subunit of a kainate type

[†] Columbia University.
[§] University of Nottingham.
[‡] University of Maryland.

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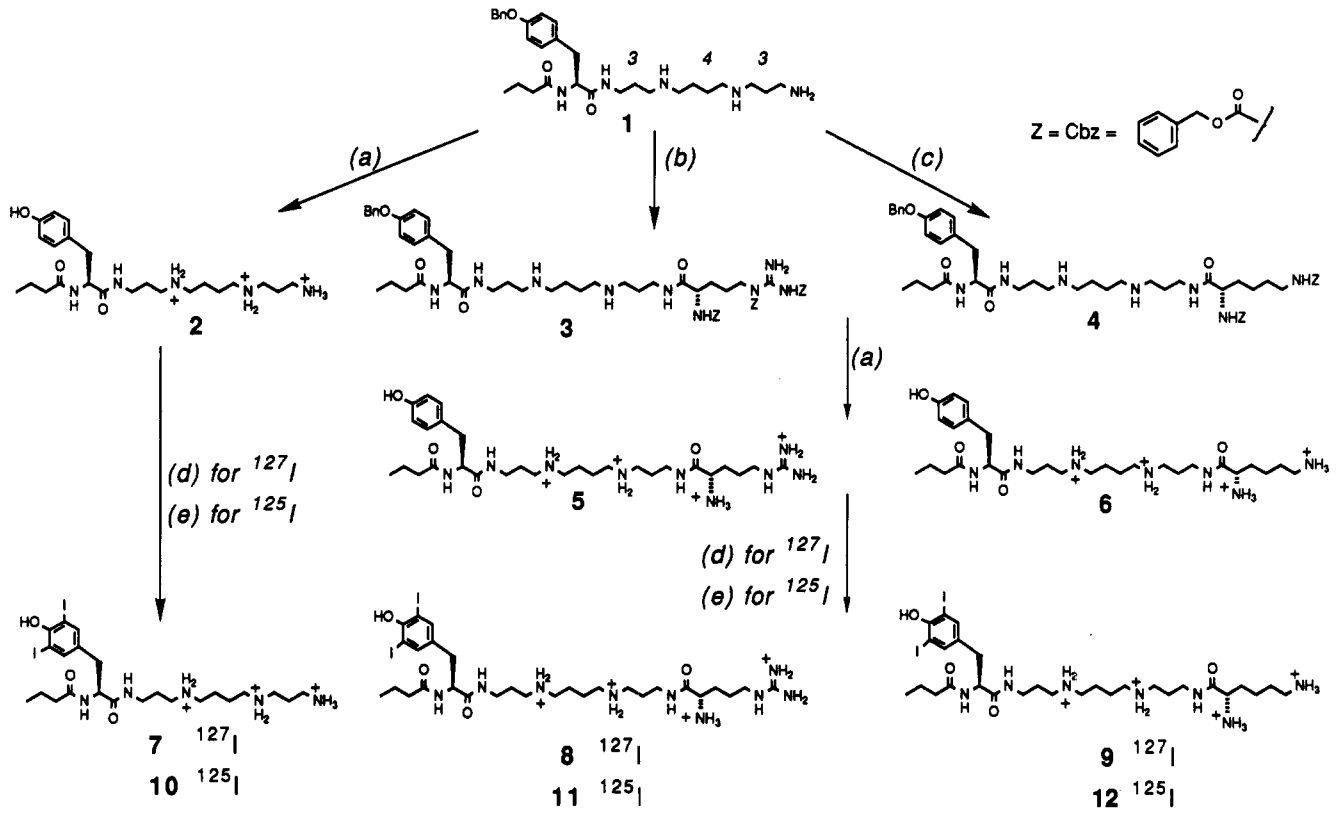
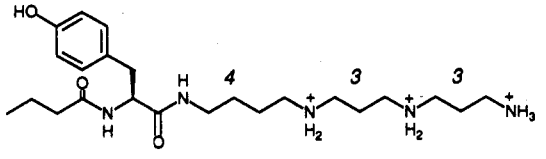
Scheme I



[a] (a) H_2, 5% Pd/C, CH_3OH; (b) N_α-N^G, $N^{G'}$-tri-CBZ-L-arginine-N-hydroxy succinimide ester, DMF; (c) N,N-di-CBZ-L-lysine *p*-nitrophenyl ester, DMF; (d) KI, NBS, K_2HPO_4, H_2O, CH_3OH; (e) $Na^{127}I$, $Na^{125}I$, K_2HPO_4, H_3PO_4, chloramine T, H_2O.

receptor has been purified[8] and its gene cloned, and the NMDA subtype of the receptor has been recently solubilized.[9] The quisqualate receptor, which is predominant in insect muscle and is found in mammalian brain, is the least known type, despite the discovery of the paralytic wasp and spider neurotoxins.[10]

Philanthotoxin (PhTX-433) is a polyamine neurotoxin made by the venom glands of the digger wasp *Philanthus triangulum*.[11] The wasp, known as the bee wolf, uses its venom to paralyze honey bee workers, its favorite prey,[12] but the venom was found to paralyze other insects as well.



Philanthotoxin-443

The active component of the venom is PhTX-433 (433 denotes the number and order of methylene groups in the polyamine moiety), which inhibits glutamate receptors of insect muscles noncompetitively.[11,13,14] Other polyamine neurotoxins, which are synthesized and used by spiders to paralyze their insect preys, have a similar mechanism of action but have higher affinity and slightly more complex structures.[10] Since assays with a glutamatergic, nerve–muscle preparation from locust leg showed little difference in activity among PhTX-433 and synthetic PhTX-343 and synthetic PhTX-334, most PhTX analogues synthesized for structure–activity studies had the 343 polyamine moiety;[15-17] in view of the presence of an arginine residue at the end of the polyamine moiety in some spider toxins,[10] analogues with terminal arginine (and lysine) residues, among others, were also prepared.[15-17] Initially, PhTX analogues were hoped to be highly selective for quisqualate receptors,[17] but it was discovered that they inhibit both NMDA[18] and kainate receptors[19] as well; in addition, they also inhibited nicotinic acetylcholine (ACh) receptors in a noncompetitive manner.[20] Despite their

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effects on other receptors, certain PhTX analogues were more selective for quisqualate receptors while others were more selective for nicotinic receptors.[21] An extensive structure–activity study showed I_2-PhTX-343-arginine (8) and I_2-PhTX-343-lysine (9) to be respectively 33 and 14 times more potent inhibitors of locust muscle quisqualate receptors than PhTX-343 (2) itself.[15,17] The compounds [$^{125}I_2$]PhTX-343 (10), [$^{125}I_2$]PhTX-343-arginine (11), and [$^{125}I_2$]PhTX-343-lysine (12) were thus synthesized, and their binding to brain was investigated to evaluate them as selective probes for quisqualate receptors. The results, however, show that $^{125}I_2$-labeled PhTX-343 analogues are poor radioligands for identification of glutamate or nicotinic receptors because of the high nonspecific binding to nonreceptor sites.[17,22] The results, however, suggest that [$^{125}I_2$]PhTX-343-arginine (11) and -lysine (12) may be excellent probes for the receptor sites of the polyamines spermine and spermidine, at least in brain. Furthermore, photoaffinity-labeled PhTX analogues may be useful in (i) purifying or isolating glutamate or nicotinic acetylcholine receptors from relatively pure sources, or (ii) clarifying the tertiary structures of receptors (see below).

Synthesis

Both radioactive and nonradioactive PhTX-343 analogues were prepared according to Scheme I. The syntheses of *O*-benzyl-PhTX-343 (1), PhTX-343 (2), *O*-benzyl-PhTX-343-tri-Cbz-arginine (3), and PhTX-343-arginine (5) have been previously reported.[16] Methods for radioactive and nonradioactive iodination were developed for PhTX-343 (2) with use of the NBS and the chloramine T reactions, respectively. Binding studies of [$^{125}I_2$]-PhTX-343 (10) indicated the need for ligands with higher binding affinities. Since structure–activity studies had indicated that I_2-PhTX-343-arginine (8) and I_2-PhTX-343-lysine (9) had higher affinities for the quisqualate receptors[19] (~33- and ~14-fold higher than PhTX-343 (2), respectively), it was decided to synthesize and measure the binding of both [$^{125}I_2$]PhTX-343-arginine (11) and [$^{125}I_2$]PhTX-343-lysine (12). Thus, the iodination methods were applied to compounds 5 and 6. Compound 6, PhTX-343-lysine, was obtained by hydrogenolysis of the product of the coupling of polyamine 1 with commercially available $N_{\alpha,\epsilon}$-di-Cbz-L-lysine *p*-nitrophenyl ester. Nonradioactive diiodo products, [$^{127}I_2$]PhTX-343-arginine (8) and [$^{127}I_2$]PhTX-343-lysine (9) were obtained after treatment of 5 and 6, respectively, with *N*-bromosuccinimide in water and methanol in the presence of potassium iodide (K^{127}I). All compounds were obtained greater than 95% pure as judged by silica gel TLC and proton and carbon-13 NMR and for compounds 5 and 6 by reversed-phase HPLC. Radioiodination of 5 and 6 was effected on a nanomolar scale with use of cold Na^{127}I as a carrier, radioactive Na^{125}I as a label, and chloramine T as an oxidizing agent[23] in phosphate buffer (pH = 6.5); the diiodo products were isolated as a single peak by reversed-phase HPLC. The putative monoiodo product was formed in detectable quantities at pH = 7.5–8.0 but could be reduced by maintaining pH = 6.5–7.0. Although the radiolabeled analogues could not be directly characterized by NMR or mass spectroscopy for reasons of safety, their identities were confirmed by comparisons of their chromatograms with those of nonradioactive reaction mixtures which were coinjected with authentic samples of $^{127}I_2$ analogues 8 and 9. The eluants containing [$^{125}I_2$]PhTX-343-arginine (11) and [$^{125}I_2$]PhTX-343-lysine (12) were lyophilized and dissolved in a methanol/benzene (1:1) solution, and a 1-μL aliquot was removed and counted before the solutions were sealed in ampules.

Results

In the initial experiments, [$^{125}I_2$]PhTX-343 (7) was incubated with rat cortical synaptic membranes containing 0.5 pmol of glutamate receptors (as determined by [^{3}H]-glutamate binding) in a volume of 1 mL of 5 mM Tris·HCl, pH 7.4, for 90 min, and then the bound and free toxins were separated by vacuum filtration over GF/B filters. Unlabeled PhTX-343 (2) at 100 μM was used as a discrimination dose to identify nonspecific binding. Unfortunately, GF/B filter controls that had no tissue had similar "binding". Typically, from a total of 1.23×10^5 cpm per assay, the cpm retained by the filter control was 7661 cpm and by the tissue sample was 6821 cpm. Nonspecific counts observed in the presence of 100 μM of unlabeled PhTX-343 (2) were 3810 cpm for the no tissue filter control and 4730 cpm for the tissue sample. These results indicated that a substantial fraction of [$^{125}I_2$]PhTX-343 (10) bound to the GF/B fiber glass filter.

Pretreatment of the GF/B filters with polyethylenimine at 0.05% (a common treatment used to reduce nonspecific binding of drugs to GF/B filters) and numerous other reagents including bovine serum albumin, ionic and nonionic detergents, salts, and positively charged drugs were unsuccessful in reducing the nonspecific binding of [$^{125}I_2$]PhTX-343 (10) to GF/B filters. The nonradioactive iodinated [$^{127}I_2$]PhTX-343 (7) bound similarly to GF/A, GF/C, and GF/F Whatman glass fiber filters. Silanization of GF/B filters with triethoxysilane also failed to reduce the nonspecific filter binding. Spermine at 0.1 mM was the only compound that successfully displaced binding of [$^{125}I_2$]PhTX-343 (10) to the GF/B filter. Centrifugal and equilibrium dialysis assays were equally unsuccessful in measuring any specific binding of [$^{125}I_2$]PhTX-343 (10) to rat cortical synaptic membranes.

As mentioned above, a structure–activity relationship study[17] indicated that I_2-PhTX-343-arginine (8) and I_2-PhTX-343-lysine (9) had higher affinities for quisqualate receptors (~33- and ~14-fold higher than PhTX-343). [$^{125}I_2$]PhTX-343-arginine (11) and [$^{125}I_2$]PhTX-343-lysine (12) still had fairly high nonspecific binding to GF/B filters, which made specific binding measurements to tissue very difficult. However, in this case, a centrifugal binding assay worked well and it was possible to measure specific binding of [$^{125}I_2$]PhTX-343-arginine (11) and [$^{125}I_2$]-PhTX-343-lysine (12) to the tissue. Since the binding of [$^{125}I_2$]PhTX-343-arginine (11) was found to be quite similar to that of [$^{125}I_2$]PhTX-343-lysine (12), binding studies were only performed with one of them, i.e., 12. Binding of [$^{125}I_2$]PhTX-343-lysine (12) was linear with the amount of tissue present in the incubation mixture. Nonspecific binding was that binding observed in the presence of 100 μM nonradioactive [$^{127}I_2$]PhTX-343-lysine (9). As shown in Table I, the specific binding of 3 nM [$^{125}I_2$]PhTX-343-lysine (12) to rat brain synaptic membranes was not significantly affected by 10^{-4} M of glutamate, the excitatory

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Table I. Effect of Glutamine Agonists and Antagonists on the "Specific" Binding of 3 nM [$^{125}I_2$]PhTX-343-lysine to Rat Cortical Membranes

ligand (100 μM)	% of control mean ± SD
control	100 ± 6.75
glutamate	99 ± 17
quisqualate	104 ± 17
kainate	103 ± 1.4
N-methyl-D-aspartate	96 ± 8.5
glycine	109 ± 21
MK-801	92 ± 12
APV (2-amino-5-phosphonovalerate)	94 ± 21
DNQX (6,7-dinitroquinoxoline-2,3-dione)	102 ± 8.5
phencyclidine	81 ± 12
spermine	14.4 ± 6.2[a]
philanthotoxin	32.7 ± 7.0[a]

[a] Significant difference ($p < 0.05$) between drug treated and total binding in the absence of drugs (Student's two-tailed *t*-test).

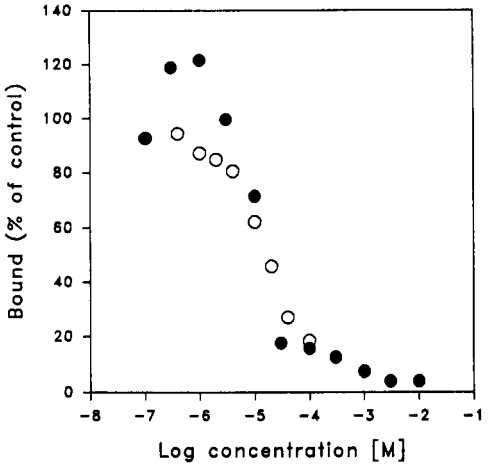

Figure 1. Inhibition of 3 nM [$^{125}I_2$]PhTX-343-lysine binding to rat cortical membrane by I_2-PhTX-343-lysine (○) and spermine (●). Symbols are the mean of three replicates ($n = 9$). SEM values were <10% of the mean.

amino acids quisqualate, kainate, and NMDA, the NMDA receptor allosteric modulator glycine, the noncompetitive blockers phencyclidine and 5-methyl-10,11-dihydro-5-methyl-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine (MK-801), the competitive antagonist 2-amino-5-phosphonovalerate (APV), and the quisqualate noncompetitive blocker 6,7-dinitroquinoxoline-2,3-dione (DNQX). However, the specific binding of 3 nM [$^{125}I_2$]PhTX-343-lysine (**12**) to rat brain synaptic membranes was inhibited significantly by PhTX-343 (**2**) and even more so by spermine.

The displacement curves of [$^{125}I_2$]PhTX-343-lysine (**12**) by spermine and [$^{127}I_2$]PhTX-343-lysine (**9**) indicated that the two ligands were equipotent (Figure 1). However, the spermine displacement curve exhibited an upward curvature at low spermine concentrations, suggesting that spermine and [$^{125}I_2$]PhTX-343-lysine (**12**) might be binding to distinct but allosterically coupled sites. Sensitivity to spermine prompted the testing of other polyamines to determine their potencies in displacing [$^{125}I_2$]PhTX-343-lysine (**12**). Spermine had a K_i ± SD value of (3.77 ± 1.4) × 10^{-5} M, spermidine was half as effective (K_i = (7.51 ± 0.77) × 10^{-5} M), while putrescine, agmatine, and cadaverine had K_i values of (4.14 ± 0.16) × 10^{-4} M, (2.69 ± 0.05) × 10^{-4} M, and (1.49 ± 0.08) × 10^{-3} M, respectively.

Saturation isotherms of [$^{125}I_2$]PhTX-343-lysine (**12**) binding to rat cortical synaptic membranes were measured by two methods. In the first method only [$^{125}I_2$]PhTX-343-lysine (**12**) was used in a limited concentration range

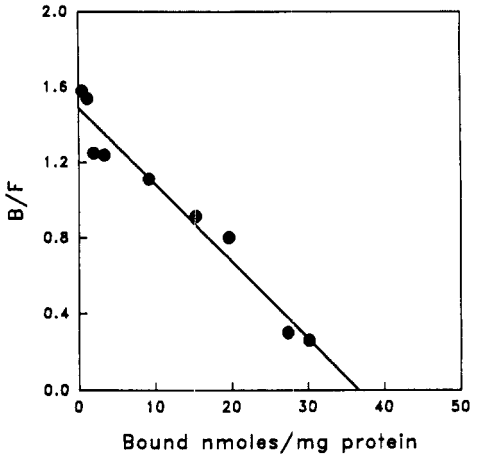

Figure 2. Scatchard plot of [$^{125}I_2$]PhTX-343-lysine saturation binding to rat cortical membrane fragments. Binding was performed by addition of increasing concentrations of unlabeled [$^{127}I_2$]PhTX-343-lysine (10 nM to 100 μM) to a single concentration (3 nM) of labeled [$^{125}I_2$]PhTX-343-lysine. The data represent averages of duplicate experiments that gave similar results. Each datum point is the mean of six measurements.

from 0.2 to 80 μM (data not shown), whereas in the second method [$^{125}I_2$]PhTX-343-lysine (**12**) was displaced by nonradioactive [$^{127}I_2$]PhTX-343-lysine (**9**). Scatchard plots of the saturation isotherms showed binding to a single population of sites present at a fairly high density (B_{max} 37.2 ± 9.1 nmol/mg of protein) and exhibiting a K_D of 11.4 ± 2 mM (Figure 2). PhTX-343 (**1**) had a K_i value of (3.27 ± 7) × 10^{-5} M.

The time course of association or dissociation of [$^{125}I_2$]PhTX-343-lysine to and from rat cortical synaptic membranes suffered from the inherent limitations of the centrifugal assay. The ligand and the tissue were in the incubation mixture for 5 min (the centrifugation time) at time zero. Taking that into account, the toxin showed very fast association rates, reaching the steady-state level of binding at the first measurement. It also showed a fairly fast rate of dissociation with total dissociation after a 5-min incubation. In all assays, 1 mM spermine was used to determine nonspecific binding. Spermine was also added to the dilution buffer in the dissociation experiments.

Discussion

The binding of [$^{125}I_2$]PhTX-343 (**10**) to 37.2 nmol/mg of protein, which is ~10000-fold of the glutamate receptor site in these cortical synaptic membranes (2–4 pmol/mg protein), is evidence that this ligand is binding to sites almost all of which are not glutamate receptors. Presumably, the binding sites of PhTX-343 (**2**) and analogues are various proteins and lipids that bind polyamines. The linearity seen in the Scatchard plot of the binding may be due to similar affinities of the different binding sites. The lack of effects of glutamate receptor ligands on [$^{125}I_2$]-PhTX-343-lysine (**12**) binding (Table I) does not preclude toxin binding to glutamate receptors. However, binding to glutamate receptors cannot be detected because of the large nonspecific binding. The [$^{125}I_2$]PhTX-343-lysine (**12**) binding sites have similar affinity for spermine as for PhTX-343 (**2**) (Table I) and slightly lower affinity for spermidine. Spermine is known to bind to NMDA receptors and modulate MK-801 binding[24] and has been shown recently to have similar effects on quisqualate receptors of insect muscles.[25]

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The data suggest that [$^{125}I_2$]PhTX-343 (10) and its derivative [$^{125}I_2$]PhTX-343-arginine (11) or [$^{125}I_2$]PhTX-343-lysine (12) are inappropriate ligands for labeling or purifying glutamate receptors. However, [$^{125}I_2$]PhTX-343-arginine (11) and/or [$^{125}I_2$]PhTX-343-lysine (12) may be good probes for the major sites that bind the polyamines spermine and spermidine in the brain.

Other polyamine-containing neurotoxins have been radiolabeled and used to study glutamate receptors. Iodinated joro toxin (JSTX), from the vemon of the joro spider *Nephila clavata*, was used to localize quisqualate receptors in lobster muscle.[26] It has been shown to block responses of hippocampal neurons to kainate and quisqualate[27] as well as to NMDA.[28] Although toxins from spider vemons have higher affinities than PhTX-343 (2) for quisqualate receptors,[10] their polyamine component may affect the specificity of their binding. A [I_2]PhTX analogue carrying the photoaffinity label azidophenyl ($N_3C_6H_4$) (instead of propyl group in [$^{127}I_2$]PhTX-343-lysine (9)) shows a 13-fold enhanced activity relative to that of PhTX-343 (2);[17] several other [I_2]PhTX analogues with photoaffinity labels at various sites have also been prepared (unpublished). In view of their increased activities, such analogues may prove to be useful in the isolation/purification of various glutamate and nicotinic acetylcholine receptors. The PhTX type molecules have an extended structure, and therefore, in the event that these receptors become available for binding studies, PhTX analogues carrying the photoaffinity label at various sites in the molecule could be very useful in tertiary structural studies of the receptors and their binding sites.

Experimental Section

High-resolution FAB-MS were obtained with a JEOL JMS-DX303HF mass spectrometer. Proton and carbon-13 NMR spectra were obtained for the free base polyamines unless otherwise indicated with a Varian VXR-300 MHz instrument. The proton and carbon spectra were measured in any of the following deuterated solvents: deuteriochloroform ($CDCl_3$) with 0.03% tetramethylsilane (TMS) and a $CDCl_3/CD_3OD$ (1:1) mixture with 0.015% TMS. Proton chemical shifts are reported in parts per million (ppm) downfield from TMS (0.00 ppm ^1H) as the internal standard. Carbon-13 NMR spectra were measured in $CDCl_3$ and were referenced with respect to the ^{13}C signal (δ = 77 ppm) of $CDCl_3$ or in D_2O and referenced with respect to the ^{13}C signal of dioxane (δ = 66 ppm) added as an internal standard. The solvents of dimethylformamide (DMF) and isopropylamine (*i*-$PrNH_2$) were distilled at atmospheric pressure over CaH_2. Reagent-grade methanol was distilled from magnesium turnings. HPLC purifications were performed with a Rainin Rabbit HP pump system, a Spectra-Physics SP8490 dual-wavelength detector, and a Linear 1200 chart recorder. The HPLC column was a YMC Packed Column AP-303 300 Å, ODS (4.5 × 250 mm). HPLC-grade solvents (Aldrich) were used for all HPLC purifications. Compounds were judged to be pure on the basis of silica gel TLC using several different solvent systems and on the basis of the proton and carbon NMR by which no impurities were detectable. Injection on the above reversed-phase HPLC column before running the radiolabeling reaction indicated compounds of greater than 95% purity.

***O*-Benzyl-PhTX-343-N_β,N_ϵ-di-Cbz-L-lysine Amide (4).** To a 5-mL DMF solution of 0.250 g (0.480 mmol) of 1 was added 0.255 g (0.485 mmol, 1.00 equiv) of N_β,N_ϵ-di-Cbz-L-lysine *p*-nitrophenyl ester (Sigma), and this solution was stirred overnight under a nitrogen (N_2) atmosphere at room temperature. The reaction mixture was concentrated by evaporation of the solvent, and the residue was dissolved in chloroform. The chloroform layer was extracted with dilute aqueous $NaHCO_3$ solution. The aqueous layers were extracted twice with chloroform. The combined chloroform layers were washed once with brine, dried with $MgSO_4$, and filtered through cotton. The filtrate was evaporated and the product was isolated after silica gel column chromatography by elution with 15% $CH_3OH/CHCl_3$ followed by 15:5:1 $CHCl_3/CH_3OH/i$-$PrNH_2$ to yield 0.399 g (91%) of the desired amide. R_f: 0.5 in (15:5:1 $CHCl_3/CH_3OH/i$-$PrNH_2$). ^1H NMR ($CD_3OD/CDCl_3$, 1:1): δ 0.84 (t, 3 H, J = 7.3 Hz), 1.38 (m, 2 H), 1.58 (m, 12 H), 1.72 (t, 2 H, J = 6.5 Hz), 2.15 (t, 2 H, J = 7.6 Hz), 2.62 (m, 12 H), (ABX system: δ_a = 2.87, δ_b = 3.02, δ_x = 4.50, J_{ab} = 13.0 Hz, $J_{ax/bx}$ = 7.3 Hz, $ArCH_2CH$, $ArCH_2CH$), 3.21 (m, 2 H), 5.04 (s, 4 H), 5.08 (s, 2 H), 6.91 (d, 2 H, J = 8.6 Hz), 7.14 (d, 2 H, J = 8.7 Hz), 7.33 (m, 15 H). ^{13}C NMR ($CDCl_3$): δ 13.5, 18.9, 22.4 ($CH_3CH_2CH_2C$=O, respectively), 27.0, 27.7, 27.9, 29.1, 32.0, 37.5, 38.1, 40.3, 46.3, 48.6, 50.2, 54.8, 54.9 (polyamine and lysine chain), 66.4, 66.7, 69.8 (benzyl methylene), 114.7, 127.3, 127.8, 128.0, 128.4, 129.1, 130.2, 136.2, 136.5, 136.8 (aromatic ring), 171.9 ($CH_3CH_2CH_2C$=O), 172.4 (lysine carbonyl), 174.7 (tyrosine carbonyl). FT-IR (thin film on NaCl plate): 3660, 3277, 1708, 1656, 1649, 1639, 1562, 1240, 1024, 734 cm^{-1}.

PhTX-343-L-lysine Amide (6). To a 10-mL CH_3OH solution of 0.399 g (0.440 mmol) of 4 was added 0.05 g of 5% Pd/C. This suspension was purged several times with hydrogen and then was allowed to stir under hydrogen atmosphere at room temperature and pressure overnight. The reaction was worked up first by filtering and washing with CH_3OH through Celite followed by evaporation of the solvent. The crude product was then loaded onto a silica gel flash column and eluted with a step gradient of 15:5:1 $CHCl_3/CH_3OH/i$-$PrNH_2$ and 4:4:1 $CHCl_3/CH_3OH/i$-$PrNH_2$ to yield 0.144 g (58%) of the desired amide. R_f: 0.26 (1:1:1 $CHCl_3/CH_3OH/i$-$PrNH_2$). ^1H NMR ($CD_3OD/CDCl_3$, 1:1): δ 0.89 (t, 3 H, J = 7.3 Hz), 1.38 (m, 2 H), 1.53 (m, 12 H), 1.72 (sextet, 2 H, J = 7.3 Hz), 2.17 (t, 2 H, J = 7.3 Hz), 2.43 (t, 2 H, J = 7.3 Hz), 2.62 (m, 10 H), (ABX system: δ_a = 2.85, δ_b = 2.94, δ_x = 4.50, J_{ab} = 11.0 Hz, $J_{ax/bx}$ = 7.3 Hz, $ArCH_2CH$, $ArCH_2CH$), 3.14 (m, 2 H), 6.72 (d, 2 H, J = 8.3 Hz), 7.04 (d, 2 H, J = 8.3 Hz). ^{13}C NMR ($CDCl_3$): δ 13.7, 19.0, 23.0 ($CH_3CH_2CH_2C$=O, respectively), 26.9, 27.0, 28.2, 29.2, 32.9, 34.9, 36.9, 38.4, 38.8, 41.6, 46.8, 47.9, 49.0, 49.3, 50.1, 55.3 (polyamine and lysine chain), 115.7, 127.5, 130.4, 156.4 (aromatic ring), 170.6 ($CH_3CH_2CH_2C$=O), 172.6 (lysine carbonyl), 175.6 (tyrosine carbonyl). FT-IR (thin film on NaCl plate): 3284, 2933, 1644, 1556, 1378, 753 cm^{-1}. HRMS (nitrobenzyl alcohol matrix) calcd for $C_{29}H_{54}N_7O_4$ $(M + 1)^+$ 564.4238, found 564.4232.

[$^{127}I_2$]PhTX-343-L-lysine (9). To a 15-mL solution of H_2O/CH_3OH (5:1) containing 50 mg (0.089 mmol) of 6, 29.4 mg (0.177 mmol, 2.0 equiv) of $K^{127}I$, and 46.5 mg (0.267 mmol, 3.0 equiv) of K_2HPO_4 was added dropwise by pipet 2 mL of a CH_3OH/H_2O (1:1) solution of 31.5 mg (0.177 mmol, 2.0 equiv) of *N*-bromosuccinimide (NBS). After the completion of the NBS addition, 3 mL of H_2O was added to the dark brown solution, which after several minutes lightened to a clear yellowish solution. The solution was allowed to stir for 45 min at room temperature under a nitrogen atmosphere. The reaction was terminated by filtration of the solution through cotton, followed by the evaporation of the solvent. The residue was dissolved in CH_3OH, 0.5 g of silica gel was added, and the solvent was evaporated. The dry, impregnated silica gel was then loaded onto a dry silica gel column, and the product was eluted by using a step gradient of 15:5:1 $CHCl_3/CH_3OH/i$-$PrNH_2$, 4:4:1 $CHCl_3/CH_3OH/i$-$PrNH_2$, and 1:1:1 $CHCl_3/CH_3OH/i$-$PrNH_2$. The desired diiodo product was obtained in 68% yield (49 mg). R_f: 0.35 (1:1:1 $CHCl_3/CH_3OH/i$-$PrNH_2$). ^1H NMR of trifluoroacetic acid (TFA) salt (CD_3OD): δ 0.85 (t, 3 H, J = 7.5 Hz), 1.54 (sextet, 2 H, J = 7.5 Hz), 1.71 (t, 2 H, J = 7.6 Hz), 1.82 (m, 12 H), 2.18 (t, 2 H, J = 7.7 Hz), 2.50–2.80 (m, 2 H), 2.94 (m, 7 H), 3.08 (m, 7 H), 3.89 (t, 1 H, J = 6.9 Hz), 4.40 (t, 1 H, J = 6.3 Hz), 7.63 (s, 2 H). ^{13}C NMR of TFA salt (D_2O, referenced on dioxane carbon signal, δ = 66.5): δ 12.8, 18.9, 22.0 ($CH_3CH_2CH_2C$=O, respectively), 26.0, 26.4, 26.8, 33.7, 35.0, 36.0, 36.3, 37.2, 39.2, 44.7, 45.3, 47.3 (polyamine and lysine chain), 88.8, 123.9, 139.3, 163.4 (aromatic ring), 170.6 ($CH_3CH_2CH_2C$=O), 173.1 (lysine carbonyl), 176.4 (tyrosine carbonyl). FT-IR (thin film on a NaCl plate): 3270, 2934, 1644,

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1552, 1435, 1296, 1031, 817 cm^{-1}. FAB-MS calcd for $C_{29}H_{52}N_7O_4I_2$ $(M + 1)^+$ 816.2170, found 816.2185.

[$^{125}I_2$]PhTX-343-L-lysine (12). To a 100-μL solution of 0.1 N NaOH of 5.4 mCi of $Na^{125}I$ (ICN Radiochemicals) contained as received in an eppendorf vial was added directly a small triangular stir bar. The following aqueous solutions were then added: 10 μL of 1.0 M Na_2HPO_4 (10 μmol), 24.7 μL of 8.0 mM $Na^{127}I$ (197.6 nmol), and 10 μL of 1.0 M H_3PO_4 (10.0 μmol) followed by 31.3 μL of 6.7 mM chloramine T (210 nmol). The solution was allowed to stir at room temperature for 30 s before the addition of 20 μL of 5.0 mM PhTX-343-L-lysine $(TFA)_3$ salt (200 nmol). The solution was then stirred for 1 min before quenching by addition of 32 μL of 8.0 mM Na_2SO_3 (256 nmol). After 2 min, half of the reaction mixture was injected onto a YMC-ODS reversed-phase analytical HPLC column and eluted isocratically with (16% CH_3CN, 0.1 % TFA)/H_2O at a flow rate of 1.5 mL/min, with detection at 274 nm. The desired diiodo product began to elute 13 min after injection. The eluant containing this product was first rotoevaporated for 30 min in a warm water bath followed by freezing of the remaining solution at −78 °C before lyophilization of the solid overnight. The resulting residue was dissolved in 1 mL of CH_3OH/benzene (1:1). An aliquot of 1 μL was removed and shaken in scintillation fluid which was measured to have 1 964 240 cpm. Thus was obtained a 1-mL solution of [$^{125}I_2$]PhTX-343-L-lysine with a total activity of 0.89 mCi and a specific activity of 21.6 Ci/mmol at a concentration of 41.2 μM.

Tissue Preparation. Brains of male Sprague-Dawley rats (250–500 g) were removed after CO_2 anesthesia and decapitation by guillotine. Cortex sections were dissected, rinsed, and homogenized in ice-cold 0.32 M sucrose with use of a glass homogenizer with a clearance of 0.11 in. The homogenate was centrifuged at 1000*g* for 10 min, and the nuclear pellet was removed. The supernatant fraction was centrifuged at 12000*g* for 20 min and the pellet resuspended in 10 mL of 0.32 M sucrose solution, and then this suspension was layered over with 20 mL of 0.8 M sucrose solution and centrifuged at 9500*g* for 30 min in a Beckman SW27 rotor. Synaptosomes dispersed in the 0.8 M sucrose layer were collected by dilution of the layer with 2–5 volumes of ice-cold water and centrifugation at 20000*g* for 20 min. The synaptosomal pellet was suspended in 40 volumes of ice-cold water to lyse them and centrifuged at 48000*g* to collect the pelleted synaptic membranes. The synaptic membranes were washed once with distilled water and then suspended in 5 mM Tris·HCl, pH 7.4, buffer at a concentration of 0.2 g of original tissue/mL. Protein concentrations of these synaptic membrane preparations were determined by the method of Lowry.[29]

Binding Assays. [$^{125}I_2$]PhTX-343-lysine was incubated with synaptic membranes (~0.25 mg of protein in 1.0 mL of 5 mM Tris·HCl) in 1.5-mL microfuge tubes. After a 30-min incubation, the tubes were centrifuged in an Eppendorf centrifuge for 5 min, and the supernatant fraction was removed. The pellet was rinsed carefully twice with 1 mL of cold buffer and then transferred to holding tubes and counted in an autogamma counter (Beckman, γ Rack-Beta II liquid scintillation counter 1216). A parallel series of microfuge tubes containing [$^{125}I_2$]PhTX-343-lysine and buffer (no tissue) was used to determine nonspecific binding. A series of four tubes was used to determine the specific binding value for each measurement. A = [$^{125}I_2$]PhTX-343-lysine + tissue but no unlabeled PhTX-343-lysine, B = [$^{125}I_2$]PhTX-343-lysine + tissue + 100 μM unlabeled PhTX-343-lysine, C = [$^{125}I_2$]PhTX-343-lysine, no tissue, no unlabeled PhTX-343-lysine, D = [$^{125}I_2$]PhTX-343-lysine, no tissue + 100 μM unlabeled PhTX-343-lysine. The specific binding was calculated from the formula (A − B) − (C − D).

Acknowledgment. This investigation was supported in part by National Institutes of Health Grants ES504977 (M.E.), AI10187 (K.N.), ES02594 (A.T.) and a British Agriculture and Food Research Council grant (to P.U.). Dr. Aristotle Kalivretenos is acknowledged for helpful discussion.

Registry No. 1, 130203-14-4; 2, 115976-93-7; 3, 130203-16-6; 4, 134419-04-8; 5, 130203-22-4; 6, 130306-76-2; 7, 130333-56-1; 8, 130306-91-1; 9, 126890-98-0; 10, 134419-05-9; 11, 134419-06-0; 12, 134419-07-1; Cbz-Lys(Cbz)-OC_6H_4-*p*-NO_2, 21160-82-7; H-Glu-OH, 56-86-0.

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