MOLECULAR PHARMACOLOGY, 28:10–16

Interactions of Microtubule-Active Agents with Nicotinic Acetylcholine Receptors

Relationship to Their Inhibition of Catecholamine Secretion by Adrenal Chromaffin Cells

DENNIS B. MCKAY,[1] ROBERT S. ARONSTAM, AND ALLAN S. SCHNEIDER

Sloan-Kettering Institute for Cancer Research and Cornell University Graduate School of Medical-Sciences, New York, New York 10021 (D.B.M., A.S.S.) and the Department of Pharmacology and Toxicology, Medical College of Georgia, Augusta, Georgia 30912 (R.S.A.)

Received June 6, 1984; Accepted April 2, 1985

SUMMARY

Several microtubule-active drugs block cholinergically mediated catecholamine secretion from adrenal chromaffin cells without affecting secretion induced by other secretagogues. Interactions of these agents with nicotinic acetylcholine receptor-ion channel complexes from *Torpedo californica* electric organs were studied using radiolabeled probes for receptor and associated ion channel-binding sites. Colchicine, taxol, and the *Vinca* alkaloids had minimal affinity for cholinergic receptor-binding sites (nicotinic or muscarinic). The *Vinca* alkaloids (vinblastine, vincristine, vindesine) and colchicine inhibited [^{3}H]perhydrohistrionicotoxin ([^{3}H]H_{12}-HTX) binding to the receptor-gated ion channel with IC_{50} values of 2–32 μM and 6 mM, respectively. The ability of the microtubule-active drugs to inhibit [^{3}H]H_{12}-HTX binding was increased by up to 5-fold in the presence of 1 μM carbamylcholine. The IC_{50} values for inhibition of [^{3}H]H_{12}-HTX binding by colchicine and three *Vinca* alkaloids were closely correlated with their abilities to inhibit acetylcholine-induced catecholamine secretion from cultured bovine adrenal chromaffin cells. As a consequence of its interaction (direct or indirect) with the ion channel, at least one *Vinca* alkaloid (vinblastine) stabilized a high agonist affinity conformation of the nicotinic receptor complex. β-Lumicolchicine, an analog of colchicine devoid of microtubule activity, also blocked ion channel binding. On the other hand, taxol, a microtubule-stabilizing agent which also selectively blocks cholinergically mediated secretion, did not affect receptor or ion channel binding. The present results indicate that interactions with the nicotinic receptor-ion channel complex may underlie the actions of certain microtubule-active agents on catecholamine secretion by adrenal chromaffin cells.

INTRODUCTION

Microtubule-active drugs block secretory processes in a variety of systems (for a review, see Ref. 1). In adrenal chromaffin cells, antimitotic agents, including colchicine, taxol, and the *Vinca* alkaloids, inhibit the catecholamine secretion that is induced by nicotinic cholinergic agonists (2–7). In contrast, chromaffin cell secretion induced by KCl, histamine, bradykinin, barium, or veratridine is not affected by these agents (3–7). This selective inhibition of cholinergically mediated secretion raises the possibility that the inhibition is a consequence of interactions with cholinergic receptor complexes rather than with microtubular structures. An alternate possibility is that microtubules or membrane tubulin plays an active role in maintaining cholinergic receptor organization and function in plasma membranes. Anticholinergic actions of microtubule-active drugs at both ganglionic (4) and skeletal muscle (8) nicotinic receptors have been described. Catecholamine secretion by bovine adrenal chromaffin cells is stimulated by activation of nicotinic acetylcholine receptors (5). Stimulation of the muscarinic receptors associated with these cells (9) elevates cyclic GMP (10) and cytosolic Ca^{2+} (11) induces phospholipid labeling (12), but does not stimulate secretion (5, 12).

In the present study, we investigated the interactions of taxol, colchicine, and *Vinca* alkaloids with nicotinic ACh[2] receptors from the electric organs of a marine

This research was supported by National Institutes of Health Grants AM-31178, T32-AM-07313, NS-17429, and DA-03303 and by American Cancer Society Grant BC346.

[1] Present address, Ohio State University, College of Pharmacy, Division of Pharmacology, Columbus, OH 43210-1291.

[2] The abbreviations used are: ACh, acetylcholine; Bgt, α-bungarotoxin; HTX, histrionicotoxin; H_{12}-HTX, perhydrohistrionicotoxin; MS, *N*-methylscopolamine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

0026-895X/85/010010–07$02.00/0

electric ray, *Torpedo californica*, using radiolabeled probes for receptor (i.e., ACh) as well as extrareceptor (allosteric or "ion channel") binding sites. These interactions were compared with the effects of the microtubule-active drugs on catecholamine secretion by adrenal chromaffin cells. While none of the microtubule-active drugs interfered with receptor binding of ^{125}I-Bgt, the microtubule-destabilizing agents colchicine, vinblastine, vincristine, and vindesine inhibited [^{3}H]H_{12}-HTX binding to receptor ion channel sites at the same concentrations at which they inhibited calcium uptake and catecholamine secretion by cultured chromaffin cells (7). Thus, the influences of these drugs on ACh-induced chromaffin cell secretion may reflect direct interactions with nicotinic receptor complexes or alterations of receptor-tubulin/microtubule interactions by the drugs. A preliminary account of these results has been presented elsewhere (13).

MATERIALS AND METHODS

Materials. ACh, colchicine, β-lumicolchicine, and vinblastine sulfate were purchased from Sigma Chemical Company (St. Louis, MO). Taxol was obtained from the National Products Branch, Division of Cancer Treatment, National Cancer Institute (Bethesda, MD) and dissolved in dimethylsulfoxide. Vinblastine, vincristine, and vindesine were provided by the Eli Lilly Corp. (Indianapolis, IN). DL-[7-^{3}H]Norepinephrine (10 Ci/mmol), [*N-methyl*-^{3}H]-scopolamine methyl chloride (53.3 Ci/mmol), and ^{125}I-Bgt (10–20 Ci/μg) were purchased from New England Nuclear Corporation (Boston, MA). [^{3}H]H_{12}-HTX (approximately 55 Ci/mmol) was provided by Dr. John W. Daly of the National Institutes of Health. All other reagents were of analytical grade.

Chromaffin cell isolation and maintenance. Bovine adrenal chromaffin cells were isolated by methods previously described (5). The experiments described here were performed on either freshly isolated chromaffin cells or chromaffin cells maintained in primary culture as previously described (7, 14).

Catecholamine secretion studies. For the secretion studies, the cell incubation medium was a physiological salt solution consisting of 137 mM NaCl, 4.4 mM KCl, 1.2 mM KH_2PO_4, 3.6 mM $NaHCO_3$, 1.2 mM $MgSO_4$, 10 mM glucose, 2 mM $CaCl_2$, 0.5% bovine serum albumin, 100 μM neostigmine, and 5 mM HEPES (pH 7.2–7.4). Cells were preincubated for 15–30 min with various concentrations of the drugs to be tested. The cells were then stimulated in the presence of the drug for 10–15 min with either 0.1 mM ACh or 65 mM K^+. When 65 mM K^+ was used to stimulate catecholamine secretion, the Na^+ content of the buffer was decreased to maintain isotonicity. Two methods were used to measure catecholamine secretion. The trihydroxyindole fluorometric method was used to measure total catecholamine content (15, 16). Cells previously loaded with tritiated norepinephrine were used to monitor release (7, 14). In all experiments, the amount of radioactivity released by a nonstimulated group was considered background and was subtracted from all groups. Results are expressed as percentage of catecholamine released relative to control (drug-free) groups. Control and test samples were run in quadruplicate for each determination. Catecholamine release stimulated by 0.1 mM ACh represented 25–30% of the total cell catecholamine content. For the secretion studies summarized in Table 1, complete concentration-response relationships were determined (data not shown) from which the IC_{50} values were extracted.

ACh receptor-binding studies. Electric organs from *T. californica* were purchased from Pacific Bio-Marine (Venice, CA) and stored in liquid nitrogen. To obtain a crude particulate fraction, tissue samples were homogenized using a glass Waring blender in 5 volumes of 50 mM Tris-HCl, pH 7.4, containing 0.1 mM phenylmethylsulfonyl fluoride to prevent proteolysis. The homogenate was filtered through cheesecloth to remove collagenous and undisrupted material, and then centrifuged at 20,000 × *g* for 20 min. The resulting pellet was suspended in 50 mM Tris-HCl, pH 7.4, at a concentration of 1–2 mg of protein/ml. Protein content was determined by the method of Lowry *et al.* (17).

To measure receptor binding, an aliquot of the suspended tissue (10–15 μg of protein) was added to an incubation medium containing 50 mM Tris-HCl, pH 7.4, and 5 nM ^{125}I-Bgt (final volume = 1 ml). After 20 min at room temperature, the reaction was quenched by addition of 0.5 ml of 10 mg/ml methylated bovine serum albumin (Sigma Chemical Co.). The suspensions were then filtered through Whatman GF/B glass fiber filters which had been soaked in the methylated albumin solution. The filters were washed twice with 5 ml of Tris buffer, and their radioactivity content was determined by γ scintillation counting. Nonspecific binding was determined in the presence of 100 μM nicotine. In drug competition experiments, the competing ligand (a microtubule-active agent or carbamylcholine) was incubated with the tissue for 20 min before the addition of ^{125}I-Bgt.

To measure steady state binding to ion channel sites, an aliquot of the suspended tissue (40–60 μg of protein) was added to an incubation medium containing 2 nM [^{3}H]H_{12}-HTX and Tris-HCl buffer in a final volume of 1 ml. Parallel experiments were run in the presence and absence of 1 μM carbamylcholine, which strongly influences ion channel-binding properties (18). Nonspecific binding was determined in the presence of 10 μM phencyclidine, a potent channel blocker (19). After a 60-min incubation at room temperature, a steady state level of binding was attained (18) and the suspensions were filtered through Whatman GF/B filters which had been wetted with a 1% organosilane preparation (Sigmacote, Sigma Chemical Co.) to reduce [^{3}H]H_{12}-HTX binding to the filters. The filters were washed twice with 5 ml of Tris buffer and their radioactivity content was determined by liquid scintillation counting.

[^{3}H]MS was used as a probe of muscarinic acetylcholine receptors from rat brain. A crude synaptosomal-microsomal pellet was prepared from the cerebral cortex as previously described (20). An aliquot (100 μg of protein) of suspended tissue was added to incubation media containing 0.1 nM [^{3}H]MS, 50 mM Tris-HCl, pH 7.4, and any drugs as required by the experiment. After a 45-min incubation at room temperature, equilibrium had been reached and the media were filtered through Whatman GF/B filters. The filters were washed and their radioactivity content was determined by liquid scintillation counting. Nonspecific binding was determined in the presence of 10 μM atropine.

RESULTS

Interactions of microtubule-active agents with nicotinic and muscarinic ACh receptors. Since microtubule-active drugs specifically inhibit cholinergically mediated catecholamine secretion by isolated chromaffin cells (7), the effects of these drugs on radiolabeled ligand binding to nicotinic and muscarinic receptors were assessed. None of the drugs inhibited the binding of 5 nM ^{125}I-Bgt to the nicotinic receptor of *Torpedo* electric organ by more than 25% (Fig. 1A), even at concentrations at which they inhibit most catecholamine secretion (Figs. 2 and 3 in Ref. 7).

The *Vinca* alkaloids and taxol were weak inhibitors of [^{3}H]MS binding to muscarinic receptors from rat cerebral cortex (Fig. 1B). All of the microtubule-active agents inhibited muscarinic binding by less than 20% at a concentration at which they inhibit most catecholamine secretion. Thus, the influence of microtubule-active agents is unlikely to be due to direct interactions with ACh-binding sites on either nicotinic or muscarinic receptors.

Interactions of microtubule-active agents with the ion channel of nicotinic ACh receptors. The microtubule-destabilizing agents colchicine and the *Vinca* alkaloids

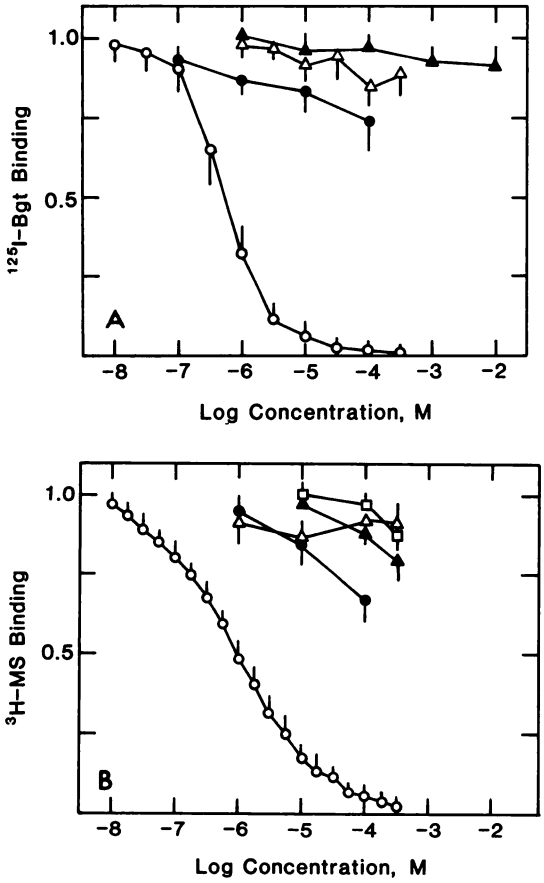

FIG. 1. *Influence of microtubule-active agents on ligand binding to nicotinic and muscarinic acetylcholine receptors*

The binding of the radiolabeled probes is expressed as fraction of total specific binding measured in the absence of competing ligands. Each point and bar represent the mean and standard deviation from three independent measurements. A, the binding of 5 nM ^{125}I-Bgt to nicotinic ACh receptors from *Torpedo* electric organ was measured in the presence of the following drugs: carbamylcholine, ○; taxol, ●, vinblastine, △; colchicine, ▲. B, the binding of 100 pM [^{3}H]MS to muscarinic ACh receptors from rat cerebral cortex was measured in the presence of the following drugs; carbamylcholine, ○, vindesine, ●; vincristine, △; vinblastine, ▲; taxol, □. Control = 1850 cpm.

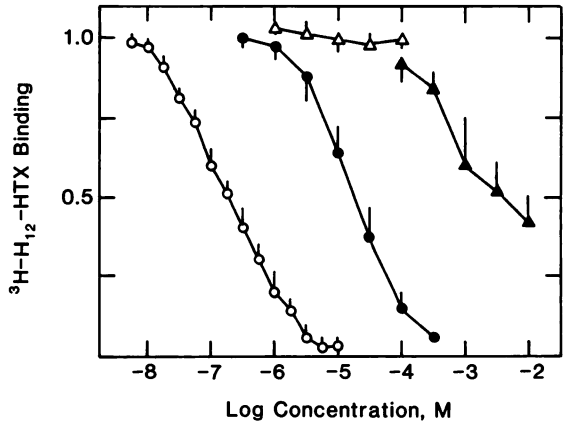

FIG. 2. *Influence of microtubule-active agents on [^{3}H]H_{12}-HTX binding to nicotinic ACh receptor-channel complexes*

The binding of 2 nM [^{3}H]H_{12}-HTX to ion channels associated with nicotinic ACh receptors in membranes prepared from *T. californica* electric organs was measured in the presence of the indicated concentrations of unlabeled H_{12}-HTX (○), vinblastine (●), colchicine (▲), and taxol (△). Binding is expressed as fractional specific (i.e., phencyclidine-sensitive) [^{3}H]H_{12}-HTX binding measured in the absence of competing ligands. No receptor ligands were included in the incubation media. Each point and bar represent the mean and standard deviation from three measurements each performed in triplicate. Control binding = 3750 cpm.

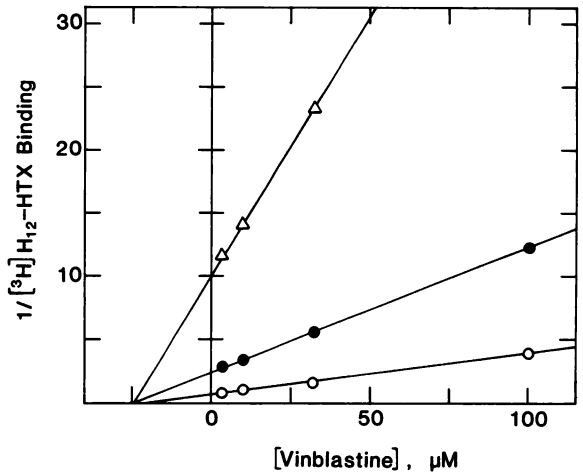

FIG. 3. *Dixon plot of vinblastine inhibition of [^{3}H]H_{12}-HTX binding to ion channel sites*

The binding of 0.5, 1, and 2 nM [^{3}H]H_{12}-HTX to *Torpedo* electric organ was measured in the presence of five concentrations of vinblastine from 3.2 to 100 μM. Carbamylcholine was not included in the binding media. Each point represents the mean from three experiments which varied by less than 15% of control binding measured in the absence of vinblastine. Lines are drawn according to linear regression analysis.

inhibited [^{3}H]H_{12}-HTX binding to sites associated with the ion channel in nicotinic receptor complexes from *Torpedo* electric organ (Fig. 2; Table 1). The *Vinca* alkaloids inhibited 2 nM [^{3}H]H_{12}-HTX binding with IC_{50} values between 2 and 32 μM. Colchicine and β-lumicolchicine were much less potent inhibitors of [^{3}H]H_{12}-HTX binding (IC_{50} = 5.6 mM; Fig. 2). However, taxol, a microtubule-stabilizing agent, did not inhibit [^{3}H]H_{12}-HTX binding at all, even when present at the limits of its solubility (100 μM). Essentially similar results were obtained using [^{3}H]phencyclidine as the ion channel probe (data not presented).

Vinblastine did not appear to be a simple competitive inhibitor of [^{3}H]H_{12}-HTX binding: Dixon plots of [^{3}H]H_{12}-HTX binding measured in the presence of five concentrations of vinblastine from 3.2 to 100 μM intersected on the x axis, indicating a K_i of 16–23 μM (Fig. 3). Vinblastine effects were reversible; the fractional inhibition of 2 nM [^{3}H]H_{12}-HTX by 100 μM vinblastine decreased from 85 to 35% after washing the tissue twice by centrifugation. Allosteric interactions were not readily apparent insofar as vinblastine did not alter the rate of dissociation of [^{3}H]H_{12}-HTX induced by a saturating concentration of HTX.

Receptor and ion channel-binding sites are allosterically linked. Access to ion channel-binding sites appears to be regulated by receptor ligands. In the presence of 1 μM carbamylcholine, a potent agonist, the ability of

TABLE 1

Inhibition of catecholamine secretion and nicotinic receptor complex binding by microtubule-active drugs

The IC_{50} values for inhibition of catecholamine secretion by adrenal chromaffin cells and for inhibition of 5 nM ^{125}I-Bgt and 2 nM [^{3}H]H_{12}-HTX binding to nicotinic receptor complexes from *Torpedo* electric organ are presented. [^{3}H]H_{12}-HTX binding was measured in the absence and presence of 1 μM carbamylcholine (Carb), as indicated. Each value represents the mean ± standard deviation from three to five separate determinations. ND, not determined. All values are inhibitory concentrations, IC_{50}, in μM.

Drug	ACh-induced Catecholamine Secretion	Receptor Binding		
		^{125}I-Bgt	[^{3}H]H_{12}-HTX	[^{3}H]H_{12}-HTX/Carb
Taxol	2	100	100	100
Vinblastine	2	320	14 ± 2	4.7 ± 0.3
Vincristine	10	320	32 ± 4	6.0 ± 0.7
Vindesine	0.5	320	2.0 ± 0.3	0.8 ± 0.1
Colchicine	500	1000	5600 ± 400	3980 ± 340
β-Lumicolchicine[a]	ND	1000	5500 ± 700	3200 ± 500

[a] β-Lumicolchicine is not a microtubule-active agent.

Vinca alkaloids to inhibit [^{3}H]H_{12}-HTX binding was increased 2.5–5.3-fold (e.g., vinblastine in Fig. 4; Table 1). The ability of colchicine and β-lumicolchicine to inhibit ion channel binding was increased to a somewhat lesser extent (1.4- and 1.7-fold, respectively; Table 1). These effects on carbamylcholine affinity cannot be ascribed to an effect of the agonist on H_{12}-HTX binding since carbamylcholine also increases H_{12}-HTX affinity for the ion channel (17).

A complementary influence of *Vinca* alkaloids and colchicine on the affinity of carbamylcholine binding to the receptor was also observed. Thus, the ability of carbamylcholine to inhibit ^{125}I-Bgt binding was enhanced 5-fold in the presence of 10 μM vinblastine (Fig. 5). This effect of vinblastine was concentration-dependent; no enhancement of carbamylcholine affinity was observed in the presence of 1 μM vinblastine, while the effect increased in a graded fashion as the vinblastine concentration was increased from 3 to 10 to 30 μM.

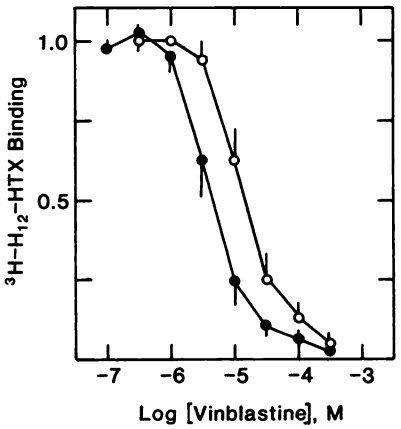

FIG. 4. *Influence of a receptor agonist on vinblastine interactions with ion channel sites in nicotinic ACh receptor complexes*

The binding of [^{3}H]H_{12}-HTX to ACh receptors on *Torpedo* electric organ membranes was measured in the presence of the indicated vinblastine concentrations in the absence (○) and presence (●) of 1 μM carbamylcholine. Binding is expressed as the fraction of total specific [^{3}H]H_{12}-HTX binding measured in the absence of vinblastine. Each point and bar represent the mean and standard deviation from three experiments. Specific binding to control samples was 10,200 and 18,700 cpm in the absence and presence of 1 μM carbamylcholine, respectively.

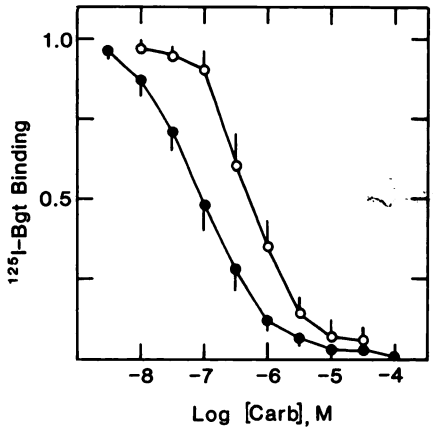

FIG. 5. *Influence of vinblastine on carbamylcholine binding to the nicotinic ACh receptor from Torpedo electric organ*

The binding of 5 nM ^{125}I-Bgt to the ACh receptor was measured in the presence of the indicated concentrations of carbamylcholine (*Carb*) in the absence (○) or presence (●) of 10 μM vinblastine. Binding is expressed as the fraction of specific (i.e., nicotine-sensitive) ^{125}I-Bgt binding measured in the absence of carbamylcholine. This control binding level (32,000 cpm) was not affected by 10 μM vinblastine. Each point and bar represent the mean and standard deviation from three experiments.

The *Vinca* alkaloids tested differed by up to 16-fold in their ability to inhibit [^{3}H]H_{12}-HTX binding in the absence of carbamylcholine, and by up to 7-fold in the presence of 1 μM carbamylcholine (Fig. 6; Table 1). Ranking according to their ability to inhibit [^{3}H]H_{12}-HTX binding, the following series was obtained: vindesine > vinblastine > vincristine. The same series was obtained in the presence or absence of 1 μM carbamylcholine. The ability of carbamylcholine to enhance *Vinca* alkaloid inhibition of [^{3}H]H_{12}-HTX binding was inversely related to the potency of the alkaloids at inhibiting [^{3}H]H_{12}-HTX binding in the absence of carbamylcholine. There is also a high correlation between the ability of microtubule-destabilizing agents to inhibit [^{3}H]H_{12}-HTX binding to the ion channel and their ability to inhibit catecholamine secretion by isolated chromaffin cells (Table 1; Fig. 7).

Influence of HTX and microtubule-active agents on catecholamine secretion by bovine adrenal chromaffin

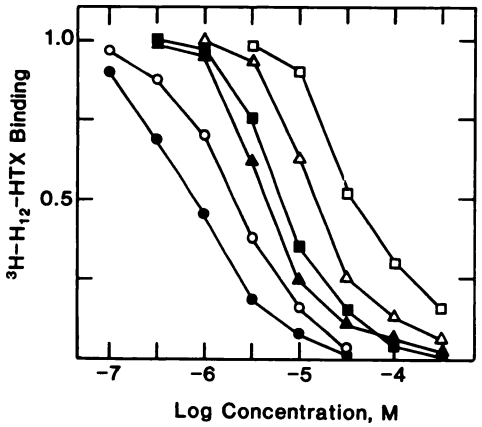

FIG. 6. *Inhibition of [^{3}H]H_{12}-HTX binding to Torpedo electric organ membranes by various Vinca alkaloids*

The binding of 2 nM [^{3}H]H_{12}-HTX to sites on the nicotinic ACh receptor-ion channel complex was measured in the presence of the indicated concentrations of vindesine (circles), vinblastine (triangles), and vincristine (squares), in the absence (open symbols) and presence (closed symbols) of 1 μM carbamylcholine. Binding is expressed as fraction of specific binding measured in the absence of any *Vinca* alkaloid. Each point represents the mean from three triplicate determinations which generally varied by less than 15% of the control binding value (10,600 and 19,250 cpm in the absence and presence of 1 μM carbamylcholine, respectively).

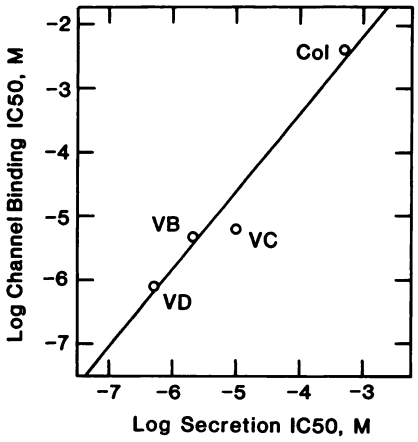

FIG. 7. *Comparison of the effects of colchicine and three Vinca alkaloids on catecholamine secretion by cultured chromaffin cells and [^{3}H]H_{12}-HTX binding to ACh receptor-channel complexes from Torpedo electric organ*

The log of the IC_{50} value for inhibition of [^{3}H]H_{12}-HTX binding in the presence of carbamylcholine (1 μM) is plotted as a function of the log of the IC_{50} value for inhibition of ACh-induced catecholamine secretion for colchicine (*Col*), vinblastine (*VB*), vincristine (*VC*), and vindesine (*VD*). Linear regression analysis indicated a strong correlation ($r = 0.97$) between these two measures.

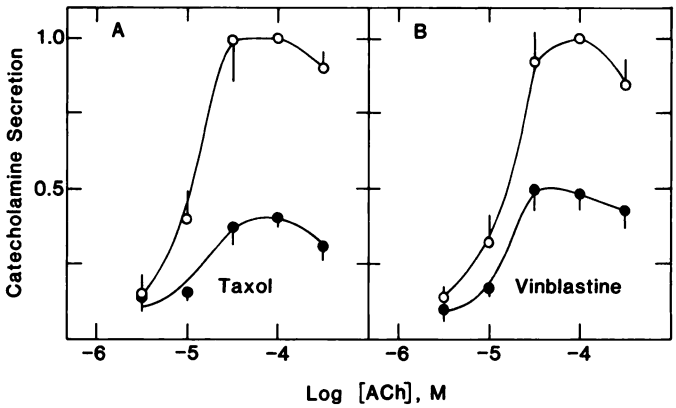

FIG. 8. *Concentration-response effects of ACh on catecholamine secretion from bovine adrenal cells in the absence and presence of taxol and vinblastine*

Adrenal chromaffin cells were preincubated for 5 min in the absence (○) or presence of either taxol or vinblastine (●). The cells were then stimulated for 10 min with varying concentrations of ACh in the presence of the same microtubule drug as in the preincubation period. Results are expressed as percentage of catecholamine secretion obtained with 0.1 mM ACh in the absence of any microtubule drug.

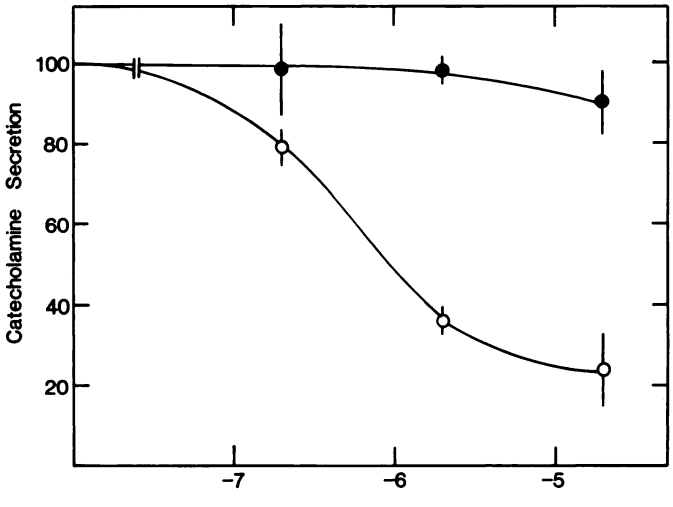

FIG. 9. *Influence of histrionicotoxin on ACh- and K$^+$-induced catecholamine secretion from bovine adrenal chromaffin cells*

Suspensions of chromaffin cells were incubated for 30 min with the indicated concentrations of histrionicotoxin. The cells were then stimulated for 15 min with 0.1 mM ACh (○) or 65 mM K^+ (●) in the continued presence of histrionicotoxin. Catecholamine secretion is expressed as percentage of catecholamine secretion obtained in the absence of histrionicotoxin. Each point and bar represent the mean and standard error from three to five experiments.

cells. To establish the nature of the inhibitory actions of vinblastine and taxol in the adrenal chromaffin system, the concentration-response relationship of ACh-induced stimulation of adrenal catecholamine secretion was determined in the absence and presence of the microtubule drugs (Fig. 8). In the absence of microtubule drugs, maximum stimulation occurred with about 50 μM ACh; half-maximum stimulation occurred with 10 μM ACh. In the presence of a fixed concentration of either vinblastine or taxol, no change in the concentration of ACh eliciting half-maximum response was seen; however, the maximum amount of secretion obtained was decreased (Fig. 8).

HTX did not inhibit catecholamine secretion by isolated chromaffin cells induced by a concentration of K^+ (65 mM) that depolarizes the cell (Fig. 9). In contrast, secretion stimulated by 0.1 mM ACh was inhibited with an IC_{50} of 1 μM (Fig. 9). This value agrees well with the affinity of HTX for [^{3}H]H_{12}-HTX-binding sites on the

nicotinic receptor ion channel ($K_i = 0.63$).[3] HTX and its reduced analog, H_{12}-HTX, have similar influences on nicotinic ACh receptor function.

DISCUSSION

Microtubule involvement in secretion is indicated in a number of different systems by the effects of microtubule-active agents on secretory responses (1). The *Vinca* alkaloids, colchicine, and taxol inhibit cholinergically mediated secretion by chromaffin cells. Poisner and Bernstein (2) postulated a role for microtubules in catecholamine secretion from adrenal chromaffin cells. However, it was subsequently demonstrated that these microtubule-active drugs do not inhibit secretion evoked by other secretagogues (3–7). For instance, K^+-induced catecholamine secretion and $^{45}Ca^{2+}$ uptake by chromaffin cells are not inhibited by taxol and vinblastine, while ACh-induced secretion and uptake are inhibited (7). This indicates that taxol and vinblastine affect a mechanism that is active before membrane depolarization and opening of voltage-sensitive Ca^{2+} channels. Since veratridine-induced secretion is not affected by the microtubule drugs (7), the site of action is not tetrodotoxin-sensitive Na^+ channels. The selectivity of these microtubule-active drugs for cholinergically mediated secretion suggests an action on some aspect of cholinergic receptor function in the chromaffin cell.

To investigate the interactions of microtubule-active agents with nicotinic ACh receptors, *Torpedo* electric organs were used as a plentiful and homogeneous source of nicotinic receptors. While these receptors are very similar in biochemical and physiological properties to those at the mammalian neuromuscular junction, some caution should be exercised when generalizing these findings to processes in chromaffin cells. The relatively low density of cholinergic receptors precluded direct measurements of [^{3}H]H_{12}-HTX binding in isolated chromaffin cells.

H_{12}-HTX and HTX are noncompetitive blockers of neuromuscular transmission. They bind with high affinity to a site that appears to be located within the central hydrophilic depression of the nicotinic receptor complex and which is probably the site of ion translocation gated by cholinergic agonists (21). As a consequence of this binding, a desensitized conformation of the receptor complex, characterized by a high affinity for receptor agonists, is stabilized (22, 23). In the presence of receptor agonists, the histrionicotoxins may sterically block ion fluxes as well as influence receptor conformation (24). Conversely, occupancy of receptor-binding sites alters [^{3}H]H_{12}-HTX binding to ion channel sites. In the absence of cholinergic receptor ligands, [^{3}H]H_{12}-HTX binding approaches equilibrium quite slowly (18). Binding is greatly acclerated in the presence of receptor ligands. This has been interpreted as indicating severe steric restrictions of [^{3}H]H_{12}-HTX access to ion channel-binding sites (18, 23). Apparently, these restrictions are eliminated upon assumption of activated (and/or desensitized) conformations by the receptor.

[3] R. S. Aronstam, C. T. King, E. X. Albuquerque, J. W. Daly, and D. M. Feigl, unpublished results.

In the present study, the properties of the *Vinca* alkaloids and colchicine were similar to those of a number of other ion channel blockers. All of the microtubule-active agents have little or no effect on ligand interactions with the ACh-binding sites of nicotinic or muscarinic receptors. The *Vinca* alkaloids and colchicine blocked the binding of [^{3}H]H_{12}-HTX to the ion channel site, and their ability to block channel binding was enhanced in the presence of carbamylcholine. At least one of these agents, vinblastine, allosterically enhanced carbamylcholine affinity for the nicotinic receptor. Vinblastine enhanced this binding in the same concentrations at which it prevented [^{3}H]H_{12}-HTX occupancy of ion channel-binding sites. HTX itself was found to be a potent and specific noncompetitive inhibitor of cholinergically mediated catecholamine secretion by chromaffin cells. Moreover, there is a good correlation between the IC_{50} values for inhibition of catecholamine secretion and [^{3}H]H_{12}-HTX binding by the *Vinca* alkaloids and colchicine (Fig. 7). It is likely, then, that the effects of *Vinca* alkaloids and colchicine on adrenal catecholamine secretion are due to interactions with ion channel sites associated with chromaffin cell nicotinic receptors.

It should be noted that a large number of structurally unrelated hydrophobic amines inhibit [^{3}H]H_{12}-HTX binding to ill defined sites on the ion channel. There are likely to be a number of "binding domains" associated with the ion channel and, as noted above, the binding of ligands to these sites depends on, and in turn influences, the conformation of the receptor complex (18, 23). Thus, binding to ion channel sites reflects both the microscopic dissociation constants which define ligand binding to each conformation of the sites and the isomerization constants which define the distribution of receptor complexes between the various conformational states. Because of these uncertainties in the mechanism of drug interactions with ion channel sites, we have expressed the effects of the microtubule agents as operational IC_{50} values for inhibition of [^{3}H]H_{12}-HTX binding. Because of the low (2 nM) concentration of [^{3}H]H_{12}-HTX used in relation to its K_D (about 100 nM), these IC_{50} values are probably a close reflection of the true inhibition constants.

The possibility that the actions of these microtubule-active agents on binding and secretion may be mediated, directly or indirectly, by membrane-associated cytoskeletal elements has not been totally excluded. α- and β-tubulin and actin are associated with chromaffin cell plasma membranes (25). Membrane-associated tubulin has been detected in other neuronal tissues (26–28). The cytoskeleton may be involved in both the topographical arrangement of the nicotinic receptor and its lateral mobility in the membrane. Prives *et al.* (29) found that the proportion of receptors on cultured embryonic muscle cells that are aggregated increases with maturation and, as the receptors aggregate, they become connected to the cytoskeleton. Similarly, Stya and Axelrod (30) found that as rat myotubes age in culture ACh receptor mobility decreases and attachment to the cytoskeleton increases.

The apparent binding constant for vinblastine's interaction with microtubule protein *in vitro* is 9 μM, and the

other *Vinca* alkaloids show similar affinities (31). These values correspond well to the concentrations of the *Vinca* alkaloids required for half-maximum inhibition of [^{3}H]H_{12}-HTX binding and catecholamine secretion. However, the apparent tubulin-binding constant for colchicine, 1 μM (31), is 2.5 orders of magnitude lower than the concentration required for half-maximum inhibition of secretion or HTX binding. Since β-lumicolchicine, which is structurally similar to colchicine but has no tubulin-binding activity, shows a similar pattern of ion channel inhibition as colchicine, it is unlikely that colchicine is blocking [^{3}H]H_{12}-HTX binding or catecholamine secretion as a consequence of an interaction with tubulin.

Taxol does not interact with nicotinic ACh or ion channel-binding sites in *Torpedo* electric organ. However, in the cultured chromaffin cell system, taxol selectively inhibits cholinergically mediated catecholamine secretion in a manner analogous to that of the *Vinca* alkaloids and colchicine (7). Since K^+-stimulated secretion is not affected, taxol apparently also acts on some step occurring between agonist binding to the cholinergic receptor and depolarization of the membrane. These results with taxol are particularly interesting since taxol does not cause depolymerization of microtubules, as do the *Vinca* alkaloids and colchicine; taxol enhances microtubule polymerization and stabilizes previously formed microtubules *in vitro* (32, 33). In the present study, only the microtubule-depolymerizing agents inhibited ligand binding to the ion channel. Whether taxol inhibition of the transduction of cholinergic signals in chromaffin cells involves alteration of a microtubule polymerization-depolymerization cycle is a question which requires further investigation.

ACKNOWLEDGMENTS

We wish to thank Dr. John W. Daly of the National Institutes of Health for providing the HTX and [^{3}H]H_{12}-HTX used in these studies, the Eli Lilly Corp. for donating the vincristine and vindesine, Drs. M. Sonenberg, A. Moore, and R. Corin of Sloan-Kettering Institute for useful comments on the manuscript, and M. Cobianchi, C. Schoedel, and L. Narayanan for excellent technical assistance.

REFERENCES

1. Hall, P. F. The role of the cytoskeleton in endocrine function, in *Cellular Regulation of Secretion and Release* (P. M. Conn, ed.). Academic Press, New York, 194–222 (1982).
2. Poisner, A. M., and J. Bernstein. A possible role of microtubules in catecholamine release from the adrenal medulla: effect of colchicine, Vinca alkaloids, and deuterium oxide. *J. Pharmacol. Exp. Ther.* **177**:102–108 (1971).
3. Douglas, W. W., and M. Sorimachi. Colchicine inhibits adrenal medullary secretion evoked by acetylcholine without affecting that evoked by potassium. *Br. J. Pharmacol.* **45**:129–132 (1972).
4. Trifaro, J. M., B. Collier, A. Lastowecka, and D. Stern. Inhibition by colchicine and vinblastine of acetylcholine-induced catecholamine release from the adrenal gland: an anticholinergic action, not an effect upon microtubules. *Mol. Pharmacol.* **8**:264–267 (1972).
5. Schneider, A. S., R. Herz, and K. Rosenheck. Stimulus-secretion coupling in chromaffin cells isolated from bovine adrenal medulla. *Proc. Natl. Acad Sci. U. S. A.* **74**:5036–5040 (1977).
6. Schneider, A. S., H. T. Cline, K. Rosenheck, and M. Sonenberg. Stimulus-secretion coupling in isolated adrenal chromaffin cells: calcium channel activation and possible role of cytoskeletal elements. *J. Neurochem.* **37**:567–575 (1981).
7. McKay, D. B., and A. S. Schneider. Selective inhibition of cholinergic receptor-mediated $^{45}Ca^{++}$ uptake and catecholamine secretion from adrenal chromaffin cells by taxol and vinblastine. *J. Pharmacol. Exp. Ther.* **231**:102–108 (1984).
8. Spoor, R. P., and F. C. Fergusen. Colchicine. IV. Neuromuscular transmission in isolated frog and rat tissue. *J. Pharm. Sci.* **54**:779–780 (1965).
9. Kayaalp, S. O., and N. H. Neff. Cholinergic muscarinic receptors of bovine adrenal medulla. *Neuropharmacology* **18**:909–911 (1979).
10. Schneider, A. S., H. T. Cline, and S. LeMaire. Rapid rise in cyclic GMP accompanies catecholamine secretion in suspensions of isolated adrenal chromaffin cells. *Life Sci.* **24**:1389–1394 (1979).
11. Kao, L.-S., and A. S. Schneider. Muscarinic receptors on bovine chromaffin cells mediate a rise in cytosolic calcium that is independent of extracellular calcium. *J. Biol. Chem.* **260**:2019–2022 (1985).
12. Fisher, S. K., R. W. Holz, and B. W. Agranoff. Muscarinic receptors in chromaffin cell cultures mediate enhanced phospholipid labeling but not catecholamine secretion. *J. Neurochem.* **37**:491–497 (1981).
13. McKay, D. B., R. S. Aronstam, and A. S. Schneider. Interaction of microtubule-active drugs with the *Torpedo* nicotinic receptor: correlations with effects on adrenal catecholamine secretion and $^{45}Ca^{++}$ uptake. *Fed. Proc.* **43**:567 (1984).
14. Kilpatrick, D. L., F. H. Ledbetter, K. A. Carson, A. G. Kirshner, R. Slepetic, and N. Kirshner. Stability of bovine adrenal medulla cells in culture. *J. Neurochem.* **35**:679–692 (1980).
15. von Euler, U. S., and I. Floding. A fluorimetric micromethod for differential estimation of adrenaline and noradrenaline. *Acta Physiol. Scand.* **33**:45–56 (Suppl. 118) (1955).
16. von Euler, U. S., and F. Lishajko. Improved technique for the fluorimetric estimation of catecholamines. *Acta Physiol. Scand.* **51**:348–356 (1961).
17. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
18. Aronstam, R. S., A. T. Eldefrawi, I. N. Pessah, J. W. Daly, E. X. Albuquerque, and M. E. Eldefrawi. Regulation of [^{3}H]perhydrohistrionicotoxin binding to *Torpedo ocellata* electroplax by effectors of the acetylcholine receptor. *J. Biol. Chem.* **256**:2843–2850 (1981).
19. Eldefrawi, M. E., A. T. Eldefrawi, R. S. Aronstam, M. A. Maleque, J. E. Warnick, and E. X. Albuquerque. [^{3}H]Phencyclidine—a probe for the ionic channel of the nicotinic receptor. *Proc. Natl. Acad. Sci. U. S. A.* **77**:7458–7462 (1981).
20. Aronstam, R. S., L. G. Abood, and W. Hoss. Influence of sulfhydryl reagents and heavy metals on the functional state of the muscarinic acetylcholine receptor in rat brain. *Mol. Pharmacol.* **14**:575–586 (1978).
21. Stroud, R. M. Acetylcholine receptor structure. *Neurosci. Comment.* **1**:124–138 (1983).
22. Burgermeister, W., W. A. Catterall, and B. Witkop. Histrionicotoxin enhances agonist-induced desensitization of acetylcholine receptor. *Proc. Natl. Acad. Sci. U. S. A.* **12**:5754–5758 (1977).
23. Heidmann, T., R. E. Oswald, and J.-P. Changeux. Multiple sites of action for noncompetitive blockers on acetylcholine receptor rich membrane fragments from *Torpedo marmorata. Biochemistry* **22**:3112–3127 (1983).
24. Oswald, R. E., T. Heidmann, and J.-P. Changeux. Multiple affinity states for noncompetitive blockers revealed by [^{3}H]phencyclidine binding to acetylcholine receptor rich membrane fragments from *Torpedo marmorata. Biochemistry* **22**:3128–3136 (1983).
25. Zinder, O., P. G. Hoffman, W. M. Bonner, and H. B. Pollard. Comparison of chemical properties of purified plasma membranes and secretory vesicle membranes from the bovine adrenal medulla. *Cell Tiss. Res.* **188**:153–170 (1978).
26. Zisapel, N., M. Levi, and I. Gozes. Tubulin: an integral protein at mammalian synaptic vesicle membranes. *J. Neurochem.* **34**:26–32 (1980).
27. Strocchi, P., B. A. Brown, J. D. Young, J. A. Benventre, and J. M. Gilbert. The characterization of tubulin in CNS membrane fragments. *J. Neurochem.* **37**:1295–1307 (1981).
28. Babitch, J. A. Synaptic plasma membrane tubulin may be an integral constituent. *J. Neurochem.* **37**:1394–1400 (1981).
29. Prives, J., A. B. Fulton, S. Penman, M. P. Daniels, and C. N. Christian. Interactions of the cytoskeletal framework with acetylcholine receptors on the surface of embryonic muscle cells in culture. *J. Cell Biol.* **92**:231–236 (1982).
30. Stya, M., and D. Axelrod. Mobility and detergent extractability of acetylcholine receptors on cultured rat myotubes: a correlation. *J. Cell Biol.* **97**:48–51 (1983).
31. Wilson, L. Microtubules as drug receptors: pharmacological properties of microtubule protein. *Ann. N. Y. Acad. Sci.* **253**:213–231 (1975).
32. Schiff, P. B., J. Fant, and S. B. Horwitz. Promotion of microtubule assembly *in vitro* by taxol. *Nature* (*Lond.*) **277**:665–667 (1979).
33. Schiff, P. B., and S. B. Horwitz. Tubulin: a target for chemotherapeutic agents, in *Molecular Actions and Targets for Cancer Chemotherapeutic Agents* (A. C. Sartorelli, J. S. Lazo, and J. R. Bertino, eds.), Vol. 2. Academic Press, New York, 483–507 (1981).

Send reprint requests to: Allan S. Schneider, Sloan-Kettering Institute for Cancer Research, Cornell University Graduate School of Medical Sciences, 1275 York Avenue, New York, NY 10021.