Journal of Neurochemistry, 2005, **94**, 926–933 doi:10.1111/j.1471-4159.2005.03223.x

Multiple calcium channels and kinases mediate α7 nicotinic receptor neuroprotection in PC12 cells

Ke Ren,* Viviana Puig,† Roger L. Papke,† Yoshitsugu Itoh,‡ Jeffrey A. Hughes* and Edwin M. Meyer†

**Department of Pharmaceutics, University of Florida, Gainesville, Florida, USA*

†*Department of Pharmacology, University of Florida, Gainesville, Florida, USA*

‡*Taiho Pharmaceuticals, Tokyo, Japan*

Abstract

α7 Nicotinic receptors are calcium permeant and provide neuroprotection against many insults. We investigated the roles of intracellular calcium ions and downstream calcium channels in this protection. The α7 agonist GTS-21 prevented pheochromocytoma cell death induced by nerve growth factor + serum deprivation over a 3-day interval. This effect was blocked by the intracellular calcium chelator 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid in a manner that did not appear to involve changes in receptor density. 1,2-Bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid blocked GTS-21-induced protein kinase C activation, a necessary process for protection. The insositol triphosphate calcium-channel blocker xestospongin C and the phospholipases C inhibitor U-73122 blocked protection, ryanodine partially attenuated protection, but the L-type channel antagonist nifedipine had no effect. ERK1/2 but not JNK and p38 were activated by GTS-21, and the ERK phosphorylation inhibitors PD98059 and U0126 blocked protection.

Keywords: calcium, neuroprotection, α7 nicotinic receptor, protein kinase.

J. Neurochem. (2005) **94,** 926–933.

Brain α7 nicotinic receptors are calcium-permeant channels (Seguela *et al*. 1993) that influence memory-related behaviors (Briggs *et al*. 1997; Meyer *et al*. 1998b; Bettany and Levin 2001; Woodruff-Pak 2003) and are found in high concentrations in hippocampus, neocortex and other regions associated with learning and memory (Marks and Collins 1982). Selective α7 receptor agonists such as GTS-21 have been developed as potential therapeutic agents for Alzheimer's disease and other neuropathological conditions (Kitagawa *et al*. 2003). These agents also appear to be neuroprotective, adding to their potential value for treating these disorders. However, the mechanisms underlying their cytoprotective effects are only beginning to be elucidated.

α7 Nicotinic receptor-induced neuroprotection has been demonstrated in models that are apoptotic, at least in part: trophic factor deprivation (Li *et al*. 1999; Jonnala and Buccafusco 2001), glutamate-induced excitotoxicity in primary rat brain neuronal cultures (Shimohama *et al*. 1998), Abeta amyloid exposure in neurons and cell lines (Kihara *et al*. 2001), and ethanol toxicity in primary neuronal cultures and PC12 cells (Li *et al*. 2002). Neuroprotection from ischemic damage (Shimohama *et al*. 1998) and lesions (Martin *et al*. 1994; Meyer *et al*. 1998a) has also been reported with GTS-21 *in vivo*. Even indirect activation of α7 nicotinic receptors by blocking cholinesterase activity is sufficient for protection (Jonnala and Buccafusco 2001).

GTS-21 and other α7 agonists increase intracellular calcium concentrations (Gueorguiev *et al*. 2000; Li *et al*. 2002) and activate the calcium-sensitive transduction processes protein kinase A (PKA; Dajas-Bailador *et al*.

Received January 24, 2005; revised manuscript received March 31, 2005; accepted April 4, 2005.

Address correspondence and reprint requests to Edwin M. Meyer, Department of Pharmacology, University of Florida, Box 100267, 1600 SW Archer Road, Gainesville, FL 32610, USA.
E-mail: meyerlab@grove.ufl.edu

Abbreviations used: BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; InsP_3, inositol triphosphate; KR, Krebs Ringer buffer; MLA, methyllycaconitine; NGF, nerve growth factor; PAGE, polyacrylamide gel electrophoresis; PC12, pheochromocytoma cells; PKA, protein kinase A; PKC, protein kinase C; SDS, sodium dodecyl sulfate.

2002b) and protein kinase C (PKC; Li *et al*. 1999), inositol triphosphate ($InsP_3$) kinase (Kihara *et al*. 2001), ERK (Dajas-Bailador *et al*. 2002b; Bell *et al*. 2004), and janus kinase (JNK; Salehi *et al*. 2004). PKC (Li *et al*. 1999), $InsP_3$ kinase (Kihara *et al*. 2001) and JNK (Salehi *et al*. 2004) are essential for α7-mediated protection against one or more apoptotic insults, whereas the roles of the other kinase pathways have not been studied. This protection through calcium-sensitive kinases suggests a protective role for the increased intracellular calcium ion concentrations seen following treatment with α7 agonists. We tested this hypothesis by investigating the effects of intracellular calcium ion chelation on both protection and PKC activation in nerve growth factor (NGF)-differentiated rat pheochromocytoma (PC12) cells. These cells express α7 receptors, undergo apoptosis following NGF removal, and provide a model for the dysfunction of ascending basal forebrain neurons associated with decreased NGF transport seen in Down's syndrome and Alzheimer's disease (Kerwin *et al*. 1992; Scott *et al*. 1995; Cooper *et al*. 2001; Salehi *et al*. 2004).

Activation of α7 receptors can increase calcium accumulation both directly and through activation of downstream L-type voltage sensitive channels, $InsP_3$ channels, and ryanodine channels (Vijayaraghavan *et al*. 1992; Gueorguiev *et al*. 2000; Shoop *et al*. 2001; Dajas-Bailador *et al*. 2002a), analogous to what is seen with metabotropic glutamate receptors (Fagni *et al*. 2000). Blocking $InsP_3$ channels with xestospongin C attenuates the long-term increase in calcium accumulation following α7-receptor activation almost completely in PC12 cells (Gueorguiev *et al*. 2000). Analysis of the calcium elevations triggered by nicotine in SH-SY5Y cells indicated that xestospongin C-sensitive $InsP_3$ channel activation appeared to be more important than nifedipine-sensitive L-type channels or ryanodine-sensitive channels for the long-term effects of α7 receptors (Dajas-Bailador *et al*. 2002a). We therefore hypothesized that these three channels may be differentially important for the long-term cytoprotective actions of α7 receptors, which we also tested in PC12 cells that express each of these calcium channels (Gafni *et al*. 1997; Tully and Treistman 2004). Finally, this study evaluated the role of the calcium-sensitive MAP kinases ERK1/2, p38, and JNK in the α7 receptor-mediated neuroprotection, because they are also differentially involved in the cytoprotective effects of other anti-apoptotic agents (Hetman and Xia 2000; Hsu *et al*. 2005; Kyosseva 2004).

Materials and methods

Cell culture

Rat PC12 cells (American Type Culture Collection, Rockville, MD, USA) were cultured and maintained as described previously at 37°C in a humidified atmosphere of 5% CO_2 (Li *et al*. 1999). They were split at a 1 : 4 ratio every 4 days for up to eight passages until the initiation of differentiation with 50 ng/mL of mouse submandibular NGF. One week later, the medium was replaced with serum-free medium with or without 50 ng/mL NGF. 1,2-Bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA-AM, final concentration 10 μM; Molecular Probes Eugene, OR, USA), nifedipine (500 nM; Sigma, St. Louis, MO, USA), xestospongin C (10 μM; Calbiochem, San Diego, CA, USA), ryanodine (10 μM, Sigma), PD98059 (10 μM, Calbiochem), U0126 (10 μM, Cell Signaling, Beverly, MA, USA), U-73122 (10 μM, Calbiochem), methyllycaconitine (MLA; 100 nM; RBI, South Natick, MA, USA), were added at specified time points in 100 μL sterile water immediately after medium replacement. GTS-21 (10 μM) was added in 50 μL sterile water. Three days after NGF + serum removal, five random photographs were taken in each plate with a Nikon microscope and analyzed in a blinded manner for cell density using the NIH IMAGE 1.55 program.

PKC assay

Total PKC activity was measured in membrane and soluble fractions of cell lysates obtained by probe sonication (100 W for 20 s) using a kit purchased from Amersham (Arlington Heights, IL, USA), which was based on the phorbol-stimulated, PKC-catalyzed transfer of [^{32}P] from gamma-[^{32}P]ATP to a PKC-selective peptide (Li *et al*. 1999). Values were expressed as the ratio of membrane/soluble PKC activity to reflect activation of the enzyme that occurs upon translocation to membrane binding sites.

[^{3}H]MLA binding

Nicotine-displaceable, high-affinity [^{3}H]MLA binding was measured as described previously (Davies *et al*. 1999). Cells were homogenized in 1 mL of ice-cold Krebs Ringer (KR) buffer (in mM: 118 NaCl, 5 KCl, 10 glucose, 1 $MgCl_2$, 2.5 $CaCl_2$, 20 HEPES; pH 7.5) with a Polytron (setting 4 for 15 s). After two 1 mL washes at 20 000 *g* with KR, membranes (0.2–0.3 mg protein) were incubated in 0.5 mL KR with 2–30 nM [^{3}H]MLA (Tocris, Bristol, UK) for 60 min at 4°C, plus or minus 5 mM (–)nicotine (Sigma). Tissues were washed three times with 5 mL cold KR buffer by filtration through Whatman GF/C filters that were pre-incubated for 30 min with 0.5% polyethylenimine. They were assayed for radioactivity using liquid scintillation counting in a Beckman LS1800 scintillation counter. Nicotine-displaceable binding was calculated for each MLA concentration in triplicate in each experiment; the resulting B_{max} values were calculated from Scatchard plots using PRISM to determine the *x*-intercept.

MAP kinase phosphorylation

NGF-differentiated PC12 cells were incubated for 5 or 180 min with 1–10 μM GTS-21, 100 nM MLA, or both drugs. They were assayed by western blot for phosphorylation of ERK1/2, JNK and p38 according to the manufacturer's instructions (Promega, Madison, WI, USA). Cells were sonicated in 20 mM Tris pH 7.4, 150 mM NaCl, 1% sodium dodecyl sulfate (SDS), 5 mM EDTA, 5 mM EGTA, 1 μg/mL each of aprotinin, pepstatin A and leupeptin, 1 mM sodium orthovanadate, 25 mM NaF, and 5 mM sodium pyrophosphate. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was conducted with 10 μg of sample protein in 12% polyacrylamide Bio-Rad Ready Gels, and samples were transferred to nitrocellulose membranes. Immunoblotting was

conducted with primary antibodies for phospho-ERK1/2 (1 : 5000), phospho-JNK (1 : 5000), or phospho-p38 (1: 2000). Secondary antibody was donkey anti-rabbit IgG (1 : 5000). Bands were assayed by chemiluminescence using Western Blotting Luminol Reagent (Santa Cruz Biotechnology) and quantified with an IBM-based image-analysis system using National Institutes of Health (Bethesda, MD) IMAGE software. The average mean absorbance for each group was calculated, and results are expressed as a percent of the untreated control value for the same time interval.

Statistical analysis

Comparisons between single treatment groups and their respective control values were conducted with the Student's *t*-test (two way). When multiple treatment groups were compared with the same control value or when multiple types of controls were compared with a single treatment, one-way analysis of variance (ANOVA) was used.

Results

Removal of NGF + serum reduced cell density over the 3-day interval compared with cells maintained in NGF-containing serum-free medium (Fig. 1). Treatment with BAPTA (10 μM) had no effect on cell survival in the absence of NGF, although it blocked the cytoprotective action of 10 μM GTS-21 (Fig. 1) when added 30 min prior to or 30 min after the receptor agonist. BAPTA at this concentration had no effect on cell survival in the presence of NGF (cell density: 110 ± 5%, mean ± SEM, of NGF-treated group; $N = 3$ plates). MLA blocked the GTS-21-induced protection when added at both time points (Fig. 1), as did pre-treatment with 50 nM α-bungarotoxin, a α7 receptor blocker with a much slower on-rate than MLA (not shown).

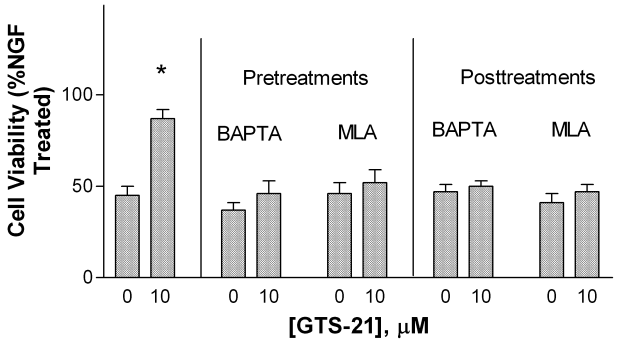

Fig. 1 Effects of intracellular calcium chelation on GTS-21-induced protection of PC12 cells during trophic factor deprivation. Cells were differentiated for 7 days with 50 ng/mL mouse NGF and then exposed to serum-free medium containing either 50 ng/mL NGF (positive control), BAPTA-AM (10 μM), GTS-21, or MLA (100 nM). BAPTA-AM and MLA were added 30 min before (pre-treatment) or 30 min after (post-treatment) the GTS-21. Cell density was measured 3 days later and expressed as the mean ± SEM of 6–8 plates per group from three experiments, normalized to the NGF-treated values for each experiment. *$p < 0.05$ compared with untreated group (one-way ANOVA).

Table 1 Effects of BAPTA and GTS-21 on α7 receptor binding density in PC12 cells

Treatment	High-affinity MLA binding (B_{max} in fmol/mg protein) 30 min	3 days
NGF + serum removal	152 ± 11	107 ± 10#
+ NGF (50 ng/mL)	165 ± 18	172 ± 15*
+ BAPTA (10 μM)	167 ± 13	144 ± 13*
+ GTS-21 (10 μM)	155 ± 17	101 ± 14#
+ BAPTA + GTS-21	172 ± 16	98 ± 12#

PC12 cells were treated differentiated for 7 days with 50 ng/mL NGF and then exposed to NGF + serum withdrawal. Specified concentrations of NGF, BAPTA, or GTS-21 were added and high-affinity [^{3}H]MLA was measured 30 min or 3 days later as described in the text. Each value is the mean ± SEM of three samples, each assayed in duplicate.

*$p < 0.05$ compared with the same time point, no drug-treatment (one-way ANOVA); #$p < 0.05$ compared to 30 min interval, same treatment.

None of the treatments affected the K_d for high-affinity MLA binding to α7 receptors (range: 1.6–2.3 nM). Removal of NGF + serum reduced the density of α7 receptors over the 3-day, but not 30-min, interval (Table 1); this reduction was not seen with the addition of 50 ng/mL NGF. Addition of BAPTA to the NGF + serum-deprived medium had no acute effect on α7 nicotinic receptor binding density, but modestly increased density over the 3-day interval compared with cells without the chelator. Neither GTS-21 nor GTS-21 + BAPTA preserved receptor density in this manner. BAPTA also blocked the concentration-dependent, GTS-21-induced, elevation in PKC membrane translocation seen in these cells at 15 min post drug treatment (Fig. 2).

The voltage-sensitive L-type calcium-channel blocker nifedipine had no effect on cell viability when applied alone or with GTS-21 (Fig. 3). Ryanodine partially attenuated the GTS-21-induced protection, also without a direct effect alone, indicating that only some cells appeared to depend on this channel activation for survival in this model. Xestospongin C, the InsP_3 channel antagonist, and U-73122, the phospholipase C inhibitor, completely blocked α7 receptor-mediated protection without affecting cell density.

ERK1/2-phosphorylation was increased by neuroprotective concentrations of GTS-21 within 5 min, and this effect increased by 3 h (Fig. 4). p38- and JNK-phosphorylation were unaffected by GTS-21 at either time interval (Fig. 4). The effect of GTS-21 on ERK1/2 phosphorylation was concentration dependent and blocked by MLA, demonstrating the role of α7 receptors (Fig. 5). Finally, GTS-21-mediated neuroprotection was blocked by pre-treatment with the PKC blocker BIM or the ERK1/2 blockers UO126 and PD98059 (Fig. 6).

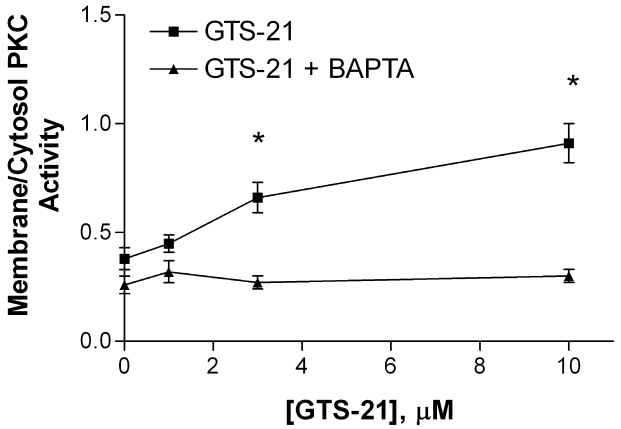

Fig. 2 Effects of intracellular calcium chelation on PKC activation by GTS-21 in PC12 cells. Cells were differentiated as in Fig. 1 and simultaneously exposed to serum and NGF removal, as well as to specified concentrations of GTS-21 in the presence or absence of 10 μM BAPTA-AM. Fifteen minutes later, cells were fractionated to membrane and soluble portions, each of which was assayed for phorbol-stimulated PKC activity per mg protein. Values are expressed as the ratios of membrane/soluble PKC activity; $N = 6$ plates/group from three separate experiments. *$p < 0.05$ compared with the BAPTA-treated group, same GTS-21 concentration (Student's *t*-test).

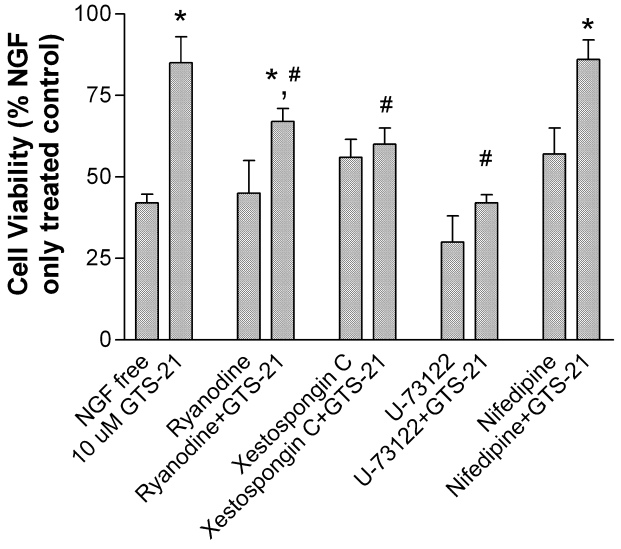

Fig. 3 Effects of calcium channel antagonists on GTS-21-induced cytoprotection in NGF-deprived PC12 cells. Cells, differentiated as in Fig. 1, were treated with 10 μM GTS-21 immediately after NGF + serum removal, with or without 10 μM ryanodine, 10 μM xestospongin C, 10 μM U-73122, or 500 nM nifedipine. Each value is the mean ± SEM of 6–8 plates/group from three separate experiments. *$p < 0.05$ compared with the same treatment without GTS-21; #$p < 0.05$ compared with GTS-21 only treatment group (one-way ANOVA).

Discussion

Previous studies have demonstrated GTS-21-induced calcium elevations (Gueorguiev *et al*. 2000; Li *et al*. 2002) and

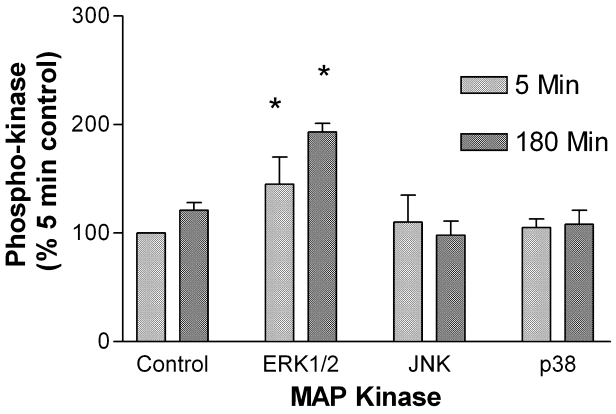

Fig. 4 Effects of GTS-21 on the phosphorylation of several MAP kinases in PC12 cells. Cells, differentiated as in Fig. 1, were treated with 10 μM GTS-21 for the specified interval upon removal of the NGF + serum. Whole-cell extracts were assayed for phospho-ERK1/2, phospho-JNK and phospho-p38 by western blotting and expressed as the mean ± SEM of four samples/group, normalized to the 5 min control value for that experiment. Each gel contained two lanes from the same 5 min control sample that were averaged and used for normalization. *$p < 0.05$ compared with controls from same time point (one-way ANOVA).

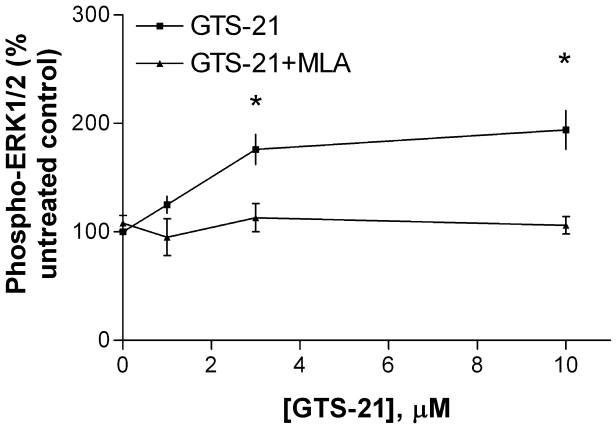

Fig. 5 MLA blocks the ERK1/2 phosphorylation triggered by GTS-21. Cells were treated as in Fig. 4, except that varying concentrations of GTS-21 were added for 180 min, plus or minus 100 nM MLA. Phospho-ERK was assayed and expressed as the mean ± SEM of four samples/group, normalized to untreated control values on the same gel. *$p < 0.05$ compared with either the corresponding MLA treatment group (Student's *t*-test).

have suggested the importance of multiple calcium-activated processes for α7 receptor-induced neuroprotection (Li *et al*. 1999; Kihara *et al*. 2001; Dajas-Bailador *et al*. 2002b; Bell *et al*. 2004; Salehi *et al*. 2004). These results show for the first time that chelation of intracellular calcium ions blocks GTS-21-induced, α7-mediated neuroprotection, demonstrating a direct role for these divalent cations in the

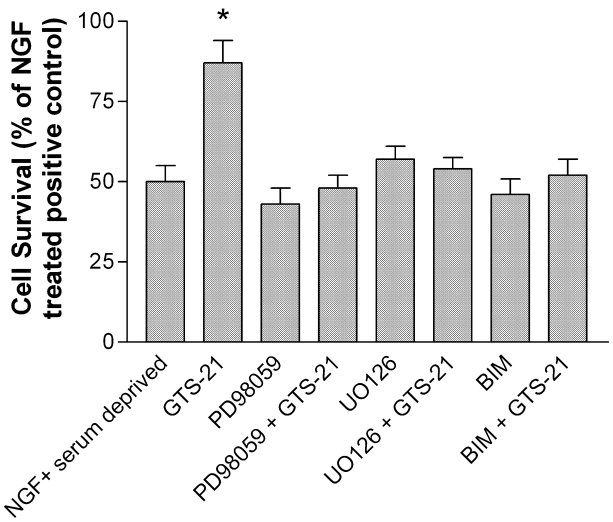

Fig. 6 GTS-21 induced cytoprotection is dependent on ERK phosphorylation and PKC activation. Differentiated PC12 cells were treated with10 μM GTS-21 for 3 days upon NGF + serum removal, with or without 10 μM PD98059, 10 μM U0126 or 500 nM bis-indole maleimide. Cell densities are expressed as the mean ± SEM of six plates/group from three experiments; $*p < 0.01$ compared with untreated control (one-way ANOVA).

receptor-modulation of cell viability. This is consistent with previous studies that chelated or removed extracellular calcium ions to attenuate α7-mediated protection or kinase activation (Donnelly-Roberts *et al*. 1996; Dajas-Bailador *et al*. 2002b). The concentration of BAPTA-AM used in our study was selected for its ability to prevent nicotine-induced elevations in both cytoplasmic calcium and tyrosine hydroxylase activity in PC12 cells (Gueorguiev *et al*. 1999). It therefore appears that the calcium elevations triggered by α7 receptor activation have important roles both for the phenotypic properties of these cells and for their long-term viability in the presence of toxic insults. It is interesting to note that a careful study of intracellular calcium chelation in hippocampal neurons recently concluded that modest increases in intracellular calcium concentrations were also associated with improved viability (Bickler and Fahlman 2004). It remains to be determined to what extent BAPTA treatment reduces calcium concentrations in mitochondria, smooth endoplasmic reticulum, and other storage sites under our experimental conditions.

The effects of α7 receptor activation on cell viability and their blockade by BAPTA were seen without any acute change in α7 nicotinic receptor-binding density and a modest increase in receptor density by 3 days. These observations are significant because α7-binding density was recently found to modulate the neuroprotection seen in PC12 cells (Jonnala and Buccafusco 2001). The reduction in α7 receptor-binding density over 3 days of NGF + serum removal is probably related to the observation that NGF increases this receptor subunit expression in PC12 cells (Takahashi *et al*. 1999). GTS-21 has not been reported to increase α7 receptor expression or lead to PC12 cell differentiation, which may account for its inability to prevent the loss of α7 receptor density over the 3 days of NGF deprivation. The lack of effect of GTS-21 on α7 receptor-binding density following the 3 days of NGF + serum deprivation was a surprising result because the less selective agonist nicotine was found to increase α7 receptor expression in these cells (Jonnala and Buccafusco 2001). MLA also increases α7 receptor density in PC12 cells (Jonnala and Buccafusco 2001), which might be expected with GTS-21 if it acted by desensitizing most α7 receptors. One possibility is that selective, low-level activation of α7 receptors is insufficient to increase their density, which is consistent with the lack of effect of chronic GTS-21 administration on α7 receptor density in neocortex *in vivo* (Meyer *et al*. 1997). Alternatively, GTS-21 may preserve a population of PC12 cells that expresses fewer α7 receptors, while simultaneously increasing their α7 receptor expression, resulting in no net change in density under these conditions.

Our results indicate that the cell viability effects of BAPTA are likely due to processes downstream from the receptor activation, since there was no decrease in binding density that would be expected to interfere with the actions of GTS-21. This is consistent with the observation that BAPTA exposure attenuated GTS-21-induced PKC activation. PKC translocation and activation were previously found to be essential for α7-mediated protection in this apoptotic model (Li *et al*. 1999). This increase in PKC activity occurs within 15 min, during the interval that neuroprotection is BAPTA sensitive, suggesting that kinase activation may be one of the relatively early steps in the protective process.

The anti-apoptotic effect of GTS-21 in PC12 cells requires extended activation of α7 receptors, because MLA blocks this cell survival when administered up to 1 h post agonist (Li *et al*. 1999). The ability of BAPTA to block protection when applied 5 min after GTS-21 suggests that a protracted elevation in intracellular calcium ions is also essential for protection. Whether this is due to a slow or rapid increase in calcium is not clear. We previously demonstrated that neuroprotective concentrations of GTS-21-caused a long-term, near steady-state calcium influx via α7 receptors, without desensitization of the overall receptor population seen at higher, non-protective agonist concentrations (Papke *et al*. 2000). However, although it is likely that this low-level receptor activation provides an early calcium transient that is important for triggering the cytoprotective pathway, it appears that downstream calcium channel activation is important as well.

The multichannel modulation of intracellular calcium by voltage-sensitive L-type calcium channels, intracellular $InsP_3$ channels, and intracellular ryanodine channels provides a potentially complex mechanism for α7 receptors to affect

cellular function and viability (Vijayaraghavan *et al*. 1992; Gueorguiev *et al*. 2000; Shoop *et al*. 2001; Dajas-Bailador *et al*. 2002a). Activation of the intracellular InsP_3 calcium channel, and to a lesser extent the ryanodine receptor, are necessary for complete α7-mediated protection in this model, based on sensitivity to antagonists. This is consistent with the protective actions of metabotropic receptors such as bradykinin (Yamauchi *et al*. 2003) and mGluR4s (Maj *et al*. 2003) that also act on InsP_3 receptors. The concentration of xestospongin C used in this study was found previously by our group to block InsP_3 receptors and reduce GTS-21-induced calcium accumulation in PC12 cells for as long as it was assayed (45 min) (Gueorguiev *et al*. 2000). This dependence on InsP_3 channels for most of the long-term elevation in intracellular calcium triggered by α7 receptors is consistent with their involvement in cytoprotection, which similarly depends on long-term receptor activation (Li *et al*. 1999). Inhibition of phospholipase C with U-73122 (Kokoska *et al*. 1998), which blocks InsP_3 diacylglycerol production, also attenuates GTS-21-induced protection, providing additional support for a role of InsP_3 receptors and PKC.

Ryanodine channels were also involved in the protection of some NGF + serum-deprived PC12 cells, although this effect was less dramatic than that seen with InsP_3 channel attenuation. Because ryanodine may only partially attenuate α7-mediated calcium elevations (Dajas-Bailador *et al*. 2002a), it is not surprising that some cells remained sensitive to GTS-21 in the presence of this channel blocker. There are multiple types of ryanodine receptor that are differentially expressed in various tissues, however, so it is conceivable that this result may be difficult to extrapolate to other neuronal models (Berridge *et al*. 2000). In brain neurons, ryanodine receptors are primarily localized to the endoplasmic reticulum of post-synaptic entities, from which they release calcium in response to increased cytoplasmic calcium. Recently, however, they have been found pre-synaptically and may be involved in modulating transmitter release (Bouchard *et al*. 2003), another well-characterized function of α7 receptors.

The nifedipine-sensitive, voltage-sensitive L-type channel underlies a significant amount of the calcium accumulation seen following α7 receptor activation. Although extracellular calcium is necessary for neuroprotection, as noted above, it appears that channels other than the L-type are involved in this action, probably the α7 receptors themselves. The observation that L-type channels are not essential for protection may be due to multiple factors. First, L-type channel openings may be for a shorter duration than is necessary for protection, e.g. if the voltage-dependent channels openings are attenuated over time through calcium-activated potassium channels. Alternatively, the calcium entry through these channels may be physically removed from the transduction processes essential for cytoprotection. A third possibility is that the intracellular calcium channels may provide sufficient calcium for protection even in the absence of voltage-sensitive calcium-channel activation.

Our results indicate for the first time that the ERK1/2 MAP kinase pathway is required for α7 receptor-mediated protection, based on its GTS-21-induced phosphorylation and attendant activation, as well as the ability of the ERK1/2 inhibitors PD98059 and U0126 to block protection. ERK1/2 phosphorylation has been associated with cytoprotection in a variety of model systems (Hetman and Xia 2000; Kyosseva 2004), and with other anti-apoptotic processes found to be triggered by α7 receptors, including bcl2 elevations, increased mitochondrial membrane potential, and reduced cytochrome *c* release (Li *et al*. 1999). Our results indicate, therefore, that calcium accumulation triggered by α7 receptor activation is necessary for this chain of kinase-mediated anti-apoptotic events. In contrast, neither the p38 nor the JNK pathway was apparently activated by a protective concentration of GTS-21. It is interesting to note that activation of ERK has been reported with very low concentrations amyloid peptides, suggesting that this may provide a dose-dependent protective role for the peptides and receptors under appropriate conditions, perhaps even in the sparing of α7 receptor-expressing cells in Alzheimer's disease.

In summary, α7 receptor activation provides cytoprotection against trophic factor deprivation and triggers PKC translocation through a mechanism that appears to involve intracellular calcium ion elevations. Among the several calcium channels triggered by α7 receptor activation, InsP_3- and, to a lesser extent ryanodine-receptor calcium channels are likely mediators of these calcium elevations and are essential for cytoprotection. Downstream ERK1/2 phosphorylation is also essential for protection, while other MAP kinases JNK and p38 are not. It now becomes important to determine the role of these various pathways in the protective actions of α7 receptors in brain *in vivo*.

References

Bell K. A., O'Riordan K. J., Sweatt J. D. and Dineley K. T. (2004) MAPK recruitment by beta-amyloid in organotypic hippocampal slice cultures depends on physical state and exposure time. *J. Neurochem.* **91**, 349–361.

Berridge M. J., Lipp P. and Bootman M. D. (2000) The versatility and universality of calcium signalling. *Nat. Rev. Mol. Cell Biol.* **1**, 11–21.

Bettany J. H. and Levin E. D. (2001) Ventral hippocampal alpha7 nicotinic receptor blockade and chronic nicotine effects on memory performance in the radial-arm maze. *Pharmacol. Biochem. Behav.* **70**, 467–474.

Bickler P. E. and Fahlman C. S. (2004) Moderate increases in intracellular calcium activate neuroprotective signals in hippocampal neurons. *Neuroscience.* **127**, 673–683.

Bouchard R., Pattarini R. and Geiger J. D. (2003) Presence and functional significance of presynaptic ryanodine receptors. *Prog. Neurobiol.* **69**, 391–418.

Briggs C. A., Anderson D. J., Brioni J. D. *et al.* (1997) Functional characterization of the novel neuronal nicotinic acetylcholine receptor ligand GTS-21 *in vitro* and *in vivo*. *Pharmacol. Biochem. Behav.* **57**, 231–241.

Cooper J. D., Salehi A., Delcroix J. D., Howe C. L., Belichenko P. V., Chua-Couzens J., Kilbridge J. F., Carlson E. J., Epstein C. J. and Mobley W. C. (2001) Failed retrograde transport of NGF in a mouse model of Down's syndrome: reversal of cholinergic neurodegenerative phenotypes following NGF infusion. *Proc. Natl Acad. Sci. USA* **98**, 10 439–10 444.

Dajas-Bailador F., Mogg A. J. and Wonnacott S. (2002a) Intracellular Ca^{2+} channels evoked by stimulation of nicotinic acetylcholine receptors in SH-SY5Y cells: contribution of voltage-operated Ca^{2+} channels and Ca^{2+} stores. *J. Neurochem.* **81**, 606–619.

Dajas-Bailador F. A., Soliakov L. and Wonnacott S. (2002b) Nicotine activates the extracellular signal regulated kinase 1/2 via the alpha7 nicotinic acetylcholine receptor and protein kinase A, in SH-SY5Y cells and hippocampal neurones. *J. Neurochem.* **80**, 520–530.

Davies A. R., Hardick D. J., Blagbrough I. S., Potter B. V., Wolstenholme A. J. and Wonnacott S. (1999) Characterisation of the binding of [^{3}H]methyllycaconitine: a new radioligand for labelling alpha7-type neuronal nicotinic acetylcholine receptors. *Neuropharmacology.* **38**, 679–690.

Donnelly-Roberts D. L., Xue I. C., Arneric S. and Sullivan J. (1996) *In vitro* neuroprotective properties of the novel cholinergic channel activator (ChCA), ABT-418. *Brain Res.* **719**, 36–44.

Fagni L., Chavis P., Ango F. and Bockaert J. (2000) Complex interactions between mGluRs, intracellular Ca^{2+} stores and ion channels in neurons. *Trends Neurosci.* **23**, 80–88.

Gafni J., Munsch J. A., Lam T. H., Catlin M. C., Costa L. G., Molinski T. F. and Pessah I. N. (1997) Xestospongins: potent membrane permeable blockers of the inositol 1,4,5-trisphosphate receptor. *Neuron.* **19**, 723–733.

Gueorguiev V. D., Zeman R. J., Hiremagalur B., Menezes A. and Sabban E. L. (1999) Differing temporal roles of Ca^{2+} and cAMP in nicotine-elicited elevation of tyrosine hydroxylase mRNA. *Am. J. Physiol.* **276**, C54–C65.

Gueorguiev V. D., Zeman R. J., Meyer E. M., Sabban E. and L. (2000) Involvement of alpha7 nicotinic acetylcholine receptors in activation of tyrosine hydroxylase and dopamine beta-hydroxylase gene expression in PC12 cells. *J. Neurochem.* **75**, 1997–2005.

Hetman M. and Xia Z. (2000) Signaling pathways mediating anti-apoptotic action of neurotrophins. *Acta. Neurobiol. Exp. (Warsaw)* **60**, 531–545.

Hsu Y. L., Kuo P. L., Lin L. T. and Lin C. C. (2005) Asiatic acid, a triterpene, induces apoptosis and cell cycle arrest through activation of extracellular signal-regulated kinase and p38 mitogen-activated protein kinase pathways in human breast cancer cells. *J. Pharmacol. Exp. Ther.* **313**, 333–344.

Jonnala R. R. and Buccafusco J. J. (2001) Relationship between the increased cell surface alpha7 nicotinic receptor expression and neuroprotection induced by several nicotinic receptor agonists. *J. Neurosci. Res.* **66**, 565–572.

Kerwin J. M., Morris C. M., Perry R. H. and Perry E. K. (1992) Hippocampal nerve growth factor receptor immunoreactivity in patients with Alzheimer's and Parkinson's disease. *Neurosci. Lett.* **143**, 101–104.

Kihara T., Shimohama S., Sawada H., Honda K., Nakamizo T., Shibasaki H., Kume T. and Akaike A. (2001) alpha7 Nicotinic receptor transduces signals to phosphatidylinositol 3-kinase to block a beta-amyloid-induced neurotoxicity. *J. Biol. Chem.* **276**, 13 541–13 546.

Kitagawa H., Takenouchi T., Azuma R., Wesnes K. A., Kramer W. G., Clody D. E. and Burnett A. L. (2003) Safety, pharmacokinetics, and effects on cognitive function of multiple doses of GTS-21 in healthy, male volunteers. *Neuropsychopharmacology.* **28**, 542–551.

Kokoska E., Smith G., Wolff A., Deshpande Y., Rieckenberg C., Banan A. and Miller T. (1998) Role of calcium in adaptive cytoprotection and cell injury induced by deoxycholate in human gastric cells. *Am. J. Physiol.* **275**, G322–G330.

Kyosseva S. V. (2004) Mitogen-activated protein kinase signaling. *Int. Rev. Neurobiol.* **59**, 201–220.

Li Y., Papke R. L., Martin E. J., He Y.-J., Millard W. J. and Meyer E. M. (1999) Characterization of the neuroprotective and toxic effects of alpha7 nicotinic receptor activation in PC12 cells. *Brain Res.* **816**, 225–230.

Li Y., Meyer E. M., Walker D. W., Millard W. J., He Y. J. and King M. A. (2002) Alpha7 nicotinic receptor activation inhibits ethanol-induced mitochondrial dysfunction, cytochrome *c* release and neurotoxicity in primary rat hippocampal neuronal cultures. *J. Neurochem.* **81**, 853–858.

Maj M., Bruno V., Dragic Z. *et al.* (2003) (-)-PHCCC, a positive allosteric modulator of mGluR4: characterization, mechanism of action, and neuroprotection. *Neuropharmacology.* **45**, 895–906.

Marks M. J. and Collins A. C. (1982) Characterization of nicotine binding in mouse brain and comparison with the binding of a bungarotoxin and quinuclindinyl benzilate. *Mol. Pharmacol.* **22**, 554–564.

Martin E. J., Panickar K. S., King M. A., Deyrup M., Hunter B. E., Wang G. and Meyer E. M. (1994) Cytoprotective actions of 2,4-dimethoxybenzylidene anabaseine in differentiated PC12 cells and septal cholinergic cells. *Drug Dev. Res.* **31**, 127–134.

Meyer E. M., Tay E. T., Papke R. L., Meyers C., Huang G. and De Fiebre C. (1997) 3-[2,4-Dimethoxybenzylidene] anabaseine (DMXB) selectively activates rat alpha7 receptors and improves memory-related behaviors in a mecamylamine-sensitive manner. *Brain Res.* **768**, 49–56.

Meyer E. M., Meyers C. and King M. A. (1998a) The selective alpha7 nicotinic receptor agonist DMXB protects against neocortical neuron loss after nucleus basalis lesions. *Brain Res.* **786**, 152–154.

Meyer E. M., Tay E. T., Zoltewicz J. A., Meyers C., King M. A., Papke R. L. and Fiebre C. M. (1998b) Neuroprotective and memory-related actions of novel alpha7 nicotinic agents with different mixed agonist/antagonist properties. *J. Pharmacol. Exp. Ther.* **284**, 1026–1032.

Papke R. L., Meyer E. M., Nutter T. and Uteshev V. V. (2000) Alpha7-selective agonists and modes of alpha7 receptor activation. *Eur. J. Pharmacol.* **393**, 179–195.

Salehi A., Delcroix J. D. and Swaab D. F. (2004) Alzheimer's disease and NGF signaling. *J. Neural Transm.* **111**, 323–345.

Scott S. A., Mufson E. J., Weingartner J. A., Skau K. A. and Crutcher K. A. (1995) Nerve growth factor in Alzheimer's disease: increased levels throughout the brain coupled with declines in nucleus basalis. *J. Neurosci.* **15**, 6213–6221.

Seguela P., Wadiche J., Dineley-Miller K., Dani J. A. and Patrick J. W. (1993) Molecular cloning, functional properties and distribution of rat brain alpha7: a nicotinic cation channel highly permeable to calcium. *J. Neurosci.* **13**, 595–604.

Shimohama S., Day A., Greenwald D., Shafron D., Simpkins C. and Meyer E. M. (1998) Alpha7 nicotinic receptor activation protects against NMDA-induced toxicity *in vitro* and focal ischemia induced neurotoxicity. *Brain Res.* **779**, 359–363.

Shoop R. R., Chang K. T., Ellisman M. H. and Berg D. K. (2001) Synaptically driven calcium transients via nicotinic receptors on somatic spines. *J. Neurosci.* **21**, 771–781.

Takahashi T., Yamashita H., nakamura S., Ishiguro H., Nagatsu T. and Kawakami H. (1999) Effects of nerve growth factor and nicotine

on the expression of nicotinic acetylcholine receptor subunits in PC12 cell. *Neurosci. Res.* **35**, 175–181.

Tully K. and Treistman S. N. (2004) Distinct intracellular calcium profiles following influx through N- versus 1-type calcium channels: role of Ca^{2+}-induced Ca^{2+} release. *J. Neurophysiol.* **92**, 135–143.

Vijayaraghavan S., Pugh P. C., Zhang Z. W., Rathouz M. M. and Berg D. K. (1992) Nicotinic receptors that bind alpha-bungarotoxin on neurons raise intracellular free Ca^{2+}. *Neuron.* **8**, 353–362.

Woodruff-Pak D. S. (2003) Mecamylamine reversal by nicotine and by a partial alpha7 nicotinic acetylcholine receptor agonist (GTS-21) in rabbits tested with delay eyeblink classical conditioning. *Behav. Brain Res.* **143**, 159–167.

Yamauchi T., Kashii S., Yasuyoshi H., Zhang S., Honda Y. and Akaike A. (2003) Mitochondrial ATP-sensitive potassium channel: a novel site for neuroprotection. *Invest. Ophthalmol. Vis. Sci.* **44**, 2750–2756.