Chromaffin Cell Catecholamine Secretion: Bisindolylmaleimide Compounds Exhibit Novel and Potent Antagonist Effects at the Nicotinic Cholinergic Receptor in Pheochromocytoma Cells

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ABSTRACT

Activation of protein kinase C (PKC) stimulates nicotine-induced catecholamine secretion. PKC down-regulation by prolonged pretreatment with phorbol 12-myristate 13-acetate diminished nicotine-induced catecholamine secretion only slightly (~16%), suggesting substantial PKC independence of nicotinic receptor activation. However, we found that bisindolylmaleimide compounds (which are also putative PKC chemical inhibitors) dramatically inhibited nicotine-induced catecholamine secretion (IC50 values of ~24–37 nM). This inhibition was specific for the nicotinic cholinergic receptor. Catecholamine secretion induced by other nicotinic agonists (such as epibatidine, anatoxin, or cytisine) was also powerfully antagonized by bisindolylmaleimide II (IC50 values of ~60–90 nM). Even high-dose nicotinic agonists failed to overcome the inhibition by bisindolylmaleimide II, suggesting noncompetitive nicotinic antagonism by this class of compounds. Nicotinic inhibition by bisindolylmaleimide seemed not to be readily reversible. Structure-activity studies of bisindolylmaleimide compounds revealed that bisindolylmaleimides I through III are the most potent nicotinic antagonists at the nicotinic cholinergic receptor in PC-12 cells (IC50 ≤37 nM), whereas bisindolylmaleimide IV and V have far less nicotinic antagonist activity (IC50 >1 μM); the active compounds I through III have cationic tails at an indole nitrogen, whereas the least potent compounds IV and V do not. By contrast, a free NH within the maleimide ring is crucial for PKC inhibition by this class of compounds. We conclude that bisindolylmaleimides I through III are some of the most potent noncompetitive neuronal nicotinic antagonists, indeed the most potent such antagonists we have observed in PC-12 cells. Nicotinic antagonism of these compounds seems to be independent of PKC inhibition.

The role of protein kinase C (PKC) in secretion of catecholamines is well documented (TerBush and Holz, 1986, 1990; TerBush et al., 1988; Bittner and Holz, 1993; Graham et al., 2000; Taylor et al., 2000). Activation of PKC enhances catecholamine release from intact (Graham et al., 2000) and membrane-permeabilized (Knight and Baker, 1983; Pocotte et al., 1985) bovine chromaffin cells, rat pheochromocytoma PC-12 cells (Taylor et al., 2000), the perfused rat adrenal gland (Wakade et al., 1986), and the splanchnic nerve-stimulated dog adrenal gland (Suzuki et al., 1994). Several mechanisms have been put forward to explain the PKC-induced augmentation of catecholamine release, such as regulation in the influx of Ca2+ through voltage-sensitive and nicotinic receptor-linked Ca2+ channels (Wakade et al., 1986), enhancement of Ca2+ sensitivity at the late MgATP-independent step in exocytosis (Bittner and Holz, 1993), disruption of cortical F-actin near the plasma membrane and increment in the number of docked vesicles (Vitale et al., 1995), an increment in the size of the readily releasable pool of secretory granules (Gillis et al., 1996), a specific hyperpolarizing shift in the activation of L-type Ca2+ channels (Taylor et al., 2000), and changes in the rate of fusion pore expansion leading to pore closure or granule retrieval (Graham et al., 2000).

Bisindolylmaleimides (I-IV) are a new generation of potent PKC inhibitors (Toullec et al., 1991) previously used to study the involvement of PKC in regulation of catecholamine secretion (Gillis et al., 1996; Graham et al., 2000; Taylor et al., 2000). Here, we found that although PKC inhibition per se has little effect upon nicotine-induced catecholamine secretion, bisindolylmaleimide compounds are nonetheless powerful inhibitors of such secretion (IC50 values ~24–37 nM), probably acting as noncompetitive antagonists at the nicotinic receptor. The specificity, potency, and mechanism of

ABBREVIATIONS: PKC, protein kinase C; DMSO, dimethyl sulfoxide; PMA, phorbol 12-myristate 13-acetate; A23187, calcimycin; Ro 31-8220, 3-1-[3-(amidinothio)propyl-1H-indol-3-yl]-3-(1-methyl-1H-indol-3-yl)maleimide.
action of bisindolylmaleimides on nicotinic signaling to catecholamine secretion are discussed herein.

**Materials and Methods**

**Cell Culture.** Rat PC-12 pheochromocytoma cells (Greene and Tischler, 1976) at passage 8 were obtained from Dr. David Schubert (Salk Institute, La Jolla, CA). The cells were grown at 37°C, 6% CO₂, in 10-cm or six-well plates, in Dulbecco's modified Eagle's medium/ high glucose medium supplemented with 5% fetal bovine serum, 10% horse serum, and 1% penicillin/streptomycin (100% stocks were 10,000 units/ml of penicillin G and 10,000 μg/ml of streptomycin sulfate; Invitrogen, Carlsbad, CA).

**Secretagogue-Stimulated Release of Norepinephrine.** To study secretion of norepinephrine, PC-12 cells were plated on poly-D-lysine–coated polystyrene dishes (Falcon Plastics, Oxnard, CA), labeled for 3 h with 1 μCi L-[3H]norepinephrine (71.7 Ci/mmol; PerkinElmer Life Sciences, Boston, MA) in 1 ml of PC-12 growth medium, washed twice with release buffer (150 mM NaCl, 5 mM KCl, 2 mM CaCl₂, and 10 mM HEPES, pH 7), and then incubated at 37°C for 30 min in release buffer with or without secretagogues, such as nicotine (60 μM) or cell membrane depolarization (55 mM KCl), as described previously (Mahata et al., 1996). Release buffer for experiments involving KCl as secretagogue had NaCl reduced to 100 mM to maintain isotonicity. After 30 min, secretion was terminated by aspirating the release buffer and lysing cells into 150 mM NaCl, 5 mM KCl, 10 mM HEPES, pH 7, and 0.1% (v/v) Triton X-100. Release medium and cell lysates were assayed for [3H]norepinephrine by liquid scintillation counting, and results were expressed as percentage secretion: [amount released / (amount released + amount in cell lysate)] × 100. Net secretion is secretagogue-stimulated release minus basal release, where basal norepinephrine release is typically 5.8 ± 0.36% of cell total [3H]norepinephrine released over 30 min (n = 10 separate secretion assays).

**Chemicals.** (-)-Nicotine, (-)-anatoxin-A fumarate, (-)-cytisine, (-)-epibatidine hydrochloride, dihydro-β-erythroidine hydrobromide, d-tubocurarine chloride, α-bungarotoxin, and dimethyl sulfoxide (DMSO) were obtained from Sigma/RBI (Natick, MA). Phorbol-12-13-acetate (PMA) and bisindolylmaleimides I through V were purchased from Calbiochem (San Diego, CA). PMA and...
activation of PKC, we tested the effect of 4 different doses (1, 10, and 100 nM) of PMA, and secretion was studied for a period of 30 min. PMA dose-dependently stimulated the release of norepinephrine (net, 20% of cell content) from PC-12 cells (Fig. 1A). To verify whether nicotine interacted with PKC in evoking catecholamine secretion, [3H]norepinephrine-loaded PC-12 cells were treated with ascending logarithmic doses (0.01, 0.1, and 1 μM) of bisindolylmaleimides I through V, and secretion was monitored for a period of 30 min. Surprisingly, bisindolylmaleimides I through V displayed potent, dose-dependent inhibition of nicotine-induced secretion of norepinephrine, with IC\textsubscript{50} values of 24, 33, and 37 nM, respectively. Bisindolylmaleimides IV and V showed far less activity (IC\textsubscript{50} >1 μM) than compounds I through III (Fig. 3).

Reversibility and Specificity of Bisindolylmaleimide II Inhibition on Nicotinic Cholinergic Stimulation of Catecholamine Secretion. Potential reversibility of secretion was examined for 3 h. L-[3H]norepinephrine secretion was stimulated by nicotine (60 μM), and cells were harvested 30 min after treatment. Results are mean value ± S.E.M.; n = 3 replicates/condition. PKC preinhibition reduced nicotinic-stimulated secretion by only ~16%.

Results

Acute Activation of PKC Stimulates Catecholamine Secretion. The phorbol ester PMA activates PKC because of its structural similarity to the endogenous lipid activator diacylglycerol (Gillis et al., 1996). To evaluate whether PKC activation stimulates secretion of catecholamines, [3H]norepinephrine-loaded PC-12 cells were treated with ascending logarithmic doses (1, 10, and 100 nM) of PMA, and secretion was studied for a period of 30 min. PMA dose-dependently stimulated the release of norepinephrine (net, 20% of cell total stores) from PC-12 cells (Fig. 1A). To verify whether PMA-induced secretion of catecholamines results from activation of PKC, we tested the effect of 4α-PMA, a phorbol ester analog that cannot activate PKC (Gillis et al., 1996); 4α-PMA did not cause catecholamine secretion from PC-12 cells (Fig. 1B). Consistent with these observations, we found that PMA-induced catecholamine secretion was completely abolished in PKC–down-regulated PC-12 cells (Fig. 1C), in which PKC down-regulation was achieved by prolonged pretreatment of PC-12 cells with PMA (200 nM; 20 h).

Lack of Interaction between Nicotine and PKC in Evoking Catecholamine Secretion. To test whether nicotine interacts with PKC in evoking catecholamine secretion, [3H]norepinephrine-loaded PC-12 cells were treated acutely with PMA (100 nM) either alone or in combination with nicotine (60 μM), and secretion was then studied over a 30-min period. Norepinephrine secretion induced by PMA plus nicotine was approximately additive (Fig. 2A), suggesting utilization of separate signaling pathways by these compounds to evoke catecholamine secretion. Nicotine-evoked catecholamine secretion was also tested in PC-12 cells after PKC down-regulation by prolonged pretreatment with PMA (200 nM; 20 h); PKC inhibition reduced nicotinic-stimulated secretion by only ~16% (Fig. 2B), which makes any substantial role for PKC in nicotine secretion unlikely.

Effect of Several Bisindolylmaleimide Compounds on Nicotinic Induced Secretion of Catecholamines. To further explore the role of PKC in the secretory process, we examined nicotine-induced catecholamine secretion by a new generation of PKC chemical inhibitors: the bisindolylmaleimide compounds. For these studies, [3H]norepinephrine-loaded cells were treated with nicotine (60 μM) either alone or in combination with ascending logarithmic doses (0.01, 0.1, and 1 μM) of bisindolylmaleimides I through V, and secretion was monitored for a period of 30 min. Surprisingly, bisindolylmaleimides I through IV displayed potent, dose-dependent inhibition of nicotine-induced secretion of norepinephrine, with IC\textsubscript{50} values of 24, 33, and 37 nM, respectively. Bisindolylmaleimides IV and V showed far less activity (IC\textsubscript{50} >1 μM) than compounds I through III (Fig. 3).
tory inhibition of bisindolylmaleimide was tested along with other peptide (catestatin and substance P) and nonpeptide (hexamethonium) nicotinic antagonists by preincubation (15 min) with a maximal inhibitory dose of antagonist, followed by extensive washing of cells with subsequent stimulation by nicotine (60 μM). Secretion inhibition (63%) by bisindolylmaleimide II persisted even after washout, whereas the effects of catestatin, substance P, and hexamethonium seemed to be readily reversible (Fig. 4A).

To verify the specificity of bisindolylmaleimide compounds in inhibition of catecholamine secretion for the physiologic (nicotinic cholinergic) secretory pathway (nicotine, 60 μM), we tested the effects of bisindolylmaleimide II on several agents that act at stages in the pathway later than the nicotinic receptor, including membrane depolarization (55 mM KCl) to open voltage-gated calcium channels, a calcium ionophore (A23187, 1 μM), an alkaline earth metal (BaCl2, 2 mM), or stimulation of P2x purinergic receptors (ATP, 100 μM). Bisindolylmaleimide II suppressed norepinephrine release only when triggered by nicotine and not when secretion was caused by agents acting later (i.e., distal to the nicotinic cholinergic receptor) in the secretory pathway (Fig. 4B).

**Potency of Bisindolylmaleimide II: Comparison with Other Noncompetitive and Competitive Nicotinic Antagonists.** The potency of bisindolylmaleimide II on nicotine-induced catecholamine secretion was compared with other noncompetitive (catestatin, substance P, mecamylamine, and hexamethonium) or competitive (dihydro-β-erythroidine, d-tubocurarine, and α-bungarotoxin) antagonists by exposing [3H]norepinephrine-loaded PC-12 cells with nicotine (60 μM) either alone or in combination with ascending logarithmic doses (0.1–1,000 μM) of each antagonist, and secretion was monitored for a period of 30 min. The results revealed the following rank order of potencies for these nicotinic antagonists: for noncompetitive antagonists (Fig. 5A), bisindolylmaleimide II (IC50 ~0.031 μM) > mecamylamine (IC50 ~0.065 μM) > catestatin (IC50 ~0.33 μM) > substance P (IC50 ~1.06 μM) > hexamethonium (IC50 ~40 μM); for competitive antagonists (Fig. 5B), bisindolylmaleimide II (IC50 ~0.031 μM) < d-tubocurarine (IC50 ~0.71 μM) < dihydro-β-erythroidine or α-bungarotoxin (both IC50 >10 μM).

**Effect of Bisindolylmaleimide II on Secretion of Catecholamines Induced by Several Different Nicotinic Agonists.** The effect of bisindolylmaleimide II on secretion of...
catecholamines induced by several different nicotinic agonists was tested by exposing [3H]norepinephrine-loaded PC-12 cells to the nicotinic agonists (epibatidine, 1 μM; anatoxin, 10 μM; or cytisine, 100 μM) either alone or in combination with ascending logarithmic doses (0.01, 0.1, or 1 μM) of bisindolylmaleimide II. Secretion was terminated after 30 min of incubation. Bisindolylmaleimide II dose-dependently inhibited secretion of catecholamines induced by all nicotinic agonists, with IC50 values of 70 nM to inhibit epibatidine, 90 nM to inhibit anatoxin, and 60 nM to inhibit cytisine (Fig. 6).

**Bisindolylmaleimide II as a Noncompetitive Nicotinic Cholinergic Antagonist.** To test whether bisindolylmaleimide compounds exerted their nicotinic antagonist action by a competitive or noncompetitive means, we stimulated PC-12 cells with ascending logarithmic doses of nicotinic agonists (nicotine, 10–1,000 μM; epibatidine, 0.01–1 μM; anatoxin, 0.1–100 μM; or cytisine, 10–1,000 μM) alone or in combination with ascending logarithmic doses of bisindolylmaleimide II (0.01–1 μM). Nicotinic agonists failed to completely overcome inhibition by bisindolylmaleimide II at any agonist dose, and nicotinic agonists were not able to reverse the blocking effects of even submaximal inhibitory concentrations of bisindolylmaleimide II. Hexamethonium was used as a positive control for noncompetitive nicotinic antagonist. This result functionally establishes bisindolylmaleimide II as a noncompetitive nicotinic antagonist (Fig. 7, A–D). However, further studies, such as agonist binding/displacement or comparison with a classical positive

![Fig. 5. Potency of bisindolylmaleimide II: comparison with other noncompetitive and competitive nicotinic antagonists. A, comparison of bisindolylmaleimide effect with noncompetitive nicotinic antagonists. L-[3H]Norepinephrine-prelabeled cells were treated with nicotine (60 μM) either alone or in combination with ascending logarithmic doses of catestatin (0.1–10 μM), substance P (0.1–10 μM), mecamylamine (0.01–1 μM), hexamethonium (1–1,000 μM), and bisindolylmaleimide II (0.01–1 μM). Cells were harvested 30 min after treatment. Control (100%) net norepinephrine release is that in the presence of nicotine (60 μM) stimulation alone, without nicotinic antagonists. Results are mean value ± S.E.M.; n = 3 replicates/condition. B, comparison of bisindolylmaleimide effect with competitive nicotinic antagonists. L-[3H]Norepinephrine-prelabeled cells were treated with nicotine (60 μM) either alone or in combination with ascending logarithmic doses of dihydro-β-erythroidine (0.1–100 μM), d-tubocurarine (0.01–100 μM), α-bungarotoxin (0.01–10 μM), and bisindolylmaleimide II (0.01–1 μM). Cells were harvested 30 min after treatment. Control (100%) net norepinephrine release is that in the presence of nicotine (60 μM) stimulation alone, without nicotinic antagonists. Results are mean value ± S.E.M.; n = 3 replicates/condition.](https://example.com/f5)

![Fig. 6. Effect of bisindolylmaleimide II on several nicotinic agonists. L-[3H]Norepinephrine-prelabeled cells were treated with nicotinic agonists (epibatidine, 1 μM; anatoxin, 10 μM; or cytisine, 100 μM) either alone or in combination with ascending logarithmic doses (0.001–1 μM) of bisindolylmaleimide II. Secretion was terminated after 30 min of incubation. Control (100%) net norepinephrine release is that in the presence of nicotinic agonists such as epibatidine, anatoxin, and cytisine without bisindolylmaleimide II. Results are mean value ± S.E.M.; n = 3 replicates/condition.](https://example.com/f6)
control competitive antagonist response in our PC-12 system, would confirm noncompetitive antagonism.

Discussion

Consistent with previous studies in chromaffin cells (Pocotte et al., 1985; Wakade et al., 1986; Graham et al., 2000; Taylor et al., 2000), we found that activation of PKC augmented catecholamine release from PC-12 cells (Figs. 1 and 2). In addition, the PKC activator PMA increased nicotine-induced secretion of catecholamines, and the results seemed to be additive (Fig. 2A). Analogous results were reported previously in isolated, perfused rat adrenal glands (Wakade et al., 1986). These findings suggest utilization of separate signaling pathways by nicotine and PMA to evoke catecholamine secretion. Nonetheless, PKC down-regulation by prolonged pretreatment (20-h) with PMA only minimally (~16%) diminished nicotine-induced catecholamine secretion (Fig. 2B), indicating that PKC activity is not crucial for nicotinic secretory pathway activation. However, a new family of putative PKC inhibitors, the bisindolylmaleimides, dramatically (IC\textsubscript{50} values ~24–37 nM) inhibited nicotine-induced catecholamine secretion from PC-12 cells (Fig. 3). Here, we sought to understand the secretion-inhibitory mechanism of the bisindolylmaleimides.

Bisindolylmaleimide II inhibition of catecholamine release was specific to nicotinic stimulation, because this compound failed to antagonize catecholamine secretion induced by agents that bypass the nicotinic receptor, such as membrane depolarization (55 mM KCl), stimulation of P\textsubscript{2x} purinergic receptor (100 \mu M ATP), calcium ionophore (1 \mu M A23187), and barium (2 mM) (Fig. 4B). Bisindolylmaleimide II inhibition seemed to be less (37%) reversible than the readily reversible (~100%) inhibition exerted by catestatin, substance P, and hexamethonium (Fig. 4A). We have reported previously that nicotinic inhibition by phencyclidine and cocaine are also less reversible (Mahata et al., 1999). Being a
small molecule, bisindolylmaleimide II might easily penetrate the nicotinic cation pore well into the lipid bilayer, perhaps making it more difficult to remove with a simple buffer wash. Other properties, such as hydrophobicity or charge, may also be important for relative irreversibility. In addition, modulation of protein kinases involved with nicotinic receptor desensitization/re-sensitization by bisindolylmaleimide II cannot be ruled out.

The present findings revealed that catecholamine secretion induced by other nicotinic agonists, such as epibatidine, anatoxin, and cytisine, is also antagonized by bisindolylmaleimide II (IC50 values ~60–90 nM) (Fig. 6), establishing bisindolylmaleimide II as a general, potent inhibitor of nicotinic antagonists. Indeed, comparison of the potency (IC50 value) of bisindolylmaleimide II with other noncompetitive (Fig. 5A) and competitive (Fig. 5B) nicotinic antagonists establish bisindolylmaleimide II as among the most potent nicotinic antagonists at the nicotinic cholinergic receptor in PC-12 cells (Bencherif et al., 1995; Mahata et al., 1999; Dwoskin and Crooks, 2001). However, it should be noted that our studies were conducted in PC-12 pheochromocytoma cells, in which nicotinic receptor expression includes only the α3, α5, α7, β2, β3, and β4 subunits (Rogers et al., 1992; Skok et al., 1999), unlike neurons, which express α4 subunits (Wonnacott, 1997). Thus, our observations on the relative potency of bisindolylmaleimide compounds might not be generalizable to other nicotinic receptor subunit compositions on other nicotinic cell types, such as neurons. Another putative PKC inhibitor, Ro 31-8220, also seems to act as a nicotinic antagonist during catecholamine secretion or tyrosine hydroxylase stimulation in bovine chromaffin cells (Marley and Thomson, 1996).

The inability of nicotinic agonists (nicotine, epibatidine, anatoxin, or cytisine) to overcome the secretory inhibition of bisindolylmaleimide II even at very high agonist doses (Fig. 7, AD) indicates noncompetitive nicotinic inhibition, although we have not established the precise site at which bisindolylmaleimide II interacts with the nicotinic receptor. However, substantial (63%) irreversible secretory inhibition by bisindolylmaleimide II (Fig. 4A) may point to the penetration of this compound deep into the nicotinic cation pore.

How do bisindolylmaleimides inhibit nicotinic activation?
The bisindolylmaleimides, which are nicotinic antagonists (I-III), contain a cationic tail anchored at an indole nitrogen (Fig. 8); its absence in inactive bisindolylmaleimides IV and V indicates that the cationic tail is crucial for nicotinic antagonism. By contrast, inhibition of PKC by bisindolylmaleimides seems to be located on physically distant portions of the molecules. Indeed, we found that nicotine-induced catecholamine secretion was only slightly abolished by PKC-inhibiting activity (Davis et al., 1992a,b). Thus, the crucial pharmacophores for these two distinct actions of bisindolylmaleimides seem to be located on physically distant portions of the molecules. Indeed, we found that nicotine-induced catecholamine secretion was only slightly (~16%) diminished in PKC down-regulated PC-12 cells (Fig. 2B), further documenting a non-PKC target for the antisecretory effects of bisindolylmaleimides I through III.

Why might the cationic tails in the indole rings of bisindolylmaleimides I through III (Fig. 8) be so important for noncompetitive nicotinic cholinergic inhibition? Noncompetitive nicotinic antagonists are often blockers of the nicotinic cation pore (Tsigelny et al., 1998). The extracellular vestibule of the nicotinic cation pore is itself relatively anionic in charge (Tsigelny et al., 1997, 1998) and may thereby provide an electrostatic target for cationic noncompetitive antagonists of the nicotinic receptor (Tsigelny et al., 1998).

In conclusion, these findings establish a novel action of bisindolylmaleimides I through III: these compounds are not only PKC antagonists but also some of the most potent nicotinic cholinergic antagonists described in PC-12 cells (Benchfer et al., 1995; Mahata et al., 1999; Dwooskin and Crooks, 2001). The nicotinic antagonist action of bisindolylmaleimides is distinct from their PKC-inhibiting properties, especially in the particular moieties that mediate the two very different effects of these molecules.

References


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