

**Mouse Beta-TC6 Insulinoma Cells: High Expression of Functional  $\alpha\beta 4$  Nicotinic Receptors Mediating Membrane Potential, Intracellular Calcium and Insulin Release**

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**Running title: Functional nicotinic receptors in an insulinoma cell line.**

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**ABBREVIATIONS:** A-85380, 3-(azetidylmethoxy)pyridine; dhbetaE, dihydro-beta-erythroidine; DMPP, dimethylphenylpiperazinium; FCCP, carbonylcyanide 4-(trifluoromethoxy)phenylhydrazone; MLA, methyllycaconitine; MRS 1845, N-propargylnitrendipine; RT-PCR, reverse transcription polymerase chain reaction; SKF 96365, 1-[ $\beta$ -(3-(4-methoxy-phenyl)propoxy)-4-methoxyphenethyl]1H-imidazole.

## ABSTRACT

Nicotine elicited membrane depolarization, elevation of intracellular calcium, rubidium efflux, and release of insulin from mouse Beta-TC6 insulinoma cells. Such responses were blocked by the nicotinic antagonist mecamylamine, but not by the muscarinic antagonist atropine. Neither the selective  $\alpha_4\beta_2$  antagonist dihydro- $\beta$ -erythroidine, nor the selective  $\alpha_7$  antagonist methyllycaconitine significantly blocked the nicotine-elicited depolarization or the calcium response. The elevation of intracellular calcium did not occur in calcium-free media, indicating that the rise in intracellular calcium was due to influx of calcium. The rank order of potency for nicotinic agonists was as follows: epibatidine > nicotine = A-85380, cytisine, dimethylphenylpiperazinium (DMPP). Cytisine and DMPP appeared to be partial agonists. The density of nicotinic receptors measured by [ $^3\text{H}$ ]epibatidine binding was 7-fold higher in membranes from Beta-TC6 cells than in rat brain membranes. No binding of [ $^{125}\text{I}$ ]A-85380 was detected, indicating absence of  $\beta_2$ -containing receptors. RT-PCR analyses indicated the presence of mRNA for  $\alpha_3$  and  $\alpha_4$  subunits, and  $\beta_2$  and  $\beta_4$  subunits in Beta-TC6 cells. The binding and functional data suggest that the major nicotinic receptor is composed of  $\alpha_3$  and  $\beta_4$  subunits. The Beta-TC6 cells thus provide a model system for pharmacological study of such nicotinic receptors.

A wide array of nicotinic acetylcholine receptors occur in the mammalian nervous system and other organs (Dani, 2001) and there have been extensive efforts to define potent and selective agonists for such subtypes (Bunnelle *et al.*, 2004; Daly, 2005; Toma *et al.*, 2004). Often multiple subtypes nicotinic receptors exist in the same cell. Thus, cells that only express one subtype or have been selectively transfected with one subtype represent valuable model systems. The rat pineal gland is one such system, since it expresses the  $\alpha3\beta4$  subtype virtually exclusively (Hernandez *et al.*, 2004). The neuromuscular subtype ( $\alpha,\beta,\gamma\delta$ ) is expressed in human TE-671 rhabdomyosarcoma cells (Lukas, 1989). The ganglionic subtypes ( $\alpha3\beta2^*$  and  $\alpha3\beta4^*$ ) are expressed in rat PC-12 pheochromocytoma cells (Avila *et al.*, 2003). Human IMR-32 (Nelson *et al.*, 2001) and human SH-SY5Y neuroblastoma cells (Dajas-Bailador *et al.*, 2002), express a ganglionic subtype, but the subunit composition are not known with certainty, since several subunits are expressed in these cells. Mammalian cells, stably transfected with different combinations of  $\alpha$  and  $\beta$  nicotinic subunits, have proven useful for many studies (Eaton *et al.*, 2003; Gopalakrishnan *et al.*, 1996; Johnson *et al.*, 1998; Meyer *et al.*, 2001; Stauderman *et al.*, 1998; Wang *et al.*, 1998; Whiting *et al.*, 1991; Xiao and Kellar, 2004). However, the properties of receptors in transfected cells may not be fully equivalent to the properties in native systems due to ancillary proteins or other membrane components.

In an effort to define insulinoma cell lines as models for pancreatic islet cells, the effects of carbamylcholine and other agonists on insulin release, membrane potential and intracellular calcium were investigated with mouse Beta-TC6, hamster HIT-T15 and rat RINm5F cells. Muscarinic agonists were well known to elicit an elevation in calcium



and insulin release in pancreatic islets and insulinoma cell lines (Gilon and Henguin, 2001; Iisman *et al.*, 2000) as was confirmed in preliminary studies with mouse Beta-TC6, hamster HIT-T15 and rat RINm5F cells (data not shown). In contrast, nicotinic agonists had not been reported to have such effects, and none was seen in the hamster and rat cell lines (data not shown). However, *in vivo* both nicotine and dimethylphenylpiperazinium (DMPP), apparently through ganglionic activation, can elicit insulin secretion (Karlsson and Ahrens, 1988). Nicotine did elicit marked increases in calcium in the mouse Beta-TC6 cell line. Here we report a detailed study of the effects of cholinergic agonists and antagonists on the mouse Beta-TC6 insulinoma cells. We found that muscarinic (oxotremorine M), nicotinic (nicotine, epibatidine, A-85380, DMPP, cytisine) and mixed cholinergic (carbamylcholine) agonists elevated intracellular calcium and caused insulin release in these cells. High levels of functional nicotinic receptors with characteristics of the  $\alpha 3\beta 4$  subtype were present. Thus, the Beta-TC6 cells represent a new model system for the study of nicotinic receptors and their involvement in the calcium-dependent release of insulin.

### Materials and Methods

Materials and Cells: Nicotine, cytisine, dimethylphenylpiperazinium (DMPP) iodide, carbamylcholine, oxotremorine M, mecamylamine, dihydro-beta-erythroidine, methyllycaconitine, atropine, scopolamine, nifedipine, A-85380, SKF 96365, and MRS 1845 were obtained from the Sigma Chemical Company (St. Louis, MO). ( $\pm$ )-Epibatidine was from Tocris Cookson Inc. (Ellisville, MO). DMEM and RPMI1640 culture medium, fetal bovine serum, penicillin/streptomycin, trypsin/EDTA, and Trizol

were from Gibco (Rockville, MD) or from Invitrogen (Carlsbad, CA). Dnase I was from Ambion (Austin, TX) and bovine serum albumin from ICN Biochemicals (Irvine, CA). [ $^3\text{H}$ ]( $\pm$ )-Epibatidine, [ $^{125}\text{I}$ ]epibatidine, [ $^{125}\text{I}$ ]A-85380, [ $^{125}\text{I}$ ]bungarotoxin, and [ $^{86}\text{Rb}$ ]rubidium chloride ( $^{86}\text{Rb}^+$ ) were supplied by Perkin Elmer Life Sciences (Boston, MA). Mouse Beta-TC6 cells and other cell lines were purchased from American Type Culture Collection (ATCC, Manassas, VA).

**Cell Culture.** Mouse Beta-TC6 cells were cultured in DMEM medium containing 20 mM glucose at 37 °C under 5% CO<sub>2</sub> condition. The cells were subcultured every week. Cells from passage 30-80 were used for all experiments. When the cells had grown to 90-95% confluency in a cell culture flask (162 cm<sup>2</sup>), the cells were striped from the bottom of the flask by adding trypsin/EDTA solution, and an aliquot of cell suspension was transferred into a new flask filled with new medium.

**Membrane Potential.** The Beta-TC6 cells were seeded in 96-well plates and cultured for 3-4 days. After reaching 90-95% confluency (1-2 x 10<sup>5</sup> cells/well), the cells were washed with Hanks balanced salt solution/HEPES buffer twice and loaded with the membrane potential kit dye (Molecular Devices Corporation, Sunnyvale, CA) for 60 min at a room temperature in the darkness. The components of Hanks/HEPES buffer were as follows: 137 mM NaCl, 5.4 mM KCl, 0.34 mM KH<sub>2</sub>PO<sub>4</sub>, 1.26 mM CaCl<sub>2</sub>, 0.5 mM MgCl<sub>2</sub>, 0.41 mM MgSO<sub>4</sub>, 0.34 mM Na<sub>2</sub>HPO<sub>4</sub>, 5.5 mM D-glucose, 20 mM HEPES, pH 7.4. Temporal changes in the membrane potential were monitored using a FLEX Station fluorescence microplate reader (Molecular Devices) with excitation at 535 nm and emission at 560 nm and were then calculated as a relative fluorescence intensity, based on analyses by SoftMax Pro software (Molecular Devices). Maximum depolarization was

elicited with 40 mM KCl as a calibrant at the end of each assay. Data obtained from each well were normalized by use of these maximum values as described (Fitch *et al.*, 2003).

**Intracellular Calcium.** Intracellular calcium measurements with Beta-TC6 cells were carried out essentially as described above for the membrane potential assay except for the fluorescence dye. The cells were loaded with the calcium ion kit dye (Molecular Devices) in the Hanks/HEPES buffer for 60 min at room temperature in darkness. Temporal changes in the intracellular calcium concentration were monitored using excitation at 485 nm and emission at 525 nm and were calculated as a relative fluorescence intensity based on analyses by SoftMax Pro software. Maximum calcium ion levels were elicited with 5  $\mu$ M ionomycin/20  $\mu$ M FCCP/100  $\mu$ M carbamylcholine as a calibrant at the end of each assay. Data obtained from each well were normalized by use of these maximum values as described (Fitch *et al.*, 2003).

**Insulin release.** After reaching 80-90 % confluency, mouse Beta-TC6 cells were washed with glucose-free Krebs/HEPES Ringer (115 mM NaCl, 24 mM NaHCO<sub>3</sub>, 5 mM KCl, 1 mM MgCl<sub>2</sub>, 2.5 mM CaCl<sub>2</sub>, 25 mM HEPES, pH 7.4) twice and preincubated at 37 °C for 30 min with the glucose-free Krebs/HEPES Ringer. After the preincubation, the cells were incubated in the Krebs/HEPES Ringer containing 1 mg/ml bovine serum albumin and the indicated concentration of glucose in the presence or absence of test agents. The antagonists and channel blockers were applied 3 min prior to addition of nicotine. An aliquot of supernatant was collected for radioimmunoassay (RIA). The amount of insulin released was measured with an RIA kit (Linco Research, St. Charles, MO).

**<sup>86</sup>Rb<sup>+</sup> efflux assays.** Functional properties of the nicotinic receptors expressed in the Beta-TC6 cells were assessed by measurements of nicotinic agonist-stimulated <sup>86</sup>Rb<sup>+</sup>

efflux, as described (Xiao *et al.*, 1998). In brief, aliquots of cells in the selection growth medium were plated into 24-well plates coated with poly-D-lysine. The plated cells were grown in medium at 37°C for 18 to 24 h to reach 70-95% of confluence. The cells were then loaded with  $^{86}\text{RbCl}$  by incubating then in growth medium (0.5 ml/well) containing  $^{86}\text{RbCl}$  (2  $\mu\text{Ci/ml}$ ) for 4 h at 37°C. The loading mixture was then aspirated, and the cells were washed four times with 1 ml/well of HEPES buffer (140 mM NaCl, 2 mM KCl, 1 mM  $\text{MgSO}_4$ , 1.8 mM  $\text{CaCl}_2$ , 11 mM glucose, 15 mM HEPES, pH 7.4). One ml of buffer, with or without agonists, was then added to each well. After incubation for 2 min, the assay buffer was collected and the amount of  $^{86}\text{Rb}^+$  efflux into the buffer was determined. Cells were then lysed by adding 1 ml of 100 mM NaOH to each well, and the lysate was collected for determination of the amount of  $^{86}\text{Rb}^+$  in the cells at the end of the efflux assay. Radioactivity of assay buffer samples and lysates was measured by liquid scintillation counting. Total amount of  $^{86}\text{Rb}^+$  loaded was calculated as the sum of the  $^{86}\text{Rb}^+$  in the assay buffer sample and in the lysate of each well. The amount of  $^{86}\text{Rb}^+$  efflux was expressed as a percentage of total  $^{86}\text{Rb}^+$  loaded. Agonist-stimulated  $^{86}\text{Rb}^+$  efflux was defined as the difference between efflux in presence of nicotinic agonists and basal efflux measured in the absence of agonists. Nonlinear regression analyses and statistical analyses were performed using Prism 3 software (GraphPad Software, San Diego, CA).

**[ $^3\text{H}$ ]Epibatidine binding assay.** Binding of [ $^3\text{H}$ ]epibatidine to nicotinic receptors was measured as described (Xiao *et al.*, 1998) with minor modifications. Briefly, cultured cells at >80% confluence were removed from their flasks (80  $\text{cm}^2$ ) with a disposable cell scraper and placed in 10 ml of 50 mM Tris HCl buffer (pH 7.4) at 4 °C. The cell

suspension was centrifuged at 1,000 x g for 5 min and the pellet was collected. The cell pellet was then homogenized in 10 ml buffer with a Brinkman polytron homogenizer (Model PT2100, 12 mm generator, 26,000 rpm, 20 seconds) and centrifuged at 36,000 g for 10 min at 4 °C. The membrane pellet was resuspended in fresh Tris buffer, and aliquots of the membrane preparation equivalent to 30 to 200 µg protein were used for binding assays. Membrane preparations were incubated with [<sup>3</sup>H]epibatidine for 4 hr at 24 °C. The volumes for saturation and competition binding assays were 1 ml/tube and 0.5 ml/tube, respectively. Nonspecific binding was assessed in parallel incubations in the presence of 300 µM nicotine. Bound and free ligands were separated by vacuum filtration through Whatman GF/C filters treated with 0.5% polyethylenimine. The filter-retained radioactivity was measured by liquid scintillation counting. Specific binding was defined as the difference between total binding and nonspecific binding. Data from saturation and competition binding assays were analysed by nonlinear least-square regressions using Prism 3 software (GraphPad Software, San Diego, CA).

**RT-PCR.** The total RNA of Beta-TC6 cells ( $1 \times 10^8$  cells) was extracted with Trizol, precipitated with isopropyl alcohol, and then treated with Dnase I. One µg of RNA was reverse-transcribed to cDNA using a GeneAMP RNA PCR kit (Applied Biosystems, Foster City, CA) and then amplified by PCR with 30 cycles. The oligo-primers for nicotinic subunits  $\alpha_3$ ,  $\alpha_4$ ,  $\beta_2$ ,  $\beta_3$ , and  $\beta_4$  and for  $\beta$ -actin (internal control) were synthesized commercially (Bioserve Biotechnology, Laurel, MD), according to sequences used previously for nicotinic subunits (Kuo *et al.*, 2002) and for  $\beta$ -actin (Knaack *et al.*, 1994). These primer sequences were as follows: nicotinic subunit  $\alpha_3$  : Sense 5'-TGGGGATTCCAAGTGGA-3', antisense 5'-CATGACCCTGGGGAGAAGGTT-3',

nicotinic subunit  $\alpha_4$  : Sense 5'-GAATGTCACCTCCATCCGCATC-3', antisense 5'-CCGGCA(A/G)TTGTC(C/T)TTGACCAC-3', nicotinic subunit  $\beta_2$ : Sense 5'-CTCCAACCTCTATGGCGCTGCT-3', antisense 5'-GAGCGGAAC TTCATGGTGCAG-3', nicotinic subunit  $\beta_3$ : Sense 5'-CTCCTCAGACATTTGTTCCAAGG-3', antisense 5'-AATGAGGTCAACCATGGT-3, nicotinic subunit  $\beta_4$ : Sense 5'-TCTGGTTGCCTGACATCGTG-3', antisense 5'-GGGTTCACAAAGTACATGGA-3' and  $\beta$ -actin: Sense 5'-GATGACGATATCGCTGCGCTGGTCGTC-3', antisense 5'-GACCCTCAGGGCATCGGAACCGCTCG-3'. Annealing temperatures for nicotinic subunits  $\alpha_3$ ,  $\alpha_4$ ,  $\beta_2$ ,  $\beta_3$ , and  $\beta_4$ , and for  $\beta$ -actin in PCR were 52, 53, 62, 49, 52 and 65 °C respectively. A portion of the PCR products were separated on a 1.5% agarose gel containing ethidium bromide (67 ng/ml) by electrophoresis. Possible contamination of genomic DNA was assessed by performing the RT-PCR in the absence of a reverse transcriptase.

**Data Analysis.** Data were expressed as the mean $\pm$ S.E.M. Statistical significance was assessed by the Student t-test. A P-value <0.05 was considered to be statistically significant.

## Results

**Functional Responses: Membrane Potential and Calcium.** Nicotine at 10  $\mu$ M elicited a marked membrane depolarization in Beta-TC6 cells (Fig. 1). A higher concentration of 100  $\mu$ M did not elicit a greater depolarization, while 1  $\mu$ M had only a slight effect.

Nicotine at a threshold concentration of about 10  $\mu$ M elicited an increase of calcium in Beta-TC6 cells, and this calcium response reached a maximum at 100  $\mu$ M nicotine (Fig. 2AB). The response to 100  $\mu$ M nicotine was virtually eliminated by the

nicotinic blocker mecamylamine at a concentration of 10  $\mu\text{M}$ , but was unaffected by a high concentration (10  $\mu\text{M}$ ) of the muscarinic antagonist atropine (Fig. 2CD). The  $\text{IC}_{50}$  value for mecamylamine was about 3  $\mu\text{M}$  (data not shown). The elevation of intracellular calcium elicited by 100  $\mu\text{M}$  nicotine was dependent on the presence of extracellular calcium. There was no significant nicotine-response in the absence of calcium, while the response was nearly maximal at 1.26 mM calcium compared to 10 mM calcium (data not shown).

The selective  $\alpha_4\beta_2$  antagonist dihydro-beta-erythrodine at 10  $\mu\text{M}$  and the selective  $\alpha_7$  antagonist methyllycaconitine at 10  $\mu\text{M}$  did not significantly block either nicotine-elicited membrane depolarization nor the elevation of intracellular calcium (Fig. 3). Both responses were nearly completely blocked by 10  $\mu\text{M}$  mecamylamine.

The calcium response to nicotine was partially blocked by a high concentration (10  $\mu\text{M}$ ) of the L-type calcium channel blocker nifedipine (Fig. 4) and by 10  $\mu\text{M}$  concentrations of the calcium-release-activated calcium (CRAC)-channel blockers SKF 96365 and MRS 1845 (data not shown). Nifedipine at such a high concentration can block nicotinic receptor channels (Donnelly-Roberts *et al.*, 1995). However, at a 1  $\mu\text{M}$  concentration that should effectively block L-type calcium channels but have little effect on nicotinic channels, nifedipine still partially inhibited the response to 100  $\mu\text{M}$  nicotine (Fig. 4).

The muscarinic agonist oxotremorine M at 10  $\mu\text{M}$  caused a similar calcium response to that elicited by 10  $\mu\text{M}$  nicotine, and a combination of nicotine with oxotremorine M caused only a marginally greater response than oxotremorine M or nicotine alone (Fig. 5). A prior nicotine stimulation greatly reduced the response to a

subsequent addition of nicotine (Fig. 6), as has been previously shown for HEK 293 cells expressing nicotinic receptor subunits (Fitch *et al.*, 2003). In contrast, a prior stimulation with nicotine had only a slight inhibitory effect on the elevation of calcium elicited by oxotremorine M, while the response to carbamylcholine was significantly reduced (Fig. 6).

Other nicotinic agonists also elicited an increase in intracellular calcium in Beta-TC6 cells (Table 1). Epibatidine, with an  $EC_{50}$  of about 20 nM, was the most potent. Nicotine, cytisine, A-85380 and DMPP were about a 1000-fold less potent with  $EC_{50}$  values of 15 to 22  $\mu$ M. Relative to nicotine, only epibatidine and A-85380 appeared to be full agonists.

**Functional Responses: Rubidium Efflux.** Both (-)-nicotine and ( $\pm$ )-epibatidine evoked a modest concentration-dependent efflux of  $^{86}\text{Rb}^+$  from Beta-TC6 cells preloaded with that radioisotope (Fig. 7A) with  $EC_{50}$  values of 17  $\mu$ M and 38 nM respectively. A maximal efflux of about 3-fold over the basal efflux was elicited by both drugs. The nicotine-stimulated efflux of  $^{86}\text{Rb}^+$  was blocked by mecamylamine in a concentration-dependent manner with an  $IC_{50}$  of about 2  $\mu$ M (Fig. 7B). The potencies of these agents were consistent with the potencies reported at  $\alpha 3\beta 4$  nicotinic receptors (Meyer *et al.*, 2001; Xiao *et al.*, 1998).

**Functional Responses: Insulin Secretion.** Nicotine at 100  $\mu$ M elicited a marked increase in insulin secretion from Beta-TC6 cells (Fig. 8). A threshold effect occurred at a nicotine concentration of 10  $\mu$ M. These results were obtained in media with a physiological concentration (5.5 mM) of glucose. In the absence of glucose, basal release of insulin was greatly decreased and even 100  $\mu$ M nicotine had no effect (Fig. 8). The



absence of extracellular calcium also prevented any response to 100  $\mu$ M nicotine (data not shown). Interestingly in the presence of a high concentration (16.7 mM) of glucose, even 100  $\mu$ M nicotine had no significant effect (Fig. 8). The glucose-elicited release of insulin in Beta-TC6 cells appeared near maximal at 1.3 mM glucose, (Fig. 8), unlike pancreatic B-cells where glucose levels of near 15 mM are required for a maximal response. A prior report with Beta-TC6 cells indicated that the maximal release of insulin occurred at about 3 mM glucose (Poitout *et al.*, 1995).

In the present study, the nicotine-elicited release of insulin was blocked by mecamylamine, but was not significantly reduced by atropine (Table 2). The nicotine-elicited release of insulin was markedly reduced by nifedipine at 3  $\mu$ M, but not at 1  $\mu$ M, and also was reduced by SKF 96365 at 10  $\mu$ M. Both carbamylcholine (10 and 100  $\mu$ M) and oxotremorine M (10  $\mu$ M) caused a marked stimulation of insulin release (data not shown). The response to oxotremorine was blocked by 1  $\mu$ M atropine. The muscarinic response in Beta-TC6 cells needs further investigation.

**Expression of messenger RNA for nicotinic receptor subunits.** Analysis of expression of mRNA for subunits of nicotinic receptors in Beta-TC6 cells indicated that there was significant expression of  $\alpha$ 3,  $\alpha$ 4,  $\beta$ 2, and  $\beta$ 4 mRNAs (Fig. 9). Subsequently, expression of  $\alpha$ 5 mRNA was detected, while mRNAs for  $\alpha$ 2,  $\alpha$ 6 and  $\alpha$ 7 were not detected (data not shown).

**Nicotinic Receptor Binding Sites.** [ $^3$ H]Epibatidine was used to detect heteromeric nicotinic receptor binding sites in cell membrane homogenates from Beta-TC6 cells. The nonspecific binding was linear and less than 20% of the specific binding throughout the [ $^3$ H]epibatidine concentration range used. The  $K_d$  value for [ $^3$ H]epibatidine was  $\sim$  150

pM, which is only slightly higher than the  $K_d$  value reported for rat  $\alpha 3\beta 4$  nicotinic receptors (~100 pM) of rat pineal gland (Hernandez *et al.*, 2004). The density of [ $^3\text{H}$ ]epibatidine binding sites in Beta-TC6 cell membrane homogenates was ~ 250 fmol/mg protein. Thus, the density was about 4-fold higher than that reported for rat forebrain membranes (Xiao *et al.*, 1998). There was no significant binding of [ $^{125}\text{I}$ ]A-85380 to membranes of Beta-TC6 cells (data not shown), indicating the absence of  $\beta_2$ -containing receptors. There was only very low binding of [ $^{125}\text{I}$ ]bungarotoxin (data not shown), indicating the near absence of  $\alpha_7$  nicotinic receptors.

Representative binding curves for four nicotinic agonists competing against 500 pM [ $^3\text{H}$ ]epibatidine are shown in Fig. 10. The  $K_i$  values of acetylcholine, (-)-nicotine, (-)-cytisine and A-85380 were 400, 320, 140 and 27 nM, respectively. Compared to affinities of these ligands at six heterologously expressed nicotinic receptor subtypes (Xiao and Kellar, 2004), the binding properties of the sites in mouse Beta-TC6 cell membrane homogenates were most similar to those reported for the rat  $\alpha 3\beta 4$  nicotinic receptors (Parker *et al.*, 1998; Xiao and Kellar, 2004).

Nicotinic receptors were not detected in membranes of the other insulinoma cells, namely hamster HIT-T15 and rat RINm5F cells, nor were nicotinic receptors detected in membranes from mouse islets (data not shown) even with [ $^{125}\text{I}$ ]epibatidine, which provides a very high level of sensitivity.

## Discussion

The mouse Beta-TC6 insulinoma cell line provides a model system in which binding to a nicotinic receptor, nicotine-elicited membrane depolarization and nicotine-elicited, increase in intracellular calcium can be investigated. The functional responses to

nicotine were inhibited by the nicotinic antagonist mecamylamine, but were not significantly affected by the muscarinic antagonist atropine. Other nicotinic agonists also elicited increases in intracellular calcium, and the relative potencies of epibatidine > nicotine  $\approx$  cytisine, A-83850, and DMPP were similar to the rank order of potencies found in other studies with these agonists at  $\alpha_3\beta_4$  receptors (see below).

Influx of calcium appeared essential for nicotine-elicited insulin release, since it did not occur in calcium-free media. There was an inhibitory effect of the L-type calcium channel blocker nifedipine, even at low concentration of 1  $\mu$ M, on the nicotine-elicited elevation of calcium and on nicotine-elicited release of insulin. Thus, the effect of nicotine on membrane potential may lead to opening of voltage-sensitive calcium channels. It should be noted that nifedipine at micromolar concentrations does have activity as a non-competitive blocker of nicotinic channels (Donnelly-Roberts *et al.*, 1995).

The subtype composition of the nicotinic receptors in Beta-TC6 cells that mediate increases in intracellular calcium and insulin release, has not been rigorously determined. Functional data on calcium increases demonstrated that cytisine and DMPP were nearly equipotent with nicotine, and that A-83850 was many-fold less potent than epibatidine (Table 1). Such data demonstrate that the functional receptor in these cells is not a neuronal  $\alpha_4\beta_2$  receptor. The binding results with [ $^3$ H]epibatidine demonstrated high levels ( $\sim$ 250 fmols/mg protein) of nicotinic receptors with high affinity ( $K_d \sim$  150 pM) for this radioligand in Beta-TC6 membranes. The  $K_i$  values for the four nicotinic agonists, derived from the binding competition studies, are consistent with those expected of an  $\alpha_3\beta_4$  subtype as were the calcium response data. The lack of significant binding of

[<sup>3</sup>H]A-85380 indicates the absence of  $\beta$ 2-containing receptors, in spite of expression of  $\beta$ 2 mRNA. The very low binding of [<sup>125</sup>I]bungarotoxin indicates that  $\alpha$ 7 receptors were not highly expressed if at all. Taken together, the functional and binding data indicate that the receptors in Beta-TC6 cells are most likely of the  $\alpha$ 3 $\beta$ 4 subtype. The RT-PCR data on expression of mRNA confirm the presence of  $\alpha$ 3 and  $\beta$ 4 subunits. However, mRNA for  $\alpha$ 4 and  $\beta$ 2 subunits also was present. This raises the question of whether  $\alpha$ 4 and  $\beta$ 2 subunits form small populations of receptors that go undetected in our assays. It should be noted that there is another tumor cell line, namely the human IMR-32 neuroblastoma, that appears to contain mainly  $\alpha$ 3 $\beta$ 4 receptors (Nelson *et al.*, 2001). Levels of  $\alpha$ 3 $\beta$ 4 receptors are much lower in such cells than in Beta-TC6 cells and there is no functional release process to assess.

The Beta-TC6 insulinoma cells appear to represent an atypical insulinoma cell line, since two other insulinoma cell lines, namely HIT-T15 and RINm5F, did not express detectable levels of nicotinic receptors as assayed with [<sup>125</sup>I]epibatidine (data not shown). In addition, nicotine did not elicit elevation of calcium or insulin release in those cells (data not shown). Membranes of mouse pancreatic islets also did not have detectable levels of nicotine receptors (data not shown). However, 100  $\mu$ M nicotine did elicit a slight increase in intracellular calcium in mouse pancreatic islets in three independent experiments (data not shown). A recent study reported that nicotine had marginal inhibitory effects on insulin release in rat and human islet cells (Yoshikawa *et al.*, 2005). Specific binding of [<sup>3</sup>H]nicotine to intact islets or to insulinoma INS-1 cells was reported to be very low (50-80 dpm/islet), as was binding of [<sup>125</sup>I]- $\alpha$ -bungarotoxin (20 dpm/islet). RT-PCR data on total RNA indicated that  $\alpha$ 2,  $\alpha$ 3,  $\alpha$ 4,  $\alpha$ 5,  $\alpha$ 7 and  $\beta$ 2 nicotinic subunits

were expressed in the INS-1 cells.

An insulinoma cell that provides an adequate model for the pancreatic  $\beta$ -cells of islets has not yet been found (Hehmeier and Newgard, 2004). The Beta-TC6 cells will not serve this purpose, since an increase in intracellular calcium and insulin release did not occur when media glucose increased from 5.5 to 16.7 mM. However, increasing glucose from 0 to 5.5 mM did increase insulin release by about 4-fold. A clonal Beta-TC6-F7 cell line has been reported to be glucose-sensitive with respect to release of insulin (Knaack *et al.*, 1994). It is not known whether that cell line retains nicotinic responses.

In summary, the Beta-TC6 cells provide an excellent model system to study expression, binding, and function of presumed  $\alpha 3\beta 4$  nicotinic receptors. Further studies of the involvement of nicotinic and muscarinic effects on membrane potential, calcium levels and insulin release in mouse Beta-TC6 insulinoma cells should provide further insights.

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## Footnotes

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## Figure Legends

**Fig. 1.** Concentration-dependent responses of nicotine on membrane potential in Beta-TC6 cells: **A.** Time course for the effect of nicotine at 1, 10 and 100  $\mu\text{M}$  in a representative assay. **B.** Calculated values (mean  $\pm$  SEM,  $n=7$ ) for membrane depolarization changes as a % of the maximal response induced by the calibrant KCl. Responses at 10 and 100  $\mu\text{M}$  nicotine compared to the absence of nicotine:  $P < 0.005$ .

**Fig. 2.** Effect of nicotine and antagonists on intracellular calcium in Beta-TC6 cells: **A.** Time course for the effect of nicotine at 10 and 100  $\mu\text{M}$  in a representative assay. **B.** Calculated values (mean  $\pm$  S.E.M.,  $n=4$ ) for intracellular calcium changes as a % of the maximal response induced by ionomycin/FCCP/carbamylcholine. Responses at 10 and 100  $\mu\text{M}$  nicotine relative to no nicotine:  $P < 0.005$ . **C.** Time course for the atropine or mecamlamine in a representative assay. Both antagonists at 10  $\mu\text{M}$  were added together with nicotine (100  $\mu\text{M}$ ) as indicated. **D.** Calculated values (mean  $\pm$  S.E.M.,  $n=4$ ) for intracellular calcium as the % of the maximal response induced by ionomycin/FCCP/carbamylcholine. Inhibition by mecamlamine:  $***P < 0.001$ .

**Fig. 3.** Effect of cholinergic antagonists on nicotinic responses in Beta-TC6 cells. **A.** Membrane depolarization. Antagonists including dihydro-beta erythroidine (dhbetaE) and methyllycaconitine (MLA) were added 100 sec before 100  $\mu\text{M}$  nicotine and the maximal response was calculated as a percentage of the calibrant response ( $n=3-4$ ,  $*P < 0.05$ ). **B.** Intracellular calcium. Antagonists were added together with 100  $\mu\text{M}$  nicotine and the maximal response was calculated as a percentage of the calibrant response ( $n=4-5$ ,  $***P$

< 0.001).

**Fig. 4.** Effect of nifedipine on nicotine-elicited increases in intracellular calcium in Beta-TC6 cells. The calculated response (mean  $\pm$  S.E.M., n=12) elicited by nicotine (100  $\mu$ M) in the presence of 0, 1, 3 and 10  $\mu$ M of nifedipine is presented as a % of the maximal response induced by the calibrant ionomycin/FCCP/carbamylcholine. \*\*\*  $P < 0.001$  compared to the absence of nifedipine.

**Fig. 5.** Effects of nicotine and oxotremorine M on intracellular calcium in Beta-TC6 cells: **A.** Time course for the effect of nicotine alone (10  $\mu$ M), oxotremorine M alone (10  $\mu$ M) and in combination (each 10  $\mu$ M) in a representative assay. **B.** Calculated values (mean  $\pm$  SEM, n=3) for intracellular calcium as a % of the maximal response induced by the calibrant ionomycin/FCCP/carbamylcholine. The combined response was significantly greater as shown: \* $P < 0.05$ .

**Fig. 6.** The effect of a prestimulation with nicotine on increases in intracellular subsequent elicited by nicotine, oxotremorine M and carbamylcholine in Beta-TC6 cells. The traces are from a representative assay. The cells were either not stimulated or were stimulated with 100  $\mu$ M nicotine prior to a subsequent addition of (A) nicotine, (C) oxotremorine M or (E) carbamylcholine. **B.** The subsequent response to nicotine was eliminated in **A** or significantly reduced: \*\* $P < 0.001$  (n=12) by the prior nicotine stimulation. **D.** The response to oxotremorine was marginally reduced: \* $P < 0.05$  (n=12). **F.** The response to carbamylcholine was reduced by the prior nicotine stimulation: \*\* $P <$

0.01 (n=12).

**Fig. 7.** Effects of nicotinic agonists and the antagonist mecamylamine on  $^{86}\text{Rb}^+$  efflux from Beta-TC6 cells. The  $^{86}\text{Rb}^+$  efflux and analysis were as described in **Materials and Methods**. The data were fit to the equation for a sigmoidal concentration-response relationship. Each data point is the mean of quadruplicate determinations. **A.**

Concentration-dependent stimulation of  $^{86}\text{Rb}^+$  efflux function by nicotinic agonists in Beta-TC6 cells. Data from a representative experiment are shown. The  $\text{EC}_{50}$  values were  $0.038 \pm 0.002$  nM for ( $\pm$ )-epibatidine and  $17.2 \pm 4.4$   $\mu\text{M}$  for (-)-nicotine (mean  $\pm$  SEM, n=3). **B.** Concentration-dependent inhibition of (-)-nicotine-stimulated  $^{86}\text{Rb}^+$  efflux from Beta-TC6 cells by mecamylamine. Data from a representative experiment are shown. The  $\text{IC}_{50}$  value of mecamylamine was  $0.46 \pm 1.1$   $\mu\text{M}$  (mean  $\pm$  SEM, n=4).

**Fig. 8.** Insulin release elicited by nicotine in Beta-TC6 cells. The cells were incubated in Krebs/HEPES buffer (1 ml, pH 7.4) containing no glucose or the indicated concentrations of glucose (1.3, 5.5 and 16.7 mM) in the absence or presence of nicotine for 90 min at 37 °C. Each bar represents the mean $\pm$ SEM (n=3 or more). \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.005$  compared to the absence of nicotine.

**Fig. 9.** RT-PCR detection of nicotinic acetylcholine receptor subunit mRNA in Beta-TC6 cells. Expression of  $\alpha 3$ ,  $\alpha 4$ ,  $\beta 2$  and  $\beta 4$  subunit transcripts was detected. PCR was performed by 30 cycles with varied annealing temperatures for all subunits. The expected PCR product lengths for  $\alpha 3$ ,  $\alpha 4$ ,  $\beta 2$ ,  $\beta 4$  subunits and  $\beta$ -actin were 679, 790,

513, 850 and 778, respectively.

**Fig. 10.** Binding competition profiles in membrane homogenates from Beta-TC6 cells. Competition binding assays were carried out and analyzed as described in **Material and Methods**, using a [<sup>3</sup>H]epibatidine concentration of ~ 500 pM. Competition curves shown are from a single representative experiment. The K<sub>i</sub> values were 400 ± 110 nM for acetylcholine, 315 ± 71 nM for (-)-nicotine, 141 ± 11 nM for (-)-cytisine and 27 ± 3 nM for A-85380 (mean ± SEM, n=3) and were calculated using the Cheng-Prusoff equation (Cheng and Prusoff, 1973).

TABLE 1. Effect of nicotinic agonists on intracellular calcium in Beta-TC6 cells.  
Efficacy relative to nicotine set equal to 100.

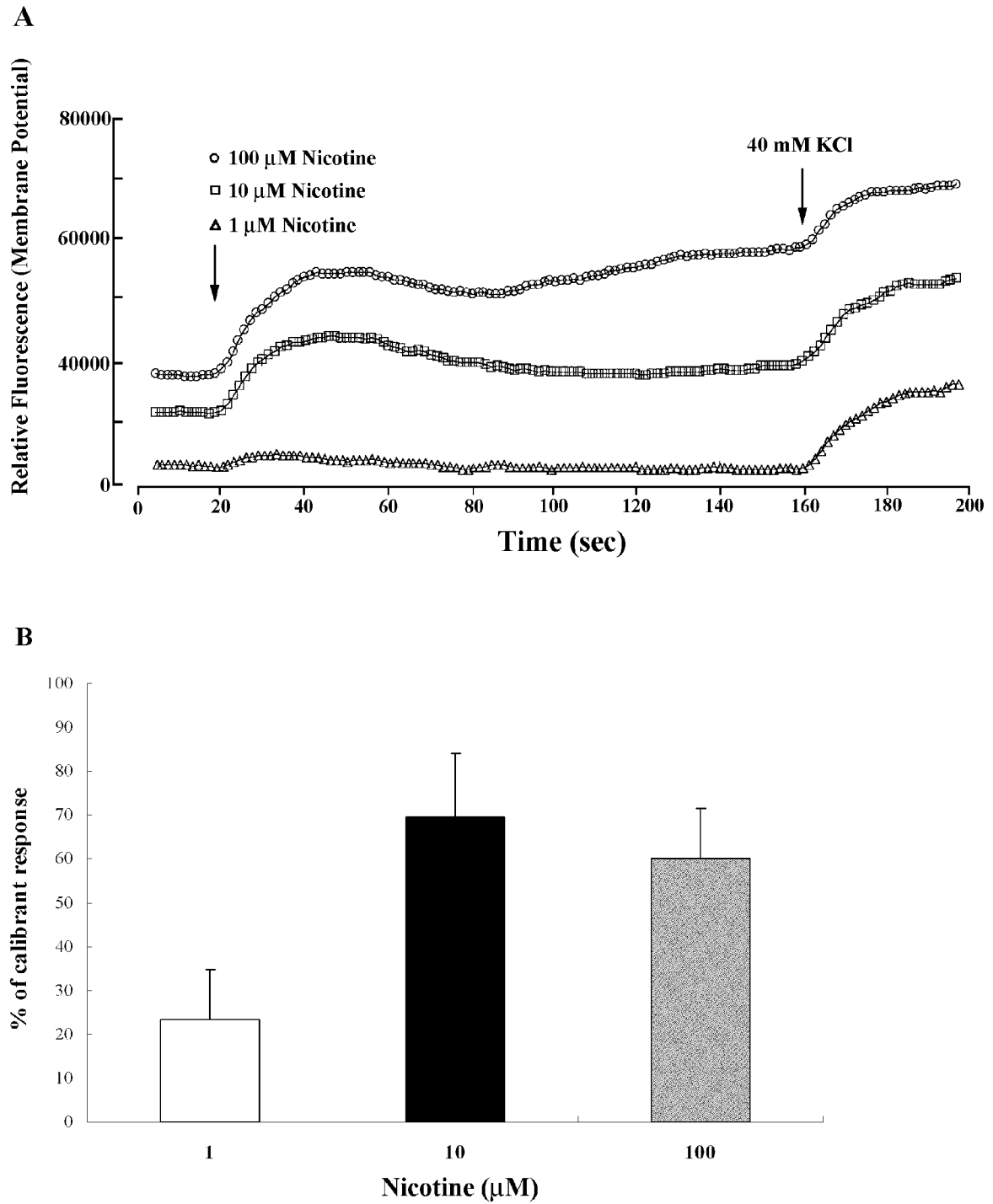
Nicotinic agonists	EC <sub>50</sub> (μM)	Efficacy
(-)-Nicotine	20 ± 5 (n=4)	100
(+/-)-Epibatidine	0.020 ± 0.030 (n=6)	95 ± 21
(-)-Cytisine	22 ± 7 (n=3)	61 ± 17
Dimethylphenylpiperazinium (DMPP)	15 ± 2 (n=5)	48 ± 9
A-85380	20 ± 8 (n=4)	86 ± 23

Table 2. Effects of a cholinergic antagonists and channel blockers on nicotine-induced insulin release in Beta-TC6 cells.

Control <sup>a</sup>	Nicotine 100 $\mu$ M	+Atropine 3 $\mu$ M	Insulin secretion (ng/ml)			
			+Mecamylamine 10 $\mu$ M	+Nifedipine 1 $\mu$ M	3 $\mu$ M	+SKF96365 10 $\mu$ M
5.9 $\pm$ 1.9 (n=27)	10 $\pm$ 2.5 (n=13)	8.2 $\pm$ 2.2 (n=3)	5.6 $\pm$ 0.9* (n=3)	9.3 $\pm$ 1.2 (n=5)	5.7 $\pm$ 0.9* (n=4)	6.4 $\pm$ 1.5* (n=4)

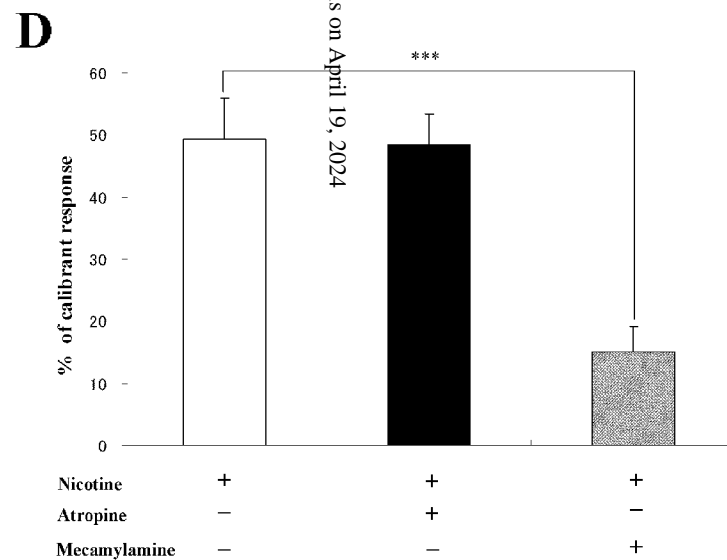
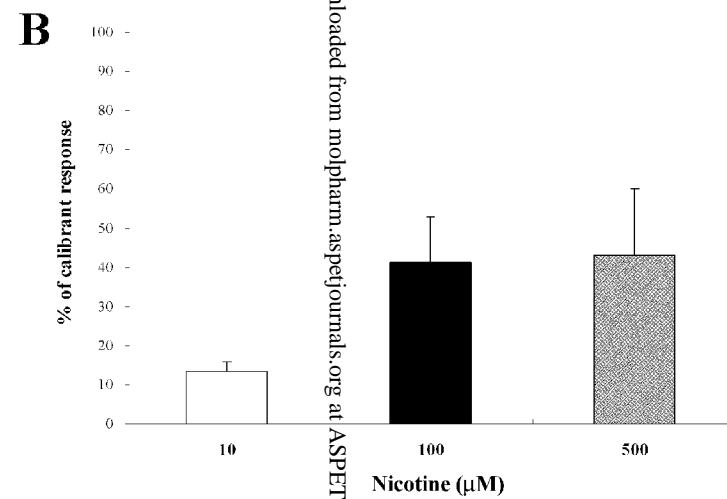
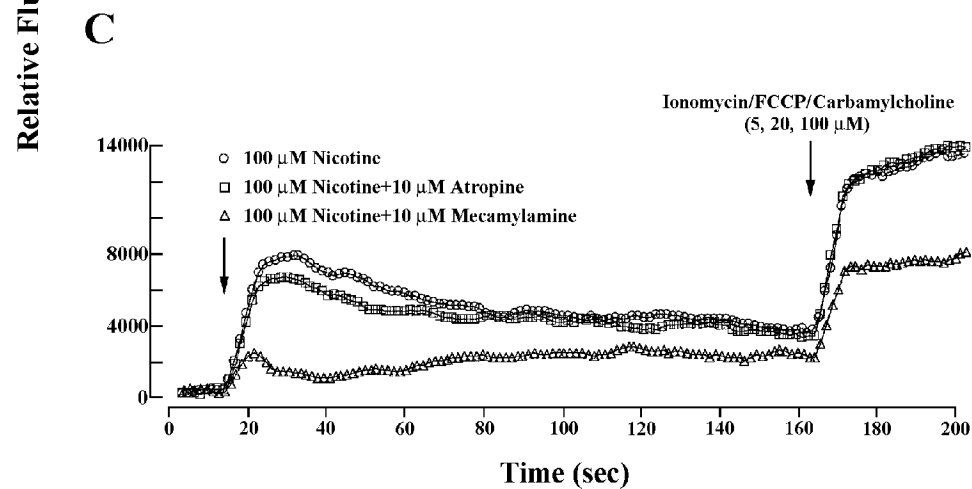
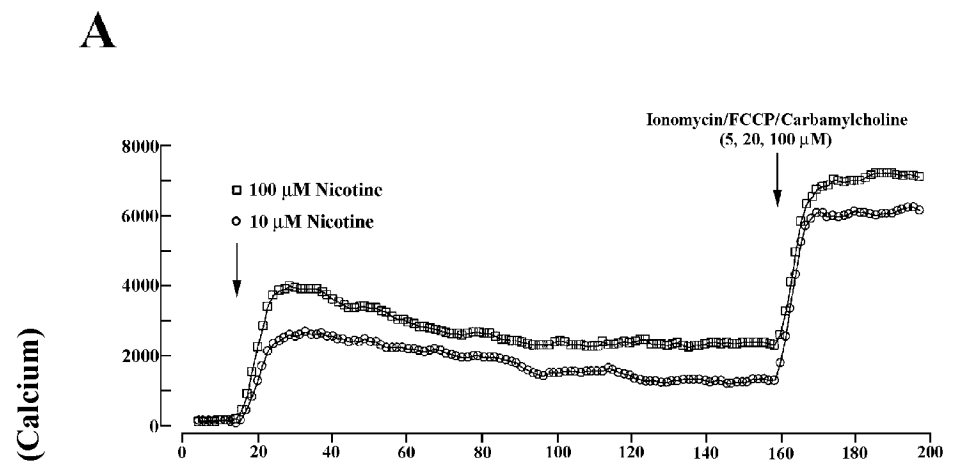
<sup>a</sup>Glucose 5.5 mM.

\*p &lt; 0.05



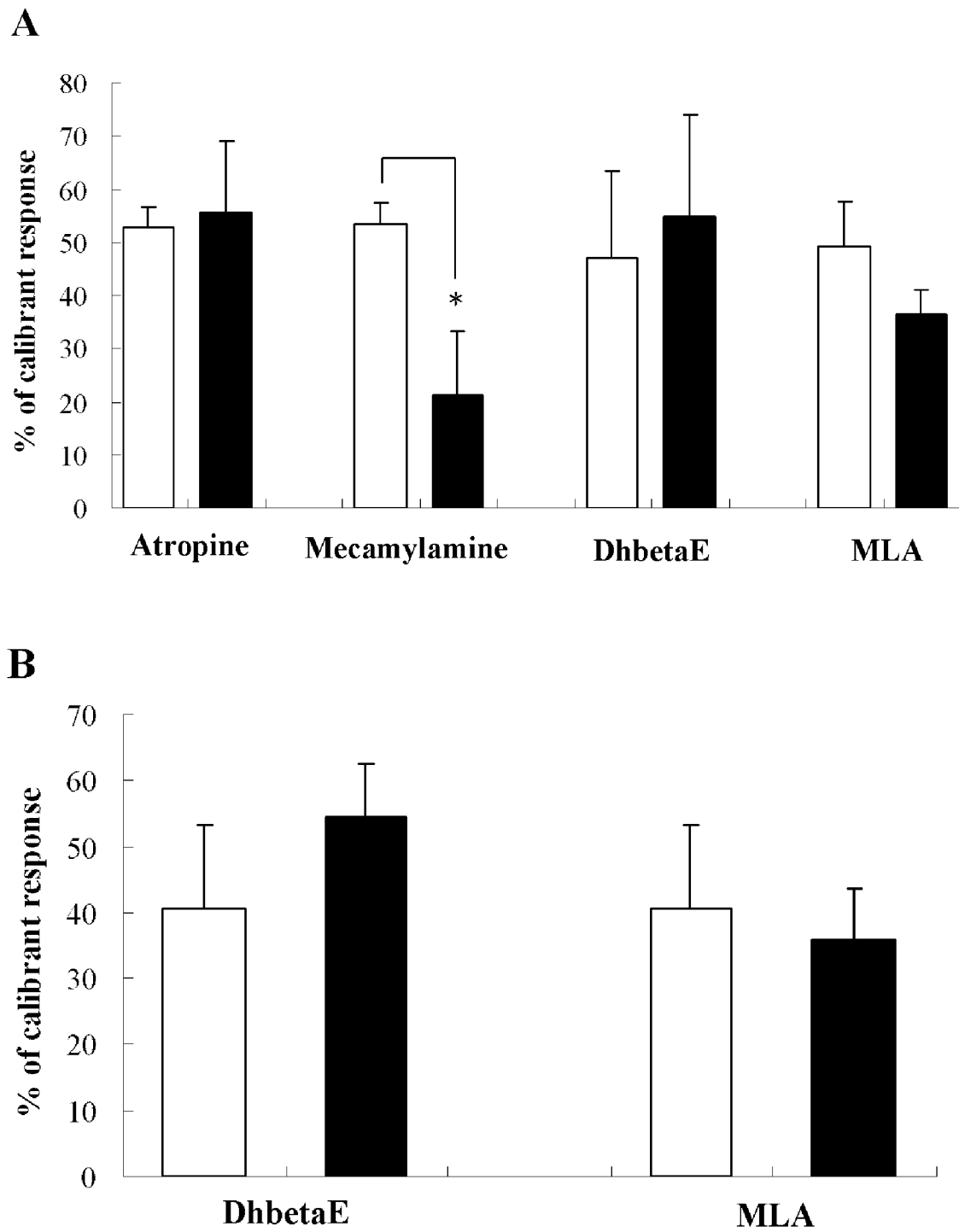
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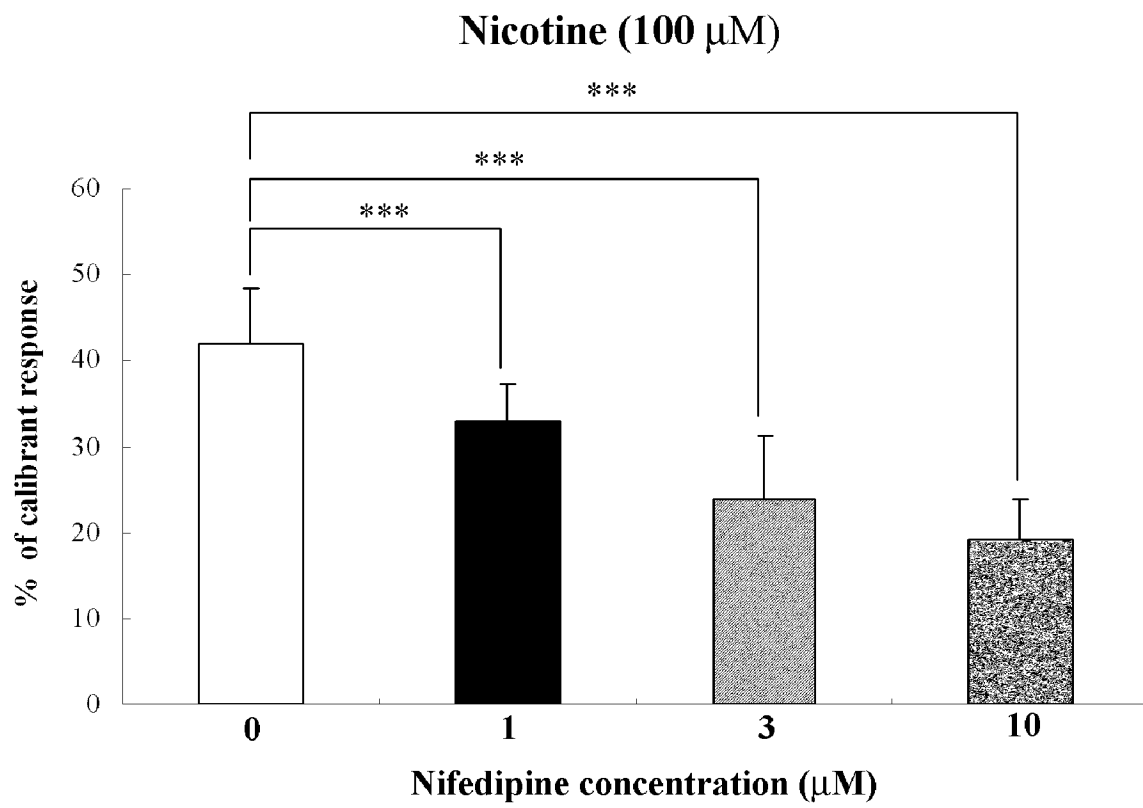


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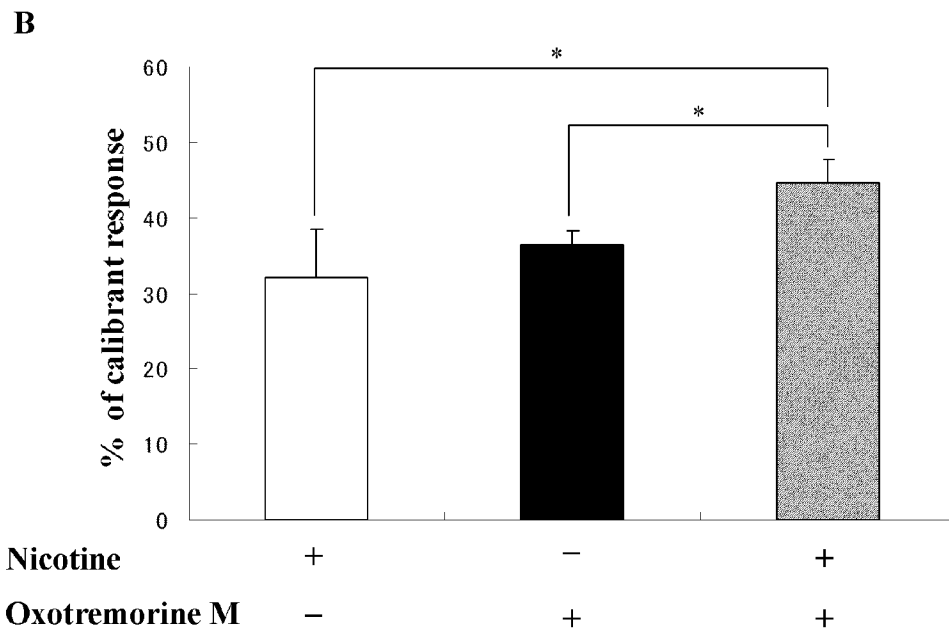
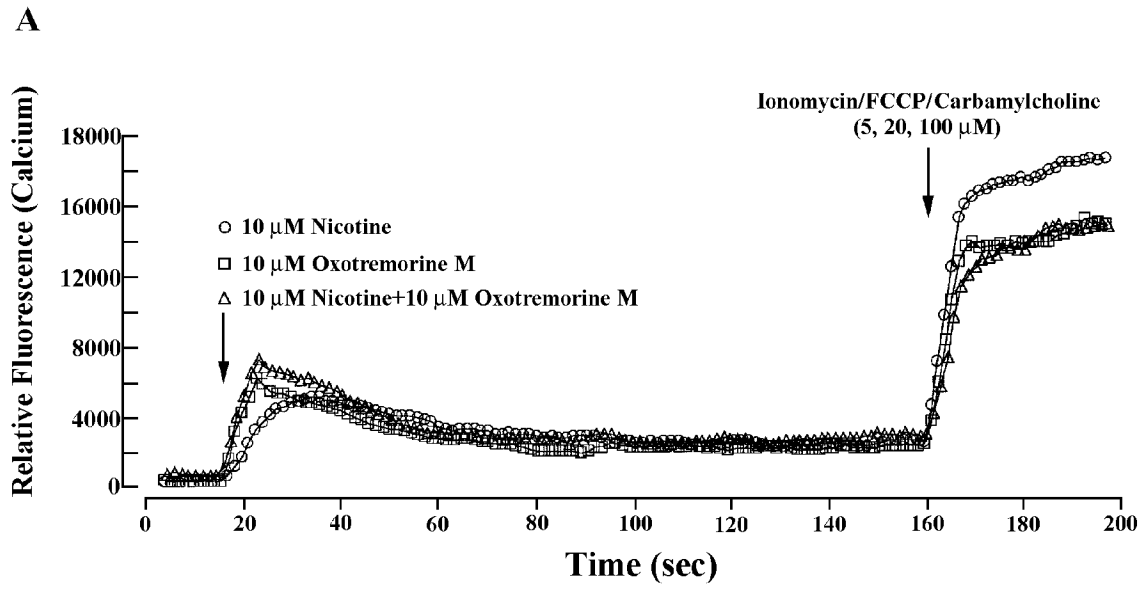
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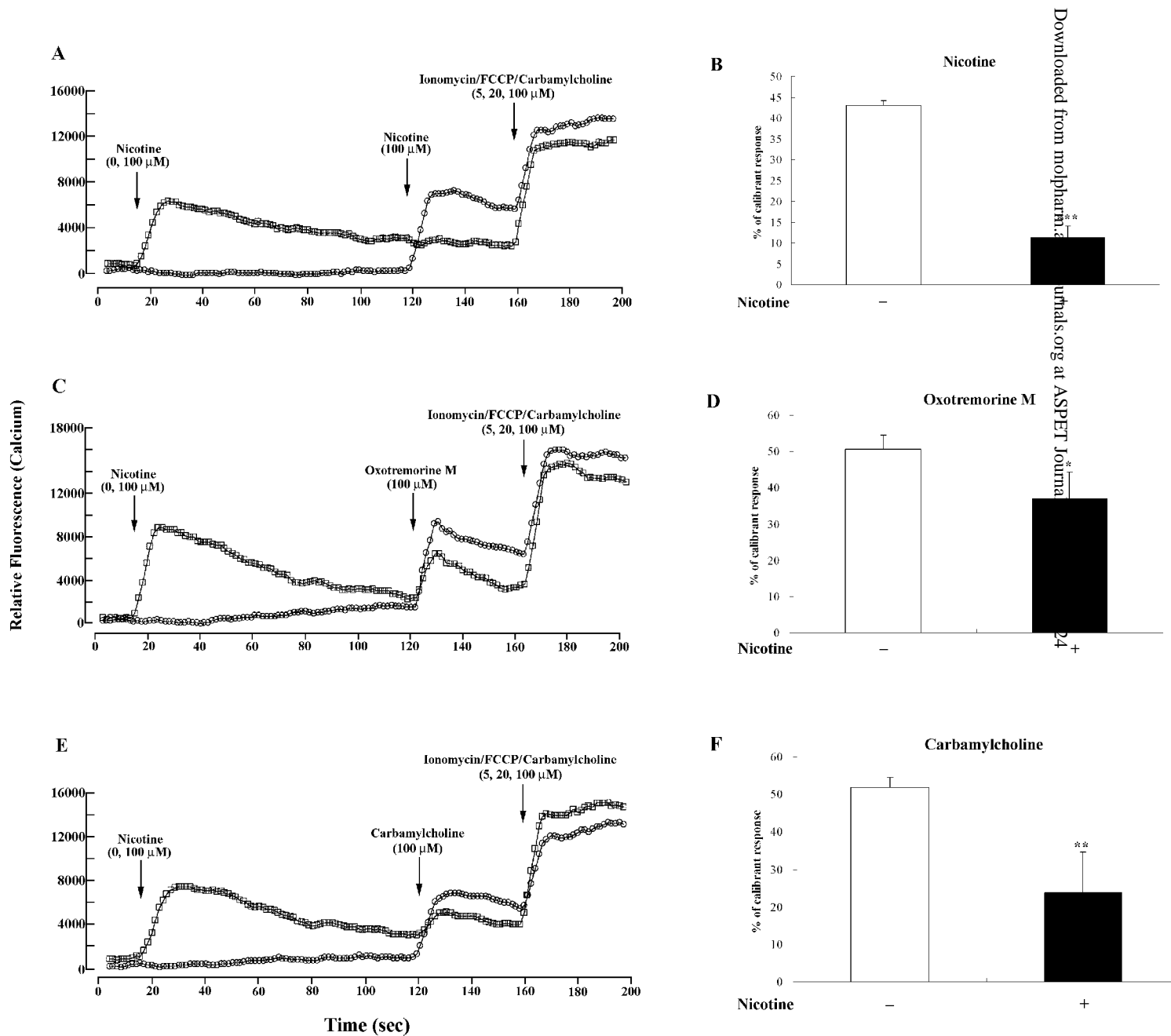
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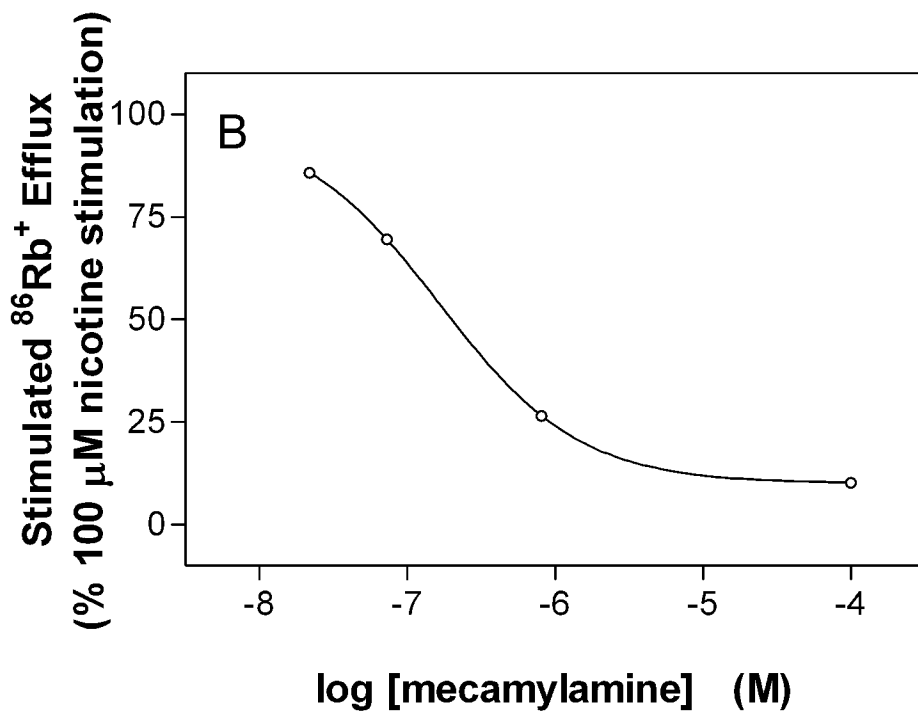
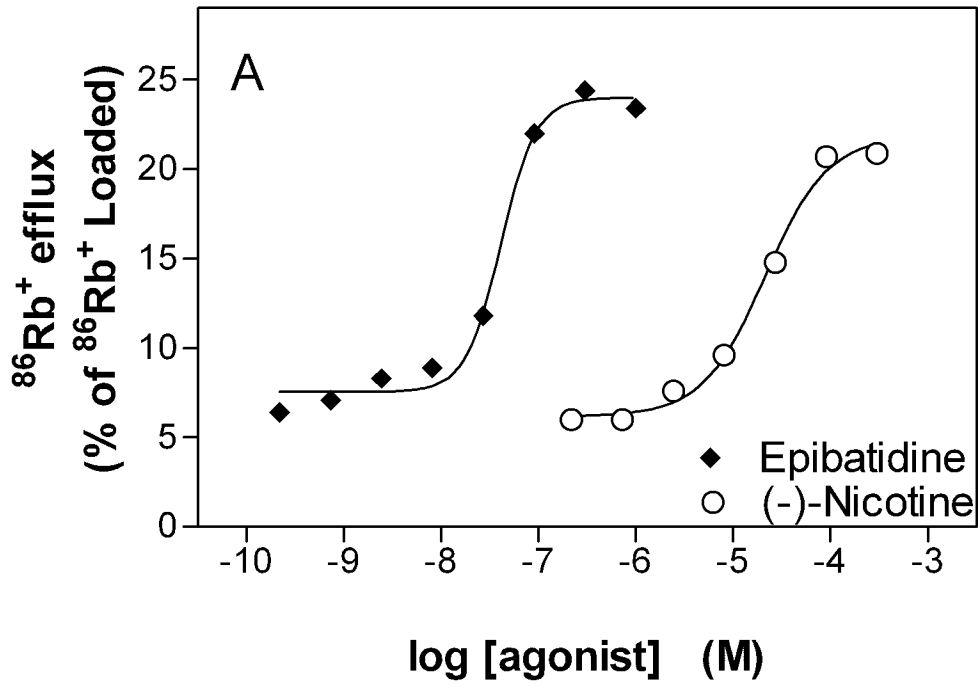
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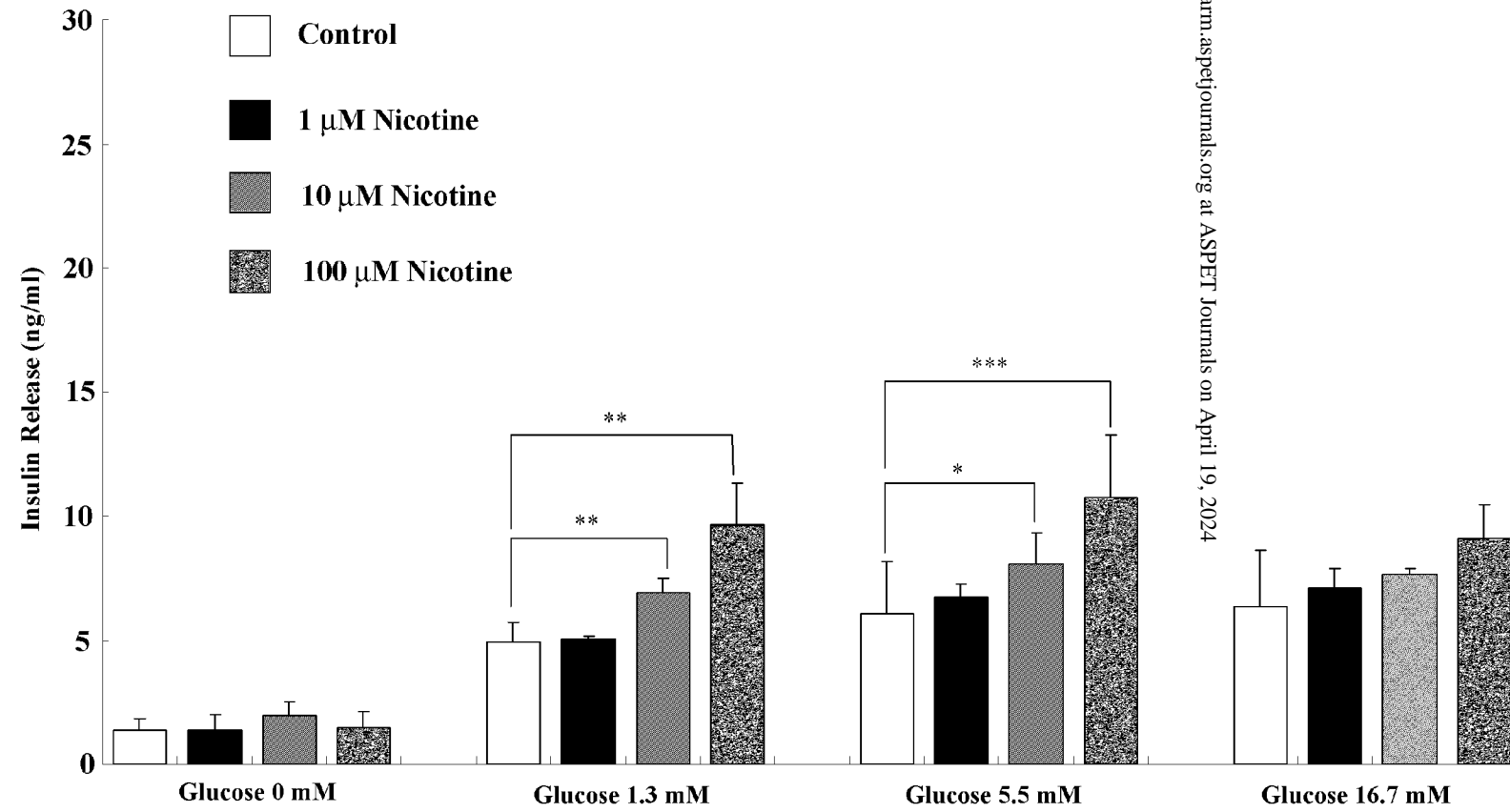


**Figure 5**

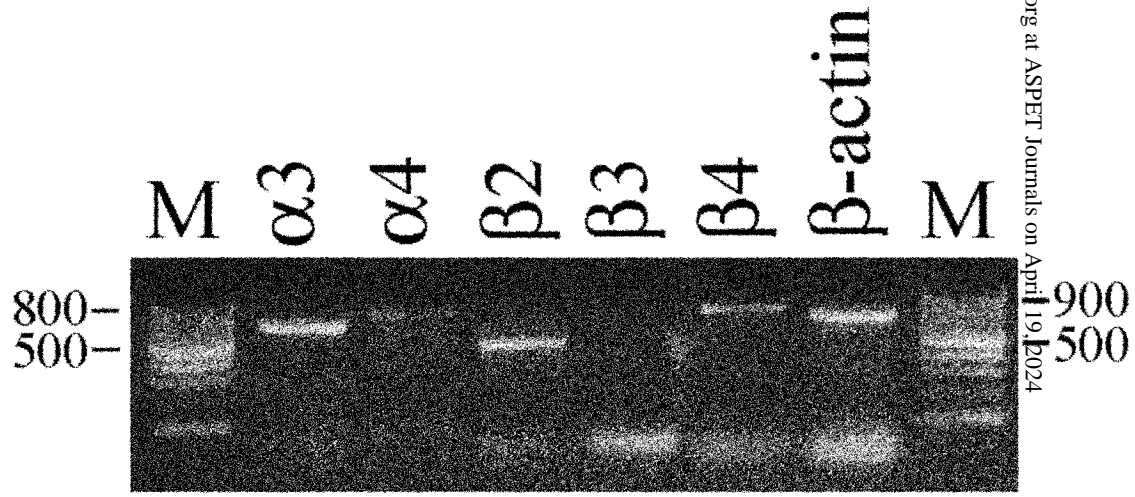


**Figure 6**





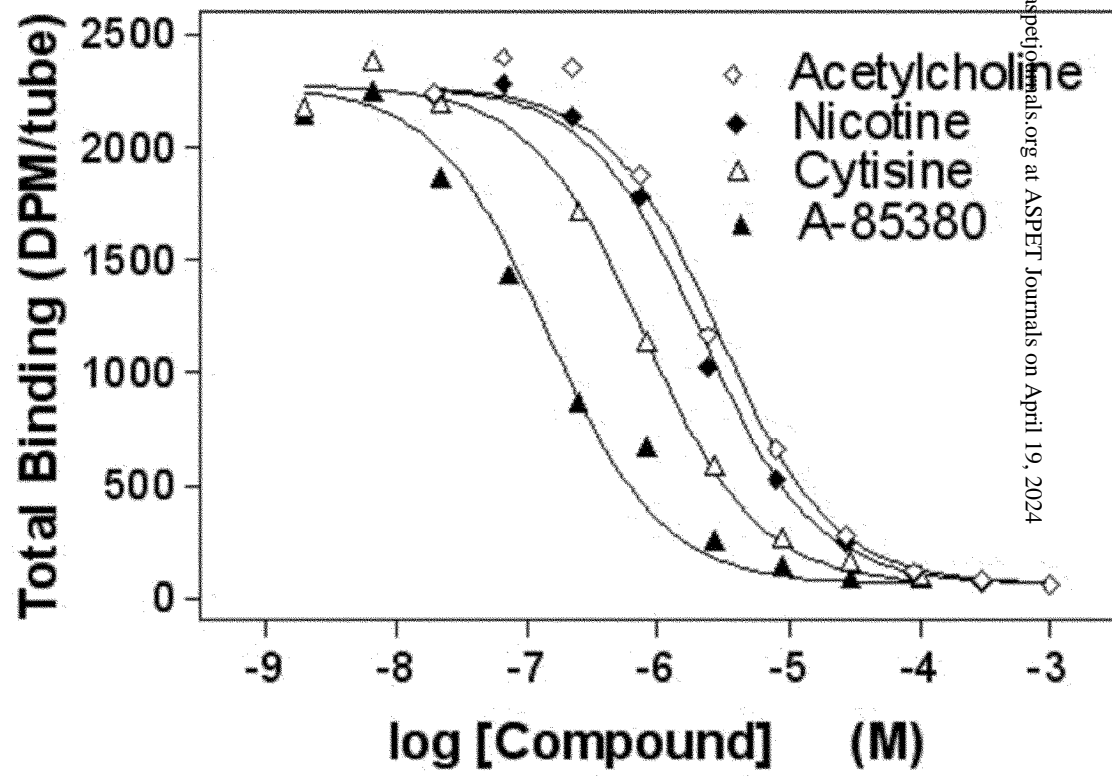
**Figure 8**



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Figure 9





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Figure 10