British Journal of Pharmacology (2002) 135, 1051–1059

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Effects of chronic drug treatments on increases in intracellular calcium mediated by nicotinic acetylcholine receptors in SH-SY5Y cells

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1 SH-SY5Y cells express α7 and α3* subtypes of nicotinic acetylcholine receptors (AChR). Numbers of these receptors are upregulated by chronic treatment with nicotinic agonists or KCl. In this study we have examined the functional consequences of these drug treatments on nicotine- or KCl-evoked increases in $[Ca^{2+}]_i$, in SH-SY5Y cells.

2 In untreated cells, nicotine increased $[Ca^{2+}]_i$ (EC_{50} 7.5 μM). Responses to 10 μM nicotine were abolished by the non-selective nicotinic antagonist mecamylamine and were partially blocked by α7-selective antagonists, the α3β2*-selective antagonist α-conotoxin-MII, and by cadmium and verapamil.

3 After treatment for 4 days with nicotinic agonists, nicotine-evoked increases in $[Ca^{2+}]_i$ were significantly decreased by about 25%. Nicotine-evoked responses were paradoxically increased in the presence of acute methyllycaconitine (MLA; an α7-selective antagonist) although other α7-selective antagonists were without effect, while α-conotoxin-MII gave a partial inhibition. The increase observed with MLA was abolished by mecamylamine but not by α-conotoxin-MII and was still observed 24 h after chronic nicotine treatment.

4 After treatment for 4 days with KCl, nicotine-evoked increases in $[Ca^{2+}]_i$ were also decreased by 25%, but acute MLA was without effect. Responses to 20 mM KCl were unchanged by prior treatment with nicotine or KCl. Treatment for 4 days with 5 μM verapamil reduced responses to both nicotine and KCl by about 50%.

5 Multiple nicotinic AChR subtypes contribute to nicotine-evoked increases in $[Ca^{2+}]_i$ in SH-SY5Y cells. Responses to acute nicotine are reduced after chronic nicotine or KCl treatment, with loss of the component attributed to the α7 subtype. However, in nicotine-treated cells this effect is reversed when nicotine stimulation is applied in the presence of acute MLA. The antagonist may assist in converting a non-functional α7 nicotinic AChR to a conducting state.

British Journal of Pharmacology (2002) **135,** 1051–1059

Keywords: Nicotinic receptor; SH-SY5Y cell line; intracellular Ca^{2+}; nicotine; DMAC; αbungarotoxin; α-conotoxin; methyllycaconitine; upregulation

Abbreviations: α-Bgt, α-bungarotoxin; BSA, bovine serum albumin; $[Ca^{2+}]_i$, concentration of intracellular calcium; CaM-kinase II, Ca^{2+}-calmodulin dependent kinase II; DMAC, 3, [(4-dimethylamino) cinnamylidene] anabaseine maleate; DMEM, Dulbecco's modified eagle's medium; DMSO, dimethylsulphoxide; FCS, foetal calf serum; MLA, methyllycaconitine; NEAAs, non-essential amino-acids; nicotinic AChR, nicotinic acetylcholine receptor; PBS, phosphate buffered saline; TSS, Tyrode's salt solution

Introduction

Nicotinic acetylcholine receptors (AChR) are a family of pentameric ligand-gated cation channels (Lukas *et al*., 1999). In the brain, nicotinic AChR are responsible for mediating the psychoactive and addictive properties of nicotine (Dani & Heinemann, 1996), and are also targets for the pharmaceutical industry, with respect to a portfolio of diverse brain disorders (Decker & Arneric, 1998). Chronic exposure to nicotine (and other nicotinic agonists) upregulates the numbers of neuronal nicotinic AChR binding sites *in vivo* and *in vitro* (Wonnacott, 1990; Lukas *et al*., 1996). The α4β2 nicotinic AChR subtype appears to be most sensitive to this influence, but other subtypes are also upregulated at higher concentrations of nicotine (Peng *et al*., 1997; Wang *et al*., 1998). The functional consequences of nicotinic AChR upregulation are controversial. Chronic nicotine treatments *in vivo* result in behavioural stimulation (typically measured as locomotor activity), with a bell shaped dose-response curve (Ksir *et al*., 1987), whereas nicotine-evoked dopamine overflow, measured by *in vivo* microdialysis, has been found to increase (Benwell & Balfour, 1992; Benwell *et al*., 1995; Marshall *et al*., 1997) or remain unchanged (Damsma *et al*., 1989; Nisell *et al*., 1994) after various nicotine treatment regimes. In contrast, nicotine-evoked prolactin release, sampled *in vivo*, was abolished after chronic nicotine injections, with a time-course that mimicked that of the upregulation of [^{3}H]-ACh

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binding sites (Hulihan-Giblin *et al*., 1990). *Ex vivo* measurements of nicotine-evoked transmitter release, after chronic nicotine treatment *in vivo*, have shown decreased (Marks *et al*., 1993) and increased responses (Yu & Wecker, 1994).

Interpretation of these studies in relation to nicotinic AChR upregulation is compromized by the complexity of the preparation, with many unknown variables influencing the effects of systemically administered nicotine. *In vitro* cell systems provide a better defined and more easily manipulated model. Chronic nicotine treatment is reported to decrease, or completely block, whole cell currents recorded from *Xenopus* oocytes in which various nicotinic AChR subunit combinations have been heterologously expressed (Hsu *et al*., 1996; Olale *et al*., 1997). In transfected HEK-293 cells stably expressing $\alpha7$ or $\alpha4\beta2$ nicotinic AChR, ACh-evoked whole cell currents were increased after chronic treatment with nicotine or with the $\alpha7$-selective antagonist methyllycaconitine (MLA) (Molinari *et al*., 1998; Buisson & Bertrand, 2001). The $\alpha4\beta2$ nicotinic AChR in HEK-293 cells gave increased responses ($^{86}Rb^+$ efflux) after chronic treatment with low agonist concentrations and decreased responses after higher concentrations (Gopalakrishnan *et al*., 1997). The over-expression of nicotinic AChR in non-neuronal cells may not provide a suitable model for studying neuronal mechanisms regulating these receptors. Neuroblastoma cell lines endogenously express native nicotinic AChR in a more relevant context, although the low levels and heterogeneity of nicotinic AChR expression complicate analyses. Short applications (up to 1 h) of high concentrations of nicotine induce 'persistent functional inactivation' of responses ($^{86}Rb^+$ influx) in the neuroblastoma SH-SY5Y cell line (Ke *et al*., 1998). This phenomenon may be the trigger for nicotinic AChR upregulation; we wished to know the magnitude of nicotinic responses after nicotinic AChR binding sites have been upregulated.

We have shown in primary hippocampal cultures and SH-SY5Y cells that a few days' exposure to nicotine or KCl upregulates $\alpha7$ nicotinic AChR binding sites, labelled with [^{125}I]-α-Bgt, through different mechanisms (Ridley *et al*., 2001), consistent with previous studies on sympathetic neurones (De Koninck & Cooper, 1995). Upregulation by KCl appears to occur through activation of L-type Ca^{2+} channels and activation of Ca^{2+}-calmodulin dependent kinase (CaM-kinase II), probably leading to an increase in transcription of $\alpha7$ RNA, whereas neither L-type Ca^{2+} channel blockers nor CaM-kinase II inhibitors prevent nicotine-induced upregulation of [^{125}I]-α-Bgt binding sites. In SH-SY5Y cells, [^{3}H]-epibatidine binding sites, corresponding to $\alpha3$* nicotinic AChR, were also upregulated after nicotine treatment, but were unaffected by chronic KCl depolarization (Ridley *et al*., 2001). The $\alpha7$-selective agonist 3,[(4-dimethylamino) cinnamylidene] anabaseine maleate (DMAC) (De Fiebre *et al*., 1995) also upregulated [^{125}I]-α-Bgt and [^{3}H]-epibatidine binding sites, in a manner indistinguishable from that of nicotine (Ridley *et al*., 2001). In the present studies we have characterized increases in $[Ca^{2+}]_i$ in response to acute application of either nicotine or KCl, as an index of nicotinic AChR-mediated responses or general responsiveness respectively, in control SH-SY5Y cells and after chronic drug treatment regimes that upregulate numbers of nicotinic AChR binding sites.

Methods

Drugs and reagents

Tissue culture media, serum and plasticware were from Gibco BRL (Paisley, Renfrewshire, Scotland). Media supplements, cadmium chloride, (−)-nicotine hydrogen tartrate, (±)-verapamil hydrochloride, mecamylamine and α-conotoxin-ImI and α-bungarotoxin (α-Bgt) were purchased from Sigma Co. (Poole, Dorset, U.K.). Nicotine, KCl and verapamil used for chronic treatment of SH-SY5Y cells were made up freshly and directly in Dulbecco's Modified Eagle's Medium (DMEM):Ham's F12 supplemented medium on day 1 of drug treatment. (±)-Anatoxin-a fumarate and methyllycaconitine (MLA) were purchased from Tocris Cookson, (Avonmouth, U.K.). 3,[(4-dimethylamino) cinnamylidene] anabaseine maleate (DMAC) was synthesized by Organon Laboratories Ltd., Newhouse, Lanarkshire, U.K. and was made up in dimethylsulphoxide (DMSO) to give a stock solution of 10 mM; the final concentration of DMSO on cultures did not exceed 0.1%. α-conotoxin-MII was synthesized as previously described (Cartier *et al*., 1996; Kaiser *et al*., 1998). Fura-2 AM and Fluo-3 AM were purchased from Molecular Probes, Eugene, Oregon, U.S.A. and stored at −20°C as stock solutions in DMSO.

Cell culture of SH-SY5Y cells

Cell culture was carried out as described in detail by Ridley *et al*. (2001). In brief, SH-SY5Y human neuroblastoma stock cultures were routinely maintained in DMEM:Ham's F12 (1 : 1) modified medium containing 1% non-essential amino-acids (NEAAs) and supplemented with foetal calf serum (FCS, 15%), glutamine (2 mM), penicillin (50 iu ml^{-1}) and streptomycin (50 μg ml^{-1}). Stock cultures were passaged 1 : 5 weekly and fed twice weekly. For fura-2 assays, cultures were sub-cultured and grown to confluency in 175 cm^2 flasks containing 25 ml of supplemented medium and maintained at 37°C in 5% CO_2/humidified air. For chronic treatments, drugs were added 3 days after the cells reached confluency. After 4 days of drug treatment, cells were washed three times over 4 h with warm Ca^{2+}-free phosphate buffered saline (PBS; 150 mM NaCl, 8 mM K_2HPO_4, 2 mM KH_2PO_4, pH 7.4, 37°C) to ensure the removal of nicotine or other drugs taken up by the cells, before measurement of $[Ca^{2+}]_i$ using fura-2. For fluo-3 assays, cultures were sub-cultured into 96 well plates in 180 μl of modified medium. To avoid the cells becoming over-confluent, chronic drug treatments were initiated immediately. After 4 days, cells in each well were washed six times with warm medium (200 μl/well) over 4 h, before measurement of Ca^{2+} using fluo-3.

Calcium fluorimetry: fura-2 assays on cells in suspension

Changes in $[Ca^{2+}]_i$ in suspensions of SH-SY5Y cells were measured essentially as described by Sharples *et al*. (2000). In brief, after extensive washing (above) cultured cells were removed from flasks by incubation for 3 min at 37°C in Ca^{2+}-free PBS. The cell suspension was centrifuged (500 × *g*, 3 min) and the cell pellet was resuspended in 5 ml of Ca^{2+}-free HEPES buffer (in mM: HEPES 10, NaCl 145, KCl 5,

$MgCl_2$-6-hydrate 1, Na_2HPO_4 0.5 and glucose 5.5) and recentrifuged ($500 \times g$, 3 min). The cells were loaded with 5 μM fura-2 AM by resuspending the pellet in 3 ml HEPES buffer containing 0.25% (w v^{-1}) BSA, to improve the solubilization of the dye. After incubating in darkness for 45 min at room temperature, excess dye was removed by centrifugation ($500 \times g$, 3 min), followed by three washes with 5 ml of Ca^{2+}-free HEPES buffer. When used, α-Bgt was added to the cells with fura-2 AM for 45 min at room temperature.

Cell density was adjusted to $1-2 \times 10^6$ cells ml^{-1} and $[Ca^{2+}]_i$ was measured in 2 ml aliquots of cell suspension in a constantly stirred cuvette, in a PTI dual-excitation photofluorimeter. The excitation wavelengths were of 340 and 380 nm, with an emission wavelength of 510 nm, 4 nm slit width, equipped with PTI software version 2.060 (Photon Technology International Inc., Deerpark Drive, South Brunswick, NJ, U.S.A.). Before the addition of nicotinic agonist or KCl, 2 mM $CaCl_2$ was introduced into the cuvette and the fura-2 fluorescence was monitored for 80 s. Where used, antagonists (mecamylamine (10 μM), MLA (10 nM), α-conotoxin-ImI (1 μM) or α-conotoxin-MII (112 nM)), or the L-type Ca^{2+}-channel blocker verapamil (5 μM) were added 5 min before the addition of nicotinic agonist or KCl. Subsequently, calibration was performed by sequentially adding 0.5% Triton X-100 to lyse the cells in order to obtain R_{max} (the fluorescence of calcium-saturated dye), followed by 20 mM EGTA to determine R_{min}, the fluorescence signal after the dye is quenched.

$[Ca^{2+}]_i$ was determined from the fluorescence ratio of fura-2 at 340/380 nm excitation (A_{340}/A_{380}; given as R below) according to the Grynkiewicz Equation (Grynkiewicz *et al*., 1985):

$$[Ca^{2+}]_i = K_D^*[(R - R_{min})/(R_{max} - R)] * (S_{f2}/S_{b2})$$

where K_D is the dissociation constant of fura-2 for Ca^{2+} binding, 224 nM (Tsien *et al*., 1982), R_{min} is the fluorescence ratio under 'zero' Ca^{2+} conditions, R_{max} is the fluorescence ratio under saturating Ca^{2+} conditions and S_{f2}/S_{b2} is the ratio of fluorescence values of Ca^{2+}-free and Ca^{2+}-saturated fura-2, measured at the wavelength used to follow Ca^{2+}-free fura-2.

Calcium fluorimetry: fluo-3 assays in 96 well plates

Culture medium was removed from the wells and the cells were washed twice with Tyrode's salt solution (TSS). Fluo-3 AM (10 μM) and 0.02% pluronic acid were added in TSS (40 μl per well) and the cells were incubated at room temperature for 1 h in the dark. After two further washes in TSS, 80 μl of TSS with or without antagonists were added, and the plate transferred to a Fluoroscan Ascent (Labsystems, Helsinki, Finland). After 10 min preincubation (or 30 min in the case of α-Bgt) at 20°C in the dark, nicotine (final concentration 30 μM unless otherwise stated) was added (20 μl per well), and fluorescence monitored at 538 nM wavelength. Maximum and minimum fluorescence was determined by the sequential addition of 20 μl Triton $\times$100 (2%, final concentration) and 20 μl $MnCl_2$ (40 mM, final concentration). Changes in intracellular calcium were calculated as a percentage of the difference between the minimum and maximum fluorescence.

Data analysis

Changes in $[Ca^{2+}]_i$ are expressed as mean$\pm$s.e.mean concentration or as a percentage of the increase in $[Ca^{2+}]_i$ (fura-2 experiments) or as a percentage of Max$-$Min fluorescence (fluo-3 experiments) measured in control cells. For concentration response curves, agonist responses were calculated as a percentage of the increase in $[Ca^{2+}]_i$ or fluorescence produced by a maximally effective concentration of the same drug, assayed in parallel. Data points were fitted to the Hill equation, using the nonlinear least squares curve fitting facility of Sigma Plot V2.0 for Windows. All data were analysed statistically using an analysis of variance (ANOVA) for repeated measures using SPSS (Statistics Package for Social Scientists, PC version).

Results

Effects of acute nicotine and KCl depolarization on $[Ca^{2+}]_i$ in SH-SY5Y cells

The resting $[Ca^{2+}]_i$ in fura-2-loaded SH-SY5Y cells was 140$\pm$4 nM (n=160). Application of nicotine produced a rapid increase in fluorescence which was followed by a

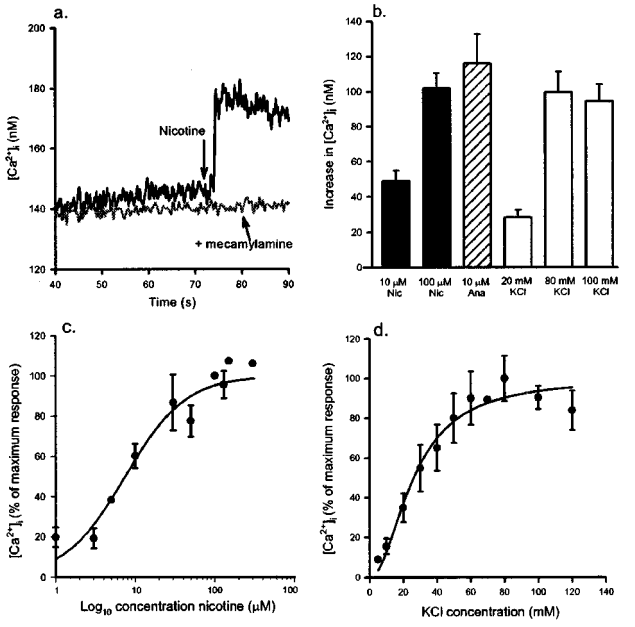

Figure 1 Increases in $[Ca^{2+}]_i$ in SH-SY5Y cells evoked by nicotinic agonists and KCl. (a) A representative trace from fura-2-loaded SH-SY5Y cells in suspension, showing the change in $[Ca^{2+}]_i$ in response to 10 μM nicotine (arrow) in the presence (lower trace) and absence (upper trace) of 10 μM mecamylamine. (b) Increases in $[Ca^{2+}]_i$ above basal levels in response to nicotine (Nic; 10 μM, 100 μM), anatoxin-a (Ana; 10 μM) and KCl (20, 80 and 100 mM). Values are the mean$\pm$s.e.mean from at least five separate cultures. (c) Concentration-response curve for nicotine-evoked increases in $[Ca^{2+}]_i$. Responses to 100 μM nicotine were taken as 100%. Data points are the mean$\pm$s.e.mean of five separate cultures and are fitted to the Hill equation giving an EC_{50} value of 7.5$\pm$4 μM. (d) Concentration-response curve for KCl-evoked increases in $[Ca^{2+}]_i$. Responses to 80 mM KCl were taken as 100%. Data points are the mean$\pm$s.e.mean of five separate cultures and are fitted to the Hill equation giving an EC_{50} value of 26$\pm$8 mM.

sustained plateau (Figure 1a). At higher concentrations of nicotine (100 μM) the plateau slowly declined over time in some cases. The initial plateau value of the response was used to derive the $[Ca^{2+}]_i$. Nicotine-evoked increases in $[Ca^{2+}]_i$ were concentration-dependent, with a maximum response produced by 100 μM nicotine, which elevated $[Ca^{2+}]_i$ by 102 ± 9 nM ($n=29$) above its resting level (Figure 1b,c). The EC_{50} value for nicotine-evoked increase in $[Ca^{2+}]_i$ was 7.5 ± 4 μM. For comparison with previous studies (Sharples *et al*., 2000), a maximally effective concentration of the nicotinic agonist anatoxin-a (10 μM) was tested in parallel with nicotine. It elicited a response comparable in magnitude to that of 100 μM nicotine (Figure 1b).

KCl also produced a rapid increase in $[Ca^{2+}]_i$ followed by a sustained plateau, similar to the response evoked by nicotine shown in Figure 1a. KCl-evoked increases in $[Ca^{2+}]_i$ were concentration-dependent, with a maximum response produced by 80 mM KCl, which increased $[Ca^{2+}]_i$ by 100 ± 12 nM ($n=12$). The EC_{50} value was 26 ± 8 mM KCl (Figure 1b,d). In subsequent experiments, approximate EC_{50} concentrations of nicotine (10 μM) and KCl (20 mM) were selected to elicit responses.

As SH-SY5Y cells express a variety of nicotinic AChR subunit mRNAs (Lukas *et al*., 1993) and nicotinic AChR subtypes (Peng *et al*., 1997), we used a selection of chemically distinct, subtype-selective nicotinic antagonists to dissect the contributions of different nicotinic AChR subtypes to the increase in $[Ca^{2+}]_i$ produced by 10 μM nicotine. None of the antagonists examined in this study had any effect on basal levels of $[Ca^{2+}]_i$. Mecamylamine (10 μM) completely blocked the response to nicotine (Figures 1a and 2), confirming that the response is nicotinic AChR-mediated. Maximally effective concentrations of three α7-selective antagonists inhibited the nicotine-evoked increase in $[Ca^{2+}]_i$ to a similar extent (Figure 2): α-Bgt (50 nM), α-conotoxin-ImI (1 μM; Pereira *et al*., 1996) and methyllycaconitine (MLA; 10 nM; Alkondon *et al*., 1992) blocked the response by $41 \pm 8\%$ ($n=5$), $39 \pm 5\%$ ($n=4$) and $40 \pm 6\%$ ($n=4$) respectively. The $\alpha 3\beta 2$-selective antagonist α-conotoxin-MII (112 nM; Cartier *et al*., 1996; Kaiser *et al*., 1998) blocked the response to nicotine by $50 \pm 5\%$ (Figure 2). Co-application of MLA (10 nM) and α-conotoxin-MII (112 nM) inhibited the nicotine-evoked increase in $[Ca^{2+}]_i$ by $69 \pm 9\%$, suggesting that their actions are partially additive, although this did not reach statistical significance. These results demonstrate that this functional nicotinic response is mediated by at least two receptor subtypes: α7 and $\alpha 3\beta 2$-containing nicotinic AChRs.

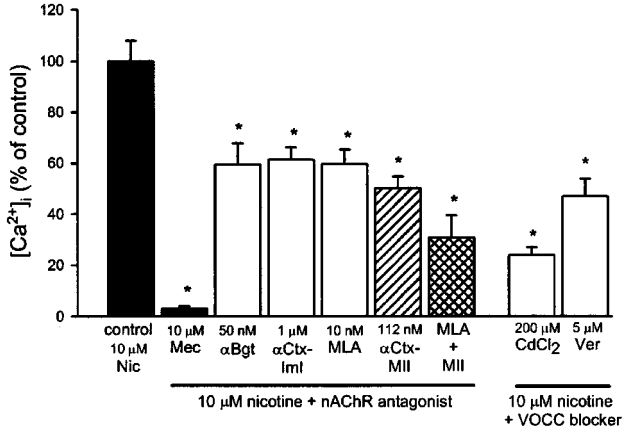

Figure 2 Effect of nicotinic antagonists and Ca^{2+} channel blockers on nicotine-evoked increases in $[Ca^{2+}]_i$ in SH-SY5Y cells. Suspensions of fura-2-loaded SH-SY5Y cells were incubated for 5 min with the nicotinic AChR antagonists mecamylamine (Mec), α-conotoxin-ImI (αCtx-ImI), MLA, α-conotoxin-MII (αCtx-MII) or blockers of voltage-operated Ca^{2+} channels: $CdCl_2$ or verapamil (Ver). α-Bungarotoxin (αBgt) was incubated with the cells, together with fura-2 AM, for 45 min. Nicotine (10 μM, Nic) was added and the increase in $[Ca^{2+}]_i$ monitored as in Figure 1. Responses are expressed as a percentage of the control response measured in parallel in the absence of antagonist. Values are the mean $\pm$ s.e.mean of at least four independent experiments carried out in triplicate (except for $CdCl_2$, where $n=2$ independent experiments and values are mean $\pm$ range). *Significantly different from control, $P<0.05$, one way ANOVA.

The increase in $[Ca^{2+}]_i$ in response to nicotinic AChR activation may be complicated by the entry of Ca^{2+} into the cell by routes other than the nicotinic AChR. Nicotine- and KCl-evoked increases in $[Ca^{2+}]_i$ were totally dependent on the presence of extracellular Ca^{2+} (data not shown). $CdCl_2$, a general blocker of voltage-operated Ca^{2+} channels that does not block nicotinic currents at the concentration used (200 μM; Rathouz & Berg, 1994), did not affect basal $[Ca^{2+}]_i$ when applied for 5 min prior to stimulation with nicotine (10 μM). However, the nicotine-evoked increase in $[Ca^{2+}]_i$ was inhibited by $76 \pm 3\%$ (mean $\pm$ range; $n=2$) by $CdCl_2$, whereas the phenylalkylamine L-type Ca^{2+}-channel blocker, verapamil (5 μM) partially inhibited nicotine-evoked increases in $[Ca^{2+}]_i$, by $53 \pm 7\%$ ($n=4$; Figure 2). Thus a major proportion of the response to nicotine arises from activation of voltage-operated Ca^{2+} channels, including L-type channels.

The effect of chronic drug treatments on nicotine- and KCl-evoked responses

Having characterized the nicotinic AChR-evoked changes in $[Ca^{2+}]_i$ in untreated SH-SY5Y cells, we next investigated if chronic treatment with nicotinic agonists or KCl, regimes that upregulate nicotinic binding sites (Ridley *et al*., 2001), would modify these functional responses. Chronic exposure (4 days) to nicotine (10 μM), the α7-selective nicotinic agonist DMAC (10 μM) and KCl (20 mM) was investigated. After thorough washing (four washes over 4 h, see Methods), increases in $[Ca^{2+}]_i$ evoked by 10 μM nicotine and 20 mM KCl were measured (Figure 3). Acute stimulation with 10 μM nicotine resulted in significantly lower responses (approximately 25% less than in untreated control cells) after chronic treatment with nicotinic agonist or KCl. Acute stimulation with KCl, on the other hand, resulted in no significant difference in the $[Ca^{2+}]_i$ response between chronically treated and control cells, examined in parallel (Figure 3a–c). DMAC could not be applied acutely to evoke changes in $[Ca^{2+}]_i$ due to its intense purple colour, even when diluted, which interfered with the fluorescence measurements. Chronic treatment of cells with 5 μM verapamil for 4 days (a treatment that has no effect on numbers of nicotinic AChR binding sites, Ridley *et al*., 2001) decreased responses to both nicotine and KCl by 50% (Figure 3d).

As nicotine and KCl treatments upregulated α7 nicotinic AChR binding sites in SH-SY5Y cells through different mechanisms (Ridley *et al*., 2001), we examined the α7 component of the functional response to nicotine stimulation in control and treated cells. In control cells, acute

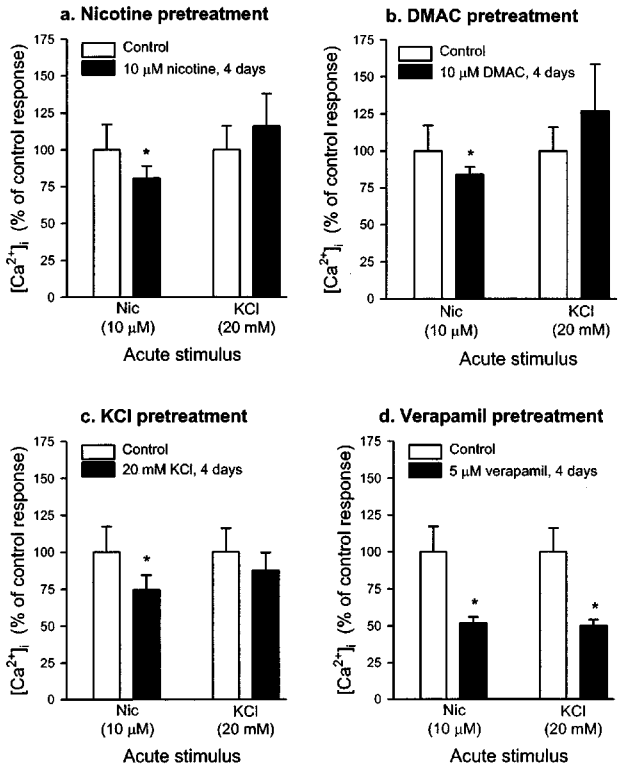

Figure 3 Effect of chronic treatment of SH-SY5Y cells with nicotinic agonists, KCl or verapamil on nicotine- and KCl-evoked increases in $[Ca^{2+}]_i$. SH-SY5Y cells were treated with (a) 10 μM nicotine, (b) 10 μM DMAC, (c) 20 mM KCl, or (d) 5 μM verapamil for 4 days, thoroughly washed, loaded with fura-2 and stimulated with nicotine (Nic; 10 μM) or KCl (20 mM). Responses from treated cells (filled columns) are expressed as a percentage of control responses (open columns) from untreated cells cultured and assayed in parallel. Values are the mean$\pm$s.e.mean of at least four independent experiments. Significantly different from control, $^*P<0.05$, one way ANOVA.

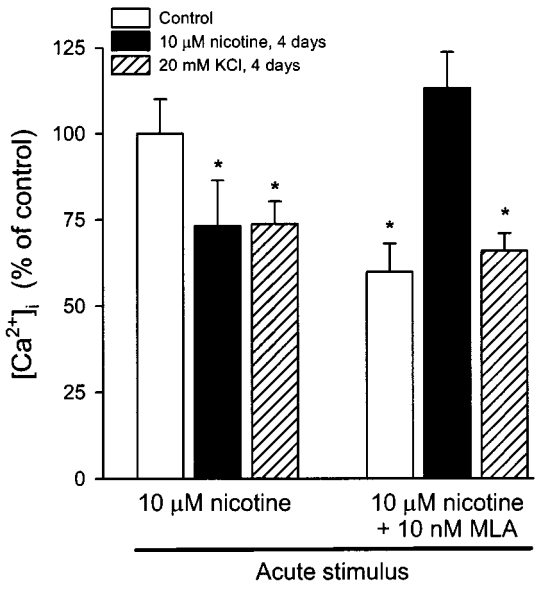

Figure 4 Effect of MLA on nicotine-evoked increases in $[Ca^{2+}]_i$ from SH-SY5Y cells chronically treated with nicotine or KCl. SH-SY5Y cells were treated with 10 μM nicotine (black columns) or 20 mM KCl (hatched columns) for 4 days, thoroughly washed, loaded with fura-2 and incubated with or without MLA (10 nM) for 5 min before stimulation with nicotine (10 μM). Responses are expressed as a percentage of control responses from untreated cells cultured and assayed in parallel in the absence of MLA. Values are the mean$\pm$s.e.mean of at least four independent experiments. *Significantly different from untreated control in the absence of MLA, $P<0.05$, one way ANOVA. #Significantly different from untreated control in the presence of MLA, $P<0.05$, one way ANOVA.

MLA (10 nM) inhibited the response to nicotine by 40$\pm$8% ($n=5$; Figure 4), in agreement with the results presented in Figure 2. After chronic nicotine treatment, the response to nicotine stimulation was reduced by 27$\pm$13% ($n=3$) compared with control cells. Surprisingly, in the presence of acute MLA, the nicotine-evoked change in $[Ca^{2+}]_i$ was enhanced, and was comparable to or greater than that elicited from untreated cells in the absence of MLA. After chronic KCl treatment, the response to nicotine stimulation was reduced by 26$\pm$7% ($n=4$) in the absence of MLA and 34$\pm$5% ($n=7$) in the presence of MLA (Figure 4). Thus MLA had no significant effect on this response in KCl-treated cells.

To explore the effects of other nicotinic antagonists on the nicotine-evoked increase in $[Ca^{2+}]_i$ in chronically nicotine-treated SH-SY5Y cells, we employed a 96 well format higher throughput assay, that uses fluo-3 instead of fura-2 to monitor Ca^{2+} levels in adherent cells (see Methods). Using this assay, the dose-response relationship for nicotine-evoked responses (Figure 5, insert; $EC_{50}=21$ μM) was similar to that derived using fura-2 (Figure 1c). A higher nicotine concentration (30 μM) was used in subsequent experiments, in order to elicit more robust and consistent increases in Ca^{2+}. These responses in control cells exhibited the same pharmacological profile (Figure 5) as previously observed (Figure 2), with approximately 30% inhibition by the α7-selective antagonists MLA, α-Bgt and α-conotoxin-ImI, 50% inhibition by α-conotoxin-MII and more than 80% inhibition by mecamylamine. In cells cultured in parallel for 4 days with 10 μM nicotine, followed by thorough washing (six washes over 4 h), the response to nicotine was decreased by 33$\pm$4% ($n=4$) whereas the response to nicotine in the presence of acute MLA (10 nM) was 127$\pm$15% of control ($n=4$; Figure 5). This assay therefore reproduces the unexpected increase in the nicotine-evoked response in the presence of MLA seen in Figure 4. This result appears to be peculiar to MLA: nicotine-evoked responses from chronically treated cells in the presence of other α7-selective antagonists, α-Bgt and α-conotoxin-ImI, were not different from responses in the absence of these agents. This suggests that the α7 nicotinic receptor no longer contributes to the nicotine-stimulated response after chronic nicotine treatment. In contrast, in the presence of the $\alpha3\beta2$*-selective antagonist α-conotoxin-MII, nicotine-evoked responses were 50$\pm$4% ($n=3$) and 46$\pm$4% ($n=3$) of the control response from untreated cells, in untreated and treated cells respectively, compared with the nicotine-evoked response of 67$\pm$4% of control ($n=4$) in treated cells (Figure 5). Thus α-conotoxin-MII inhibits nicotine-evoked responses in treated cells to the same level as observed in control preparations. Similarly, mecamylamine reduced responses in treated cells to a level comparable to that observed in control cells (Figure 5).

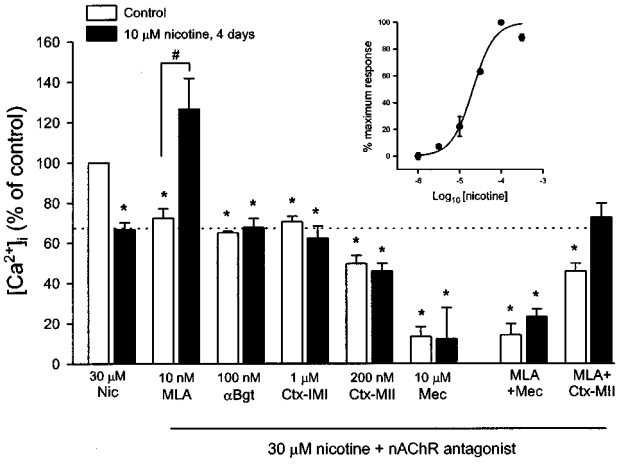

Figure 5 Comparison of the effects of nicotinic AChR antagonists on nicotine-evoked increases in $[Ca^{2+}]_i$ in SH-SY5Y cells chronically treated with nicotine. SH-SY5Y cells were grown in 96 well plates and treated with (black columns) or without (open columns) 10 μM nicotine for 4 days, thoroughly washed (six times over 4 h) and loaded with fluo-3 AM. Cells were incubated with antagonist: MLA, α-Bgt, α-conotoxin-ImI (αCtx-ImI), α-conotoxin-MII (αCtx-MII) or mecamylamine (Mec) for 10 min before stimulation with nicotine (30 μM, Nic). Changes in Ca^{2+} in fluo-3-loaded SH-SY5Y cells were monitored using a 96 well format assay (see Methods). Responses are expressed as a percentage of the response to nicotine in untreated cells cultured and assayed in parallel, in the absence of antagonist. The dashed line indicates the response to nicotine in nicotine-treated cells, assayed in the absence of antagonist. Values are the mean$\pm$ s.e.mean of at least three independent experiments. *Significantly different from untreated control in the absence of MLA, $P<0.05$, #significantly different from untreated control in the presence of MLA, $P<0.05$, one way ANOVA. Insert: concentration-response curve for nicotine-evoked increases in Ca^{2+} in untreated SH-SY5Y cells, monitored using the 96 well format assay. Data points are the mean$\pm$range for two independent experiments, each with six replicates for every concentration, and are fitted to the Hill equation, giving an EC_{50} value of 20.9 μM.

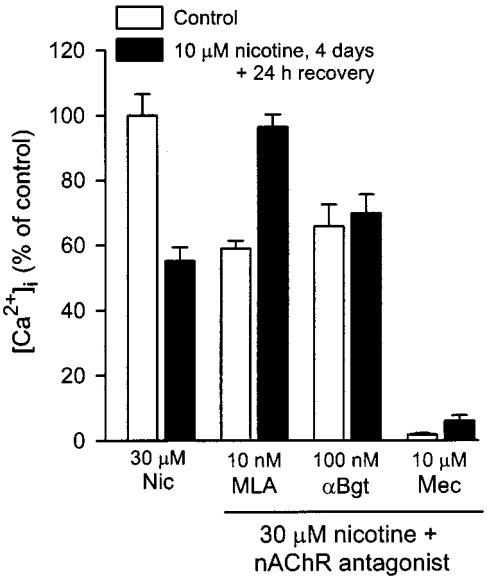

Figure 6 Nicotine-evoked increases in $[Ca^{2+}]_i$ in SH-SY5Y cells measured 24 h after chronic treatment with nicotine. SH-SY5Y cells were treated with (black columns) or without (open columns) 10 μM nicotine for 4 days, thoroughly washed (six times over 4 h) and incubated in normal medium for 20 h before loading with fluo-3-AM and measurement of $[Ca^{2+}]_i$. Cells were incubated with MLA, α-Bgt or mecamylamine (Mec) for 10 min before stimulation with nicotine (30 μM, Nic). Changes in Ca^{2+} in fluo-3-loaded SH-SY5Y cells were monitored using a 96 well format assay (see Methods). Responses are expressed as a percentage of the response to nicotine in untreated cells cultured and assayed in parallel, in the absence of antagonist. Results are from one experiment with six replicates for each condition.

To ascertain if the paradoxical increase observed in the presence of MLA was nicotinic AChR-mediated, MLA was applied in combination with mecamylamine. In this case, nicotine-evoked responses were not significantly different between treated and control cells, and did not differ significantly from nicotine-evoked responses in the presence of mecamylamine alone (Figure 5). However, MLA in combination with α-conotoxin MII did result in a somewhat higher nicotine-evoked response in treated cells compared with untreated cells (Figure 5), suggesting that MLA can still facilitate an increased response when $\alpha3\beta2$* nicotinic AChR are blocked.

To examine the reversibility of the effects of chronic nicotine treatment, and the enhancement by MLA of nicotine-evoked responses, SH-SY5Y cells were treated with nicotine for 4 days, thoroughly washed as before and then incubated in normal medium for 20 h before measuring nicotine-evoked Ca^{2+} responses (Figure 6). It is clear from this experiment that the reduced response to nicotine persisted, and the pharmacology of responses from control and treated cells was the same as observed in assays carried out immediately after the removal of chronically applied nicotine (Figures 4 and 5). In particular, the enhanced response to nicotine in the presence of MLA in chronically treated cells was preserved (Figure 6).

Discussion

In this study we have shown that both $\alpha7$ and $\alpha3$* subtypes of nicotinic AChR contribute to nicotine-evoked increases in $[Ca^{2+}]_i$ in SH-SY5Y cells. We have examined the effects of chronic treatments with nicotinic agonists and KCl on subsequent nicotine-evoked increases in $[Ca^{2+}]_i$. Functional responses in nicotine- or DMAC-treated cells were decreased, compared with responses from untreated control cells. This appears to reflect, predominantly, the loss of the $\alpha7$ subtype-mediated component of the response judged by the loss of antagonism by α-conotoxin-lml and α-Bgt, although the $\alpha7$-selective antagonist MLA provoked an unexpected increase in nicotine-evoked Ca^{2+} fluorescence. This action of MLA appeared to be nicotinic AChR-mediated as it was prevented in the presence of mecamylamine. Responses to nicotine were also diminished in cells treated with KCl for 4 days, but in this case MLA had no effect.

The human neuroblastoma SH-SY5Y cell line gave robust and reproducible increases in $[Ca^{2+}]_i$ in response to nicotine and KCl, in agreement with previous studies (Lambert *et al*., 1990). SH-SY5Y cells endogenously express $\alpha3$,$\alpha5$,$\alpha7$,$\beta2$ and $\beta4$ nicotinic AChR subunits (Lukas *et al*., 1993; Peng *et al*., 1994), which form a number of receptor subtypes (for example $\alpha3\beta2$, $\alpha3\alpha5\beta2$, $\alpha3\beta4$, $\alpha3\alpha5\beta4$, $\alpha3\beta2\beta4$ and $\alpha3\alpha5\beta2\beta4$, in addition to $\alpha7$; Wang *et al*., 1996). Three $\alpha7$-selective antagonists and the $\alpha3\beta2$*-selective antagonist α-conotoxin-MII provide evidence that both of these types of nicotinic AChR contribute to the observed increase in $[Ca^{2+}]_i$

in response to 10 μM nicotine (Figure 2). The incomplete blockade of the nicotine-evoked response by MLA and α-conotoxin-MII together (at concentrations that should maximally inhibit α7 and α3β2* nicotinic AChR respectively, Alkondon *et al*., 1992; Kaiser *et al*., 1998) suggests that α3β4* receptors also participate. Despite the rapid desensitization of neuronal nicotinic AChR (especially the α7 subtype, Séguéla *et al*., 1993), nicotine-induced increases in $[Ca^{2+}]_i$ were sustained (Figure 1a), as recently observed in CNS neurones (Dajas-Bailador *et al*., 2000; Tsuneki *et al*., 2000). The long lasting response may reflect the fact that most of the increase in $[Ca^{2+}]_i$ could be attributed to voltage-operated Ca^{2+} channels (including verapamil-sensitive L-type channels), activated in response to nicotinic AChR-induced membrane depolarization. Furthermore, a contribution from internal Ca^{2+} stores may also participate in the observed response (Tsuneki *et al*., 2000, Dajas-Bailador *et al*., 2001).

Exposure to 20 mM KCl for 4 days upregulates [^{125}I]-α-Bgt binding sites in SH-SY5Y cells by about 50% but has no effect on numbers of [^{3}H]-epibatidine binding sites (Ridley *et al*., 2001). The upregulation of [^{125}I]-α-Bgt sites occurs through a voltage-operated Ca^{2+} channel and CaM-kinase II dependent mechanism (Ridley *et al*., 2001), and probably involves an increase in α7 gene transcription (De Koninck & Cooper, 1995). Despite the increase in numbers of surface [^{125}I]-α-Bgt binding sites (Ridley *et al*., 2001), there was a significant (26%) decrease in the nicotine-evoked elevation of $[Ca^{2+}]_i$ after chronic KCl treatment, whereas KCl-evoked responses were unchanged (Figure 3). As nicotine-evoked responses from KCl-treated SH-SY5Y cells were insensitive to acute MLA and of the same magnitude as those observed in untreated cells assayed in the presence of MLA (Figure 4), the α7 nicotinic AChR-mediated component of the nicotine-evoked increase in $[Ca^{2+}]_i$ appears to have been selectively lost. This interpretation is consistent with the selective effect of chronic KCl treatment on α7 (but not α3*) nicotinic AChR binding sites (Ridley *et al*., 2001). The mechanism whereby chronic KCl depolarization leads to the elimination of α7 nicotinic AChR-mediated responses (either inactivation of the α7 receptor itself or of a point in its downstream pathway that leads to increases in $[Ca^{2+}]_i$), remains to be elucidated.

It is likely that chronic depolarization would affect a number of cellular processes that might influence the regulation of $[Ca^{2+}]_i$. One candidate is the voltage-operated Ca^{2+} channel: in PC12 cells, exposure to elevated extracellular KCl (50 mM) for 4 days caused concomitant decreases in numbers of [^{3}H]-nitrendipine binding sites and depolarization-dependent $^{45}Ca^{2+}$ entry (De Lorme *et al*., 1988), implicating a modification of L-type Ca^{2+} channels. Nevertheless, activation of L-type Ca^{2+} channels underlies the KCl-induced upregulation of α7 mRNA (De Koninck & Cooper, 1995) and [^{125}I]-α-Bgt binding sites (Ridley *et al*., 2001), as shown by its inhibition by verapamil. In the present study, KCl-evoked increases in $[Ca^{2+}]_i$ were not significantly changed after chronic KCl treatment. On the other hand, chronic verapamil treatment (which is anticipated to down-regulate L-type Ca^{2+} channels, Panza *et al*., 1985, but which has no effect on numbers of [^{125}I]-α-Bgt or [^{3}H]-epibatidine binding sites in SH-SY5Y cells, Ridley *et al*., 2001), provoked substantial decreases (50%) in responses to both nicotine and KCl depolarization (Figure 3). These observations are difficult to reconcile and more direct measures of ion channel function are required to clarify the relationship. The possibility that verapamil may interact with nicotinic AChR (especially the α3β4* subtype; Herraro *et al*., 1999) further complicates the analysis.

Chronic nicotine and DMAC treatments, in contrast to KCl, upregulate the numbers of both [^{125}I]-α-Bgt and [^{3}H]-epibatidine binding sites in SH-SY5Y cells, by a mechanism independent of voltage-operated Ca^{2+} channels (Ridley *et al*., 2001). After these treatments, responses to acute stimulation with 10 μM nicotine (but not to KCl) were significantly decreased by about 25% (Figures 3 and 4), similar to the decrease seen after chronic KCl treatment. The diminished response was equivalent to that observed in untreated cells assayed in the presence of α7 nicotinic AChR antagonists (Figure 5). Indeed, α-Bgt and α-conotoxin-ImI failed to inhibit responses from nicotine treated cells, whereas α-conotoxin-MII still produced some inhibition. These observations are consistent with the selective loss of the α7 nicotinic AChR-mediated component of the nicotine-evoked increase in $[Ca^{2+}]_i$.

However, in the presence of acute MLA, nicotine provoked an unexpected increase in intracellular Ca^{2+}, compared with responses to nicotine alone, in nicotine-treated cells only (Figures 4 and 5); this effect was observed using two fluorescence assays to measure Ca^{2+}. Thus MLA acts differently from the other α7-selective antagonists employed in this study. MLA does not appear to achieve this unexpected response through a non-specific action (for example, by interacting directly with voltage-operated ion channels) as its effect was abolished by co-application with the non-selective, non-competitive nicotinic antagonist mecamylamine (but not by the α3β2*-selective antagonist α-conotoxin-MII, Figure 5). This is consistent with an α7 nicotinic AChR-mediated action. Also, the lack of reversibility (within 24 h after the removal of chronic nicotine) of the diminished responsiveness to nicotine alone and its enhancement in the presence of acute MLA suggests a common process.

Chronic treatment with MLA (albeit at relatively high concentrations) upregulates numbers of α7 nicotinic AChR binding sites in hippocampal neurones (Ridley *et al*., 2001) and in transfected HEK-293 cells (Molinari *et al*., 1998). In the latter study, nicotine-evoked whole cell currents recorded from the transfected cells were increased after chronic nicotine or MLA treatment, suggesting that upregulated nicotinic AChR can be functional. Concentrations of nicotine (1 mM) and MLA (10 μM) that elicited comparable levels of upregulation of [^{125}I]α-Bgt binding sites (approximately 3 fold) resulted in different degrees of responsiveness, with 2 fold and 6 fold increases in whole cell currents, respectively (Molinari *et al*., 1998). This differential modulation by agonist and antagonist ligands was suggested to reflect their stabilization of different states of the nicotinic AChR. In the present study, α7 nicotinic AChR upregulated by nicotine (but not those upregulated by KCl) may be in a conformation which is converted by interaction with MLA to a conducting state upon agonist binding. The persistence of the decreased responsiveness to nicotine (in the absence of MLA) for at least 20 h after removal of the chronically applied agonist (Figure 6) implies sustained inactivation, rather than reversible desensitization, of the response. It is not clear why enhanced responses to nicotine were observed by Molinari *et al*. (1998) after chronic nicotine treatment. Differences from the present study include over-expression of

α7 nicotinic AChR in a non-neuronal cell line, and a more direct measurement of receptor activity using whole cell recording techniques. The ability of MLA to block nicotine-evoked currents recorded from the chronically treated HEK-293 α7-expressing cells was not described, but would be of interest in the light of the present results: MLA may be a useful ligand to probe the relationship between different conformations of the α7 nicotinic AChR.

In this study we have characterized the functional responses to stimulation with nicotine and KCl, after chronic drug treatments previously shown to modulate nicotinic AChR numbers. None of the treatment regimes that increased receptor numbers produced any increase in the $[Ca^{2+}]_i$ response stimulated by 10 μM nicotine or 20 mM KCl, compared with untreated controls. Rather, chronic nicotine and KCl treatments abolished the α7 nicotinic AChR-mediated component of the nicotine-evoked increase in intracellular Ca^{2+}. The α7-selective antagonist MLA, acutely applied, was unique in producing a paradoxical enhancement of nicotine-evoked response, (after chronic nicotine but not chronic KCl), thus nicotine and KCl treatments exert subtle differential effects on nicotinic AChR in SH-SY5Y cells.

This study was supported by a MRC Collaborative Studentship (to D. Ridley) in conjunction with Organon Laboratories, Newhouse, Scotland, and a grant from BAT Co. (to S. Wonnacott). J. Pakkanen was supported by the Socrates-Erasmus Programme of the Commission of the European Communities. We are grateful to Organon Laboratories for the provision of DMAC.

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(Received August 7, 2001
Revised November 7, 2001
Accepted November 15, 2001)