Biochemical Pharmacology, Vol. 52, pp. 933–939, 1996.

ISSN 0006-2952/96/$15.00 + 0.00
PII S0006-2952(96)00464-9

Spiropyrrolizidines: A New Class of Blockers of Nicotinic Receptors

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ABSTRACT. The spiropyrrolizidine oximes **236** and **222** and a related spiropyrrolizidine alkaloid, nitropolyzonamine, block nicotinic receptor channels in rat pheochromocytoma PC12 cells and in human medulloblastoma TE671 cells. In PC12 cells with an $\alpha_3\beta_{4(5)}$-nicotinic receptor, both the spiropyrrolizidine oxime **236** and nitropolyzonamine had IC_{50} values of about 1.5 μM, while spiropyrrolizidine oxime **222** had an IC_{50} value of 2.6 μM versus carbamylcholine-elicited sodium-22 influx. In TE671 cells with an $\alpha_1\beta_1\gamma\delta$ nicotinic receptor, the spiropyrrolizidine oximes **236, 222,** and nitropolyzonamine had IC_{50} values of 9.5, 14, and 67 μM, respectively. The inhibitions by the spiropyrrolizidine oxime **236** and nitropolyzonamine appeared to be noncompetitive in nature in both cell lines. In rat cerebral cortical membranes, binding of [^{3}H]nicotine to $\alpha_4\beta_2$ nicotinic receptors was not inhibited significantly by 10 μM concentrations of the spiropyrrolizidine oxime **236,** or by nitropolyzonamine, as expected for a noncompetitive blocker. Both compounds at 10 μM had marginal effects on a variety of central receptors, but did inhibit binding of [^{3}H]1,3-di(2-tolyl)guanidine to sigma receptors in mouse brain membranes with IC_{50} values of about 0.5 μM. The spiropyrrolizidine oxime **236** at 10 μM had no effect on batrachotoxin-elicited sodium influx in guinea pig cerebral cortical synaptoneurosomes or on ATP-elicited calcium influx in PC12 cells. Such spiropyrrolizidines represent a new structural class of blockers of nicotinic receptor channels with selectivity for ganglionic-type receptors. BIOCHEM PHARMACOL 52;6:933–939, 1996.

KEY WORDS. cholinergic receptors; noncompetitive antagonists; nicotine

Nicotinic receptors represent a large family of pentameric ion channels [1], which are functionally modulated by (i) acetylcholine binding sites through which carbamylcholine, nicotine, and other agonists activate the channel and for which there are a limited number of competitive antagonists, (ii) a novel channel activating site at which physostigmine and other compounds act, and (iii) so-called noncompetitive blocking sites at which histrionicotoxins, amantadine, phencyclidine, and a variety of local anaesthetics act. All these modulatory sites appear to be present at the various subclasses of nicotinic receptors, such as the $\alpha_1\beta_1\gamma\delta$ (neuromuscular), $\alpha_3\beta_{4(5)}$ (ganglionic), $\alpha_4\beta_2$ (central neuronal), and α_7 (central neuronal) receptors. The present paper describes a new class of noncompetitive blockers of nicotinic receptor channels, which in a comparison of neuromuscular-type and ganglionic-type receptors appear selective for the latter. The structures of these spiropyrrolizidine compounds are shown in Fig. 1.

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‡ *Abbreviations:* DTG, 1,3-di(2-tolyl)guanidine; HEPPSO, *N*-2-hydroxyethylpiperazine,*N*′-2-hydroxypropanesulfonic acid; and GABA, γ-aminobutyric acid.

Received 26 February 1996; accepted 29 April 1996.

MATERIALS AND METHODS

Materials

Spiropyrrolizidine oxime **236** and nitropolyzonamine were synthesized as previously described [2]. The spiropyrrolizidine oxime **222** (formerly proposed to be an amidine), allodihydrohistrionicotoxin, and batrachotoxin were isolated from skin extracts of dendrobatid frogs [3–5]. The dinitro byproduct was isolated from the preparation of 1-(2-nitroethyl)-2,2-dimethylcyclopentanecarboxaldehyde as reported by Hutchinson *et al.* [2]. ^{1}H NMR (300 MHz, $CDCl_3$) d 7.32 (s, 1H), 3.37–3.50 (m, 1H), 2.96–3.10 (m, 1H), 2.18–2.25 (m, 1H), 1.60–2.03 (m, 8H), 0.89–1.05 (m, 6H), EIMS 253 (M–H, 40%), 198 (100%), 190 (50%), 161 (32%); CIMS (NH_3), 272 (M + 18), 258, 256, 225, 217, 178.

The radioligands [^{3}H]nicotine (75.7 Ci/mmol), [^{3}H]quinuclidinyl benzilate (45.4 Ci/mmol), and [^{3}H]DTG‡ (38.3 Ci/mmol) were from New England Nuclear (Boston, MA), and ^{22}NaCl (0.2 mCi/mL) and $^{45}CaCl_2$ (0.2 mCi/mL) were from Amersham Life Science (Arlington Heights, IL). Dihydro-β-erythroidine and (−)-nicotine ditartrate were from Research Biochemicals International (Natick, MA), carbamylcholine and tetrodotoxin from the Sigma Chemical Co. (St. Louis, MO), and *d*-tubocurarine from Boehringer Mannheim (Mannheim, Germany). Other compounds were from standard commercial sources.

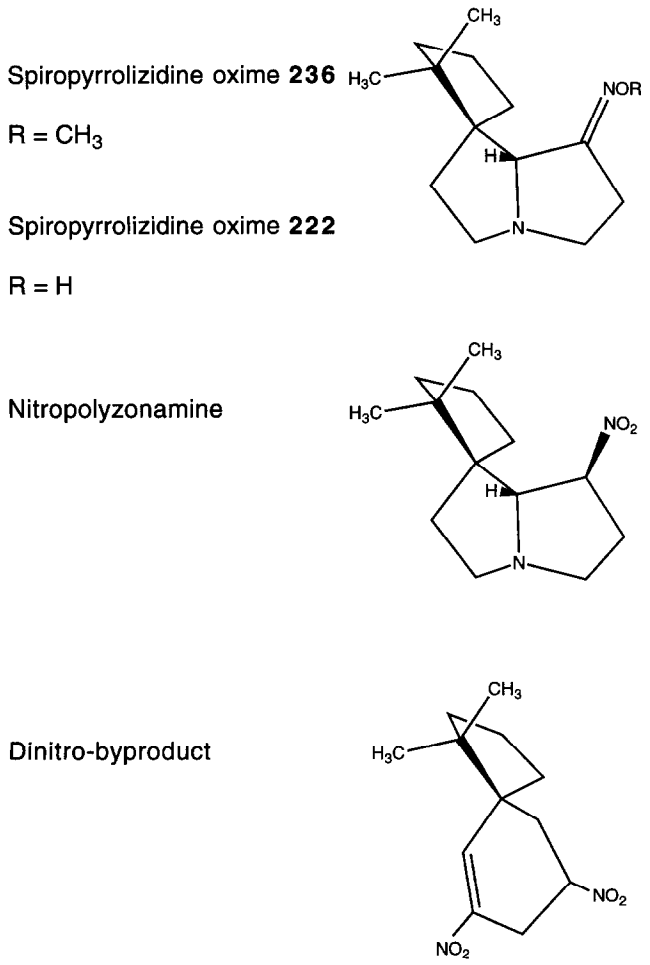

FIG. 1. Structures of the spiropyrrolizidine oxime 236 and related compounds.

Cultured Cells

Pheochromocytoma PC12 cells were provided by Dr. G. Guroff (NIH, Bethesda, MD). Medulloblastoma TE671 and Swiss 3T3 fibroblast cells were from the American Type Culture Collection (Rockville, MD). Pheochromocytoma PC12 cells were grown in Dulbecco's modified Eagle's medium with 6% fetal bovine serum, 6% horse serum, penicillin (100 U/mL) and streptomycin (100 mg/mL). Medulloblastoma TE671 and Swiss 3T3 fibroblast cells were grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 mg/mL). Cells were grown at 37° in an atmosphere enriched in CO_2.

Synaptoneurosome Preparation

Guinea pig cerebral cortical synaptoneurosomes were obtained as previously described [6]. Briefly, the cortex of one brain was homogenized in 10 mL of sodium-free buffer (pH 7.4) in a glass–glass homogenizer (Five strokes). The composition of the sodium-free buffer was as follows (concentrations in mM): choline chloride, 130; HEPES (adjusted to pH 7.4 with 50 mM Tris), 50; glucose, 5.5; $MgSO_4$, 0.8; KCl, 5.4. The suspension was centrifuged at 1000 *g* for 10 min, the supernatant was decanted, and the pellet was reconstituted in 35 mL of buffer. The suspension was filtered first through two layers of nylon material (100 mesh) and then through Millipore filters (LCWP-047, 10 mm pore size). After centrifugation for 10 min at 1000 *g*, the new pellet was reconstituted in an appropriate volume of fresh sodium-free buffer, as indicated in each case.

Ion Flux Assays

Stimulation of sodium-22 influx was assayed in cultured cells as described previously [7]. Cells were plated in 6-well culture plates (poly-D-lysine coated) and cultured with [^{3}H]leucine-containing medium for 24 hr (TE671) and 48 hr (PC12). Medium was removed by aspiration, and 0.5 mL of preincubation buffer (concentrations in mM: NaCl, 150; KCl, 5.4; $CaCl_2$, 2; HEPES/Tris, pH 7.4, 50; glucose, 5) was added at 22°. After 10 min, the preincubation buffer was replaced with influx buffer (concentration in mM: NaCl, 50; KCl, 5.4; $CaCl_2$, 2; HEPES/Tris, pH 7.4, 50; glucose, 5; sucrose, 179; ouabain, 5) containing ^{22}NaCl (0.7 μCi) and carbamylcholine or nicotine. Antagonists were present both in preincubation and influx buffers. In experiments with the spiropyrrolizidine oxime **222,** the influx buffer was modified to improve sensitivity (concentration in mM; NaCl, 5; KCl, 5.4; $CaCl_2$, 2; HEPES/Tris, pH 7.4, 50; glucose, 5; sucrose, 224; ouabain, 1). After 2 min at 22°, the influx buffer was removed by aspiration, and the cells were washed three times with wash buffer (same composition as for preincubation buffer). Cells were solubilized with 0.5 mL of 1% sodium dodecyl sulfate in 0.5 N NaOH for 30–60 min, and then pipetted into counting vials with 5 mL of Hydrofluor and 0.25 mL of 1 N HCl; radioactivity (^{3}H, $^{22}Na^+$) was determined in a scintillation counter.

Stimulation of calcium-45 influx was assayed in PC12 cells as described for sodium-22. Composition of preincubation buffer was similar to that described above. Influx buffer (concentration in mM: NaCl, 150; KCl, 5; $CaCl_2$, 2; HEPES/Tris, pH 7.4, 50; glucose, 5; sucrose, 224) containing $^{45}CaCl_2$ (0.5 μCi) was added and assayed as described for sodium-22.

Sodium flux into the synaptoneurosome preparation was based on the method previously described for synaptosomes [8]. Aliquots of synaptoneurosome suspensions (containing 200–300 mg protein) were preincubated for 10 min at 37° in 100 μL of incubation buffer containing 1 μM batrachotoxin and other test agents. Incubation buffer consisted of fresh sodium-free buffer (see "*Synaptoneurosome Preparation*" for composition) and 1 mg/mL bovine serum albumin. ^{22}NaCl (0.2 μCi) was then added in 150 μL of influx buffer (concentrations in mM: NaCl, 2.66; HEPES/Tris, pH 7.4, 50; choline chloride, 128; KCl, 5.4; $MgSO_4$, 0.8; glucose, 5.5; ouabain, 5; and 1 mg/mL bovine serum albumin) containing batrachotoxin and other test agents in the same concentrations as were present in the preincubated samples.

The final volume was 250 μL. Influx of $^{22}Na^+$ was stopped after 10 sec by the addition of 3 mL of cold wash buffer (concentrations in mM: HEPES/Tris, pH 7.4, 5; choline chloride, 163; $MgSO_4$, 0.8; $CaCl_2$, 1.8, and 1 mg/mL bovine serum albumin). Samples were collected on Gelman filters (GN-6, 0.45-μm pore size) and washed twice with 3 mL wash buffer. Filters were dissolved in Filtron-X (National Diagnostics) for liquid scintillation counting. The specific uptake of $^{22}Na^+$ was determined by subtracting nonspecific uptake obtained in the presence of 5 μM tetrodotoxin from total uptake.

Phosphoinositide Breakdown

Swiss 3T3 fibroblast cells (5×10^5 cells/well) were subcultured in 12-well plates in the presence of 10 mCi/mL *myo*-[^{3}H]inositol for 24 hr. Medium was removed by aspiration, and the cells were washed twice with incubation buffer (concentrations in mM: NaCl, 108; KCl, 4.7; LiCl, 10; $MgSO_4$, 1.2; KH_2PO_4, 1.2; EDTA, 0.5; glucose, 10; HEPES, 20; pH 7.4). Cells were then preincubated for 10 min at 37° in incubation buffer containing 3 mM $CaCl_2$. Agents were added, and incubation was continued for 30 min in a final volume of 0.5 mL. Incubation was stopped by the addition of 12% trichloroacetic acid. Cell homogenates were scraped and transferred to microfuge tubes for centrifugation at 12,000 *g* for 5 min. Anion exchange chromatography was performed to elute [^{3}H]inositol phosphates from the supernatants according to Berridge *et al.*[9].

Membrane Preparation

Brains from rats obtained from Pel Freez Biological (Rogers, AK) or from NIH Swiss mice were placed in ice-cold 50 mM Tris-HCl buffer (pH 7.4). Rat cerebral cortical tissue or whole brain from mice (including cerebellum) was homogenized using a Polytron (setting 6, 10 sec). The homogenate was centrifuged for 15 min at 35,000 *g* at 4°. The pellet was washed once by suspension and recentrifugation in Tris buffer. The final pellet was resuspended in the Tris buffer and stored at −70° until needed. Aliquots were suspended in the appropriate buffer and used for receptor binding assays.

Binding Assays

Membranes were diluted to a concentration of 1–5 mg/mL for binding assays. Protein concentrations were determined by the bicinchoninic acid protein assay (Pierce Chemical Co., Rockford, IL), using bovine albumin as a standard.

[^{3}H]Nicotine receptor binding was assayed with rat cerebral cortical membranes as described previously [10, 11] in 20 mM HEPES buffer (pH 7.4), containing 1 mM $MgCl_2$, 120 mM NaCl, 5 mM KCl, and 2 mM $CaCl_2$. Each assay contained the test agent, a suspension of the rat cerebral cortical membranes (100 μL containing 200–300 μg protein), 200 μM diisopropyl fluorophosphate, and 2 nM [^{3}H]nicotine in a final volume of 0.5 mL. Each assay was for 120 min at 0–4° and was performed in triplicate. Nonspecific binding was determined with 10 μM nicotine. Binding reactions were terminated by filtration through Whatman GF/B filters using a Brandel M24R Cell Harvester (Brandel, Gaithersburg, MD). Filters were washed twice with 5 mL of ice-cold buffer and placed in scintillation vials with 5 mL of Hydrofluor scintillation fluid, followed by counting for tritium. The filters were presoaked in 0.3% polyethylenimine to reduce nonspecific binding.

Muscarinic receptor binding was assayed with rat cerebral cortical membranes as previously described [12] in 20 mM HEPES buffer (pH 7.4), containing 100 mM NaCl and 10 mM $MgCl_2$. Each assay contained 5 μL of the test agent, a suspension of rat cerebral cortical membranes (100 μL containing 200–300 μg of protein), and 2 nM [^{3}H]quinuclidinyl benzilate in a final volume of 0.5 mL. Each assay was for 30 min at 37° and was performed in triplicate. Nonspecific binding was determined in the presence of 1 μM atropine. Filtration, washing, and scintillation counting were as described above for the [^{3}H]nicotine binding assay.

Binding of [^{3}H]DTG to sigma receptor was assayed using mouse brain membranes as previously described [13, 14] in 5 mM HEPPSO buffer (pH 8.1), containing 10 μM EDTA, 10 μM EGTA, 25 μg/mL chymostatin, and 25 μg/mL leupeptin. Each assay contained the test agent, a suspension of mouse brain membranes (100 μL containing 400–500 μg of protein) and 5 nM [^{3}H]DTG in a final volume of 1 mL. Each assay was for 120 min at 25° and was performed in triplicate. Nonspecific binding was determined with 50 μM haloperidol. Filtration, washing, and scintillation counting were as described above for the [^{3}H]nicotine binding assay. Filters were presoaked in 0.3% polyethylenimine to reduce nonspecific binding.

RESULTS

Ion Flux Assays

Concentration-response curves for the inhibition of carbamylcholine-elicited sodium-22 influx are shown for the spiropyrrolizidine oximes **236,** and **222** and for nitropolyzonamine in PC12 cells (ganglionic-type nicotinic receptor) and TE671 cells (muscle-type nicotinic receptor) in Fig. 2. Table 1 shows the IC_{50} values for these spiropyrrolizidines and other nicotinic antagonists in both cell lines. Spiropyrrolizidine oxime **236** was about 7-fold more potent than nitropolyzonamine at the muscle-type nicotinic receptor, but of equal potency at the ganglionic-type nicotinic receptor. Spiropyrrolizidine oxime **222** was about 5-fold more potent than nitropolyzonamine at the muscle-type nicotinic receptor, but less potent than either **236** or nitropolyzonamine at the ganglionic-type nicotinic receptor. Spiropyrrolizidine **236, 222** and nitropolyzonamine were more potent at the ganglionic-type receptor than at the muscle-type receptor. A dinitro byproduct in the synthesis of nitropolyzonamine was less potent than spiropyrrolizidine ox-

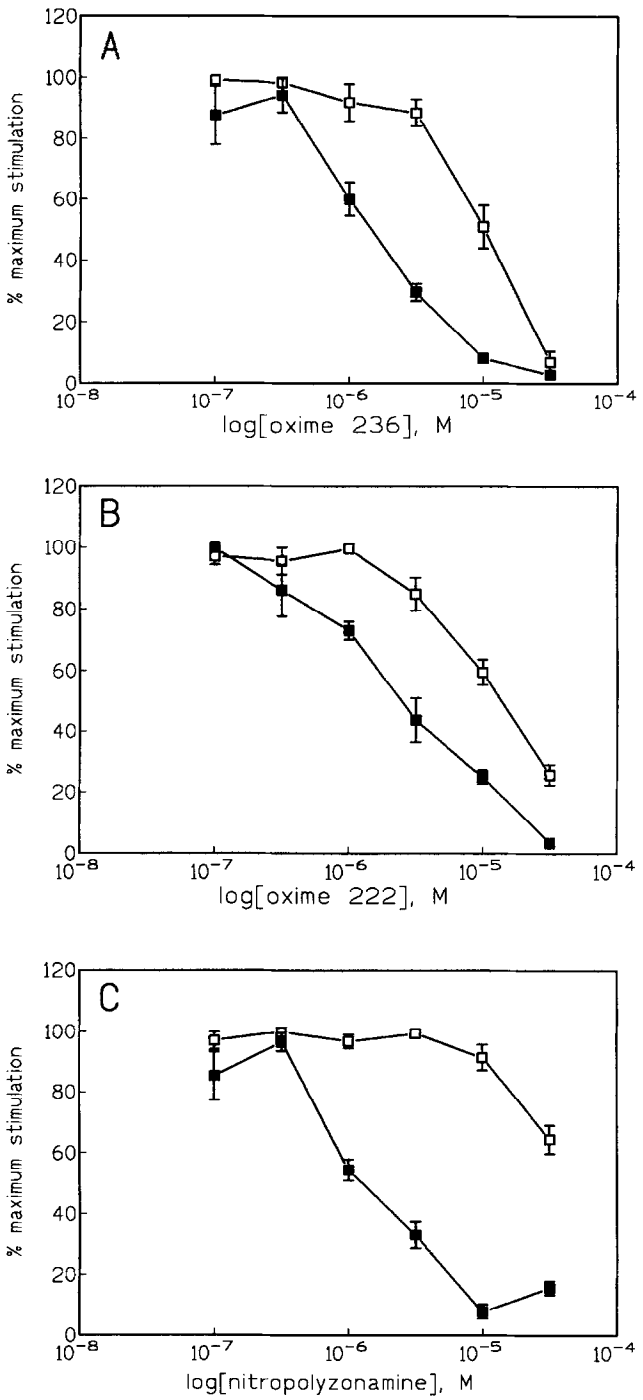

FIG. 2. Inhibition of carbamylcholine-elicited sodium-22 influx in rat pheochromocytoma PC12 cells (■) and human medulloblastoma TE671 cells (□) in the presence of (A) spiropyrrolizidine oxime 236, (B) spiropyrrolizidine oxime 222, and (C) nitropolyzonamine. Each value is reported as a percentage of stimulation obtained with 2 mM carbamylcholine and is the mean ± SEM of three experiments. Typically, 2 mM carbamylcholine-elicited sodium-22 influx results in 500–600 cpm/10,000 PC12 cells and 3,000–4,000 cpm/10,000 TE671 cells. Basal sodium-22 influx is 50–100 cpm/10,000 PC12 cells and 200–400 cpm/10,000 TE671 cells.

TABLE 1. Inhibition of carbamylcholine-elicited sodium-22 influx in cultured cells

	IC_{50} (μM)	
	PC12 cells	**TE671 cells**
Spiropyrrolizidine oxime **236**	1.5 ± 0.3	9.5 ± 1.4
Spiropyrrolizidine oxime **222**	2.6 ± 0.4	14 ± 2
Nitropolyzonamine	1.5 ± 0.2	67 ± 14
Dinitro byproduct	12 ± 2	29 ± 5
Allodihydrohistrionicotoxin	0.11 ± 0.04	0.15 ± 0.04
d-Tubocurarine	2 ± 0.8	0.4 ± 0.1
Dihydro-β-erythroidine	>100	>100

Assay was as described in Materials and Methods and was in the presence of 2 mM carbamylcholine. Values are means ± SEM of three experiments.

imes **236** and **222** at both receptors, but more potent than nitropolyzonamine at the ganglionic-type nicotinic receptor. Allodihydrohistrionicotoxin, a noncompetitive nicotinic antagonist, was more potent than the spiropyrrolizidines at both receptors. *d*-Tubocurarine, a competitive nicotinic antagonist at the neuromuscular nicotinic receptors, proved to be selective for the muscle-type receptor of TE671 cells, where it was many-fold more potent than the spiropyrrolizidines. At the ganglionic-type receptors of PC12 cells, *d*-tubocurarine was comparable in potency to the spiropyrrolizidines. β-Dihydroerythroidine, a competitive nicotinic antagonist, which is selective for central neuronal ($\alpha_4\beta_2$) nicotinic receptors, was, as expected, very weak at the muscle-type receptor of TE671 cells and the ganglionic-type receptor of PC12 cells.

The effects of various concentrations of the spiropyrrolizidine oxime **236**, nitropolyzonamine, allodihydrohistrionicotoxin, and *d*-tubocurarine on concentration–response curves for nicotine-elicited sodium-22 flux in PC12 and TE671 cells are shown in Figs. 3–5. Inhibitions of nicotine-elicited sodium-22 influx by spiropyrrolizidine oxime **236** and nitropolyzonamine appeared largely noncompetitive, since they were not overcome with increasing concentrations of the agonist nicotine (Fig. 3). The inhibitory effect of allodihydrohistrionicotoxin was, as expected for this noncompetitive antagonist, not overcome by increasing concentrations of nicotine (Fig. 4). *d*-Tubocurarine, a clas-

TABLE 2. Inhibition of [^{3}H]nicotine binding to rat cerebral cortical membrane

	K_i (μM)
Spiropyrrolizidine oxime **236**	–*
Nitropolyzonamine	–*
Dinitro byproduct	–*
Allodihydrohistrionicotoxin	32 ± 4
d-Tubocurarine	13 ± 1
Dihydro-β-erythroidine	0.02 ± 0.003

Assays are as described in Materials and Methods. Values are the means ± SEM of three experiments.

* The percent inhibition of [^{3}H]nicotine binding at 10 μM was only 9–10%.

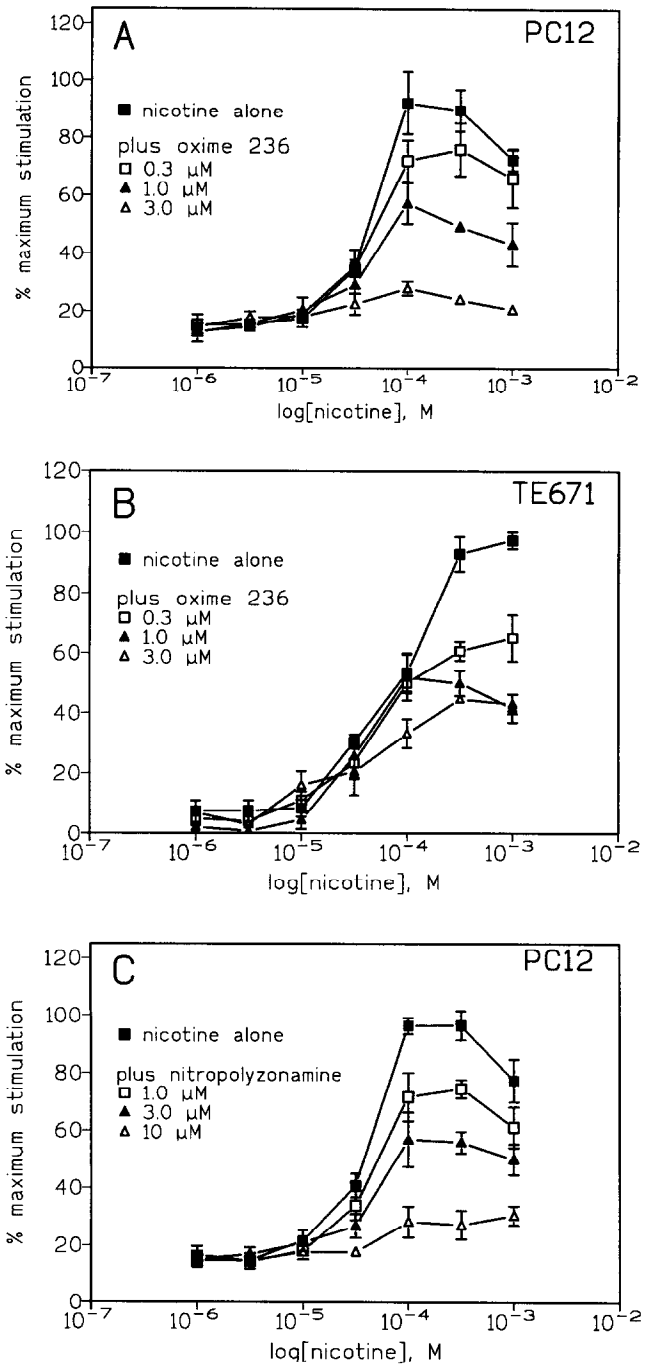

FIG. 3. Competition curves versus nicotine-elicited sodium-22 influx in the presence of pyrrolizidine oxime 236 (A and B) and nitropolyzonamine (C) in cultured cells. Each value is reported as a percentage of maximum stimulation obtained with nicotine and is the mean ± SEM of three experiments. Typically, maximum response to (-)-nicotine results in 1,000–1,500 cpm/10,000 PC12 cells and 1,000–2,000 cpm/10,000 TE671 cells. Basal sodium-22 influx is 50–100 cpm/10,000 PC12 cells and 200–400 cpm/10,000 TE671 cells.

sical competitive neuromuscular nicotinic antagonist, appeared competitive at the muscle-type nicotinic receptor, of TE671 cells, but not at the ganglionic-type nicotinic receptor of PC12 cells (Fig. 5).

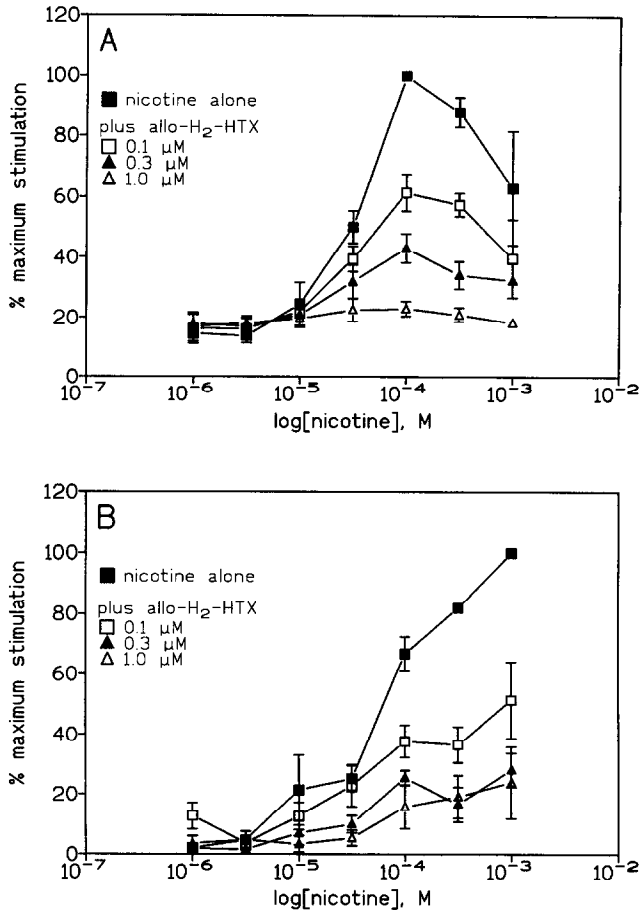

FIG. 4. Competition curves versus nicotine-elicited sodium-22 influx of allodihydrohistrionicotoxin (allo-H_2-HTX) in (A) rat pheochromocytoma PC12 cells, and (B) human medulloblastoma TE671 cells. Each value is reported as a percentage of maximum stimulation obtained with nicotine and is the mean ± SEM of three experiments. See legend of Fig. 3 for control values.

The spiropyrrolizidine oxime **236** at 10 μM had no effect on batrachotoxin-elicited sodium-22 influx in guinea pig synaptoneurosomes or on ATP-elicited calcium-45 influx in PC12 cells (data not shown). Much higher concentrations of the spiropyrrolizidine oxime **236** did inhibit the ATP-elicited influx of calcium-45 (IC_{50} value 180 ± 14 μM), which occurs through activation of a P_{2x}-type receptor channel in PC12 cells [15].

Binding Assays

Inhibition of binding of [^{3}H]nicotine to rat cerebral cortical membranes by the spiropyrrolizidine oxime **236**, nitropolyzonamine, allodihydrohistrionicotoxin, *d*-tubocurarine, and β-dihydroerythroidine were examined. The K_i values are shown in Table 2. Both the spiropyrrolizidine oxime **236** and nitropolyzonamine were weak inhibitors of [^{3}H]nicotine binding. Neither the spiropyrrolizidine oxime **236** nor nitropolyzonamine at 10 μM caused any inhibition of bind-

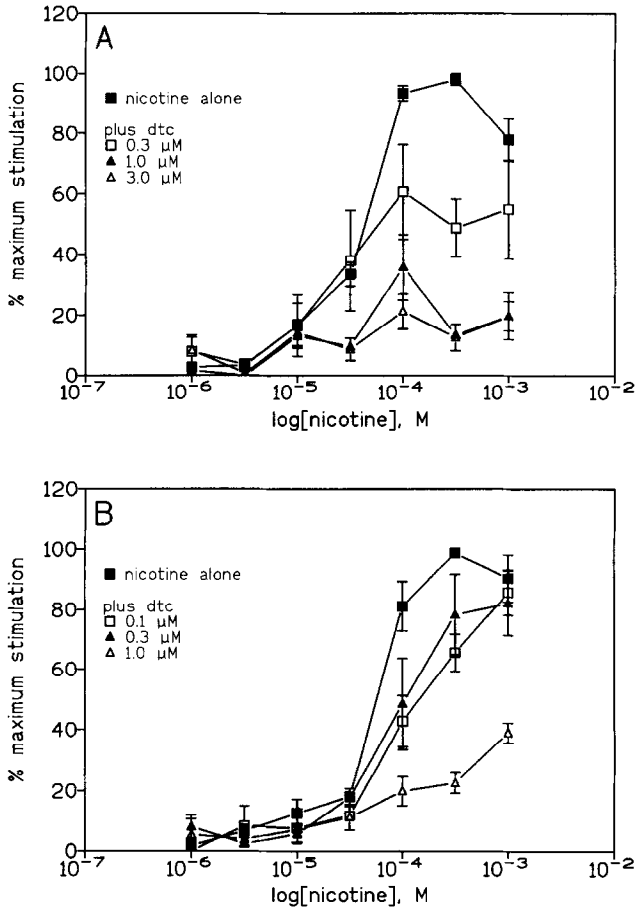

FIG. 5. Competition curves versus nicotine-elicited sodium-22 influx of *d*-tubocurarine (dtc) in (A) rat pheochromocytoma PC12 cells, and (B) human medulloblastoma TE671 cells. Each value is reported as a percentage of maximum stimulation obtained with nicotine and is the mean ± SEM of three experiments. See legend of Fig. 3 for control values.

ing of [^{3}H]quinuclidinyl benzilate to muscarinic receptors (data not shown).

Nitropolyzonamine was assayed in the NIMH NOVA Screen (Hanover, MD) at 10 μM. No significant effect was seen at a variety of receptors including those for dopamine, GABA, serotonin, glutamate, glycine, substance P, vasoactive intestinal peptide, and angiotensin or at the nifedipine-binding sites that are associated with L-type calcium channels. Nitropolyzonamine at 10 μM did inhibit binding of Tyr-4-bombesin to brain bombesin receptors by about 50%. In Swiss 3T3 cells, 10 μM nitropolyzonamine caused only a marginal (20%) inhibition of bombesin-elicited phosphoinositide breakdown, and had no effect alone (data not shown).

Both the spiropyrrolizidine oxime **236** and nitropolyzonamine inhibited binding of [^{3}H]DTG to sigma receptors with K_i values of 0.53 ± 0.20 μM (Hill coefficient 0.77 ± 0.06) and 0.43 ± 0.13 μM (Hill coefficient 0.39 ± 0.06), respectively. The low Hill coefficient for nitropolyzonamine suggests multiple sites of interaction with [^{3}H]DTG binding or multiple binding sites for [^{3}H]DTG.

DISCUSSION

The spiropyrrolizidine oximes **236** and **222** and a structurally related spiropyrrolizidine alkaloid, nitropolyzonamine, represent relatively rigid molecules differing only in the replacement of a polar O-methyl oxime or oxime function in **236** or **222** with a less polar nitro function in nitropolyzonamine. The spiropyrrolizidine oximes **236** and **222** have been found in alkaloid fractions from skin extracts of the Central American poison-frog *Dendrobates pumilio* [16], whereas nitropolyzonamine occurs, along with another alkaloid, polyzonimine, as a defensive substance in a North American millipede [17]. These spiropyrrolizidine alkaloids have now been found to block carbamylcholine- and nicotine-elicited ion flux in cultured cells (Figs. 2–5). Analysis of the nature of the blockade indicates it to be primarily noncompetitive, since it is not overcome with high concentrations of the agonist nicotine (Fig. 3). Thus, these compounds represent a new and novel class of noncompetitive blockers of nicotinic receptor channels. Spiropyrrolizidine oxime **236** and nitropolyzonamine at 1–100 μM had no effect alone on sodium influx in the two cell lines (data not shown). Based on activity in PC12 and TE671 cells, the spiropyrrolizidine oximes and to a much greater extent nitropolyzonamine are selective for the ganglionic-type, as opposed to neuromuscular-type nicotinic receptor channels (Table 1). In contrast, a histrionicotoxin showed no selectivity toward either subtype of nicotinic receptor (Table 1) and, as expected [18], was a noncompetitive blocker (Fig. 4). The classical competitive nicotinic antagonist, *d*-tubocurarine, while showing competitive blockade, as expected, at the neuromuscular-type receptor (Fig. 5B), appeared to cause an insurmountable noncompetitive blockade at the ganglionic-type receptor (Fig. 5A). *d*-Tubocurarine has been reported to have some noncompetitive blocking actions, even in neuromuscular preparations [19, 20].

The spiropyrrolizidines, namely the spiropyrrolizidine oximes **236** and **222** and nitropolyzonamine, provide yet another class of noncompetitive blockers of nicotinic receptors. Other classes of such noncompetitive blockers include the phenothiazines [21], the histrionicotoxins (spiropiperidines) [18, 22], phencyclidine [23], amantadine [24], and various local anesthetics [25]. Many of these are relatively lipophilic molecules with great flexibility in the structure or in the side chains. The structures of these noncompetitive blockers are in marked contrast to the present spiropyrrolizidine alkaloids, which are relatively rigid and, at least in the case of the oxime, relatively polar. Many, if not all, of the other noncompetitive nicotinic blockers also block voltage-sensitive ion channels, and thus have local anesthetic activity. The spiropyrrolizidine oxime **236** had no effect on activation of voltage-dependent sodium channels and only marginal (IC_{50} 180 μM) effects on P_{2x}-type receptor channels in PC12 cells (see Results). Further electrophysiological studies on the nature of the blockade (open versus closed channel and effect on desensitization) by the spiropyrrolizidines are warranted. The lack of significant

activity of the pyrrolizidine oxime **236** and nitropolyzonamine versus [^{3}H]nicotine binding to central $\alpha_4\beta_2$ nicotinic receptors is not surprising if they are noncompetitive blockers at these central receptors. Further functional studies are needed at central neuronal $\alpha_4\beta_2$ and α_7 nicotinic receptors.

The pyrrolizidine oxime **236** and nitropolyzonamine both have significant activity (K_i = 0.5 μM) in inhibiting binding of [^{3}H]DTG to central sigma receptors, albeit much lower activity than many of the very potent sigma ligands, which have K_i values in the nanomolar range [13, 26]. It is noteworthy that a variety of such sigma ligands inhibit nicotinic receptor (ganglionic) function in adrenal chromaffin cells [27]. Nitropolyzonamine and presumably the pyrrolizidine oxime **236** had no effect on ligand binding at a variety of other receptors, although weak effects on bombesin receptors were noted (see Results). Thus, spiropyrrolizidines represent new and relatively selective probes for investigation of the diverse set of nicotinic receptor channels involved in peripheral and central functions.

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