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Curcumin Potentiates the Function of Human α7-nicotinic Acetylcholine Receptors Expressed in SH-EP1 Cells

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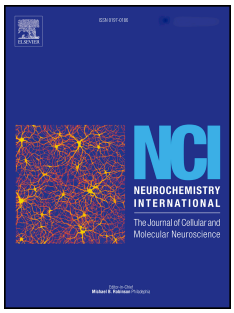

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Curcumin potentiates the function of human α_7-nicotinic acetylcholine receptors expressed in SH-EP1 cells.

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Running title: Effects of curcumin on α_7-nACh receptors

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Abstract

Effects of curcumin, a biologically active ingredient of turmeric, were tested on the Ca^{2+} transients induced by the activation of α_7 subunit of the human nicotinic acetylcholine (α_7 nACh) receptor expressed in SH-EP1 cells. Curcumin caused a significant potentiation of choline (1 mM)-induced Ca^{2+} transients with an EC_{50} value of 133 nM. The potentiating effect of curcumin was not observed in Ca^{2+} transients induced by high K^+ (60 mM) containing solutions or activation of $\alpha_4\beta_2$ nACh receptors and the extent of curcumin potentiation was not altered in the presence of Ca^{2+} channel antagonists nifedipine (1 µM), verapamil (1 µM), ω-conotoxin (1 µM), and bepridil (10 µM). Noticeably the effect of curcumin was not observed when curcumin and choline were co-applied without curcumin pre-incubation. The effect of curcumin on choline-induced Ca^{2+} transients was not reversed by pre-incubation with inhibitors of protein C, A, and CaM kinases. Metabolites of curcumin such as tetrahydrocurcumin, demethylcurcumin, and didemethylcurcumin also caused potentiation of choline-induced Ca^{2+} transients. Notably, specific binding of [^{125}I]-bungarotoxin was not altered in the presence of curcumin. Collectively, our results indicate that curcumin allosterically potentiate the function of the α7-nACh receptor expressed in SH-EP1 cells.

Keywords: *Nicotinic receptors; curcumin; intracellular calcium; choline*

1. Introduction

Curcumin, a poly phenolic compound isolated from turmeric, has been demonstrated to have various pharmacological activities ranging from anti-inflammatory, anti-cancer, anti-oxidant effects to anti-atherosclerotic, anti-microbial, and wound healing actions (Kunnumakkara et al., 2016). Importantly, in recent years, curcumin has been shown to have beneficial effects in alleviating cognitive deficits in neurodegenereative disorders such as Alzheimer's and Parkinson's diseases (Ji and Shen, 2014; Goozee et al., 2016; Kunnumakkara et al., 2016). Although therapeutic effects of curcumin on several pathological conditions and animal disease models have been well documented, the molecular mechanism of curcumin effects in cells are poorly understood.

These diverse pharmacological activities of curcumin are based on its chemical features and complex molecular structure, as well as its ability to interact with multiple signaling molecules (Zhang et al., 2014). To date, several membrane proteins including protein kinases, enzymes, transporters and ion channels have been identified as targets of curcumin (Zhang et al., 2014; Kunnumakkara et al., 2016).

Nicotinic acetylcholine (nACh) receptors are important members of the ligand-gated ion channel family that includes $GABA_A$, glycine, and $5\text{-}HT_3$ receptors. The homomeric α_7 nACh receptor subtype is abundantly expressed in the central nervous system and periphery and plays a key role in synaptic plasticity and disease (Albuquerque et al., 2009). Neuronal α_7-nACh receptors are recognized targets for drug development in several pre-clinical models of neuro-degenerative disorders including Alzheimer's disease (Thomsen et al., 2010). In the present study, we have investigated the effects of curcumin on human α_7-nACh receptors expressed in SH-EP1 cells. We

provide novel evidence that curcumin potentiates intracellular Ca^{2+} transients of the ligand-gated α_7-nACh receptors.

2. Materials and Methods

2.1. SH-EP1 cell culture: The SH-EP1 cells stably expressing the human α7 nAChR were a gift from Dr. R Lukas (Barrow Neurological Institute, Phoenix, AZ). Culturing of SH-EP1 cells was described earlier (Spivak et al., 2007, Sultan et al., 2017). Briefly, cells were grown on 35-mm dishes in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat inactivated horse serum, 5% fetal bovine serum, 1 mM sodium pyruvate, 100 U/ml penicillin G, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin B, 0.4 mg/ml hygromycin B, and 0.25 mg/ml Zeocin (all from Invitrogen). For measurements of intracellular [Ca^{2+}], cells were plated at a density of $2x10^5$ cells per well into 96-well plates and were kept for 2-3 days at 37 °C in an atmosphere of 5% CO_2 saturated with H_2O. Experiments were performed at room temperature (24 ± 2 °C).

2.2. Measurement of intracellular [Ca^{2+}]:

The SH-EP1 cells were loaded with Krebs-HEPES (in mM: 144 NaCl, 5.9 KCl, 1.2 $MgCl_2$, 2 $CaCl_2$, 11 D-glucose, 10 HEPES, pH 7.4) containing 10 µM fluo-4 AM (Molecular Probes, Life Technologies, Paisley, UK) for 45 min at 37 °C in the dark. After this incubation period, cells were washed twice with Krebs-HEPES at room temperature in the dark. Atropine (1µM) was included in all test and incubation solutions. Changes in fluorescence (excitation 485 nm, emission 520 nm) were measured using a fluorescent plate reader (Fluostar, BMG Labtech Inc., Cary, NC, USA). Basal levels of fluorescence were monitored before adding stimulation solution (1 mM choline or 100 µM ACh) by using an automatic dispenser. After stimulation, changes in fluorescence were measured for 30 sec. To normalize fluo-4 signals, responses from each well were calibrated by measuring maximum and minimum fluorescence values. At the end of each experiment, addition

of 75 µl of 5% Triton X-100 (F_{max}) was followed by addition of 50 µl of 1 M $MnCl_2$ (F_{min}). Data were calculated as a percentage of F_{max} - F_{min} and presented as % of F_{max} - F_{min} or area under fluorescence curve (AUC). Go-6983, KT-5720, KN-62; nifedipine, verapamil, bepridil, ω-conotoxin MVIIC, and α-bungarotoxin were purchased from Tocris (Tocris- Bio-Techne, Minneapolis, MN). Curcumin metabolites were purchased from Sigma (Sigma, St. Louis, MO).

2.3. Radioligand binding experiments:

The SH-EP1 cells grown to confluence in 35-mm dishes were collected by scraping in 50 mM HEPES buffer, pH 7.4, containing 1 mM $MgCl_2$, 2.5 mM $CaCl_2$, 0.1% (w/v) bovine serum albumin, 0.025% (w/v) bacitracin and 0.025% (w/v) sodium azide. Subsequently, cells were centrifuged at 1200 r.p.m. for 15 min at 4 °C. After removal of the supernatant, the cells were frozen at -80 °C until the day of the experiment. For each binding assay experiment, the cells were resuspended in 50 mM Tris-HCl buffer (pH 7.4) containing 120 mM NaCl, 5 mM EDTA, 1.5 mM $MgCl_2$ and 5 mM KCl, using a Polytron tissue homogenizer at setting 4 for 20 s. Cell suspension (150 µl) was added to 96-well microtitre plates containing 50 µl radioligand [^{125}I]-α-bungarotoxin (2200 Ci/mmol; Perkin-Elmer, Inc. Waltham, MA, USA) and 50 µl test compound. Non-specific binding was determined in the presence of 3 µM α-bungarotoxin. The plates were incubated at room temperature for 45 min, rapidly filtered through Packard Unifilter-96, GF/C plates and washed twice with 500 µl ice-cold 10 mM Tris-HCl buffer containing 150 mM NaCl, pH 7.4. Filter-bound radioactivity was counted in 50 µl MicroScint 40 (Perkin-Elmer, Inc. Waltham, MA, USA) in Packard TopCount scintillation counter. Assays were performed in triplicate.

2.4. Statistical analysis:

Data were calculated as the mean ± standard error means (S.E.M.). One way ANOVA was

conducted to determine statistical significance between measurements in different groups. When differences existed, pair-wise post-hoc comparisons using the Bonferroni correction were completed. The *P* values < 0.05 were considered significant. Concentration-response curves were obtained by fitting the data to the logistic equation,

$$y = E_{max}/(1+[x/EC_{50}]^{-n}),$$

where x and y are concentration and response, respectively, E_{max} is the maximal response, EC_{50} is the half-maximal concentration, and n is the slope factor (apparent Hill coefficient).

3. Results

We examined the effects of curcumin in Fluo-4 loaded SH-EP1 cells stably transfected with human α_7-nACh receptor. Application of curcumin alone in the concentrations up to 100 µM for 30 sec. did not induce any detectable change in intracellular Ca^{2+} levels in (n=14 from 3 separate experiments). Application of choline (1 mM), a selective agonist for α_7-nACh receptor (Albuquerque et al., 2009) for 30 sec. induced a rapid increase in intracellular Ca^{2+} concentrations (Fig. 1A, control). Application of non-selective cholinergic agonist, ACh was also found to produce a similar rise in intracellular Ca^{2+} (n=12; data not shown). Calcium transients induced by choline was completely inhibited by 10 min pre-incubation with 10 µM methyllycaconitine, a selective antagonist for α_7-nACh receptor (Figs 1A and 1B).

Pre-incubation with 1 µM curcumin (for 10 min) was associated with a significant potentiation (2.2 fold, n=12, ANOVA, P=0.001) of the choline-induced Ca^{2+} transient in the cells (Figs 1A and 1B). The effects of curcumin were concentration-dependent showing an IC_{50} value of 133 nM (Fig. 1C). Notably, 10 min. applications of 10 µM curcumin, did not alter the magnitudes of Ca^{2+} transients induced by the application of high-K^+ in the bath (60 mM KCl, n=14, ANOVA, P=0.579) or Ca^{2+} transients induced by 200 µM ACh in Fluo-4 AM loaded SH-EP1 cells expressing human $\alpha_4\beta_2$-nACh receptor (n=14; ANOVA, P=0.078; Fig 1D).

Entrance of Ca^{2+} through endogenously expressed voltage-gated Ca^{2+} channels in SH-EP1 cells can contribute to Ca^{2+} transients induced by choline. Therefore, we have tested effects of curcumin in the presence of L-type Ca^{2+} channel antagonists nifedipine (1 µM) and verapamil (1 µM), N-type Ca^{2+} channel antagonist ω-conotoxin MVIIC (1 µM), and nonselective Ca^{2+} channel antagonist, bepridil (10 µM). In the presence of these antagonists (n=15, P=0.068 for nifedipine

and n=19, P=0.086 for verapamil; n=11, P=0.074 for ω-conotoxin and n=14, P=0.083 for bepridil) curcumin (10 µM) continued to potentiate choline-induced Ca^{2+} transients (Figure 2A, and 2B). In the concentrations used in this study, these Ca^{2+} channel antagonists themselves did not have any effect on choline-induced Ca^{2+} transients (n=11-14, ANOVA, P>0.05).

Co-application of curcumin and choline, without pre-incubation with curcumin, was not associated with a rise in the choline-induced Ca^{2+} transients (n=18, ANOVA, P=0.512) suggesting that the effects of curcumin on nACh receptor Ca^{2+} signal require receptor priming by additional cellular mechanisms such as kinases. We therefore investigated the involvement of protein kinases A, C, and Ca^{2+}-calmodulin dependent kinase (CaM-kinase), which are known to regulate functions of ligand-gated ion channels (Zhang et al., 1995; Talwar and Lynch, 2014), in curcumin's effect on α_7-nACh receptor Ca^{2+} response. A 30 min. pretreatment with 3 µM Go-6983 (Go; protein kinase C specific inhibitor; Young et al., 2005), 10 µM KT-5720 (KT; protein kinase A specific inhibitor; Cabell and Audesirk, 1993), or 10 µM KN-62 (KN; CaM kinase II specific inhibitor, Tokumitsu et al., 1990) was found to have no effect on curcumin (10 µM) mediated potentiation of the choline (1 mM)-induced Ca^{2+} response (ANOVA; n=18-21; P=0.001 for Go, KT, and KN groups). The results of these experiments were presented in Figure 3A. After 30 min. pretreatment, protein kinase inhibitors (Go, KT, KN) at the concentrations used in this study did not cause any alterations on the choline-induced Ca^{2+} transients (n=12-14).

In vivo curcumin is known to be metabolized to compounds such as tetrahydrocurcumin (THC), demethylcurcumin (DMC), and didemethylcurcumin (DDMC) (Anand et al., 2007). We tested the effects of these curcumin metabolites on choline-induced Ca^{2+} transients in SH-EP1 cells. Our findings indicate that these curcumin metabolites (at 10 µM concentrations) can also

significantly (ANOVA; n=12-16; P=0.001 for THC, DMC, and DDMC groups) potentiate choline-induced Ca^{2+} transients in this cell line (Fig 3B).

[^{125}I] α-bungarotoxin competes with ACh, an endogenous activator of α_7-nACh receptors by binding to the ACh binding site on the receptor (Albuquerque et al., 2009). For this reason, the effect of curcumin was investigated on the specific binding of [^{125}I] α-bungarotoxin. Saturation curves for the binding of [^{125}I] α-bungarotoxin, in the presence and absence (controls) of curcumin are presented in Fig. 4A. At a concentration of 10 μM, curcumin did not cause a significant alteration in the specific binding of [^{125}I] α-bungarotoxin. The apparent affinity (K_D) of the receptor for [^{125}I] α-bungarotoxin was 0.71 ± 0.16 and 0.76 ± 0.21 pM for controls and curcumin, respectively (n= 9 measurement from 3 experiments; ANOVA, P=0.089). Further analysis of saturation binding data by Scatchard analysis (Fig 4B) indicated that, in the absence and presence of curcumin (10 μM), there is no significant alteration in a B_{max} values. In the absence and presence of curcumin, B_{max} values were 0.746 ± 0.065 pmol/mg and 0.781 ± 0.087 pmol/mg, respectively (n= 9 measurement from 3 experiments, ANOVA, P=0.073).

4. Discussion

In the present study, we provide novel evidence that curcumin potentiates the function of human α_7-nACh receptors expressed in SH-EP1 cells. Potentiation by curcumin occurs in a time and concentration dependent manner, but appears independent of channel phosphorylation by protein A, C, and CaM type kinases. The findings underscore a role for curcumin in the regulation of α_7-nACh receptor signaling in various cells.

Our findings suggest that the application of curcumin alone is not sufficient to promote a detectable change in intracellular Ca^{2+} within cells. However, 10 min. pre-incubation with curcumin can significantly potentiate the Ca^{2+} transients response of the ligand activated α_7-nACh receptors suggesting that curcumin functions as a positive modulator of α_7-nACh receptors. Since, Ca^{2+} transients induced by high K^+ containing solutions and activation of $\alpha_4\beta_2$ nACh receptors were not altered by pre-incubation with 10 μM curcumin, the effects of curcumin appear specific to α_7-nACh receptors.

Interestingly, these functional effects of curcumin appeared dependent on its application prior to the receptor ligand as evidenced by our finding that co-application of curcumin and choline does not alter the choline-induced Ca^{2+} transients. Although the kinetics of curcumin effect were not studied in detail in this report, our comparison of the effect of curcumin application (before and during) choline stimulation of the receptor indicates that the potentiating effects of curcumin are achieved after several seconds of ligand preexposure. This time dependent effect of curcumin on the choline response of the nACh receptor suggests that additional cellular pathways upstream of the receptor are engaged by curcumin in these cells. However, inhibition of key nACh receptor modulating kinases such as protein kinase A, C, and CaM by specific inhibitors did not reverse the

effect of curcumin suggesting that phosphorylation by the kinases is not required for curcumin's effects on the nACh receptor. In the future studies, it will be important to test the effects of curcumin on other pathways including those pathways that have been demonstrated to be activated by curcumin such as several integral membrane proteins including enzymes, transporters, and ion channels (Zhang et al., 2014).

Notably, the results of radioligand binding experiments indicate that curcumin does alter specific binding of [^{125}I] α-bungarotoxin suggesting that curcumin may act as allosteric modulator of α_7-nACh receptor. In this study, curcumin was applied in the concentration range of 10 nM to 10 µM and was found that it can enhance the function of α_7-nACh receptors in a concentration-dependent manner with EC_{50} value of 231 nM. Interestingly, even the major metabolites of curcumin tetrahydrocurcumin, demethylcurcumin, and didemethylcurcumin (Anand et al., 2007) can also potentiate the choline-induced Ca^{2+} transients, suggesting bioactivity of these curcumin metabolites at the α_7-nACh receptor site. The concentration of curcumin in plasma and its ability to pass the blood brain barrier following oral and intravenous administration has been studied previously (Shoba et al., 1998; Anand et al., 2007). When curcumin is given orally at a dose of 2 g/kg to rats, maximum serum concentration of 1.35 µg/ml (3.5 µM) was attained (Shoba et al., 1998). Since curcumin is a highly lipophilic compound with LogP (octanol–water partition coefficient) value of 3.3, its membrane concentration is expected to be considerably higher than blood levels. Therefore, the functional modulation of α_7-nACh receptors demonstrated in this study can be pharmacologically relevant.

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FIGURE LEGENDS

Figure 1. The effects of curcumin on intracellular Ca^{2+} transients induced by activation of the human α_7 nACh receptors expressed in SH-EP1 cells. **(A)** The effect of curcumin (1 µM) and methyllycaconitine (MLA, 10 µM), a selective antagonist for α_7-nACh receptor, on choline (1 mM)-induced intracellular Ca^{2+} transients in SH-EP1 cells loaded with 10 µM Fluo-4 AM. **(B**) Summary of the effects of curcumin and MLA on the area under choline-induced Ca^{2+} transients. Bars represent the mean± S.E.M. n=18-21. **(C)** Concentration-inhibition curve for the effect of curcumin on the area under choline (1 mM)-induced Ca^{2+} transients. Each data point presents the normalized means and S.E.M. n=12-16. The curve is the best fit of the data to the logistic equation described in the methods section. **(D)** The effect of curcumin on the area under Ca^{2+} transients elicited by the activation (200 µM ACh) of $\alpha_4\beta_2$ nACh.receptors and by the application of high K^+ (60 mM) in SH-EP1 cells. Bars represent the mean % potentiation ± S.E.M. n=14.

Figure 2. The effects of Ca^{2+} channel inhibitors on choline-induced Ca^{2+} transients in SH-EP1 cells expressing α_7-nACh receptor. **(A)** The effect of L-type Ca^{2+} channel antagonists nifedipine and verapamil on curcumin (10 µM) potentiation of choline-induced Ca^{2+} transients in SH-EP1 cells expressing α_7-nACh receptor. SH-EP1 cells were pre-incubated for 10 min with nifedipine (1 µM), and verapamil (1 µM). Control included 10 µM curcumin and the vehicle (0.01% DMSO). Bars represent the mean % potentiation ± S.E.M. n=15-19. **(B)** The effects of ω-conotoxin and bepridil on curcumin (10 µM) potentiation of choline-induced Ca^{2+} transients in SH-EP1 cells expressing α_7-nACh receptor. SH-EP1 cells were pre-incubated for 10 min with ω-conotoxin (1 µM), and bepridil (10 µM). Control included 10 µM curcumin and the vehicle (0.01% DMSO).

Bars represent the mean % potentiation ± S.E.M. n=11-14.

Figure 3. The effects of protein kinase inhibitors and curcumin metabolites on choline-induced Ca^{2+} transients in SH-EP1 cells expressing α_7-nACh receptor. **(A)** The effect of protein kinase inhibitors on curcumin (10 μM) potentiation of choline-induced Ca^{2+} transients in SH-EP1 cells expressing α_7-nACh receptor. SH-EP1 cells were pre-incubated for 30 min with Go-6983 (specific protein kinase C inhibitor, Go; 3 μM), KT-5720 (specific protein kinase A inhibitor, KT; 3 μM), and KN-62 (specific inhibitor of CaM kinase II, KN; 10 μM). Bars represent the mean % potentiation ± S.E.M. n=18-21. **(B)** The effects of curcumin metabolites on choline-induced Ca^{2+} transients in SH-EP1 cells expressing α_7-nACh receptor. SH-EP1 cells were pre-incubated with 10 μM of curcumin metabolites including tetrahydrocurcumin (THC), demethylcurcumin (DMC), and didemethylcurcumin (DDMC) for 30 min. Bars represent the mean % potentiation ± S.E.M. n=12-16.

Figure 4. The effect of curcumin on the specific binding of [^{125}I] α-bungarotoxin in SH-EP1 cells. **(A)** The effect of curcumin on the saturation curves for [^{125}I] α-bungarotoxin binding. [^{125}I] α-bungarotoxin concentrations represent free ligand. Membranes from SH-EP1 cells was incubated with the indicated concentrations of [^{3}H]-nicotine for 45 min in the absence (filled circles) and presence (open circles) of curcumin (10 μM) at room temperature. Non-specific binding was determined by the inclusion of unlabeled bungarotoxin (3 μM) in the incubation buffer. Data are from a single experiment. **(B):** Scatchard analysis of the effects of curcumin on saturation binding of [^{125}I] α-bungarotoxin. Units for x and y axis are fmol/mg protein and fmol/mg protein/nM, respectively.

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Figure 1

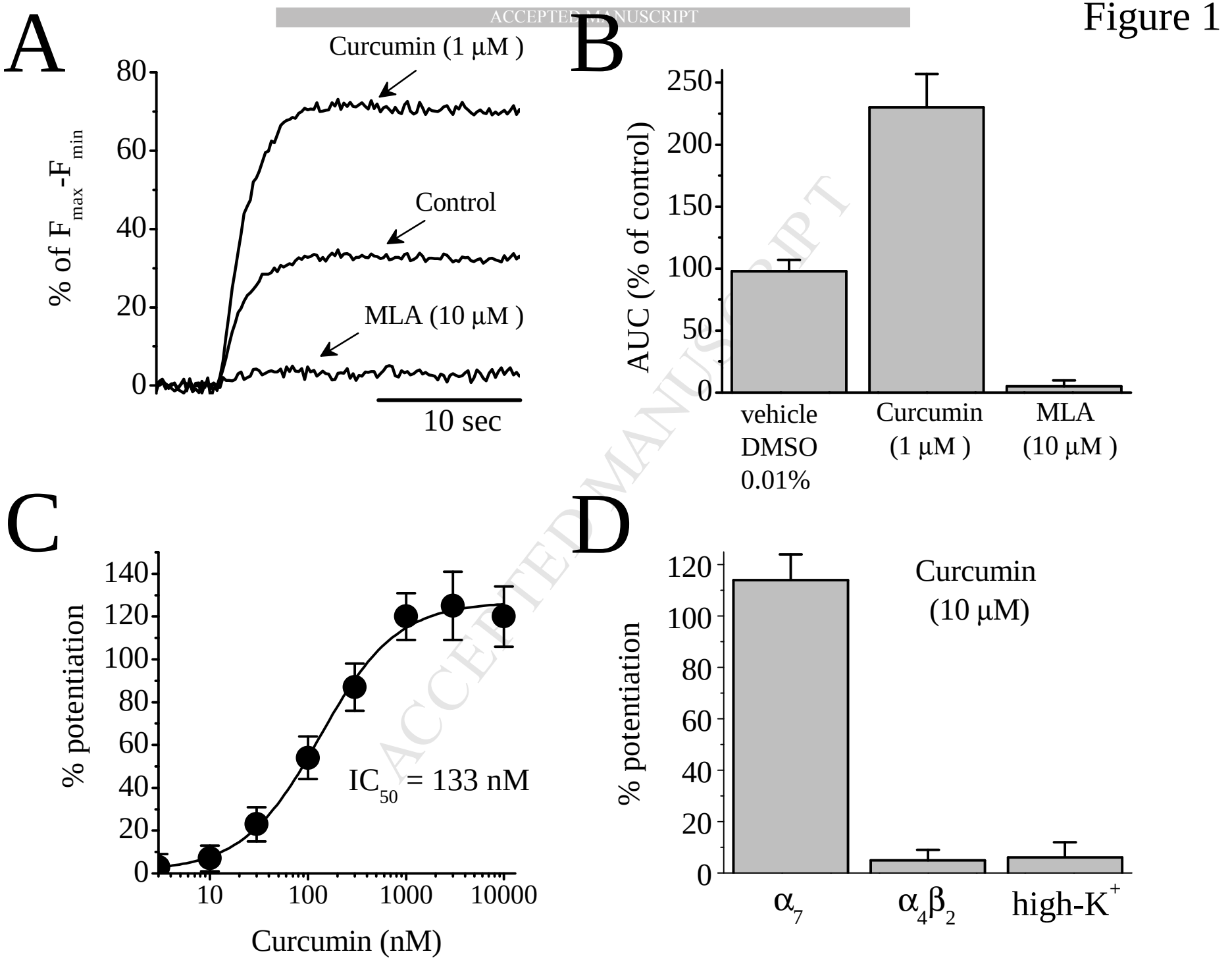
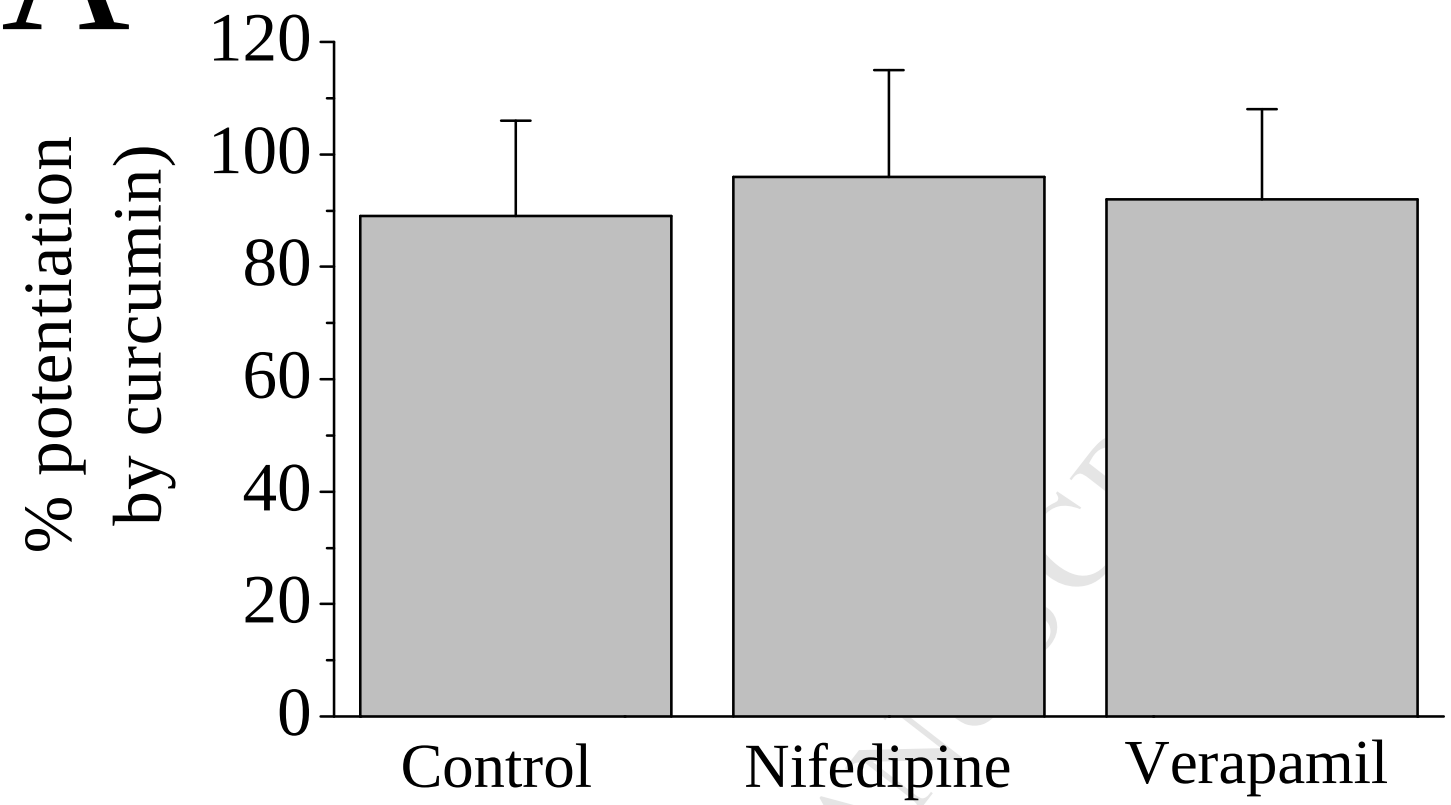


Figure 2

A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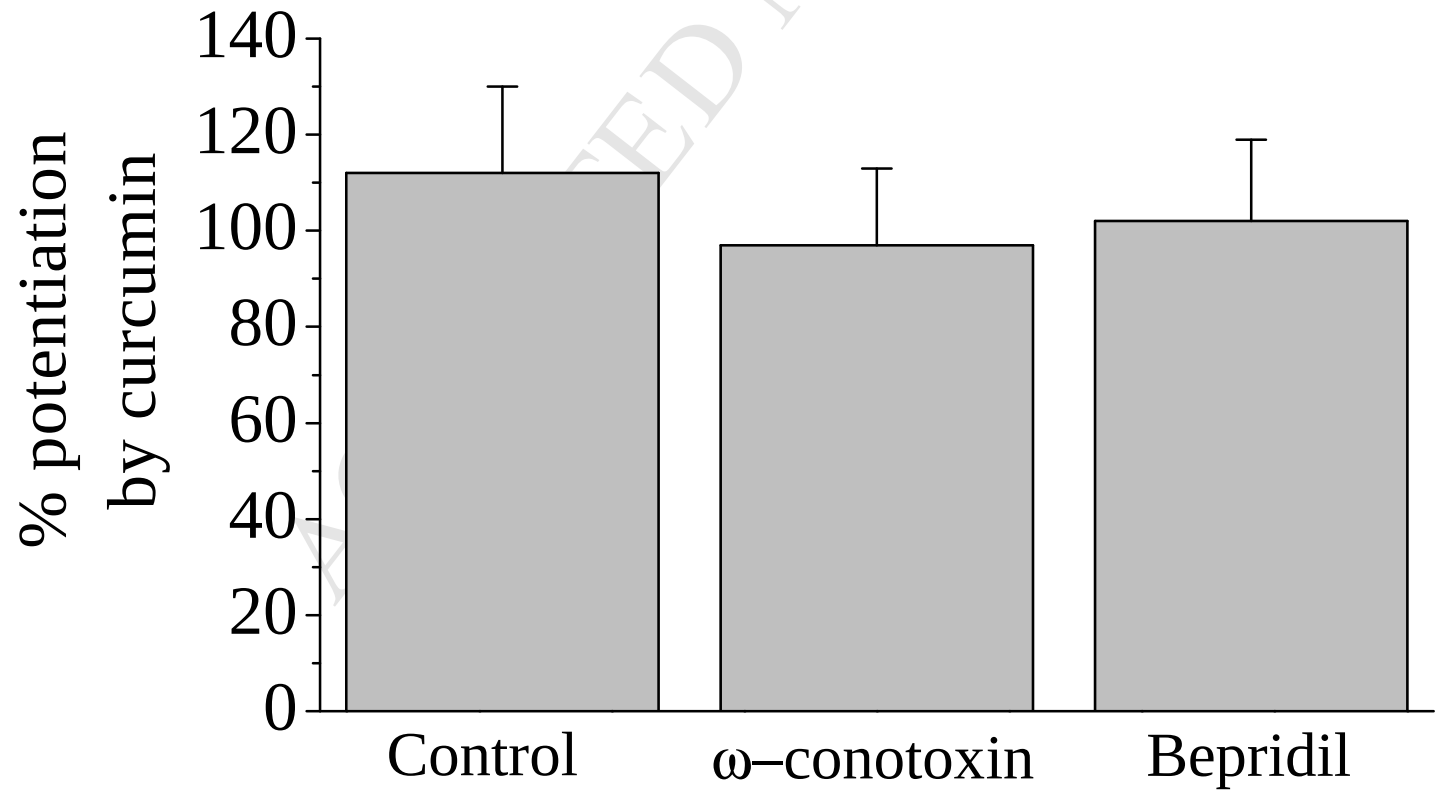
Control
Nifedipine
Verapamil


% potentiation by curcumin
140
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0
Control
ω–conotoxin
Bepridil

Figure 3

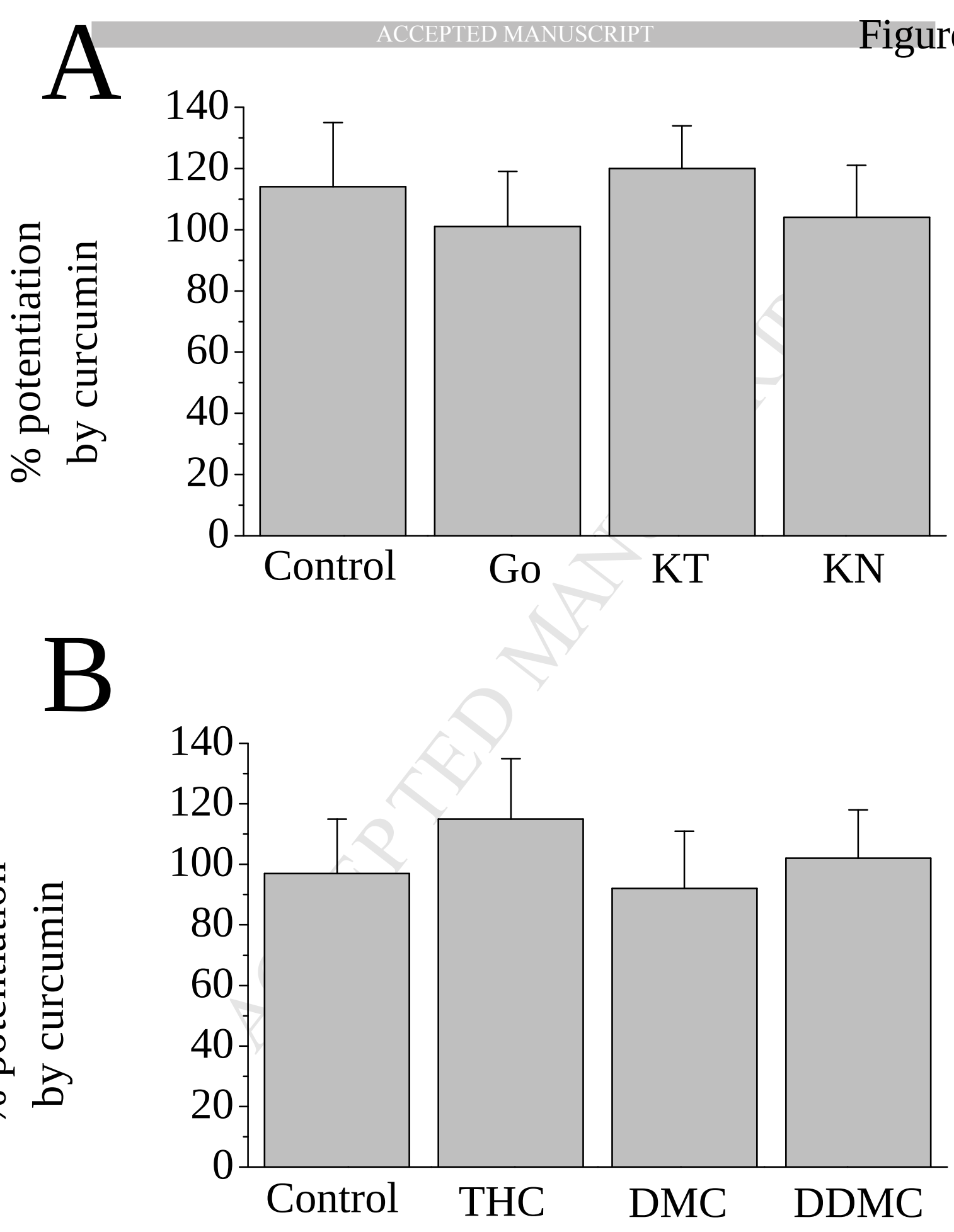
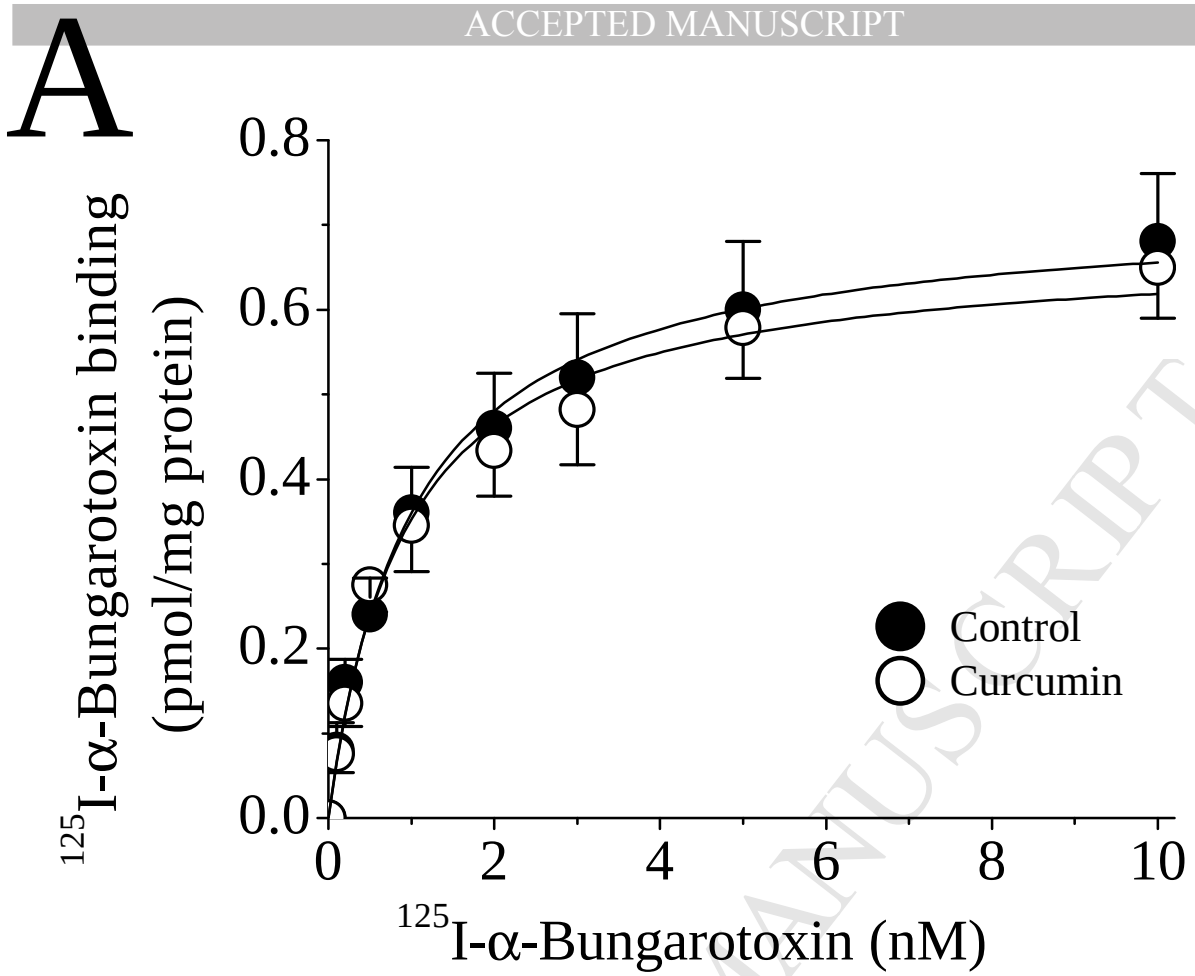
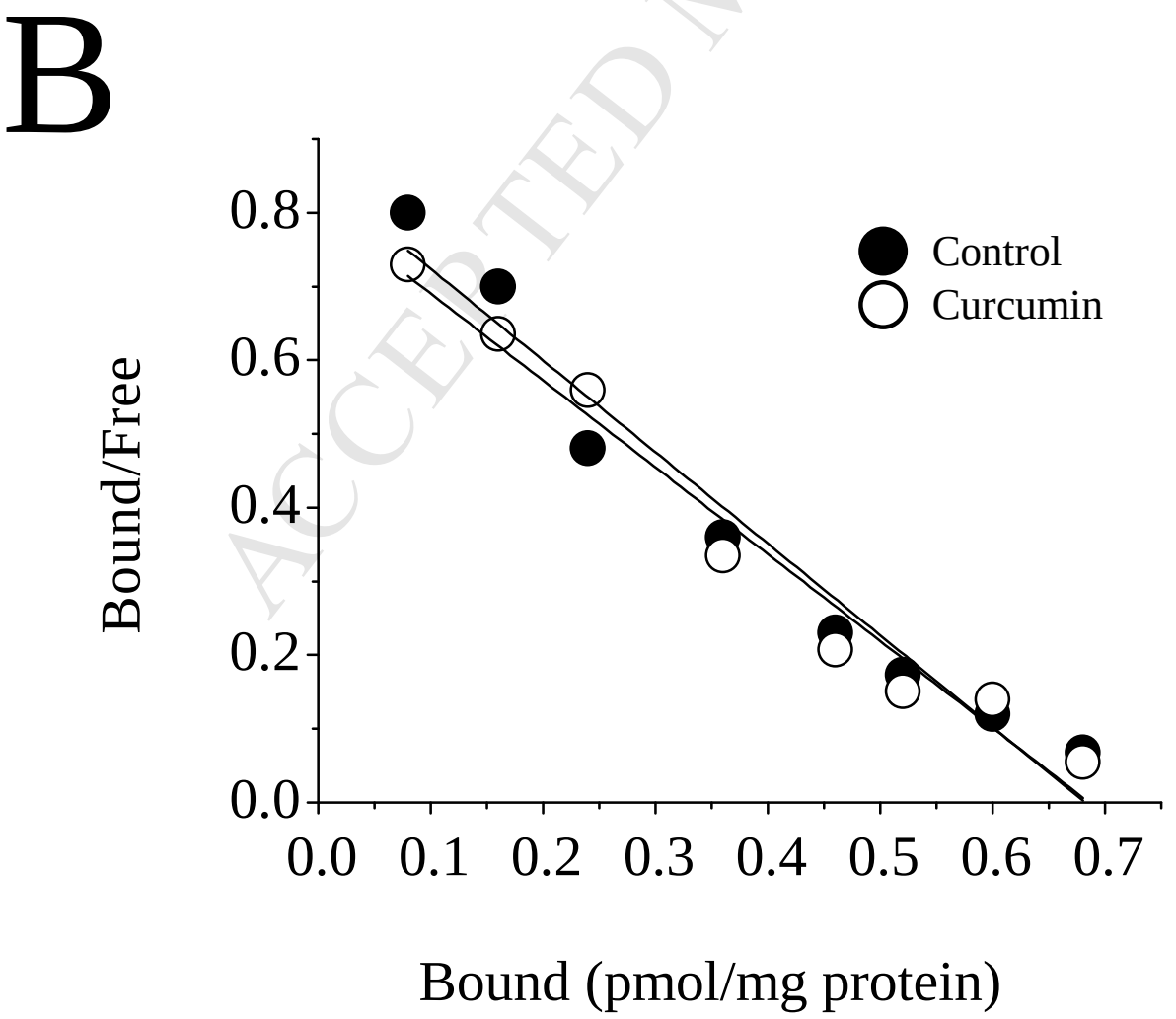


Figure 4





- Curcumin potentiated Ca^{2+} transients induced by the activation of α_7 nACh receptor in SH-EP1 cells.
- Effect of curcumin was not reversed by pre-incubation of inhibitors of protein C, A, and CaM kinases.
- Metabolites of curcumin caused potentiation of choline-induced Ca^{2+} transients.
- Specific binding of [^{125}I]-bungarotoxin was not altered in the presence of curcumin