The Allosteric Potentiation of Nicotinic Acetylcholine Receptors by Galantamine Is Transduced into Cellular Responses in Neurons: Ca^{2+} Signals and Neurotransmitter Release.

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ABSTRACT

Neuronal nicotinic acetylcholine receptors (nAChR) modulate a variety of cellular responses, including Ca^{2+} signals and neurotransmitter release, which can influence neuronal processes such as synaptic efficacy and neuroprotection. In addition to receptor activation through the agonist binding site, an allosteric modulation of nAChR has also been described for a novel class of allosteric ligands. Of these, the acetylcholinesterase inhibitor and Alzheimer drug galantamine represents the prototypical allosteric ligand, based on its potentiation of nAChR-evoked single-channel and whole-cell currents. The aim of this study was to establish whether the allosteric potentiation of nAChR currents is transduced in downstream cellular responses to nAChR activation, namely increases in intracellular Ca^{2+} and [^{3}H]noradrenaline release. In SH-SY5Y cells, galantamine potentiated nicotine-evoked increases in intracellular Ca^{2+} and [^{3}H]noradrenaline release with a bell-shaped concentration-response profile; maximum enhancement of nicotine-evoked responses occurred at 1 μM galantamine. This potentiation was blocked by mecamylamine, whereas galantamine had no effect on these measures in the absence of nicotine. Galantamine did not compete for radioligand binding to the agonist binding sites of several nAChR subtypes, consistent with an allosteric mode of action. Unlike galantamine, the acetylcholinesterase inhibitors rivastigmine and donepezil did not potentiate nAChR-mediated responses, whereas donepezil was a reasonably potent inhibitor of nicotine- and KCl-evoked increases in Ca^{2+}. nAChR-mediated [^{3}H]noradrenaline release from hippocampal slices was also potentiated by galantamine, with an additional component attributable to acetylcholinesterase inhibition and subsequent increase in acetylcholine. These results indicate that the allosteric regulation of nAChR results in the potentiation of receptor-dependent cellular processes relevant to many of the physiological consequences of nAChR activation.

Neuronal nicotinic acetylcholine receptors (nAChR) are ligand-gated cation channels permeable to both Na$^+$ and Ca^{2+} and are considered modulators of brain function. The generation of complex Ca^{2+} signals and the facilitation of transmitter release by nAChR are two major actions responsible for their modulatory functions. Although activation of nAChR is achieved by the binding of agonists to the agonist recognition site, novel allosteric potentiating ligands (APLs) provide an additional mechanism for modulation, defined to date by electrophysiological studies (Pereira et al., 2002). APLs are purported to bind to a distinct site in the extracellular N-terminal domain of the nAChR, identified by the specific monoclonal antibody FK1 (Schrattenholz et al., 1996). Thus, APLs provide a potential regulatory mechanism for nAChR that is analogous to the well-defined allosteric mechanisms of other ligand-gated ion channels, such as the interaction of benzodiazepines and glycine with GABA-A and N-methyl-D-aspartate receptors, respectively (Maelicke et al., 2000).

Galantamine, a well-characterized nAChR APL (Pereira et al., 2002; Samochocki et al., 2003), is one of several acetylcholinesterase inhibitors (AChEI) currently used in the treatment of Alzheimer's disease (AD); it induces single-channel activity of nAChRs through a process not blocked by classic competitive antagonists (Pereira et al., 1993, 1994; Storch et al., 1995). Although galantamine, and other APLs, fail to elicit discernible macroscopic currents themselves, they potentiate whole-cell currents evoked by submaximal concentrations of nAChR agonists (Schrattenholz et al., 1996; Storch et al., 1995; Santos et al., 2002). At present, the neurochemical and cellular consequences of this allosteric interaction remain unknown, although they are assumed to be clinically relevant (Woodruff-Pak et al., 2002).

Nicotinic agonists enhance cognitive function in humans and experimental animals (Levin, 2000), whereas the

ABBREVIATIONS: nAChR, nicotinic acetylcholine receptors; AChEI, acetylcholinesterase inhibitor; AD, Alzheimer's disease; APL, allosteric potentiating ligand; VOCC, voltage operated Ca^{2+} channels; AM, acetoxymethyl ester; PMSF, phenylmethylsulfonyl fluoride; MLA, methyllycaconitine; ANOVA, analysis of variance.

nAChR-mediated activation of Ca^{2+}-dependent signaling cascades has been implicated in memory processing (Dineley et al., 2001). The stimulation of nAChR can increase cytoplasmic Ca^{2+} through permeation of the nAChR channel per se and by recruiting voltage-operated Ca^{2+} channels (VOCC) and/or intracellular stores (Rathouz and Berg, 1994; Dajas-Bailador et al., 2002a). Thus, nAChR can couple to specific downstream Ca^{2+}-dependent processes, such as the extracellular signal-regulated/mitogen-activated protein kinase regulating both short and long-term cellular events (Dineley et al., 2001; Dajas-Bailador et al., 2002b). The nAChR-mediated elevation of intracellular Ca^{2+} can also facilitate transmitter release, a major function of nAChR in the central nervous system (Wonnacott, 1997) that is pertinent to memory processing in the hippocampus (Radcliffe and Dani, 1998). The cognitive decline in AD is accompanied by the loss of basal forebrain cholinergic projections and presynaptic cholinergic markers, including nAChR (Levin, 2000).

The aim of this study was to establish whether the allosteric potentiation of nAChR currents is observed in downstream cellular responses to nAChR activation. This was investigated by monitoring nicotine-evoked increases in intracellular Ca^{2+} and [3H]noradrenaline release. In addition, comparison of galantamine with the related AChEI and APL physostigmine and with other AChEI currently prescribed for the treatment of AD (rivastigmine and donepezil) shows that although each drug has a unique activity profile, only galantamine and physostigmine potentiate nicotine-evoked responses.

Materials and Methods

Adult male Sprague-Dawley rats were obtained from the University of Bath Animal House breeding colony. [7,8-3H]Noradrenaline (2.5 Ci/mmol) was purchased from Amersham Biosciences (Buckinghamshire, UK). Tissue culture media, serum, and plasticware were obtained from Invitrogen (Paisley, Renfrewshire, UK). Media supplements, cadmium chloride, (−)-nicotine hydrogen tartrate, mecamylamine, and physostigmine were purchased from Sigma Chemical (Poole, Dorset, UK). Fluo-3 AM and pluronic F127 were purchased from Molecular Probes (PoortGebouw, The Netherlands). Galantamine was provided by Sanochemia Pharmazeutika AG (Vienna, Austria), whereas donepezil and rivastigmine were donated by Intelligen Corp. (Cold Spring Harbor, NY). Human neuroblastoma SH-SY5Y cells were from ECACC (Salisbury, UK) and Lα4β2 cells (mouse L929 cells stably transfected with rat α4 and β2 nAChR subunit cDNAs) were provided by Dr. N. Millar (Department of Pharmacology, University College, London, UK).

Cell Culture

SH-SY5Y cells (passages 14–20) were cultured as described previously (Ridley et al., 2001). In brief, cultures were maintained in Dulbecco's modified Eagle's medium/Ham's F12 medium, supplemented with 15% fetal bovine serum, 2 mM L-glutamine, 1% nonessential amino acids, 190 U/ml penicillin, and 0.2 mg/ml streptomycin. Cells were plated (1:5 dilution) into 96-well Primaria plates (Falcon, Franklin Lakes, NJ), and experiments were performed 72 h later with confluent cultures.

Lα4β2 cells were cultured as described previously (Cooper et al., 1999). Cultures were maintained in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. Cells were plated (1:5 dilution) into 96-well Primaria plates. Expression of nAChR was induced by 1 μM dexamethasone for 7 days. Because expression of surface α4β2 nAChR is enhanced by lowering the

temperature (Cooper et al., 1999), cells were transferred to a 33°C incubator 24 h before the experiment.

Binding Studies

Membrane Preparations. Rat brain P2 membranes were prepared by differential centrifugation as described previously (Davies et al., 1999). The washed pellet was resuspended in 50 mM phosphate buffer, pH 7.4, containing 1 mM EDTA, 0.1 mM PMSF, and 0.01% sodium azide (2.5 ml/g original weight) and stored in 5-ml aliquots at −20°C.

SH-SY5Y cell membranes were prepared as described previously (Sharples et al., 2000). In brief, confluent 75-cm^2 flasks of cells were washed with PBS, pH 7.4, and scraped into ice-cold 50 mM phosphate buffer, pH 7.4, containing 0.1 mM PMSF, and 0.01% sodium azide. Cells were washed by centrifugation (MSE, Micro Centaur, UK) for 3 min at 500g and resuspended in 10 ml of ice-cold 50 mM phosphate buffer. The suspension was sonicated (3 × 10 s) and centrifuged for 35 min at 45,000g. The pellet was resuspended in 50 mM phosphate buffer (0.5 ml per original flask of cells). Protein was determined using the Markwell method with bovine serum albumin as standard.

Saturation Binding Assays. Saturable binding of [3H]nicotine or [3H]MLA to rat brain P2 membranes was determined by incubating increasing concentrations of radioligand with 0.25 or 0.5 mg of membrane protein, respectively, in a total volume of 250 μl of HEPES buffer (20 mM HEPES, pH 7.4, containing 118 mM NaCl, 4.8 mM KCl, 2.5 mM CaCl$_2$, 200 mM Tris, 0.1 mM PMSF, and 0.01% sodium azide) for [3H]nicotine binding (Sharples et al., 2000) or 50 mM phosphate buffer, pH 7.4, for [3H]MLA binding (Davies et al., 1999). Nonspecific binding was defined in the presence of 100 μM or 1 mM nicotine. Saturable binding of [3H]epibatidine to SH-SY5Y cell membranes was determined by incubating increasing concentrations of radioligand with 40 μl (95 μg of protein) of membranes in a total volume of 1 ml of 50 mM phosphate buffer, pH 7.4 (Sharples et al., 2000). Nonspecific binding was defined in the presence of 1 mM nicotine. Assays were conducted with or without the presence of 1 μM galantamine. Incubations were carried out at room temperature for 30 min followed by 60 min at 4°C. Bound radioligand was separated by rapid filtration on Whatman GFA/E filter paper (presoaked overnight in 0.3% polyethylenimine in PBS, pH 7.4), using a Brandell cell harvester. Filters were counted for radioactivity in a PerkinElmer Tri-Carb liquid scintillation counter 1500 (counting efficiency 45%).

Competition Assays. Competition binding assays were performed as described previously (Sharples et al., 2000, 2002). Rat brain P2 membranes were incubated with serial dilutions of (−)-nicotine or galantamine and [3H]nicotine (10 nM) or [3H]MLA (2 nM), and SH-SY5Y cell membranes were incubated with serial dilutions of (−)-nicotine or galantamine and [3H]epibatidine (150 pM), as described above.

Data Analysis. Dissociation constant (K_d) and maximum binding (B_{max}) values from saturation binding experiments were determined by nonlinear regression, fitting data points to a single-site ligand-binding model. IC_{50} values were derived from competition binding data by fitting data points to the Hill equation using a nonlinear least-squares curve fitting facility of Sigma Plot 2.0 for Windows (SPSS Inc., Chicago, IL): % Bound = $100\%/(1 + ([Ligand]/IC_{50})^{n_H})$, where n_H is the Hill number, [Ligand] is the concentration of the competing ligand, and IC_{50} is the concentration of competing ligand that displaces 50% of specific radioligand bound. K_i values were calculated from IC_{50} values (Cheng and Prusoff, 1973), assuming K_d values determined in the corresponding saturation binding assays.

Calcium Fluorometry

Ca^{2+}-mediated increases in fluo-3 fluorescence were monitored as described previously (Dajas-Bailador et al., 2002a). After removal of the medium from the confluent SH-SY5Y cultures, cells were washed

twice with Tyrode's salt solution (137 mM NaCl, 2.7 mM KCl, 1.0 mM MgCl$_2$, 1.8 mM CaCl$_2$, 0.2 mM NaH$_2$PO$_4$, 12 mM NaHCO$_3$, and 5.5 mM glucose, pH 7.4) and incubated with the membrane-permeable, Ca^{2+}-sensitive dye fluo-3 AM (10 μM) and 0.02% pluronic F127 for 1 h at room temperature in the dark. Cells were washed twice with Tyrode's salt solution, then 80 μl of buffer, with or without drugs, was added to each well. After 10 min of preincubation at room temperature in the dark, changes in fluorescence (excitation, 485 nm; emission, 538 nm) were measured using a Fluoroskan Ascent fluorescent plate reader (Labsystems, Helsinki, Finland). Basal fluorescence was monitored for 5 s before addition of stimulus (nicotine, KCl, or drug; 20 μl) and changes in fluorescence were monitored for a further 20 s. Responses in Lα4β2 cells were measured using the same protocol, except that responses were monitored in a high Ca^{2+} buffer (25 mM HEPES, 35 mM sucrose, 75 mM CaCl$_2$, pH 7.4) (Cooper and Millar, 1997) to amplify nicotine-evoked Ca^{2+} signals in cells that lack VOCC. To normalize fluo-3 signals, responses from each well were calibrated by determination of the maximum and minimum fluorescence values. At the end of each experiment, addition of 0.2% Triton (F_{max}) was followed by 40 mM MnCl$_2$ (F_{min}). Data were calculated as a percentage of $F_{max} - F_{min}$ (Dajas-Bailador et al., 2002a). Values were expressed as a percentage of the response to 30 μM nicotine included in all experiments, unless otherwise stated. Maximum responses to nicotine were approximately 20% of the $F_{max} - F_{min}$ and thus not close to saturation of the dye.

[^{3}H]Noradrenaline Release

SH-SY5Y Cells. SH-SY5Y cells were cultured in 96-well plates until confluent. After removal of culture medium, [^{3}H]noradrenaline (0.07 μM, 2.5 μCi/ml) was added (60 μl/well) in oxygenated Krebs-bicarbonate buffer (118 mM NaCl, 2.4 mM KCl, 2.4 mM CaCl$_2$, 1.2 mM KH$_2$PO$_4$, 1.2 mM MgSO$_4$, 25 mM NaHCO$_3$, 10 mM D-glucose, 1 mM ascorbic acid, and 10 μM pargyline, pH 7.4), and the cells were incubated for 1 h at 37°C. After loading the cells with [^{3}H]noradrenaline, they were washed twice with Krebs buffer with added nomifensine (0.5 μM) to prevent reuptake of released [^{3}H]noradrenaline. Cells were then incubated for 5 min in the same buffer, with or without mecamylamine. The buffer was replaced with Krebs buffer plus nomifensine containing nicotine and/or galantamine (80 μl/well). After 5 min, the medium containing released [^{3}H]noradrenaline was transferred to a 96-well Optiplate (PerkinElmer Life Sciences, Zaventem, Belgium). Microscint-40 (170 μl; PerkinElmer) was added to each well and radioactivity was counted using a microbeta liquid scintillation counter (Wallac MicroBeta TriLux 1450; PerkinElmer Life Sciences, Espoo, Finland; counting efficiency, 31%). Radioactivity remaining in the cultures was determined by addition of 80 μl of 0.5 M perchloric acid and incubation for 1 h at 37°C, followed by scintillation counting. The total amount of [^{3}H]noradrenaline present in the cells at the point of stimulation was equivalent to the tritium released plus tritium remaining. Released [^{3}H]noradrenaline was calculated as a percentage of total radioactivity in the corresponding wells, and results were then expressed as a percentage of basal release (buffer stimulation). The average values of basal release and corresponding total radioactivity were 3189 ± 80 and 39,424 ± 809 cpm (mean ± S.E.M., n = 60) respectively. Therefore, basal release corresponds to 8.0% of the radioactivity present in culture wells at the beginning of the experiment.

Hippocampal Slices. The measurement of [^{3}H]noradrenaline release from rat hippocampal slices was based on the method of Anderson et al. (2000). Rats (250–350 g) were killed by cervical dislocation, and hippocampi were rapidly dissected. Hippocampal slices (0.25 mm) were prepared using a McIlwain tissue chopper, washed twice with Krebs-bicarbonate buffer (as above), and loaded with [^{3}H]noradrenaline (0.07 μM, 2.5 μCi/ml) for 30 min at 37°C. After four washes with Krebs buffer plus nomifensine (0.5 μM), slices were dispersed into 96-well Multiscreen filter plates (Millipore, Bedford, MA). Hippocampi from two animals were sufficient for one 96-well plate. Buffer was removed using a vacuum filtration unit

(Millipore), leaving tissue slices on well filters. Subsequently, 70 μl of Krebs buffer containing nomifensine, with or without drugs, was added to each well and allowed to incubate for 5 min at 37°C in an atmosphere of 95% O$_2$ and 5% CO$_2$. Basal and stimulated release was collected via vacuum filtration into a 96-well Optiplate (PerkinElmer Life Sciences) and radioactivity was counted as described above. To estimate radioactivity remaining in the slices at the end of the experiment, filters were removed from the 96-well plate and counted for radioactivity in a PerkinElmer Life Sciences Tri-Carb 1500 liquid scintillation counter (counting efficiency, 44%). Total radioactivity present in the slices at the start of the stimulation was calculated as the sum of disintegrations per minute of tritium released plus disintegrations per minute of tritium radioactivity present in slices at the end of experiment, after correction for counting efficiency. Release was calculated as a percentage of total radioactivity. Values were then expressed as a percentage of the basal response (buffer stimulation). The average values of basal release and corresponding total radioactivity were 1182 ± 40 and 72,860 ± 2652 cpm (mean ± S.E.M., n = 60) respectively. Therefore, basal release corresponds to 1.62% of radioactivity present in the tissue at the beginning of the experiment.

Acetylcholinesterase (AChE) Activity

The potencies of AChEI with respect to rat brain AChE activity were assayed in P2 membranes based on the colorimetric method of Ellman et al. (1961). This measures the change in the rate of hydrolysis of a thiocholine ester, which exchanges with 5,5′-dithiobis(2-nitrobenzoate) to produce a yellow product, 5-mercapto-2-nitrobenzoate. Test compounds (final concentration, 0.1 nM-10 μM) were preincubated with P2 membranes (10 μl) in 2.5 ml of 0.1 M NaH$_2$PO$_4$, pH 8.0, for 20 min, before addition of 5,5′-dithiobis(2-nitrobenzoate) (final concentration, 0.4 mM) and the substrate acetylthiocholine iodide (0.5 mM, final concentration). The change in absorbance at 412 nm was monitored, and AChE activity was calculated according to the relationship micromoles hydrolyzed per minute per milliliter = $\Delta E/1.36 \times 10^4 \times$ dilution factors, where ΔE is the change in absorbance measured and 1.36×10^4 is the molar extinction coefficient. Activity in the presence of drug was calculated as a percentage of the activity determined in the absence of drug. IC$_{50}$ values were determined by fitting data points to the Hill equation, as described above for ligand binding.

Statistics

Data are the mean ± S.E.M. of three or more independent experiments, each with three (binding and AChE assays), four (Ca^{2+} studies), or eight (transmitter release) replicates. Statistical significance was determined using one-way ANOVA (post hoc Dunn's or Tukey's test, as indicated in figure legends) for comparison between groups and Student's t test for comparisons between treatments and respective controls. Values of $p < 0.05$ were taken to be statistically significant.

Results

Binding Studies

Electrophysiological recordings of single-channel and whole-cell currents have shown that APLs, such as galantamine and physostigmine, can potentiate the effects of agonists at many subtypes of nAChR, and this effect has been attributed to an interaction with an allosteric site on the nAChR (Pereira et al., 2002). However, it has also been suggested that some AChEI interact competitively with the agonist site of fetal muscle (Cooper et al., 1996) or α4β4 nAChR expressed in *Xenopus laevis* oocytes (Zwart et al., 2000). To address the possibility of a competitive interaction,

galantamine was examined for its ability to displace radioligand from the agonist binding site of three nAChR subtypes (Fig. 1A). Galantamine did not compete for [³H]nicotine binding to rat brain $\alpha4\beta2$ nAChR, [³H]epibatidine binding to $\alpha3^*$ nAChR in human SH-SY5Y cell membranes, or [³H]MLA binding to rat brain $\alpha7$ nAChR, whereas nicotine displaced radioligand binding in each case, with K_i values in the expected range (Fig. 1A). These results are consistent with the argument that galantamine acts at a site near, but distinct from, the ACh binding site on nAChR (Pereira et al., 2002; Schrattenholz et al., 1996). Through an allosteric interaction via this site, galantamine could influence agonist binding by increasing the nAChR affinity for agonist. This was examined by carrying out saturation binding assays with the same three radioligands in the presence or absence of 1 μM galantamine (Fig. 1B). Neither affinity (K_d) nor maximum binding capacity (B_{max}) was altered in the presence of galantamine (Table 1). These results indicate that there is no shift in nAChR affinity for agonist in response to galantamine; it is thus plausible that galantamine enhances the coupling of agonist binding to channel opening without affecting agonist binding per se. However, an alternative explanation is that saturation binding assays reflect the desensitized state of the receptor, which may not be relevant to the functional potentiation of nAChR responses.

Nicotine-Evoked Ca²⁺ Responses

To determine whether the allosteric potentiation of nAChR was transduced into relevant cellular responses, we measured the effect of galantamine on nicotine-evoked increases in intracellular Ca²⁺ in SH-SY5Y cells loaded with the Ca²⁺-sensitive dye fluo-3 AM. As reported previously (Dajas-Bailador et al., 2002a), we observed a rapid and significant increase in fluorescence after stimulation with 30 μM nicotine (Fig. 2A), which approximates to the EC_{50} value for this assay (Ridley et al., 2002). Preincubation with the general nAChR antagonist mecamylamine at a concentration (40 μM) to block all nAChR subtypes, completely prevented this nicotine-evoked increase in fluorescence, confirming that nAChRs mediate the response (data not shown; see Dajas-Bailador et al., 2002a). Preincubation for 5 min with a range of galantamine concentrations (0.1 - 50 μM) resulted in a bell-shaped profile of potentiation of responses to 30 μM nicotine (Fig. 2B). Galantamine at 0.5, 1, and 3 μM significantly increased the nicotine-evoked rise in fluorescence, whereas lower and higher concentrations failed to produce any significant effect compared with nicotine alone. The maximum increase of 16.9 $\pm$ 3.2% above the response to nicotine alone was produced by 1 μM galantamine. Potentiation by 0.5, 1, and 3 μM galantamine was evident throughout the 20-s time course (illustrated in Fig. 2A for 3 μM galan-
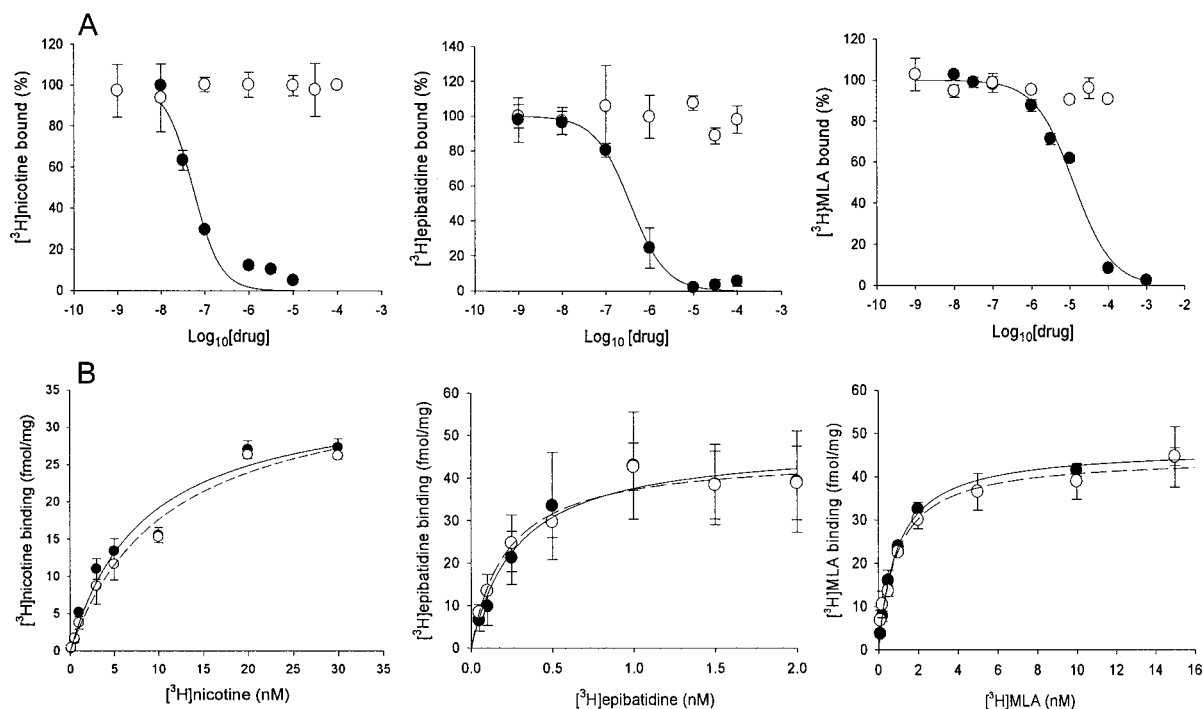
Fig. 1. Ligand binding assays. A, competition binding assays were carried out with radioligand in the absence (total binding) or presence of serial dilutions of (−)-nicotine (●) or galantamine (○) as described under *Materials and Methods*. [³H]Nicotine (10 nM) binding to rat brain membranes defined $\alpha4\beta2$ nAChR; [³H]epibatidine (150 pM) binding to SH-SY5Y cell membranes defined $\alpha3^*$ nAChR; [³H]MLA (2 nM) binding to rat brain membranes defined $\alpha7$ nAChR. Nonspecific binding was determined in the presence of 100 μM, 1 mM, and 1 mM nicotine, respectively, and subtracted to give specific binding. Data points are fitted to the Hill equation and IC_{50} values derived using the nonlinear least-squares curve fitting facility of Sigma Plot 2.0 for Windows. K_i values were determined by the method of Cheng and Prusoff (1973). Nicotine inhibited [³H]nicotine, [³H]epibatidine, and [³H]MLA binding with K_i values of 27.3 $\pm$ 1.7 nM, 122 $\pm$ 36 nM, and 12.3 $\pm$ 0.86 μM, respectively. B, saturation binding assays for [³H]nicotine binding to rat brain membranes ($\alpha4\beta2$ nAChR), [³H]epibatidine binding to SH-SY5Y cell membranes ($\alpha3^*$ nAChR), and [³H]MLA binding to rat brain membranes ($\alpha7$ nAChR) were carried out in the absence (●) or presence (○) of 1 μM galantamine. Nonspecific binding was defined in the presence of 100 μM, 1 mM, and 1 mM nicotine, respectively, and subtracted to give specific binding. Data points are fitted to the Hill equation for a single binding site, and binding constants determined by nonlinear regression. Data are summarized in Table 1. Values are the mean $\pm$ S.E.M. from three assays, with each point assayed in triplicate.

TABLE 1
Binding data

	Without Galantamine		With Galantamine	
	B_{max}	K_d	B_{max}	K_d
	fmol/mg protein	nM	fmol/mg protein	nM
[³H]Nicotine	37.6 ± 6.05	10.3 ± 4.4	39.0 ± 5.5	12.8 ± 4.6
[³H]Epibatidine	48.5 ± 12.8	0.31 ± 0.05	45.5 ± 7.9	0.22 ± 0.02
[³H]MLA	48.8 ± 6.4	1.07 ± 0.31	43.5 ± 4.4	0.95 ± 0.19

tamine). Stimulation with galantamine alone (in the absence of nicotine) had no effect on fluorescence levels (data not shown).

SH-SY5Y cells express multiple nAChR subunits (Lukas et al., 1993; Peng et al., 1994; Wang et al., 1996) and have functional $\alpha3^*$ and $\alpha7$ nAChR that contribute to nicotine-evoked Ca^{2+} increases (Dajas-Bailador et al., 2002a; Ridley et al., 2002) but do not express $\alpha4\beta2$ nAChR, which is the predominant subtype in the brain. To examine the ability of galantamine to modulate increases in intracellular Ca^{2+} initiated by activation of this subtype, we used a mouse fibroblast cell line expressing rat $\alpha4\beta2$ nAChR (L$\alpha4\beta2$ cells; Cooper et al., 1999). Preincubation with galantamine also potentiated nicotine-evoked increases in fluo-3 fluorescence in these cells, and the effect was observed over a similar range of galantamine concentrations as in SH-SY5Y cells (Fig. 2C). The maximum potentiation (25.9 ± 9.4% increase) was produced by 1 μM galantamine.

Galantamine was compared with other AChEI for their abilities to modulate nicotine-evoked Ca^{2+} responses in SH-SY5Y cells. Preincubation with physostigmine (the first compound recognized as an APL at nAChRs; Shaw et al., 1985; Pereira et al., 1993, 2002) also potentiated nicotine-evoked increases in fluorescence but only at a single concentration (1 μM; Fig. 3A). The magnitude of the increase in nicotine-evoked fluorescence in the presence of physostigmine (18.9 ± 4.9% above the response to nicotine alone) was comparable with that observed with 1 μM galantamine (Fig. 2B). However, unlike galantamine, high concentrations of physostigmine (>30 μM) produced a significant decrease in nicotine-

evoked fluorescence (Fig. 3A). To investigate whether the potentiation of nAChR function was related to the ability to inhibit AChE, we examined nicotine-evoked responses after incubation with donepezil and rivastigmine, two structurally unrelated AChEI presently used in the treatment of AD. Neither rivastigmine nor donepezil potentiated responses to nicotine. At the highest concentration tested, rivastigmine (50 μM) produced a partial inhibition of the nicotine-evoked increase in fluo-3 fluorescence (Fig. 3B), whereas preincubation with donepezil generated a significant reduction of nicotine-evoked responses at concentrations of 3 μM and above, with an almost complete block at the highest concentrations tested (Fig. 3C). The IC_{50} value for this inhibition by donepezil was 3.9 μM.

The nicotine-evoked Ca^{2+} signals in SH-SY5Y cells are generated through the sequential activation of various sources of extracellular and intracellular Ca^{2+}, including VOCC and intracellular stores (Dajas-Bailador et al., 2002a). To assess if the potentiation or inhibition of nicotine-evoked responses was occurring through the modulation of downstream processes not necessarily dependent on nAChR activation, we compared the effect of AChEIs (0.5–30 μM) on the increases in fluorescence evoked by a general depolarising stimulus (KCl, 40 mM) in SH-SY5Y cells. The magnitude of the response to this concentration of KCl was comparable with that elicited by 30 μM nicotine (Ridley et al., 2002). Neither galantamine nor physostigmine nor rivastigmine produced any significant effect on the KCl-evoked increase in fluorescence, whereas in the presence of 10 and 30 μM donepezil, KCl-evoked responses were significantly decreased, to
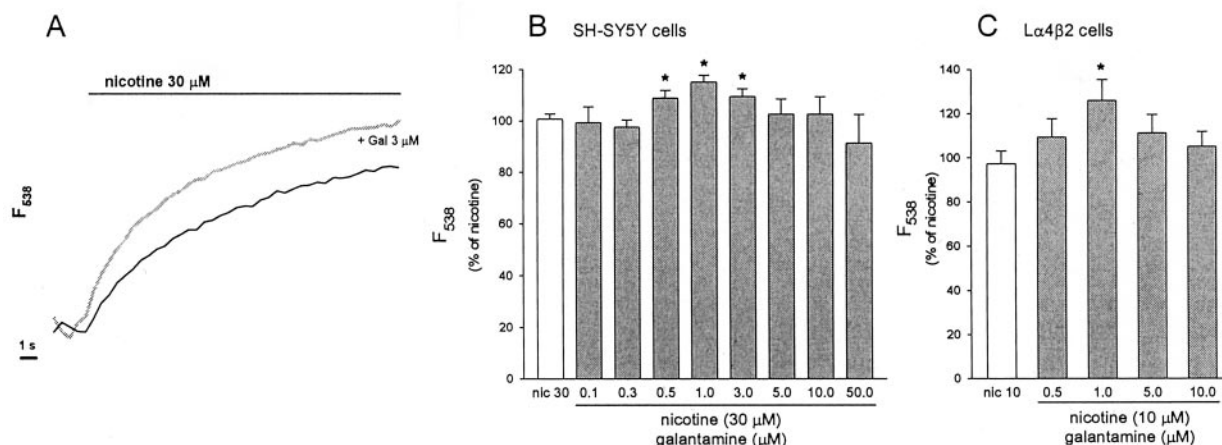
Fig. 2. Nicotine-evoked increases in intracellular Ca^{2+}: potentiation by galantamine. A, representative traces from SH-SY5Y cells loaded with fluo-3 AM and stimulated with 30 μM nicotine in the presence or absence of 3 μM galantamine. Where present, galantamine was preincubated with the cells for 5 min before addition of nicotine. Changes in fluorescence were monitored for 20 s. The nicotine-evoked increase in fluorescence in SH-SY5Y cells (B) and L$\alpha4\beta2$ cells (C) was determined after preincubation for 5 min with a range of galantamine concentrations. Nicotine (30 μM or 10 μM) was added and fluorescence changes after 20 s were recorded. Data are expressed as a percentage of the response to nicotine stimulation in the absence of galantamine and represent the mean ± S.E.M. of at least four independent experiments, each carried out in quadruplicate. Significantly different from nicotine stimulation; *, $p < 0.05$, Student's t test.

75 ± 3.9% and 53.2 ± 6.1% of KCl control, respectively (data not shown). Thus, of the four AChEI examined, only donepezil exhibited any effect on nAChR-independent increases in Ca^{2+}.

[³H]Noradrenaline Release

SH-SY5Y Cells. One of the many functional consequences of increasing intracellular Ca^{2+} concentrations is the exocytosis of neurotransmitter. Therefore, we examined whether the allosteric modulation of nAChR could be observed downstream of the evoked Ca^{2+} signals by analyzing the effects of galantamine and other AChEI on nicotine-evoked [³H]noradrenaline release from SH-SY5Y cells. Nicotine produced a significant and concentration-dependent increase of [³H]noradrenaline release (see Fig. 4) that was Ca^{2+}-dependent (data not shown) and blocked by the nAChR antagonist mecamylamine (see below). Coincubation with galantamine (0.3–30 μM) resulted in a bell-shaped potentiation of [³H]noradrenaline release evoked by 5 μM nicotine (Fig. 4A), reminiscent of the effects of galantamine on nicotine-evoked Ca^{2+} responses in these cells (Fig. 2B). Significant potentiation was observed with 0.5, 1 and 3 μM galantamine in both assays. However, the extent of potentiation was relatively greater than that observed for the Ca^{2+} responses, with 0.5, 1, and 3 μM galantamine producing an increase in [³H]noradrenaline release that was approximately 75% above that evoked by 5 μM nicotine alone. At a lower concentration of nicotine (3 μM), galantamine produced a similar potentiation (Fig. 4B), whereas responses to a higher nicotine concentration (10 μM) were unaffected by the presence of galantamine (Fig. 4C). The responses to 5 μM nicotine alone and 5 μM nicotine plus galantamine (0.5, 1, and 3 μM) were prevented by mecamylamine (40 μM; Fig. 4A), indicating that the potentiation occurred through nAChR. Consistent with this view, galantamine (1 μM) had no significant effect on [³H]noradrenaline release in the absence of nicotine (Fig. 4A).

In contrast to galantamine, neither physostigmine, rivastigmine, nor donepezil (0.3–30 μM) produced any significant modulation of [³H]noradrenaline release from SH-SY5Y cells stimulated with 5 μM nicotine (data not shown). This was unexpected in the case of donepezil, in view of its inhi-

bition of nicotine- (Fig. 3C) and KCl-evoked increases in intracellular Ca^{2+}. However, if some of donepezil's inhibitory effect is caused by the blockade of a secondary source of Ca^{2+}, and VOCC would be a prime candidate, it is possible that VOCC do not contribute to exocytosis in response to 5 μM nicotine in SH-SY5Y cells; this would explain the lack of any significant block by donepezil. To explore this possibility, we compared the effects of donepezil (30 μM) and $CdCl_2$ (100 μM; sufficient to block VOCC without inhibiting nAChR; Rathouz and Berg, 1994) on [³H]noradrenaline release evoked by increasing concentrations of nicotine (Fig. 5). At 5 μM nicotine, neither $CdCl_2$ nor donepezil affected the amount of nicotine-evoked [³H]noradrenaline release, consistent with a lack of involvement of VOCC. At 10 μM nicotine, $CdCl_2$ had no effect, whereas donepezil inhibited the response to nicotine by 34%. The response to 100 μM nicotine was significantly reduced by both $CdCl_2$ and donepezil, by 46% and 73%, respectively. These results suggest that VOCC contributes only to nicotine-evoked [³H]noradrenaline release at the highest agonist concentration tested, when $CdCl_2$ and donepezil are both effective blockers. The greater inhibition observed with donepezil, compared with that of $CdCl_2$, suggests that it has alternative modes of action: possibly a direct effect on nAChR and/or an indirect effect at a downstream target governing a secondary source of Ca^{2+}.

Hippocampal Slices. To determine whether the allosteric potentiation of nAChR-mediated transmitter release observed in SH-SY5Y cells is also pertinent to the central nervous system, we examined the effect of galantamine on nAChR-evoked [³H]noradrenaline release from rat hippocampal slices. In this experimental model, stimulation with nicotine (1, 5, and 10 μM) produced a significant and concentration-dependent increase in [³H]noradrenaline release (Fig. 6) that was much greater (relative to basal release) than in the cell line (Fig. 4). Stimulation with 1 and 5 μM nicotine in the presence of galantamine (0.5, 1, and 3 μM) resulted in a significant potentiation of [³H]noradrenaline release compared with the response induced by nicotine alone (Fig. 6). In agreement with the data from SH-SY5Y cells, galantamine did not potentiate release evoked by a higher concentration of nicotine (10 μM; Fig. 6). However, in
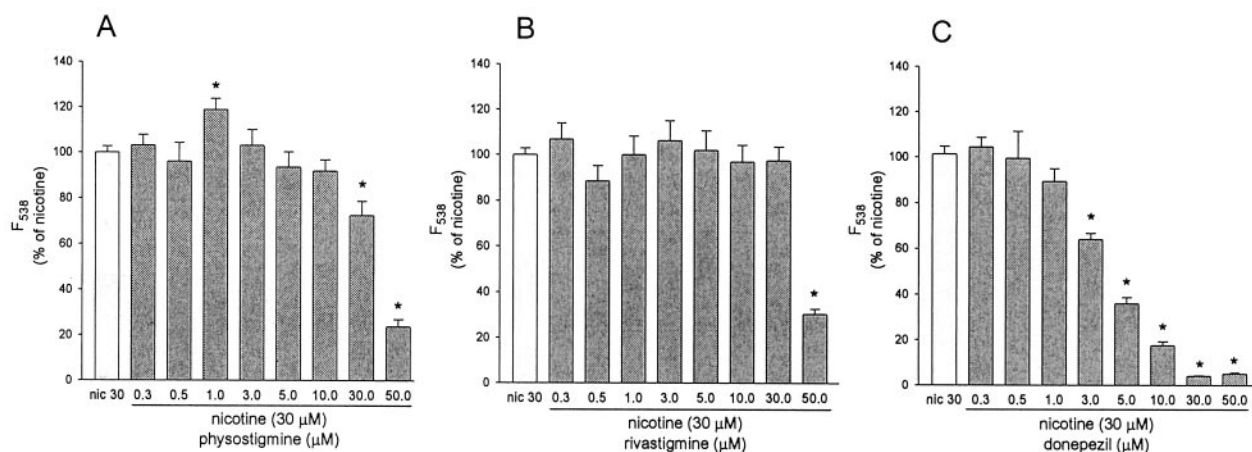
Fig. 3. The effect of AChEI on nicotine-evoked increases in intracellular Ca^{2+}. SH-SY5Y cells were loaded with fluo-3 AM and preincubated for 5 min with a range of concentrations (0.3–50 μM) of physostigmine (A), rivastigmine (B), or donepezil (C). Nicotine (30 μM) was added and fluorescence changes after 20 s were recorded. Data are expressed as a percentage of the response to nicotine stimulation in the absence of AChEI, and represent the mean ± S.E.M. of at least four independent experiments, each carried out in quadruplicate. Significantly different from nicotine stimulation; *, $p < 0.05$, Student's t test.

contrast to studies with the cell line, galantamine (1 μM) alone produced a significant increase in [³H]noradrenaline release in the absence of nicotine (Fig. 7; 31 ± 9.4% increase above basal). This release could be a consequence of AChE inhibition, such that any ACh released from the hippocampal slices would be preserved and hence could activate presynaptic nAChR (or muscarinic receptors). Evidence for the participation of nAChR in the release of [³H]noradrenaline evoked by galantamine alone was provided by the partial blockade by mecamylamine (Fig. 7A). This mechanism would augment the effects of submaximal nicotine concentrations and confound interpretation of the potentiation observed with galantamine. Simply subtracting the galantamine-alone response from the release of [³H]noradrenaline provoked by nicotine plus galantamine would be inappropriate, because nicotine will release further ACh from the hippocampal preparation (Wilkie et al., 1996); hence, the AChEI-dependent component will be increased.

Consistent with the hypothesis that AChE inhibition results in the release of [³H]noradrenaline, rivastigmine, physostigmine, and donepezil (1 μM) all significantly increased the amount of tritium released (Fig. 7). The potencies of these compounds as AChEI were determined in parallel for rat brain cholinesterase (Table 2); rivastigmine and donepezil were 8- and 20-fold more potent, respectively, than galantamine, which is in general agreement with literature values (Perry et al., 1988; Santos et al., 2002). At the concentration employed in the [³H]noradrenaline release assay (1 μM, Fig. 7), all of the compounds would have completely blocked AChE. To dissect the contribution of AChE inhibition to the observed galantamine potentiation of nicotine-evoked [³H]noradrenaline release in hippocampal slices, we examined galantamine's effects in the presence of 1 μM rivastigmine. The rationale for this experiment is that rivastigmine is essentially without effect on nAChR-mediated responses (Fig. 2B) but is a potent inhibitor of AChE (Table 2). Thus, incubation with rivastigmine would inhibit AChE without directly compromising nAChR activation, allowing any nicotinic action of galantamine to be revealed. As shown in Fig. 7B, galantamine still potentiated nicotine-evoked [³H]noradrenaline release in the presence of rivastigmine. However, the effect was considerably less than in the absence of the

independent inhibition of AChE; 1 μM galantamine enhanced nicotine-evoked [³H]noradrenaline release by 43 ± 9.5% and 14 ± 3.0% in the absence or presence, respectively, of rivastigmine.

Discussion

The present study shows that the allosteric modulation of nAChR can be translated into downstream cellular mechanisms. Galantamine potentiated nicotine-evoked increases in intracellular Ca^{2+} and [³H]noradrenaline release in SH-SY5Y cells with a bell-shaped concentration-response profile. In the absence of nicotine, galantamine had no effect on these measures, and its potentiation of nicotine-evoked responses could be blocked by mecamylamine. Galantamine did not compete for radioligand binding to the agonist binding sites of several neuronal nAChR subtypes, consistent with an allosteric mode of action. The AChEIs rivastigmine and donepezil did not potentiate nAChR-mediated responses; donepezil was a reasonably potent inhibitor of nicotine- and KCl-evoked increases in Ca^{2+}. nAChR-evoked [³H]noradrenaline release from hippocampal slices was also potentiated by galantamine, although this included a component attributable to cholinesterase inhibition and consequent increase in ACh.

Galantamine and related APLs have been well characterized as allosteric potentiators of neuronal nAChR subtypes from single-channel and whole-cell recordings (Pereira et al., 2002). Although these studies provide clear evidence for allosteric potentiation at the level of the nAChR itself, we wished to establish whether this effect is also manifested in downstream nAChR-dependent cellular responses. For this purpose, we selected as a model system the SH-SY5Y cell line, which has the advantage of providing a relatively homogenous population of cells expressing native nAChRs. Importantly, SH-SY5Y cells are predominantly monoaminergic (Vaughan et al., 1997), which minimizes the potential confounding effects arising from the anti-AChE activity of galantamine and other AChEIs. SH-SY5Y cells express $\alpha3$, $\alpha5$, $\alpha7$, $\beta2$, and $\beta4$ nAChR subunits and hence have a heterogeneous population of nAChRs, comprising $\alpha3^*$ and $\alpha7$ subtypes (Lukas et al., 1993; Peng et al., 1994; Wang et al., 1996). Each of these nAChR subtypes contributes to nicotine-
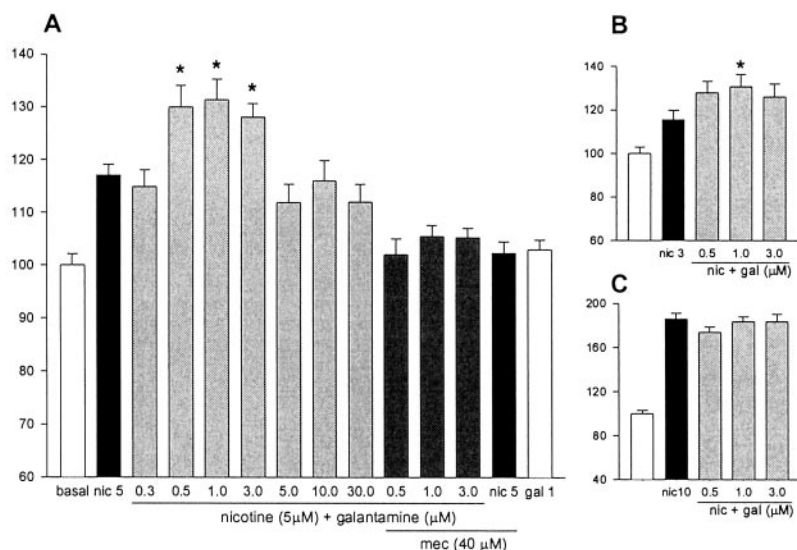
Fig. 4. The effect of galantamine on nicotine-evoked [³H]noradrenaline release in SH-SY5Y cells. SH-SY5Y cells were loaded with [³H]noradrenaline and the release of tritium evoked by incubation for 5 min with increasing concentrations of nicotine [5 μM (A), 3 μM (B), and 10 μM (C)] as described under *Materials and Methods*. Nicotine-evoked [³H]noradrenaline release was determined in the absence or presence of a range of galantamine concentrations. Galantamine (1 μM) was tested in the absence of nicotine (A). The effect of 5 μM nicotine on [³H]noradrenaline release in the presence and absence of galantamine was also examined in the presence of mecamylamine (40 μM; A). Data are expressed as a percentage of basal release in the absence of nicotine or galantamine and represent the mean ± S.E.M. of at least five independent experiments, each with eight replicates. Significantly different from nicotine stimulation; *, $p < 0.05$, Student's t test. In the presence of mecamylamine (A), there was no significant difference in release between nicotine, nicotine plus galantamine, and basal condition (Student's t test). There was no significant increase in [³H]noradrenaline release after incubation with galantamine alone (A), compared with basal release or with release in the presence of nicotine plus mecamylamine (Student's t test).

evoked increases in intracellular Ca^{2+} (Dajas-Bailador et al., 2002a; Ridley et al., 2002).

The allosteric modulation by galantamine and physostigmine of nAChR-evoked Ca^{2+} signals in SH-SY5Y cells (Fig. 2B, Fig. 3A), with no effect detected in the absence of nicotine, displayed a bell-shaped dose-response relationship and maximum potentiation at 1 μM for galantamine and physostigmine. These features correspond well with those described in electrophysiological recordings (Pereira et al., 1993, 2002; Storch et al., 1995; Santos et al., 2002). Previous studies have found that all nAChR subtypes examined are susceptible to potentiation by galantamine and related allo-

Fig. 5. The effect of donepezil and $CdCl_2$ on nicotine-evoked [³H]noradrenaline release in SH-SY5Y cells. SH-SY5Y cells were loaded with [³H]noradrenaline and the release of tritium was evoked by 5-min incubation with 5, 10, or 100 μM nicotine in the presence or absence of donepezil (30 μM) and $CdCl_2$ (100 μM). Significantly different from nicotine-evoked release in the absence of drugs; *, $p < 0.05$, Student's t test. Significantly different from response in the presence of $CdCl_2$; #, $p < 0.05$, one-way ANOVA (post hoc Dunn's test). In all cases, data represent the mean $\pm$ S.E.M. of at least five independent experiments, each with eight replicates.

Fig. 6. Effect of galantamine on nicotine-evoked [³H]noradrenaline release from hippocampal slices. Rat hippocampal slices were loaded with [³H]noradrenaline, and the release of tritium was evoked by 5-min incubation with 1, 5, or 10 μM nicotine in the presence or absence of increasing concentrations of galantamine. Data are expressed as a percentage of basal release and represent the mean $\pm$ S.E.M. of at least six independent experiments, each with eight replicates. Significantly different from nicotine stimulation; *, $p < 0.05$, Student's t test.

steric modulators (Pereira et al., 2002; Samochocki et al., 2003), and this was endorsed by the comparable potentiation by galantamine of nicotine-evoked increases in Ca^{2+} in cells expressing the $\alpha4\beta2$ nAChR (Fig. 2C). In the case of rivastigmine and donepezil, their inability to potentiate nicotine-evoked Ca^{2+} increases in SH-SY5Y cells (Fig. 3, B and C) is in accordance with their failure to potentiate ACh-induced whole-cell currents in HEK cells expressing defined nAChR subtypes (Samochocki et al., 2003) or the amplitude of excitatory postsynaptic currents recorded from hippocampal neurons (Santos et al., 2002). In the present study, the negative result with these potent AChEIs confirms that inhibition of AChE does not contribute to the observed potentiation of nicotinic responses in SH-SY5Y cells. The failure of both galantamine and physostigmine to enhance responses to a general depolarizing stimulus, KCl, further supports a nAChR-specific allosteric mechanism able to potentiate receptor-mediated cellular responses.

Nevertheless, a competitive interaction of galantamine and physostigmine with the nAChR agonist binding site has also been advocated, based on the observation that physostigmine's potentiation of ACh-evoked whole-cell currents in $X.$ $laevis$ oocytes expressing $\alpha4\beta4$ nAChR was surmountable with increasing ACh concentration (Zwart et al., 2000). However, although surmountable effects are characteristic of competitive interactions, they are also compatible with an allosteric modulation of receptor function (Samochocki et al., 2003). Physostigmine was found to block [³H]epibatidine binding to $\alpha4\beta4$ nAChR in oocyte membranes (Zwart et al., 2000), but the K_i for inhibition of binding (40 μM) was about 10-fold higher than the concentration of physostigmine producing half-maximal potentiation of macroscopic currents in $X.$ $laevis$ oocytes. Although physostigmine was previously reported to weakly inhibit both nicotinic and muscarinic binding sites (Perry et al., 1988), it did so at concentrations well above those shown to potentiate functional responses. In the present study, the failure of galantamine to compete for agonist binding sites in competition assays, even at 100 μM concentrations (Fig. 1A), is consistent with an action at a site on the nAChR that is distinct from the agonist binding site (Schrattenholz et al., 1996).

Increases in intracellular Ca^{2+} provide a signal that can regulate many biochemical events (Berridge et al., 1998). As a downstream Ca^{2+}-dependent process, we chose to monitor [³H]noradrenaline release. This is a relevant measure, because the enhancement of transmitter release is an important factor in the modulatory actions ascribed to nAChR and is also a therapeutic goal for symptomatic improvement in AD. In SH-SY5Y cells, galantamine increased nicotine-evoked [³H]noradrenaline release with the same bell-shaped profile and concentration dependence as observed for the Ca^{2+} responses (Fig. 4). Galantamine was only effective at lower nicotine concentrations, consistent with a shift of the concentration-response curve to the left, with no change in maximum response, as previously observed for ACh-evoked whole-cell currents in HEK cells expressing defined nAChR subtypes (Samochocki et al., 2003).

Taken together, the potentiation of Ca^{2+} responses and [³H]noradrenaline release provides evidence that the potentiation of nAChR currents by APLs is also translated into the cellular responses initiated by nAChR activation. This property was not shared by the other AChEIs currently used in
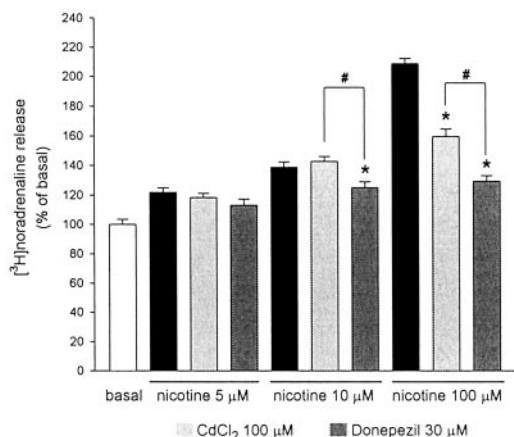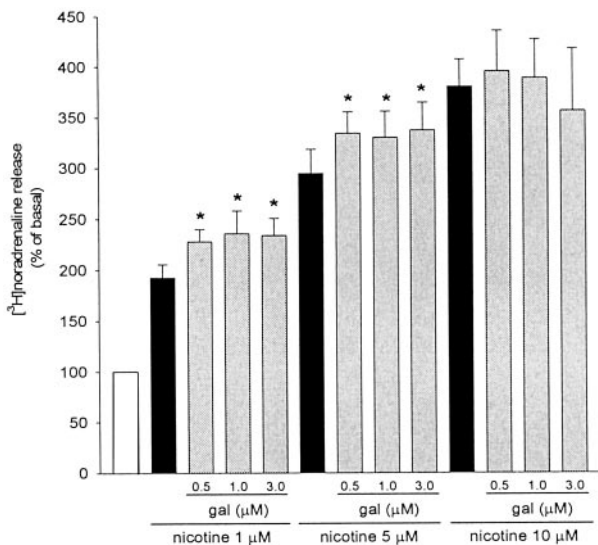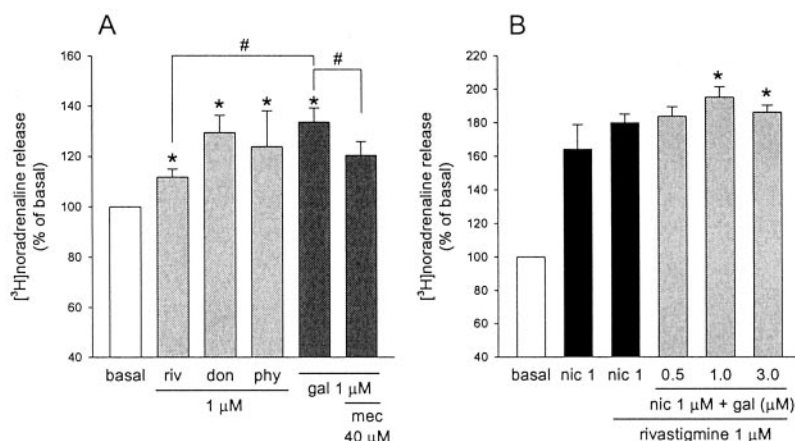
Fig. 7. Effect of AChEI on basal [^{3}H]noradrenaline release (A) and nicotine-evoked [^{3}H]noradrenaline release (B) from rat hippocampal slices. Rat hippocampal slices were loaded with [^{3}H]noradrenaline and incubated for 5 min with (A) rivastigmine, donepezil, physostigmine or galantamine (1 μM). Galantamine was examined in the presence and absence of mecamylamine (40 μM). The tritium released was expressed as a percentage of basal release in the absence of drugs. All of the AChEI tested significantly increased the release of [^{3}H]noradrenaline above basal levels (*, $p < 0.05$, Student's t test); the effect of galantamine was significantly greater than that of rivastigmine (#, $p < 0.05$, one-way ANOVA, post hoc Tukey's test). In the presence of mecamylamine, the release evoked by galantamine was significantly less than that evoked by galantamine alone (#, $p < 0.05$, Student's t test). B, rat hippocampal slices were incubated with rivastigmine (1 μM) to inhibit AChE before stimulation for 5 min with nicotine (1 μM) in the presence or absence of galantamine (0.5, 1, and 3 μM) and measurement of [^{3}H]noradrenaline release. In parallel, slices were stimulated with nicotine in the absence of any AChE. Significantly different from the response to nicotine + rivastigmine; *, $p < 0.05$, Student's t test. Data represent the mean ± S.E.M. of at least five independent experiments, each with eight replicates.

TABLE 2

Inhibition of cholinesterase activity in rat brain membranes

AChEI	IC$_{50}$ ($n = 3$)
	nM
Galantamine	346.1 ± 73.9
Physostigmine	73.7 ± 23.3
Rivastigmine	43.4 ± 14.9
Donepezil	14.5 ± 2.3

the treatment of AD, namely rivastigmine and donepezil, which exhibited distinct profiles of activity. Rivastigmine had no effect on either nicotine- or KCl-evoked increases in Ca^{2+} (except for a partial inhibition of the former by 50 μM rivastigmine, probably indicative of channel block; Fig. 3B) or on nicotine-evoked [^{3}H]noradrenaline release from SH-SY5Y cells. In the case of donepezil, this is the first report to show that it is an effective inhibitor of nAChR-evoked Ca^{2+} responses (Fig. 3C), with an IC$_{50}$ of 3.9 μM. This latter observation does not distinguish between direct inhibition of nAChRs (e.g., by channel block) and/or inhibition of secondary sources of Ca^{2+} (VOCC or internal stores; Dajas-Bailador et al., 2002a), although the block of KCl-evoked responses by donepezil might indicate a dual mode of action. This possibility was further analyzed in [^{3}H]noradrenaline release studies; the progressive inhibition of nicotine-evoked release by donepezil accompanied susceptibility to block by CdCl$_2$, supporting the involvement of VOCC only at high nicotine concentrations (Fig. 5; Kulak et al., 2001). Although this is compatible with a block of VOCC by donepezil, it is not sufficient to fully explain these results, because donepezil is more effective than CdCl$_2$ on responses evoked by 10 and 100 μM nicotine, and a nAChR subtype-selective inhibition by donepezil might also contribute.

The ability of galantamine to increase nicotine-evoked [^{3}H]noradrenaline release is also relevant to the central nervous system, because galantamine potentiated nicotine-evoked [^{3}H]noradrenaline release from hippocampal slices (Figs. 6 and 7B). The hippocampus receives a relatively rich noradrenergic innervation, and previous studies have described the nicotinic modulation of noradrenaline release from hippocampal synaptosomes (Clarke and Reuben, 1996; Kulak et al., 2001) and slices (Sacaan et al., 1995; Vizi and Kiss, 1998; Leslie et al., 2002). Both direct (nAChR on noradrenergic terminals) and indirect (via nicotine-induced release of another transmitter) mechanisms have been implicated in nicotine-stimulated [^{3}H]noradrenaline release from hippocampal slices (Leslie et al., 2002). The interpretation of cholinergic modulation in this system could be further complicated by inhibition of AChE, as would occur in the presence of galantamine. Indeed all the AChEIs examined in this study significantly increased [^{3}H]noradrenaline release from hippocampal slices (Fig. 7A), in contrast to the lack of a comparable effect in SH-SY5Y cells. In the slice preparation, AChE inhibition presumably results in the preservation of ACh released tonically or by leakage, and this ACh (acting via nAChR or muscarinic receptors) can augment the release of [^{3}H]noradrenaline (and other transmitters). In the case of galantamine, [^{3}H]noradrenaline release was partially attributable to nAChRs, as shown by its sensitivity to mecamylamine (Fig. 7A). Muscarinic mechanisms are likely to account for the residual release. The potentiation of nAChR-evoked [^{3}H]noradrenaline release by galantamine, revealed in the presence of rivastigmine (Fig. 7B) suggests that the direct allosteric action on nAChR does impact on levels of transmitter release in hippocampal slices.

The nicotinic modulation of transmitter release in the hippocampus, and its potentiation by galantamine, is pertinent to the treatment of AD (Santos et al., 2002). In particular, noradrenaline has been ascribed a modulatory role in promoting hippocampal synaptic plasticity that may contribute to memory formation (Katsuki et al., 1997). Indeed, the interaction of cholinergic and noradrenergic systems is proposed to determine the tonic levels of noradrenaline in the hippocampus (see Vizi and Kiss, 1998). Both cholinergic and

noradrenergic activities are diminished in AD (see Palmer, 1996) but may be enhanced by the dual effects of AChE inhibition and nAChR potentiation produced by galantamine.

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