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Direct Inhibition of 5-Hydroxytryptamine$_3$ Receptors by Antagonists of L-type Ca^{2+} Channels

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SUMMARY

Homopentameric complexes of either the A or As subunit of the 5-hydroxytryptamine$_3$ receptor form Ca^{2+}-permeable channels that can be activated by the selective agonist 1-(*m*-chlorophenyl)-biguanide (mCPBG). In both N1E-115 neuroblastoma cells and human embryonic kidney 293 cells stably expressing the 5-HT$_3$ receptor As subunit, (+)-verapamil, (−)-verapamil, diltiazem, and nimodipine caused reversible and concentration-dependent (IC_{50} = 2.5–6.5 μM) inhibition of the increases in cytosolic [Ca^{2+}] evoked by mCPBG. In voltage-clamped human embryonic kidney 293 cells stably expressing the 5-HT$_3$ receptor As subunit, similar concentrations of the Ca^{2+} channel antagonists (IC_{50} = 3.0–6.8 μM) accelerated the rate at which 5-HT-evoked currents decayed without affecting the amplitude of the peak current. In equilibrium competition binding assays to membranes from Sf9 cells infected with the 5-HT$_3$ receptor As subunit, [^{3}H]mCPBG and [^{3}H]granisetron were displaced by (+)-verapamil, (−)-verapamil, and diltiazem; (+)-verapamil was ~10-fold more potent than (−)-verapamil and ~30-fold more potent than diltiazem. Nimodipine neither displaced [^{3}H]granisetron binding nor affected its displacement by diltiazem and (+)-verapamil. The stereoselectivity of verapamil binding, which contrasts with the similar potency of each isomer in functional assays, was maintained when the incubations were performed at 20° or when an antagonist of the 5-HT$_3$ receptor, [^{3}H]granisetron, was used as the radioligand. The interaction between verapamil and either [^{3}H]mCPBG or [^{3}H]granisetron binding was not competitive. We conclude that the inhibition of [^{3}H]mCPBG binding by diltiazem and verapamil is mediated by a site that is distinct from both the agonist-binding site and from the site through which nimodipine inhibits 5-HT$_3$ receptor function. Our results provide evidence for allosteric regulation of agonist binding to 5-HT$_3$ receptors and the first example of a ligand-gated ion channel whose function is directly inhibited by members of all three major classes of L-type Ca^{2+} channel antagonists.

Ligand-gated ion channels comprise a family of receptors that share many structural and functional characteristics (1). Within this family, 5-HT$_3$ receptors are particularly amenable to structural analysis because pentamers of five identical subunits form functional receptors (2, 3), thereby greatly facilitating expression studies. Indeed, from sequence analyses it has been suggested that the 5-HT$_3$ receptor may be most closely related to a primordial homo-oligomeric ligand-gated ion channel (4). Although the molecular cloning of all other members of this family of receptors was rapidly followed by identification of related subunits leading to enormous potential for receptor diversity, no such diversity has emerged for 5-HT$_3$ receptors. The 5-HT$_3$RA subunit was cloned 5 years ago from the NCB-20 neuroblastoma cell line (5), but despite evidence for both pharmacological and biophysical variations between tissues and species (6), no further 5-HT$_3$ receptor subunits have been identified. 5-HT$_3$ receptor subunits have been cloned from additional neuroblastoma cell lines [NG-108-15 (7) and N1E-115 (8)] and from mouse (9) and human (10) brain, but all are homologs of the original 5-HT$_3$RA subunit. The presence in mouse, although not in the human gene, of an alternative splice site generates two forms of the 5-HT$_3$ receptor subunit: a long (A) and a short form (As) that lacks six amino acids in the large intracellular loop (8). The two forms of the receptor differ in their distribution in rodent brain (11), in their levels of expression during development (11) or differentiation (12), and in the

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ABBREVIATIONS: 5-HT, 5-hydroxytryptamine; $[Ca^{2+}]_i$, cytoplasmic free Ca^{2+} concentration; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HBM, HEPES-buffered medium; HEK, human embryonic kidney; HEK/5-HT$_3$RAs, HEK 293 cells transfected/infected with the As form of the 5-hydroxytryptamine$_3$ receptor subunit; Sf9/5-HT$_3$RAs, Sf9 cells transfected/infected with the As form of the 5-hydroxytryptamine$_3$ receptor subunit; I_p, peak current amplitude; mCPBG, 1-(*m*-chlorophenyl)-biguanide; NMDA, *N*-methyl D-aspartate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

efficacy of some agonists (13, 14), suggesting that alternative splicing may be of physiological significance, at least in rodents. In addition to its utility in allowing analyses of the essential properties of ligand-gated ion channels, the 5-HT$_3$ receptor is an important therapeutic target. Antagonists of 5-HT$_3$ receptors are used in the treatment of emesis, and the possibility that they may also be useful as anxiolytic or antipsychotic drugs has aroused further interest (15).

Mouse N1E-115 neuroblastoma cells, in which 5-HT$_3$ receptors are expressed at high levels, have been extensively used in electrophysiological (16), radioligand binding (17), purification (18), and cloning (8) studies. Although electrophysiological analyses of neuroblastoma cell lines and neurons concur in demonstrating that the 5-HT$_3$ receptor is a cation-selective channel with similar permeability to Na^+ and K^+ (19), they provide conflicting evidence on its Ca^{2+} permeability (16, 20–22). We recently demonstrated, using single-cell imaging of Fura-2-loaded cells, that the native 5-HT$_3$ receptors of N1E-115 neuroblastoma cells and both the long (A) and short (As) forms of the cloned 5-HT$_3$ receptor are Ca^{2+} permeant (23).

In our initial studies of Ca^{2+} signaling in N1E-115 cells, we observed that dihydropyridine antagonists of L-type Ca^{2+} channels abolished the Ca^{2+} signals evoked by activation of 5-HT$_3$ receptors, despite our demonstration that the receptor is itself permeable to Ca^{2+} (23). In the current study, we used a combination of single-cell video-imaging of Fura-2-loaded cells, whole-cell voltage-clamp recording, and radioligand binding to examine the effects of antagonists of L-type Ca^{2+} channels on 5-HT$_3$ receptors.

Materials and Methods

Reagents. N1E-115 neuroblastoma cells and HEK 293 cells were obtained from the European Collection of Animal Cell Cultures (Porton Down, UK). All cell culture reagents were obtained from GIBCO BRL (Paisley, UK), except fetal calf serum, which was from Advanced Protein Products (West Midlands, UK). Fura-2 AM, Fura-2 pentapotassium salt, and Ca^{2+} standard solutions were from Molecular Probes (Eugene, OR). Nimodipine and mCPBG were from Tocris Cookson Chemicals (Southampton, UK). Both isomers of verapamil, *S*-(−)-Bay K 8644 and 5-HT hydrochloride, were from Research Biochemicals (St. Albans, UK). Ondansetron and granisetron were gifts from Glaxo Group Research (Ware, UK) and SmithKline Beecham Pharmaceuticals (Welwyn, UK), respectively. [^{3}H]Granisetron (85 Ci/mmol) and [^{3}H]mCPBG (26 Ci/mmol) were from Dupont (Stevenage, UK). All other reagents, including diltiazem, were obtained from Sigma Chemical (Poole, UK).

Cell culture and selection of stable cell lines. We previously demonstrated that the Ca^{2+} signals evoked by mCPBG were similar in transiently transfected HEK/5-HT$_3$RA and HEK/5-HT$_3$RAs cells (23) and therefore concentrated on the stable HEK/5-HT$_3$RAs cells because the short subunit is the most abundantly expressed subunit in N1E-115 cells (8) and brain (11). N1E-115 cells and HEK 293 cells were cultured as described previously (23). HEK 293 cells were transfected with the eukaryotic expression vector pRc/CMV (InVitrogen, Abingdon, UK) with or without the complete coding sequence for the 5-HT$_3$RAs subunit from N1E-115 cells.[2] In brief, DNA liposomes were prepared by mixing (30 min, 20°) plasmid DNA (6 μg) with lipofectamine (15 μl) and OptiMEM (385 μl) before diluting the mixture 5-fold into OptiMEM. Cells (10^6/10-cm dish) that had been cultured for 24 hr and reached 50–60% confluence were washed with OptiMEM (twice with 5 ml) and then incubated with the DNA-liposome mixture. After 6 hr, the medium was replaced by F-12/Dulbecco's modified Eagle's medium containing serum (10%), L-glutamine (4 mM), and nonessential amino acids; after an additional 18 hr, the medium was replaced by a similar medium supplemented with antibiotics (50 units/ml penicillin, 50 μg/ml streptomycin). After 48 hr, stable cell lines were selected by diluting the transfected cells 10-fold and replating then into selection medium, which consisted of F-12/Dulbecco's modified Eagle's medium containing serum, L-glutamine, nonessential amino acids (1%), antibiotics, and geneticin (600 μg/ml). The medium was changed after 7 days. After 14 days, surviving colonies were individually removed and further cultured for 14 days in multiwell dishes containing 1 ml of selection medium. Thereafter, the cells were maintained in selection medium, and frozen stocks of cells (10^6/ml) were stored in liquid nitrogen in fetal calf serum containing dimethylsulfoxide (8%). From the four cell lines selected from each transfection condition (5-HT$_3$RAs and pRc/CMV vector only), all of the cells transfected with the vector pRc/CMV and one of the cell lines transfected with 5-HT$_3$RAs failed to respond to mCPBG. One cell line from those expressing functional 5-HT$_3$RAs receptors was selected for further study. The fraction of the stable HEK/5-HT$_3$RAs cells that gave detectable increases in $[Ca^{2+}]_i$ in response to mCPBG (75–80%) was similar in the freshly isolated cell line and in cells recovered from frozen stocks.

Sf9 cells were cultured and infected using a baculovirus transfer vector as described previously (3).

Measurement of $[Ca^{2+}]_i$. The method used to load cells with Fura-2, which ensures that ≥95% of the cellular Fura-2 is both cytosolic and hydrolyzed to the Ca^{2+}-sensitive Fura-2 free acid, has been described previously (23). Cells were incubated in either HBM (115 mM NaCl, 10 mM KCl, 1 mM KH_2PO_4, 0.5 mM $MgSO_4$, 1.25 mM $CaCl_2$, 25 mM HEPES, 15 mM glucose, pH 7.4 at 20°) or, usually, in Na^+-free HBM in which the NaCl of HBM was replaced with 115 mM *N*-methyl-D-glucamine.

The equipment used to record the Fura-2 fluorescence of single cells was similar to that used previously (23). In brief, cells on a round coverslip were mounted in a chamber held on the stage of an inverted epifluorescence microscope. A 100-W xenon lamp provided the light used to excite the Fura-2 fluorescence, and changes in excitation wavelength (λ_{ex}) were achieved by rapid rotation of a wheel housing narrow band interference filters (340 and 380 nm). Emitted light was captured by an ISIS-M charge-coupled device camera (Photonic Science, Robertsbridge, UK) after passage through a dichroic mirror (400 nm) and high pass barrier filter (480 nm). Digitized images were stored and processed using an Improvision image analysis system running IonVision III software (Improvision, Coventry, UK). In most experiments, a fluorescence ratio ($R_{340/380}$) was collected at 2-sec intervals, although this interval was extended to 4 sec for more-prolonged recordings. Corrections for background fluorescence and the calibration of fluorescence ratios to $[Ca^{2+}]_i$ with the use of a table created from Ca^{2+} standard solutions were as reported previously (23). The perfusion system allowed the media bathing the cells to be exchanged with a half-time of ~4 sec, and exchange was essentially complete within 12 sec (23).

Electrophysiological recordings. Whole-cell currents were recorded at 20–22° using an EPC-9 amplifier (HEKA Elektronik, Darmstadt, Germany) controlled by a Macintosh Power PC 7100/66 computer running Pulse software (HEKA version 7.85). Briefly, HEK/5-HT$_3$RAs cells cultured on a 35-mm dish were continuously perfused with extracellular medium (140 mM NaCl, 5.4 mM KCl, 2 mM $MgCl_2$, 1.8 mM $CaCl_2$, 30 mM glucose, 10 mM HEPES, pH 7.2 at 20°). Agonists were applied by perfusion from a perforated tube as described previously (24). Patch pipettes (3–5 MΩ) were back-filled with filtered (0.2 μm, Millipore, Bedford, MA) intracellular medium (140 mM KCl, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 10 mM EGTA, ~10 nM free Ca^{2+}, 10 mM HEPES, pH 7.2 at 20°). Currents were recorded from cells held at −60 mV unless otherwise stated.

[2] 5-HT$_3$RAs cDNA was cloned using the polymerase chain reaction from cDNA of N1E-115 cells.

Radioligand binding. N1E-115 or HEK/5-HT_3RAs cells were washed twice with HBM and then scraped into HEPES buffer (10 mM, pH 7.5) containing 1 mM EDTA, 50 μg/ml soybean trypsin inhibitor, 50 μg/ml bacitracin, and 0.1 mM phenylmethylsulfonyl fluoride. The suspension was centrifuged (20,000 × *g*, 10 min, 0°) and the membranes were then washed twice in HEPES buffer (10 mM) and used immediately for binding experiments.

Membranes were prepared from infected Sf9 cells by lysing cells in a Potter homogenizer in medium containing 10 mM HEPES, pH 7.0 (0°), 1 mM EDTA, 5 μg/ml leupeptin, 2.5 μg/ml pepstatin A, and 1 mM phenylmethylsulfonyl fluoride (3). The homogenate was centrifuged three times (1,000 × *g*, 15 min), the combined supernatants from each step were then recentrifuged (130,000 × *g*, 45 min), and the pellet was resuspended in HEPES buffer and stored at −70°.

Equilibrium competition binding and kinetic analyses were performed in a final volume of 500 μl of HEPES buffer (10 mM, pH 7.5) that included membranes (20 μg of Sf9 membrane protein or 40 μg of HEK 293 membrane protein) and, unless otherwise stated (Fig. 6) either 1 nM [^{3}H]mCPBG or 0.1 nM [^{3}H]granisetron. The same buffer and final concentration of membranes were used in saturation binding studies, but the final incubation volume was reduced to 250 μl and the concentrations of [^{3}H]mCPBG or [^{3}H]granisetron varied between 0.006 and 10 nM. Nonspecific binding was defined using 50 μM ondansetron. Except where specified in the text, incubations were for 2 hr at 0°. Incubations were terminated by rapid filtration through Whatman GF/B filters using a Brandel receptor binding harvester, followed by three washes with 4 ml of ice-cold HEPES buffer (5 mM, pH 7.5). Protein concentrations were determined (25) using bovine serum albumin as the standard.

Analysis of results. Concentration-response relationships were fitted by nonlinear least-squares regression analysis to a logistic equation using Kaleidagraph (Abelbeck Software, Reading, PA):

$$\text{Response} = \frac{E_{max}[A]^{n_H}}{EC_{50}{}^{n_H} + [A]^{n_H}}$$

and inhibition data were fitted to the logistic equation:

$$\text{Response} = \frac{E_{max} - E_{min}}{1 + (IC_{50}/[I])^{n_H}}$$

where E_{max} and E_{min} are maximal and minimal responses, respectively; [*A*] and [*I*] are concentrations of agonist and inhibitor, respectively; n_H is the Hill coefficient; and EC_{50} and IC_{50} have their usual meanings. The same methods were used to fit the results of equilibrium radioligand binding studies to the analogous logistic equations and to fit the results of kinetics experiments. The time constants (τ) of the current decays were derived by fitting monoexponential equations by least-squares regression using Igor (WaveMetrics, Lake Oswego, OR).

Results are presented as mean ± standard error. Where appropriate, Student's *t* test for paired or unpaired data was used, and values of $p \leq 0.05$ were regarded as significant.

Results

Ca^{2+} channel antagonists inhibit mCPBG-evoked increases in $[Ca^{2+}]_i$. Our previous results (23) and other reports (20–22) have established that 5-HT_3 receptors are Ca^{2+} permeable, yet the Ca^{2+} signals evoked by 5-HT_3 receptors in N1E-115 cells were almost completely (>85%) abolished by ligands of L-type Ca^{2+} channels (<10 μM). Both antagonists (nimodipine and (±)-verapamil) and an agonist of L-type Ca^{2+} channels (Bay K 8644) caused concentration-dependent (IC_{50} = 1–5 μM) inhibition of the Ca^{2+} signals evoked by the selective 5-HT_3 receptor agonist mCPBG (400 nM) (not shown). Although both nimodipine (10 μM) and Bay K 8644 (1–10 μM) inhibited the Ca^{2+} signals evoked by 5-HT_3 receptors, depolarization-evoked Ca^{2+} signals were inhibited by nimodipine but potentiated by Bay K 8644.[3] These results suggest a direct effect of L-type Ca^{2+} channel ligands on 5-HT_3 receptors. To explore their effects in the absence of any possible contribution from Ca^{2+} channels, we expressed 5-HT_3 receptors in cells that lack voltage-gated Ca^{2+} channels.

The addition of mCPBG to stably transfected HEK/5-HT_3RAs cells in Na^+-free HBM caused a concentration-dependent increase in $[Ca^{2+}]_i$ (Fig. 1a); in two independent experiments, the half-maximal effects occurred when the mCPBG concentrations were 275 and 287 nM (n_H = 4.0 and 4.4) (Fig. 1c). These results, and the ~95% inhibition of the response when Na^+ (115 mM) was present in the extracellular medium (Fig. 1b), confirm our previous results with transiently transfected HEK/5-HT_3RAs cells (23). Depolarization of HEK/5-HT_3RAs cells by increasing the extracellular K^+ concentration to 75 mM consistently failed to evoke an increase in $[Ca^{2+}]_i$ in either the presence or absence of extracellular Na^+ (Fig. 1b). In three independent experiments, depolarization of voltage-clamped HEK/5-HT_3RAs cells from −60 mV to +50 mV failed to evoke a detectable Ca^{2+} current, as has been previously reported for HEK 293 cells (26, 27).[4] These results establish that voltage-gated Ca^{2+} channels do not contribute to the Ca^{2+} signals or currents recorded from stimulated HEK/5-HT_3RAs cells.

In stably transfected HEK/5-HT_3RAs cells, nimodipine, diltiazem, (+)-verapamil, and (−)-verapamil caused concentration-dependent inhibition of the increases in $[Ca^{2+}]_i$ evoked by 400 nM mCPBG (Fig. 2 and Table 1). Maximal concentrations of each of the antagonists completely inhibited the mCPBG-evoked increase in $[Ca^{2+}]_i$. (−)-Verapamil was significantly more potent than nimodipine ($p < 0.05$), but there were no significant differences between the potencies of the other compounds. The inhibition caused by a single application (100 sec) of either (+)-verapamil (10 μM) or nimodipine (10 μM) was almost completely reversed after washout for 10 min: the mCPBG-evoked Ca^{2+} signals recovered to 92 ± 4% (eight experiments) and 93 ± 5% (six experiments) of their pretreatment levels, respectively (Fig. 2f). After several sequential (>4) applications of each antagonist, the inhibition of the mCPBG-evoked $[Ca^{2+}]_i$ responses was only partially reversed after washout (10 min). Under such conditions, the Ca^{2+} signals evoked by mCPBG (400 nM) in cells treated with (+)-verapamil, (−)-verapamil, nimodipine, and diltiazem, recovered to only 49 ± 9%, 82 ± 6%, 68 ± 6%, and 49 ± 5% (three experiments) of their pretreatment levels, respectively.

Ca^{2+} channel antagonists accelerate the decay of currents through 5-HT_3 receptors. Sustained application of maximally effective concentrations of either 5-HT (30 μM)

[3] In N1E-115 cells, depolarization-evoked increases in $[Ca^{2+}]_i$ were poorly reproducible. In two independent experiments with cell populations and single cells in which cells were sequentially challenged with mCPBG (400 nM) and increased extracellular K^+ concentration (75 mM), the increases in $[Ca^{2+}]_i$ evoked by mCPBG were abolished by (−)-Bay K 8644 (10 μM), whereas those evoked by depolarization were potentiated by 10-fold. Although N1E-115 cells frequently failed to respond to depolarization, Bay K 8644 never inhibited depolarization-evoked increases in $[Ca^{2+}]_i$ and invariably inhibited those evoked by mCPBG.

[4] We also failed to detect Ca^{2+} currents in HEK/5-HT_3RAs cells using the same methods that were successfully used to detect depolarization-evoked Ca^{2+} currents in HEK 293 cells stably transfected with cDNA encoding subunits of human N-type Ca^{2+} channels (53).

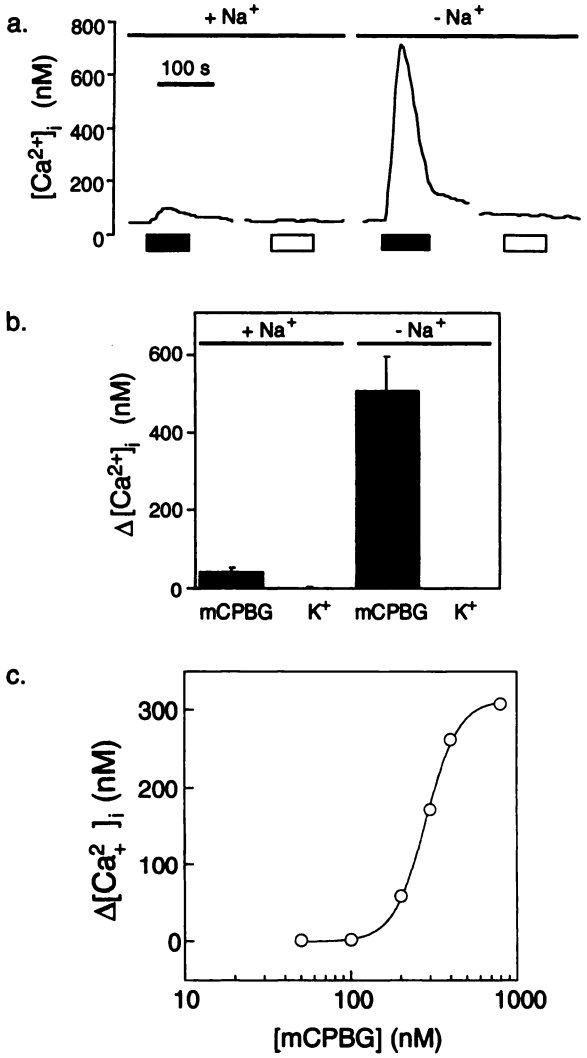

Fig. 1. Removal of extracellular Na^+ potentiates mCPBG-evoked increases in $[Ca^{2+}]_i$ in HEK/5-HT$_3$RAs cells. a, Representative *trace* from a single cell, showing the increases in $[Ca^{2+}]_i$ stimulated by 400 nM mCPBG (*filled bars*) and the potentiating effect of replacing extracellular Na^+ with 115 mM *N*-methyl-D-glucamine. Depolarization by increasing the extracellular K^+ concentration to 75 mM (*open bars*) had no effect on $[Ca^{2+}]_i$ in the presence or absence of extracellular Na^+. b, Results from experiments similar to those shown in a. Each value represents the mean ± standard error of three independent fields of cells within each of which the responses from four to eight cells were averaged. c, HEK/5-HT$_3$RAs cells were stimulated with the indicated concentrations of mCPBG in Na^+-free HBM. The results, representative of two similar experiments, show the average increases in $[Ca^{2+}]_i$ from a field of six cells.

or mCPBG (3 μM) to HEK/5-HT$_3$RAs cells clamped at −60 mV rapidly evoked an inward current that was completely abolished by the selective 5-HT$_3$ receptor antagonist ondansetron (100 nM). The current had a reversal potential of −4.7 ± 1.6 mV (five experiments), and it decayed monoexponentially (τ = 3.4 ± 0.5 sec, five experiments) (Fig. 3a). These electrophysiological characteristics are essentially similar to those previously recorded from native 5-HT$_3$ receptors (24).

Ca^{2+} channel antagonists had no effect on the currents recorded from unstimulated HEK/5-HT$_3$RAs cells, but the current evoked by 5-HT (30 μM) was inhibited by simultaneous application of nimodipine, diltiazem, (+)-verapamil, or (−)-verapamil (Fig. 3). Each antagonist increased the rate at which the 5-HT-evoked current decayed. The concentrations of each antagonist required to cause a half-maximal (IC_{50}) decrease in τ were similar to each other (Figs. 3, c–f) and similar to the concentrations required to inhibit mCPBG-evoked Ca^{2+} signals (Fig. 2 and Table 1). The antagonists had minimal effects on the peak current (I_p). During the 10 successive applications of 5-HT (30 μM) used to establish the concentration-effect relationships for each of the Ca^{2+} channel antagonists (Fig. 3, c–f), I_p changed to 109 ± 6% (nimodipine), 79 ± 5% (diltiazem), 83 ± 9% [(+)-verapamil], and 91 ± 8% [(−)-verapamil] of its value in the absence of antagonist (three experiments).

The inhibitory effect of a single application (10 μM, 2 sec) of any of the antagonists was completely reversed after washout (Fig. 3, a and b), but after three or more sequential applications there was only partial reversal. After 10 applications (2 sec) of 1–100 μM nimodipine, diltiazem, (+)-verapamil, or (−)-verapamil, each followed by washing for 3 min, responses to 5-HT recovered to 55 ± 5%, 33 ± 4%, 45 ± 5%, and 34 ± 6% (three experiments) of their pretreatment levels, respectively. Prolonging the washing period to 12 min with repeated applications of 5-HT (30 μM for 2 sec) at intervals of 3 min did not further improve recovery (not shown). These results may be a consequence of the lipophilic Ca^{2+} channel antagonists partitioning into the membrane from where they may gain access to 5-HT$_3$ receptors but be only slowly washed out.

The effect of (+)-verapamil on 5-HT-evoked currents was not voltage dependent; the concentration-dependent decrease in τ with no significant effect on I_p was similar in HEK/5-HT$_3$RAs cells clamped at −60 or +50 mV (Fig. 4).

The inhibition caused by (+)-verapamil (30 μM) was the same whether it was simultaneously applied with 5-HT or when the 5-HT was applied with verapamil after preincubation with verapamil for 1 min (not shown). Fig. 5 shows the effects of repeated applications of 5-HT (50 μM) in the continued presence of (+)-verapamil. During the first application of 5-HT, τ significantly decreased to 46 ± 5% (three experiments) of its value in the presence of 5-HT alone, but there was no further decrease after the second (44 ± 7%), third (43 ± 8%), or fourth (49 ± 9%) application of 5-HT. Subsequent application of 5-HT in the presence of a higher concentration of (+)-verapamil (10 μM) caused a further decrease in τ to 31 ± 9% of its control value.

Displacement of [^{3}H]mCPBG from 5-HT$_3$ receptors by Ca^{2+} channel antagonists. Table 2 summarizes the results of equilibrium saturation and kinetic analyses of the binding of [^{3}H]mCPBG and [^{3}H]granisetron to membranes prepared from Sf9 cells infected with 5-HT$_3$RAs. Each 5-HT$_3$ receptor-selective radioligand bound to a single class of high affinity binding site, each identified similar numbers of sites, and the specific binding of each radioligand was completely displaced by the heterologous ligand (50 μM). There was no detectable specific binding of these ligands to membranes prepared from cells infected with only the vector (3). The affinities for granisetron and mCPBG of 5-HT$_3$RAs receptors expressed in Sf9 cells were similar to those determined for native 5-HT$_3$ receptors in N1E-115 cells or for 5-HT$_3$RAs receptors expressed in HEK 293 cells (Table 2).

Because the ligand recognition properties of the 5-HT$_3$RAs receptor seem to be similar whether it is expressed in mammalian or insect cells (Table 2) and the large numbers of cells

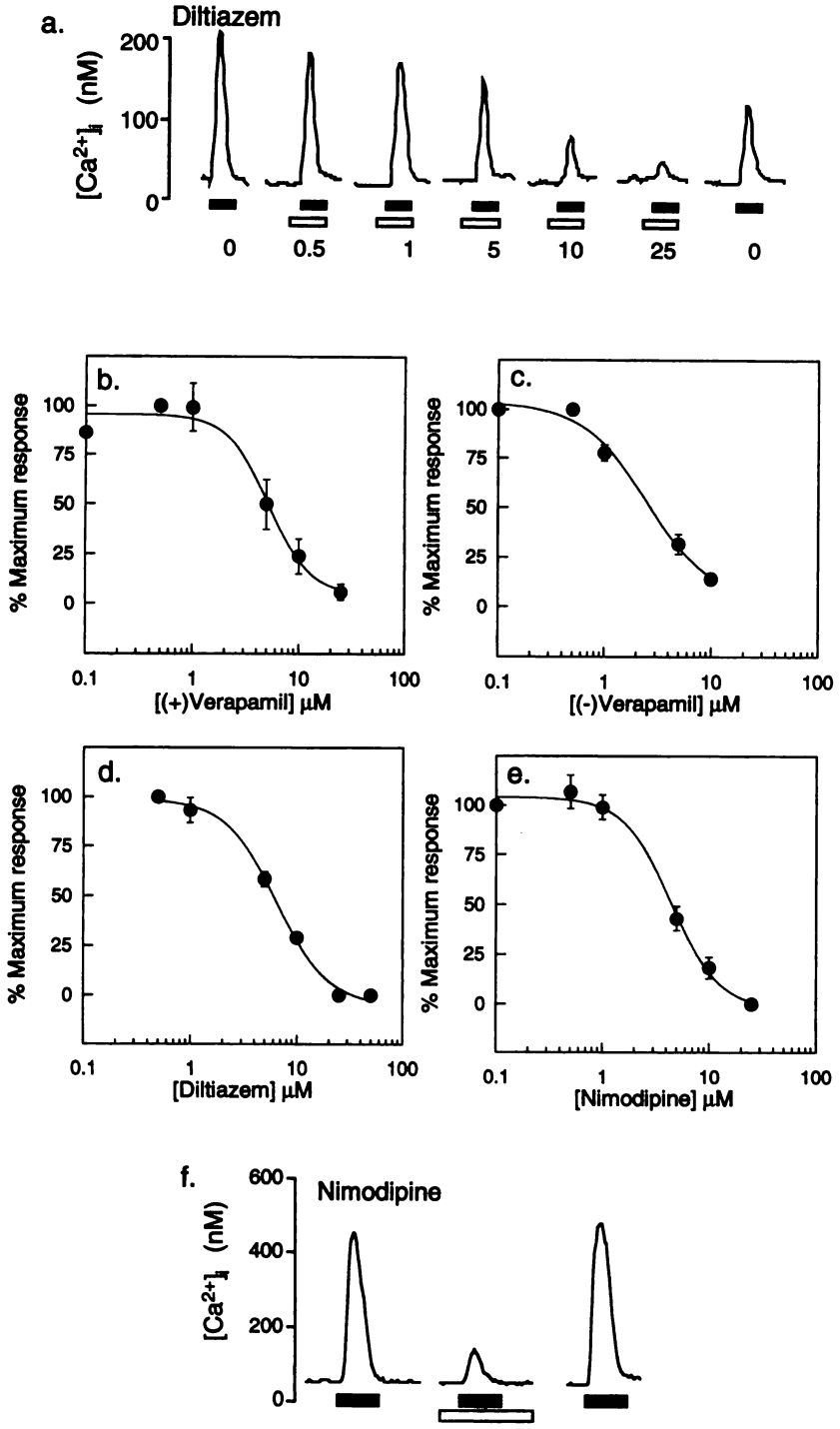

Fig. 2. Antagonists of L-type Ca^{2+} channels reversibly inhibit mCPBG-evoked rises in $[Ca^{2+}]_i$ in HEK/5-HT_3RAs cells. a, *Traces*, responses of a single cell stimulated with mCPBG (400 nM; *filled bars*) in Na^+-free HBM containing diltiazem (*open bars*); *numbers below the bars*, concentrations of diltiazem (µM). A washout period of 7 min was allowed between each addition of mCPBG. The traces are representative of three to six cells from each of three independent fields. b–e, Results from experiments similar to those shown in a are summarized, showing the inhibitory effects of increasing concentrations of (b) (+)-verapamil, (c) (−)-verapamil, (d) diltiazem, and (e) nimodipine on responses to mCPBG (400 nM). Each value represents the mean ± standard error of three independent fields of cells within each of which the responses from three to six cells were averaged. f, *Traces*, responses of single cells stimulated with mCPBG (400 nM; *filled bars*) in Na^+-free HBM, with or without nimodipine (10 µM; *open bar*). A washout period of 7 min was allowed between each addition of mCPBG. *Traces* are representative of three to six cells from each of 2 independent fields. Similar results were observed with (+)-verapamil.

needed for radioligand binding are more easily prepared from Sf9 cells, we used membranes prepared from Sf9 cells infected with the 5-HT_3RAs receptor to examine the effects of Ca^{2+} channel antagonists on the binding of [^{3}H]mCPBG and [^{3}H]granisetron to the receptor. In equilibrium competition binding experiments using membranes from Sf9/5-HT_3RAs cells, [^{3}H]mCPBG (1 nM) was displaced, in a concentration-dependent manner, by (+)-verapamil, (−)-verapamil, and diltiazem (Fig. 6a and Table 3). (+)-Verapamil was significantly more potent ($p < 0.05$) at displacing [^{3}H]mCPBG than (−)-verapamil or diltiazem. Nimodipine (≤300 µM) had no effect on [^{3}H]mCPBG binding (six experiments). Similar results were obtained when [^{3}H]granisetron was used as the radioligand (Table 3) and with membranes prepared from HEK/5-HT_3RAs cells (Fig. 6b).

The stereoselectivity of verapamil binding was maintained when incubations with [^{3}H]mCPBG (1 nM) and Sf9/5-HT_3RAs cell membranes were performed at 20°, the temperature at which measurements of both $[Ca^{2+}]_i$ and currents were obtained: IC_{50} = 2.6 ± 0.3 µM and 13.1 ± 0.9 µM for (+)-verapamil and (−)-verapamil, respectively ($p < 0.05$, three experiments). The stereoselectivity of verapamil binding was also maintained when the 5-HT_3 receptor antagonist [^{3}H]granisetron (0.1 nM) was used as the radioligand: IC_{50} =

TABLE 1

Inhibition of agonist-evoked currents and increases in $[Ca^{2+}]_i$ in HEK/5-HT$_3$RAs cells by L-type Ca^{2+} channel antagonists

Results from experiments similar to those shown in Figs. 2 and 3 are summarized. The concentration of each antagonist required to cause half-maximal inhibition (IC_{50}) of the $[Ca^{2+}]_i$ signal evoked by 400 nM mCPBG is shown as the mean ± standard error of three independent fields of cells, within each of which the responses from three to six cells were averaged. The IC_{50} values for inhibition of the currents evoked by 5-HT (30 μM) are the concentrations required to cause a 50% decrease in τ. Results are mean ± standard error of three or four independent experiments.

	Current (τ)	Ca^{2+} signal
	μM	
(+)-Verapamil	4.1 ± 0.5	5.2 ± 1.8
(−)-Verapamil	3.0 ± 0.9	2.5 ± 1.4
Diltiazem	4.9 ± 0.9	4.4 ± 0.5
Nimodipine	6.8 ± 1.1	6.5 ± 0.6

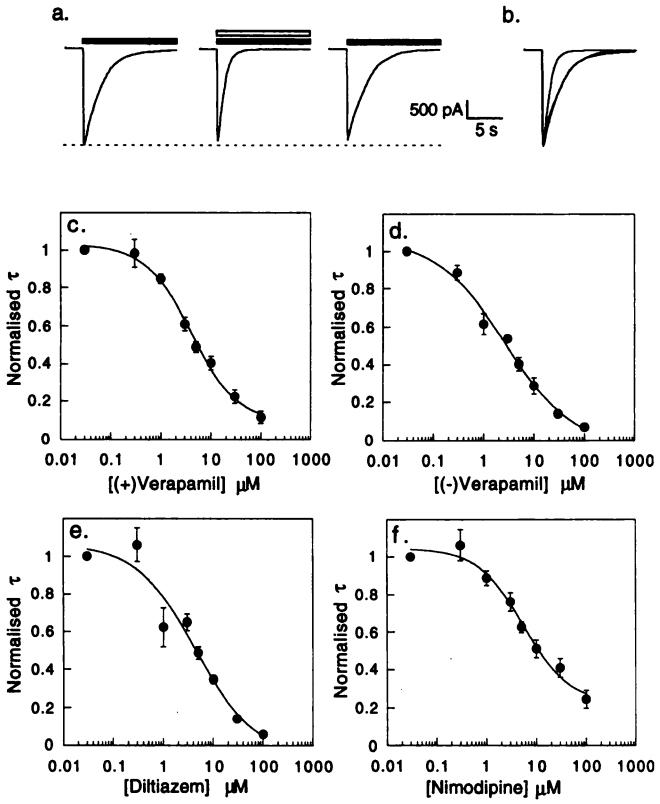

Fig. 3. Antagonists of L-type Ca^{2+} channels reversibly accelerate the rate of decay of currents evoked by 5-HT in HEK/5-HT$_3$RAs cells. a, Whole-cell currents were recorded from HEK/5-HT$_3$RAs cells held at −60 mV during perfusion with medium containing 5-HT (30 μM, *filled bar*) with or without (+)-verapamil (10 μM, *open bar*) for the periods shown. A washout period of 3 min was allowed between each application of 5-HT. The results are from one cell and are typical of five independent experiments. b, The three *traces* shown in a are superimposed. c–f, Results from experiments similar to those shown in a are summarized for each of four Ca^{2+} channel antagonists. The effects of (c) (+)-verapamil, (d) (−)-verapamil, (e) diltiazem, and (f) nimodipine on the currents evoked by 5-HT (30 μM) are shown. Results (mean ± standard error of three or four independent experiments) are expressed relative to the values obtained in the absence of antagonist in each cell to give normalized values of τ (●).

1.9 ± 0.2 μM and 16.5 ± 1.2 μM for (+)-verapamil and (−)-verapamil, respectively ($p < 0.05$, three experiments). The results of these equilibrium competition binding experiments are summarized in Table 3.

In equilibrium competition displacement experiments performed using three concentrations of either [^{3}H]mCPBG (Fig. 7) or [^{3}H]granisetron, the IC_{50} for (+)-verapamil increased as the concentration of radioligand increased, but with both radioligands there were also significant reductions in the n_H values of the displacement curves (Fig. 7 and Table 4).

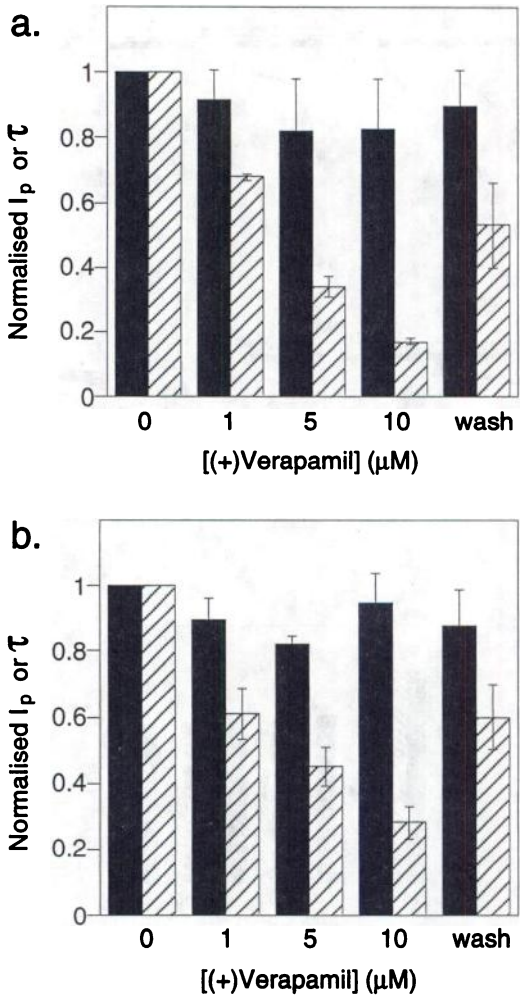

Fig. 4. Voltage-independent inhibition of 5-HT evoked currents by (+)-verapamil. The effects of (+)-verapamil on τ (*hatched columns*) and I_p (*filled columns*) on currents evoked by 5-HT (50 μM) at (a) −60 mV and (b) +50 mV are shown. Results are mean ± standard error of four independent experiments.

When Sf9/5-HT$_3$RAs membranes were equilibrated with [^{3}H]mCPBG (1 nM) for 60 min and its dissociation was then observed after the addition of ondansetron (50 μM) with or without a simultaneous addition of 100 μM (+)-verapamil, there was no significant difference in the rate of [^{3}H]mCPBG dissociation: k_{-1} = 0.284 ± 0.012 min^{-1} and 0.257 ± 0.011 min^{-1} in the absence and presence of (+)-verapamil, respectively (three experiments).

Effects of nimodipine on displacement of [^{3}H]granisetron from 5-HT$_3$ receptors by (+)-verapamil and diltiazem. Because each of the Ca^{2+} channel antagonists inhibited mCPBG-evoked Ca^{2+} signals (Fig. 2) and 5-HT-evoked currents (Fig. 3), whereas only verapamil and diltiazem antagonized the binding of mCPBG and granisetron (Table 3), we attempted to determine whether the Ca^{2+} channel antagonists share a binding site. In equilibrium competition binding experiments using [^{3}H]granisetron and Sf9/5-HT$_3$RAs membranes,

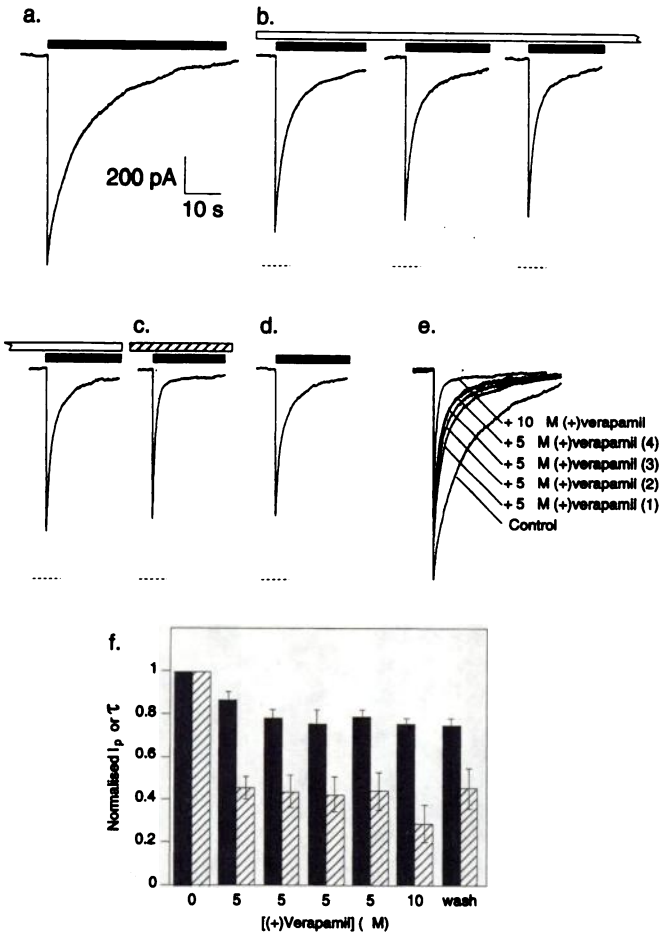

Fig. 5. Repeated application of 5-HT does not enhance the inhibitory effects of (+)-verapamil. A HEK/5-HT_3RAs cell was held at −60 mV and stimulated with 5-HT (50 μM, *filled bars*) during the following sequence of medium changes. a, 5-HT alone. b, (+)-Verapamil (5 μM, *open bar*) was continuously present in the superfusate while the cell was stimulated at 5-min intervals with four applications of 5-HT. c, The superfusate contained 10 μM (+)-verapamil (*hatched bar*), and the cell was then stimulated with 5-HT. d, After a 10-min washout period, the cell was again stimulated with 5-HT. e, Overlay of *traces* a–c, which are representative of three similar recordings. f, Results from experiments similar to those shown in a–d, showing the effects of prolonged application of (+)-verapamil on I_p (*filled columns*) and τ (*hatched columns*) of currents evoked by 5-HT. Results are mean ± standard error of responses from three cells.

nimodipine (100 μM) had no effect on the displacement curves for either (+)-verapamil or diltiazem (Fig. 8). These results indicate that nimodipine does not bind to the site or sites through which verapamil and diltiazem mediate their inhibition of ligand binding to the 5-HT_3 receptor.

Discussion

Antagonists of L-type Ca^{2+} channels directly inhibit 5-HT_3 receptors

Both the native 5-HT_3 receptors of N1E-115 cells and homo-oligomeric complexes of either the long (A) or short (As) form of the receptor are Ca^{2+} permeable (23) (Fig. 1), yet the Ca^{2+} signals evoked by activation of 5-HT_3 receptors were completely abolished by Ca^{2+} channel antagonists (Fig. 2). Although it is unlikely that 5-HT_3 receptors would be inhibited by clinically effective concentrations of Ca^{2+} channel antagonists (typically ≤ 0.3 μM) (28, 29), the inhibition of 5-HT_3 receptors occurred at modest concentrations of the antagonists (IC_{50} = 2.5–6.8 μM) (Table 1) and was fully reversible after a single incubation with antagonist (Figs. 2f and 3a), although only poorly reversed after multiple incubations. Despite the ability of compounds from three unrelated classes of Ca^{2+} channel ligand (28) to inhibit mCPBG-evoked Ca^{2+} signals, the inhibition was not mediated by their effects on L-type Ca^{2+} channels. Several lines of evidence, in addition to the demonstrated Ca^{2+} permeability of 5-HT_3 receptors (20–23), suggest that the inhibition is a consequence of direct effects of Ca^{2+} channel antagonists on 5-HT_3 receptors. First, in N1E-115 cells, Bay K 8644, an agonist of L-type Ca^{2+} channels (30), potentiated the signals evoked by depolarization but inhibited those evoked by mCPBG. Second, mCPBG-evoked Ca^{2+} signals were completely blocked by Ca^{2+} channel antagonists in HEK/5-HT_3RAs cells (Fig. 2), which lack voltage-gated Ca^{2+} channels (Fig. 1, a and b) (26, 27). Third, N1E-115 cells, which do express L-type Ca^{2+} channels (23, 31), and HEK/5-HT_3RAs cells were similarly sensitive to inhibition of their mCPBG-evoked Ca^{2+} signals by verapamil and nimodipine. Fourth, radioligand binding indicated an inhibitory interaction be-

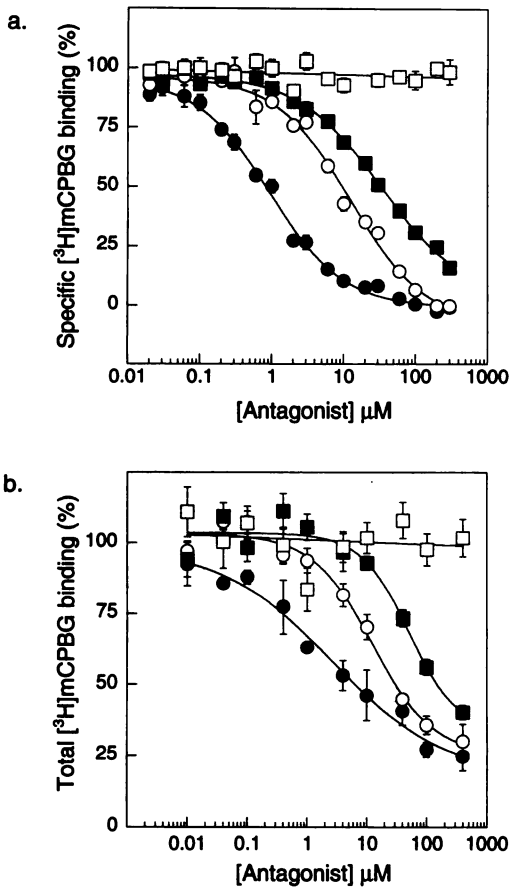

Fig. 6. Equilibrium-competition binding between [^{3}H]mCPBG and L-type Ca^{2+} channel antagonists. Membranes were incubated for 2 hr on ice with [^{3}H]mCPBG (1 nM) and the indicated concentrations of (+)-verapamil (●), (−)-verapamil (○), diltiazem (■), or nimodipine (□). a, Membranes from Sf9/5-HT_3RAs cells. Results are mean ± standard error of six independent experiments. b, Membranes from HEK-293/5-HT_3RAs cells. Results are mean ± standard error of three independent experiments

TABLE 2

Equilibrium binding of granisetron and mCPBG to Sf9/5-HT$_3$RAs, HEK/5-HT$_3$RAs, and N1E-115 cells

Results are the mean ± standard error of varying independent experiments.

	Sf9/5-HT$_3$RAs			K_d	
	K_d	n_H	B_{max}	HEK/5-HT$_3$RAs	N1E-115
	nM		pmol/mg of protein	nM	
[^{3}H]Granisetron	0.66 ± 0.08 (n = 3)	1.12 ± 0.13	15.7 ± 0.7	0.51 ± 0.05 (n = 3)	0.24 ± 0.005 (n = 3)
[^{3}H]mCPBG	1.46 ± 0.13 (n = 3)	1.18 ± 0.08	14.5 ± 0.5	0.95 ± 0.24 (n = 5)	1.22 ± 0.22 (n = 3)

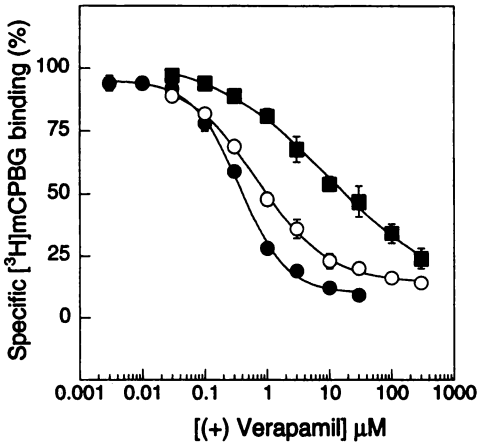

Fig. 7. Displacement of [^{3}H]mCPBG by (+)-verapamil from Sf9/5-HT$_3$RAs membranes is not competitive. Membranes were incubated for 2 hr on ice with the indicated concentrations of (+)-verapamil and 0.1 nM (●), 1 nM (○), or 10 nM (■) [^{3}H]mCPBG. Results are shown as mean ± standard error from three independent experiments.

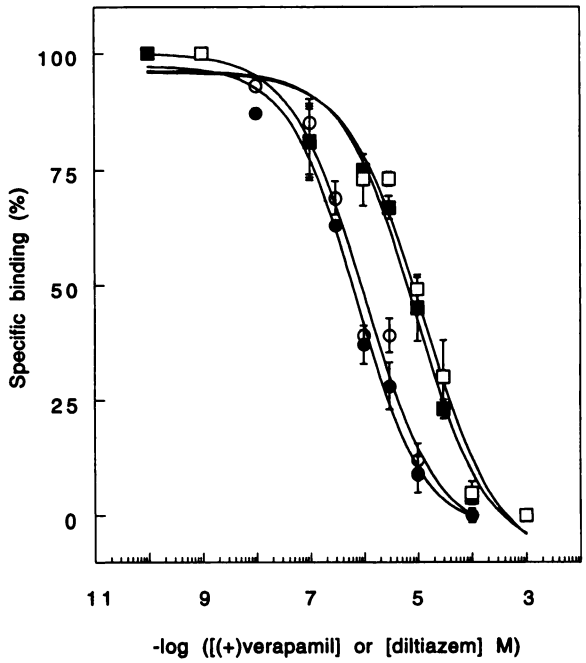

Fig. 8. Nimodipine does not bind to the site through which diltiazem and verapamil inhibit [^{3}H]granisetron binding. Membranes from Sf9/5-HT$_3$RAs cells were incubated for 2 hr on ice with [^{3}H]granisetron (0.1 nM) and the indicated concentrations of (+)-verapamil (●○) or diltiazem (■□) with (●■) or without (○□) 100 μM nimodipine. Results are the mean ± standard error of three independent experiments.

tween the sites to which agonists and antagonists of 5-HT$_3$ receptors bind and the sites to which certain Ca^{2+} channel antagonists bind (Figs. 6–8 and Tables 3 and 4). Fifth, in voltage-clamped cells, 5-HT-evoked currents were inhibited by similar concentrations of the Ca^{2+} channel antagonists (Fig. 3 and Table 1). Finally, the stereoselectivity of the site to which the isomers of verapamil bound to displace ligands from 5-HT$_3$ receptors (Fig. 6) was the opposite of that displayed by L-type Ca^{2+} channels (32).

Interactions of Ca^{2+} channel antagonists with other targets. The three major classes of Ca^{2+} channel antagonists are structurally unrelated. Each binds to its own site on the α1 subunit of the L-type Ca^{2+} channel, although the sites interact (28, 32, 33), and each has distinct effects on the behavior of the Ca^{2+} channel. It is therefore remarkable that 5-HT$_3$ receptors and L-type Ca^{2+} channels should both be inhibited by members of each of these three classes of antagonist. Ca^{2+} channel antagonists are known to bind to many targets other than L-type Ca^{2+} channels, including the multidrug-resistance-related P-glycoprotein, calmodulin, and others (for a review, see Ref. 34). Many of these targets bind both dihydropyridines and phenylalkylamines, but most, with the exception of P-glycoprotein, lack a benzothiazepine-binding site. The binding of Ca^{2+} channel antagonists to these additional targets is typically ≥1000-fold weaker than is their binding to L-type Ca^{2+} channels, and it usually lacks the stereoselectivity of the binding to Ca^{2+} channels (34).

More recently, ligand-gated ion channels have also been shown to be targets of dihydropyridines. Ca^{2+} influx evoked by activation of the NMDA receptors of cerebellar granule cells was partially inhibited by nitrendipine (1–10 μM) (35), which seems to bind to a site that is exposed only after receptor activation (36), leading to block of the open channel. That interpretation gains further support from the observation that in mouse brain slices, binding of [^{3}H]MK-801, another open channel blocker of NMDA receptors (37), is displaced by nitrendipine, although not by other dihydropyridines or verapamil (38). Although the functional effects of other Ca^{2+} channel antagonists have not been investigated, additional circumstantial support for the selectivity of this NMDA receptor site for nitrendipine over other dihydropyridines is provided by the lack of effect of nifedipine on NMDA receptors in rat granule cells (39). Nicotinic acetylcholine receptors of bovine adrenal chromaffin cells are also inhibited by dihydropyridines (40).

Nitrendipine was ~10-fold more potent at NMDA receptors (35) than was nimodipine at 5-HT$_3$ receptors (Fig. 2 and Table 1), but 5-HT$_3$ and nicotinic receptors (40) seem to be similarly sensitive to dihydropyridines. There have been no previous reports of direct effects of either phenylalkylamines or benzothiazepines on ligand-gated ion channels, although verapamil blocks cardiac delayed rectifier K$^+$ channels (41), and *l-cis*-diltiazem stereoselectively blocks the cGMP-gated cation channels of photoreceptors (42).

TABLE 3

Equilibrium binding of L-type Ca^{2+} channel antagonists to 5-HT_3 receptors

The ability of Ca^{2+} channel antagonists to displace specific binding of 1 nM [^{3}H]mCPBG or 0.1 nM [^{3}H]granisetron was tested. Nonspecific binding was determined using 50 μM ondansetron. Each value is the mean ± standard error of varying independent experiments.

Expression system	Radioligand (^{3}H)		(+)-Verapamil	(−)-Verapamil	Diltiazem	Nimodipine
Sf9 cells (n = 6)	mCPBG at 0°	IC_{50} (μM)	0.9 ± 0.1	12.5 ± 2.0	33.8 ± 6.9	No effect at 300 μM
		n_H	0.9 ± 0.1	0.8 ± 0.1	0.7 ± 0.1	
Sf9 cells (n = 3)	mCPBG at 20°	IC_{50} (μM)	2.6 ± 0.3	13.1 ± 0.9	Not determined	Not determined
		n_H	0.8 ± 0.1	1.2 ± 0.1		
Sf9 cells (n = 3)	Granisetron at 0°	IC_{50} (μM)	1.9 ± 0.2	16.5 ± 1.2	30.0 ± 5.6	No effect at 300 μM
		n_H	0.8 ± 0.1	1.0 ± 0.1	0.9 ± 0.1	
HEK cells (n = 3)	mCPBG at 0°	IC_{50} (μM)	1.5 ± 0.4	11.9 ± 3.6	51.1 ± 9.1	No effect at 300 μM
		n_H	0.6 ± 0.2	0.8 ± 0.2	1.1 ± 0.2	

TABLE 4

Effects of (+)-verapamil on equilibrium binding of different concentrations of [^{3}H]mCPBG and [^{3}H]granisetron to Sf9/5-HT_3RAs membranes

Results (mean ± standard error of three independent experiments) from experiments similar to those shown in Fig. 6 show the IC_{50} and Hill coefficients of the equilibrium competition binding curves between (+)-verapamil and the indicated concentrations of [^{3}H]mCPBG and [^{3}H]granisetron.

[^{3}H]mCPBG		0.1 nM	1 nM	10 nM
	IC_{50} (μM)	0.37 ± 0.03	0.67 ± 0.10	14.3 ± 6.0
	n_H	1.14 ± 0.02	0.68 ± 0.08	0.47 ± 0.05
[^{3}H]granisetron		0.05 nM	0.5 nM	5 nM
	IC_{50} (μM)	1.6 ± 0.7	2.9 ± 0.4	7.6 ± 0.6
	n_H	1.10 ± 0.14	0.82 ± 0.06	0.52 ± 0.03

Although Ca^{2+} channel antagonists are less potent at 5-HT_3 receptors than at the L-type Ca^{2+} channels of cardiac or smooth muscle, 5-HT_3 receptors are more sensitive than either N- or T-type Ca^{2+} channels (43, 44). Furthermore, because the L-type Ca^{2+} channels of N1E-115 cells (31) and neurons (45) are far less sensitive to Ca^{2+} channel antagonists than are those of smooth muscle, the 5-HT_3 receptors of neurons may be more sensitive to Ca^{2+} channel antagonists than are their voltage-gated Ca^{2+} channels.

Binding of Ca^{2+} channel antagonists to 5-HT_3 receptors. Equilibrium competition binding studies to membranes from both HEK/5-HT_3RAs and Sf9/5-HT_3RAs cells indicate that verapamil and diltiazem, but not nimodipine, displace both agonists and antagonists from the 5-HT_3 receptor (Figs. 6–8 and Table 3). Thus, there are significant disparities between the results of our functional and radioligand binding analyses. First, nimodipine blocked mCPBG-evoked Ca^{2+} signals (Fig. 1) and 5-HT-evoked currents (Fig. 3), yet it did not bind to the site through which diltiazem and verapamil inhibited agonist and antagonist binding to the 5-HT_3 receptor (Fig. 8). Second, there was no significant difference in the potency of the stereoisomers of verapamil in functional assays (Figs. 2 and 3 and Table 1), whereas (+)-verapamil was ~10-fold more potent than (−)-verapamil in displacing [^{3}H]mCPBG from the 5-HT_3 receptor of Sf9/5-HT_3RAs cells (Fig. 6 and Table 3). The stereoselectivity of the binding site for verapamil was confirmed in HEK/5-HT_3RAs cells (Table 3); it persisted regardless of whether an agonist or antagonist was used as the radioligand; and it was evident when the binding experiments were performed at 20° rather than at 0° (Fig. 6b and Table 3). (±)-Verapamil is a potent competitive antagonist of 5-HT_2 receptors, but that antagonism is not mimicked by dihydropyridines or diltiazem (46). It is unlikely that verapamil has a similar action at 5-HT_3 receptors because (+)-verapamil displacement of either [^{3}H]mCPBG (Fig. 7) or [^{3}H]granisetron from Sf9/5HT_3RAs membranes was not competitive (Table 4).

Mechanisms of action of Ca^{2+} channel antagonists at 5-HT_3 receptors. Each of the Ca^{2+} channel antagonists increased the rate of decay (τ) of the current through 5-HT_3 receptors (Fig. 3 and Table 1). The antagonists seem to bind to an active conformation of the 5-HT_3 receptor because the effect of (+)-verapamil was similar when cells were preincubated with (+)-verapamil before the addition of 5-HT or when 5-HT and verapamil were applied simultaneously (Fig. 5), and the Ca^{2+} channel antagonists had little or no effect on the peak current amplitude (I_p) (Fig. 3). The effects of the Ca^{2+} channel antagonists are consistent with them binding

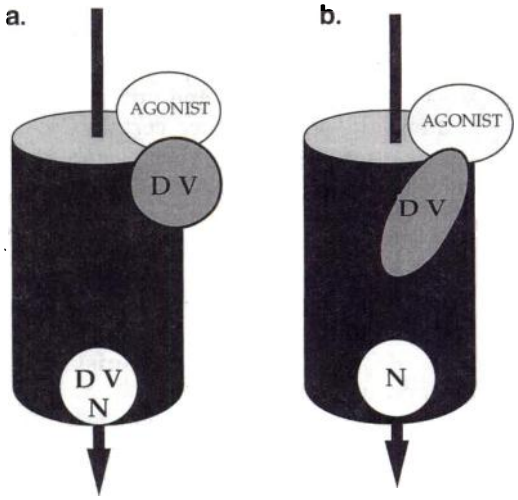

Fig. 9. Possible interactions between Ca^{2+} channel antagonists and 5-HT_3 receptors. Two possible explanations are illustrated for the different effects of Ca^{2+} channel antagonists on the binding of ligands to 5-HT_3 receptors and on receptor function. a, Diltiazem (*D*), verapamil (*V*), and nimodipine (*N*) share a common site to which each binds with similar affinity and which fails to distinguish between the isomers of verapamil. Occupancy of this site prevents ion flux through the 5-HT_3 receptor, possibly by occluding the open channel. A second site is allosterically linked to the agonist-binding site of the receptor and binds only verapamil and diltiazem; (+)-verapamil is more potent than (−)-verapamil at this site because it either binds with greater affinity or is more efficacious in modulating ligand binding. b, Each Ca^{2+} channel antagonist has only one binding site. Nimodipine binds to a site that prevents ion flux through the channel without affecting ligand binding; open channel block would again provide a plausible mechanism. The second site recognizes both diltiazem and verapamil, and its occupancy leads to both inhibition of ion flux and allosteric inhibition of ligand binding. To accommodate the stereoselective effect of verapamil on ligand binding, (+)-verapamil is proposed to be more efficacious than (−)-verapamil in inhibiting ligand binding.

to a site on the 5-HT$_3$ receptor either to cause open channel block or to accelerate receptor desensitization. The two mechanisms are very difficult to distinguish (47, 48). Both have been invoked for antagonists of NMDA receptors that increase the rate of current decay: nitrendipine seems to cause open channel block (35–38), whereas (+)-HA-966, a partial agonist of the glycine-binding site (49), has been postulated to act by decreasing the affinity of the glutamate-binding site for its agonists (50).

An additional feature of open channel block is use dependence (47). During periodic stimulation of HEK/5-HT$_3$RAs cells with 5-HT in the continued presence of (+)-verapamil, the inhibition of the 5-HT-evoked current was similar in each of four sequential challenges (Fig. 5). These experiments are, however, compromised by the need to allow several minutes between successive applications of 5-HT, during which time verapamil may slowly dissociate from closed or desensitized conformations of the receptor. The effects of voltage can provide additional support for an open channel blocking mechanism (47), but the block of 5-HT-evoked currents by (+)-verapamil was unaffected by voltage (Fig. 4). Because verapamil is uncharged, its mobility would not be influenced by membrane potential, but the results do establish that the direction of ion movement through 5-HT$_3$ receptors does not influence the effects of verapamil.

If the alternative explanation, acceleration of either receptor desensitization or channel closure, is the mechanism underlying the effects of the Ca^{2+} channel antagonists, then it would be unlikely to involve Ca^{2+}-regulated desensitization because that would be voltage sensitive (51), whereas in our experiments τ was similarly affected by verapamil at −60 mV or +50 mV (Fig. 4).

Despite the very similar effects of each of the Ca^{2+} channel antagonists on 5-HT-evoked currents (Fig. 3), our results do not allow us to conclude whether they each have the same site or mode of action. Our apparently discrepant results from radioligand and functional assays could be reconciled by suggesting that there are two distinct sites associated with the 5-HT$_3$ receptor to which Ca^{2+} channel antagonists can bind (Fig. 9).

Nimodipine inhibits 5-HT$_3$ receptor-evoked currents and Ca^{2+} signals by binding to a site that is distinct from both the agonist-binding site of the receptor (Fig. 6 and Table 3) and from the site through which verapamil and diltiazem inhibit ligand binding (Fig. 8). The discrepant stereoselectivity of the functional and radioligand binding assays for verapamil (Tables 1 and 3) and the noncompetitive nature of its inhibition of agonist and antagonist binding (Fig. 7 and Table 4) suggest that the effects of verapamil are also mediated by a site that is distinct from the agonist/antagonist-binding site of the receptor. All three Ca^{2+} channel antagonists might therefore bind to a common site to decrease ion fluxes, whereas only verapamil and diltiazem might also bind to a second site to allosterically inhibit ligand binding (Fig. 9a). The stereoselective effects of verapamil in binding but not functional assays would then be explained by assuming that only the second verapamil-binding site is stereoselective.

Alternatively, a single binding site may mediate the inhibitory effects of verapamil and diltiazem on both radioligand binding and the function of the channel, with nimodipine acting through a second site (Fig. 9b). The discrepant results with verapamil could then be explained by proposing that the two isomers bind with similar affinity to the site and are therefore equally effective in functional assays (Table 1), but (+)-verapamil is more efficacious than (−)-verapamil in allosterically regulating ligand binding (Table 3). One method of distinguishing these possibilities would be by the binding of radiolabeled Ca^{2+} channel antagonists, but their relatively low affinity for the sites through which they mediate inhibition has prevented us from detecting such sites.

Conclusions. We conclude that 5-HT$_3$ receptors, in common with NMDA and nicotinic receptors, are inhibited by organic Ca^{2+} channel antagonists. 5-HT$_3$ receptors are, however, unusual among the additional targets of these drugs (34–36, 38–42) in being inhibited by members of each of the three major classes of L-type Ca^{2+} channel antagonist. The binding sites for each of these antagonists are likely to be part of the 5-HT$_3$ receptor itself but distinct from the agonist-binding site. Competitive antagonists of 5-HT$_3$ receptors are of proven clinical value (15), but drugs targeted to the site through which Ca^{2+} channel antagonists attenuate 5-HT$_3$ receptor function could also be useful because they would selectively target active receptors. This property might then allow selective attenuation of only those pathways in which 5-HT$_3$ receptors were overactive, as might be the case for the limbic or mesolimbic systems in certain psychiatric disorders (52).

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