Mol. Cells *30*, 245-253, September 30, 2010
DOI/10.1007/s10059-010-0117-9

Molecules and Cells

Quercetin Enhances Human α7 Nicotinic Acetylcholine Receptor-Mediated Ion Current through Interactions with Ca^{2+} Binding Sites

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The flavonoid quercetin is a low molecular weight substance found in fruits and vegetables. Aside from its antioxidative effect, quercetin, like other flavonoids, has a wide range of neuropharmacological actions. The α7 nicotinic acetylcholine receptor (α7 nAChR) has a Ca^{2+}-binding site, is highly permeable to the Ca^{2+} ion, and plays important roles in Ca^{2+}-related normal brain functions. Dysfunctions of α7 nAChR are associated with a variety of neurological disorders. In the present study, we investigated the effects of quercetin on the ACh-induced inward peak current (I_{ACh}) in *Xenopus* oocytes that heterologously express human α7 nAChR. I_{ACh} was measured with the two-electrode voltage clamp technique. In oocytes injected with α7 nAChR cRNA, the effects of the co-application of quercetin on I_{ACh} were concentration-dependent and reversible. The ED_{50} was 36.1 + 6.1 μM. Quercetin-mediated enhancement of I_{ACh} caused more potentiation when quercetin was pre-applied. The degree of I_{ACh} potentiation by quercetin pre-application was time-dependent and saturated after 1 min. Quercetin-mediated I_{ACh} enhancement was not affected by ACh concentration and was voltage-independent. However, quercetin-mediated I_{ACh} enhancement was dependent on extracellular Ca^{2+} concentrations and was specific to the Ca^{2+} ion, since the removal of extracellular Ca^{2+} or the addition of Ba^{2+} instead of Ca^{2+} greatly diminished quercetin enhancement of I_{ACh}. The mutation of Glu195 to Gln195, in the Ca^{2+}-binding site, almost completely diminished quercetin-mediated I_{ACh} enhancement. These results indicate that quercetin-mediated I_{ACh} enhancement human α7 nAChR heterologously expressed in *Xenopus* oocytes could be achieved through interactions with the Ca^{2+}-binding site of the receptor.

INTRODUCTION

Nicotinic acetylcholine receptors (nAChRs) are members of the Cys-loop family of ligand-gated ion channels, which also includes 5-HT_3, $GABA_A$, and glycine receptors (Jensen et al., 2005). nAChRs are widely expressed in the brain. Eleven different nAChR subunits are currently known, and subunits of nAChR α ($\alpha_{2\text{-}10}$) and β ($\beta_{2\text{-}4}$) have been identified (Nashmi and Lester, 2006). Neuronal nAChRs containing $\alpha_{2\text{-}6}$ subunits are usually expressed as heteromers in combination with $\beta_{2\text{-}4}$ subunits (Boulter et al., 1986, 1987; Karlin, 2002) and are found in the central and peripheral nervous systems (Gotti and Clementi, 2004). In contrast, the α_7 and α_9 subunits can form homomeric receptors (Couturier et al., 1990; Elgoyhen et al., 1994; Gotti et al., 1994; Karlin, 2002). Homomeric α7 nAChRs are the major binding site for α-bungarotoxin in the mammalian central nervous systems and are predominantly expressed in cortical and limbic areas including the hippocampus. These nAChRs are known to play an important role in normal brain function and drug development (Gotti et al., 2000).

Many lines of evidence have indicated that most nAChRs are permeable to cations but different subtypes of neuronal nAChR exhibit differential permeability to Ca^{2+} (Bertrand et al., 1993). In addition, subsets of nAChR channel activity are also potentiated by increasing extracellular Ca^{2+} concentrations in neuronal cells (Amador and Dani, 1995; Eisele et al., 1993; Sine et al., 1990; Vermino et al., 1992). In cells expressing nAChRs, extracellular Ca^{2+} increases ACh-elicited inward peak currents (I_{ACh}). Ca^{2+}-mediated I_{ACh} potentiation was independent of intracellular calcium chelators injected into cells and was insensitive to membrane voltage (Amador and Dani, 1995; Eisele et al., 1993; Sine et al., 1990; Vermino et al., 1992). Galzi et al. (1996) also identified the Ca^{2+}-binding site at the N-terminal domain. This binding site is closely related to the Ca^{2+}-mediated potentiation of I_{ACh} in α7 nAChR using the chimera receptor of α7 nAChR and the 5-HT_3 receptors expressed in *Xenopus* oocytes. Thus, although Ca^{2+}-binding at extracelluar Ca^{2+}-binding sites of nAChRs might play an important role in nAChRs-mediated signalling transduction through the regulation of I_{ACh}, relatively little is known about agent(s) acting on Ca^{2+}-binding sites other

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Received April 20, 2010; revised June 10, 2010; accepted June 14, 2010; published online August 23, 2010

Keywords: α7 nAChR, Ca^{2+}, Ca^{2+}-binding site, flavonoids, quercetin

than calcium ions (Le Novère et al., 2002).

In previous reports, we have shown that the application of quercetin, a flavonoid, inhibits 5-HT- and glycine-induced peak inward currents ($I_{5\text{-}HT}$ and I_{Gly}) of mouse 5-HT_{3A} and human glycine α receptor channels expressed in *Xenopus laevis* oocytes, respectively. Inhibition of $I_{5\text{-}HT}$ by quercetin was competitive and voltage-independent, whereas inhibition of I_{Gly} by quercetin was non-competitive and voltage-dependent (Lee et al., 2005; 2007). As noted above, α7 nAChR plays an important role in nervous systems and is a member of the same Cys-loop as 5-HT_{3A} and glycine receptors, which are all homomeric ligand-gated ion channels. However, relatively little is known about the effects of quercetin on α7 nAChR channel activity.

In this study, we investigated the regulation of quercetin on the α7 nAChR channel activity expressed in *Xenopus* oocytes. For this study, we first expressed neuronal human α7 nAChR cRNAs in *Xenopus* oocytes and examined the effect of quercetin on I_{ACh}. We used this system because: (1) *Xenopus laevis* oocytes have been used widely as a tool to express the membrane proteins encoded by exogenously administered cDNAs or cRNAs including receptors, ion channels, and transporters (Dascal, 1987); and (2) nAChR channels expressed in *Xenopus* oocytes by the injection of nAChR cRNAs subunits are well studied and well characterised (Chavez-Noriega et al., 1997). We found that co- or pre-application of quercetin with ACh enhanced I_{ACh}. The enhancement of I_{ACh} by quercetin is independent of ACh concentration and voltages. In addition, quercetin further enhanced Ca^{2+}-mediated potentiation of I_{ACh}, which was dependent of extracellular Ca^{2+} concentration. However, the mutation of the Ca^{2+} binding site Glu195 to Gln195 almost completely diminished quercetin-mediated I_{ACh} enhancement. In the present study, we demonstrate that quercetin is a novel agent that regulates Ca^{2+}-mediated potentiation of I_{ACh} by interacting with the Ca^{2+}-binding site.

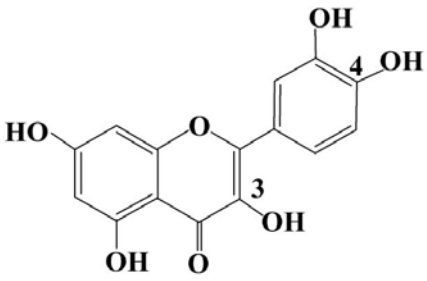
MATERIALS AND METHODS

Materials

Human wild-type α7 nAChR cDNA was kindly provided by Dr. S. Heinemann (Salk Institute, USA). Quercetin (Fig. 1) and all other reagents were purchased from Sigma-Aldrich (USA).

Preparation of *Xenopus laevis* oocytes and microinjection

X. laevis frogs were purchased from Xenopus I (Ann Arbour, USA). Animal care and handling were in accordance with the highest standards of institutional guidelines. To isolate oocytes, frogs were anesthetised with an aerated solution of 3-amino benzoic acid ethyl ester, and the ovarian follicles were removed. The oocytes were separated with collagenase followed by agitation for 2 h in a Ca^{2+}-free medium containing 82.5 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES, 2.5 mM sodium pyruvate, 100 units/ml penicillin, and 100 μg/ml streptomycin. Stage V-VI oocytes were collected and stored in a ND96 medium (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, and 5 mM HEPES, pH 7.5) supplemented with 50 μg/ml gentamicin. The solution containing the oocytes was maintained at 18°C with continuous gentle shaking and was replaced daily. Electrophysiological experiments were performed five to six days after oocyte isolation, during which time chemicals were applied to the bath. For α7 nAChR experiments, α7 nAChR-encoding cRNAs (40 nl) were injected into the animal or vegetal pole of each oocyte 1 day after isolation using a 10-μl microdispenser (VWR Scientific, USA) fitted with a tapered glass pipette tip (15-20 μm in diameter; Lee et al., 2005).



Quercetin

Fig. 1. Chemical structure of quercetin

Site-directed mutagenesis of human α7 nAChR and *in vitro* transcription of α7 nAChR cDNAs

Single amino acid substitutions were made using the QuikChange XL site-directed mutagenesis kit (Stratagene, USA) along with Pyrococcus furiosus DNA polymerase and both sense and antisense primers encoding the desired mutations. Overlap extension of the target domain by sequential polymerase chain reaction (PCR) was carried out according to the manufacturer's protocol. The final PCR products were transformed into the *Escherichia coli* strain DH5α, screened by PCR, and confirmed by sequencing of the target regions. The mutant DNA constructs were linearised at the 3′ ends by digestion with NotI, and run-off transcripts were prepared using the methylated cap analogue $m^7G(5')ppp(5')G$. The cRNAs were prepared using an mMessage mMachine transcription kit (Ambion, USA) with T7 RNA polymerase. The absence of degraded RNA was confirmed by denaturing agarose gel electrophoresis followed by ethidium bromide staining. Recombinant plasmids containing α7 nAChR cDNA inserts were linearised by digestion with the appropriate restriction enzymes, and cRNAs were obtained using the mMessage mMachine *in vitro* transcription kit with T7 polymerase. The final cRNA products were re-suspended at a concentration of 1 μg/μl in RNase-free water and stored at -80°C (Lee et al., 2005).

Data recording

A custom-made Plexiglas net chamber was used for two-electrode voltage-clamp recordings, as previously reported (Lee et al., 2005). A single oocyte was constantly superfused with a ND96 medium in the absence or presence of acetylcholine or quercetin during recording. The microelectrodes were filled with 3 M KCl and had a resistance of 0.2-0.7 MΩ. Two-electrode voltage-clamp recordings were obtained at room temperature using an Oocyte Clamp (OC-725C, Warner Instrument) and were digitised using Digidata 1200A (Molecular Devices, USA). Stimulation and data acquisition were controlled using pClamp 8 software (Molecular Devices). For most electrophysiological experiments, the oocytes were clamped at a holding potential of -80 mV, and 300-ms voltage steps were applied from -100 to +50 mV to assess the relationship between current and voltage. Linear leak and capacitance currents were corrected by means of the leak subtraction procedure. Because α7 nAChRs have a high relative permeability to Ca^{2+} (Castro and Albuquerque, 1995; Seguela et al., 1993), oocytes were incubated in 100 μM BAPTA-AM for 4 h before recording to avoid α7 nAChR-mediated endogenous Ca^{2+}-activated Cl^- currents.

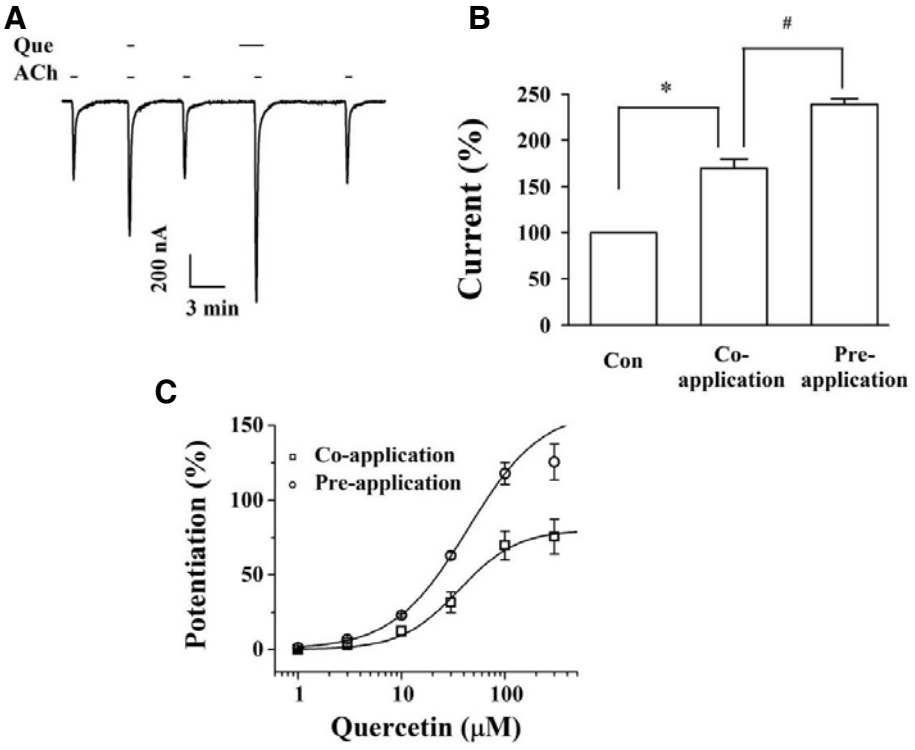


Fig. 2. Effect of quercetin on I_{ACh} in oocytes expressing wild-type α7 nAChRs. (A) Acetylcholine (ACh; 200 μM) was applied first, followed by co- or pre-application of quercetin (Que) and ACh. Co-application of 100 μM quercetin with ACh enhanced I_{ACh} and pre-application of 100 μM quercetin with ACh further enhanced I_{ACh}. Traces represent six separate oocytes from three different batches of frogs. (B) Summary of I_{ACh} enhancement by co- or pre-application of quercetin (*p < 0.001, compared to the control; #p < 0.001, compared to the co-application of quercetin. Each point represents the means ± S.E.M; n = 9-12/group). (C) Concentration-dependent effects of quercetin co- or pre-application on I_{ACh}.

Data analysis

To obtain the concentration-response curve for the effect of quercetin on the inward peak I_{ACh} mediated by the α7 AChR, the I_{ACh} peak was plotted at different concentrations of quercetin. Origin software (OriginLab Corp., USA) was used to fit the plot to the Hill equation: $I/I_{max} = 1/[1 + (ED_{50}/[A])^{nH}]$, where I_{max} is maximal current obtained from each ED_{50} value of acetylcholine in wild-type receptors, ED_{50} is the concentration of quercetin required to decrease the response by 50%, [A] is the concentration of quercetin, and nH is the Hill coefficient. All values are presented as means ± S.E.M. The differences between the means of control and treatment data were determined using the unpaired Student's t-test or one-way ANOVA. A value of p < 0.05 was considered to be statistically significant.

RESULTS

Effect of quercetin on I_{ACh} in oocytes expressing wild-type α7 nAChRs

As shown in a previous study (Lee et al., 2009), the addition of ACh (200 μM) to the bathing solution induced a large inward current (I_{ACh}) in oocytes injected with human α7 AChR cRNA (Fig. 2A). In H_2O-injected control oocytes, the application of ACh did not induce any inward current as shown previously (data not shown; Lee et al., 2009). Quercetin (100 μM) itself also had no effect in oocytes expressing α7 AChR at a holding potential of -80 mV (Fig. 2A). However, the co-application of quercetin (100 μM) with ACh (200 μM) for 30 s enhanced I_{ACh} in oocytes expressing α7 AChR (Fig. 2A, n = 15 from three different frogs). Thus, the co-application of quercetin with ACh induced enhancement of I_{ACh} by 69.7 ± 6.7% (Fig. 2B, *P < 0.005 compared with control oocytes that were not treated with quercetin). Interestingly, the pre-application of quercetin (100 μM) alone for 1 min before co-application with ACh (200 μM) induced a much larger enhancement of I_{ACh} in oocytes expressing α7 nAChR than the enhancement observed after co-application (Fig. 2A and 2B, #P < 0.005, compared to co-treatment). In concentration-dependent experiments with quercetin, co- or pre-application with quercetin for 1 min increased I_{ACh} in a concentration-dependent manner in oocytes expressing α7 nAChR (Fig. 2C). Thus, co-application of quercetin increased I_{ACh} by 0.1 ± 0.8, 3.0 ± 0.97, 12.4 ± 3.1, 31.6 ± 6.5, 69.9 ± 10.1, and 75.7 ± 11.6% at 1, 3, 10, 30, 100, and 300 μM in oocytes expressing α7 AChR, respectively. Pre-application of quercetin for 1 min increased I_{ACh} by 1.3 ± 1.2, 6.9 ± 0.9, 23.1 ± 1.2, 62.9 ± 2.2, 117.9 ± 7.4, and 125.6 ± 11.9% at 1, 3, 10, 30, 100, and 300 μM in oocytes expressing α7 AChR, respectively. The EC_{50s} of I_{ACh} were 36.1 ± 6.16 and 43.5 ± 2.7 μM for quercetin co- and pre-application in oocytes expressing the α7 AChR receptor, respectively (n = 10-11, with samples taken from three different frogs for each point; Fig. 2C). Since the pre-application of quercetin more strongly increased I_{ACh} than the co-application of quercetin, we examined the time-dependent effects on quercetin pre-application. As shown in Fig. 3A, pre-application of quercetin mediated further enhancement of I_{ACh} and was time-dependent, while the time-dependent effects of quercetin were almost saturated at 1 min pre-application (Fig. 3B).

Concentration-dependent effects of ACh and the relationship between current and voltage in the quercetin-mediated enhancement of I_{ACh}

To further study the mechanism by which the pre-application of quercetin enhances I_{ACh} in oocytes expressing α7 nAChR, we analysed the effect of quercetin on I_{ACh} evoked by different ACh concentrations (Fig. 4A). Pre-application of quercetin for 60 s with different concentrations of ACh did not significantly shift the concentration-response curve of ACh to the right (EC_{50} values were changed from 63.8 ± 11.9 to 58.3 ± 14.3 μM, *P < 0.08, while the Hill coefficient changed from 1.1 to 1.2) in oocytes expressing α7 nAChR. Thus, quercetin-mediated I_{ACh}-enhancing

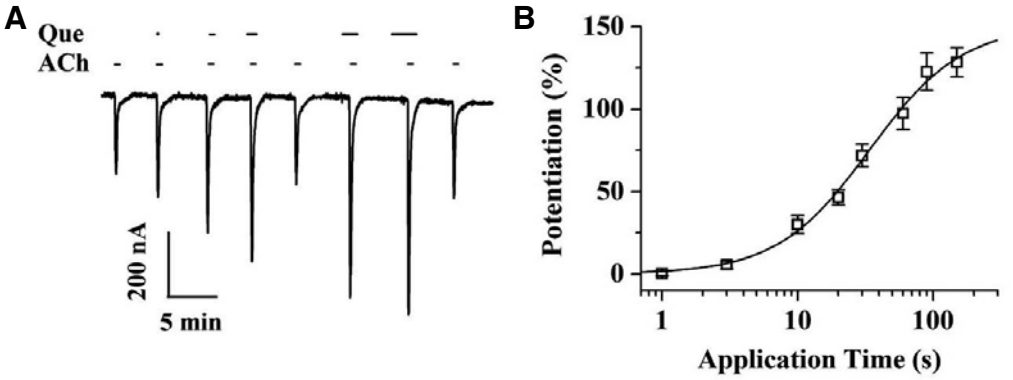


Fig. 3. Time-dependent effects of pre-application of quercetin on I_{ACh} in α7 nAChRs. (A) Quercetin (100 μM)-mediated enhancement on I_{ACh} is pre-application-time dependent. Traces represent six separate oocytes from three different batches of frogs. I_{ACh} in oocytes expressing α7 nAChRs was elicited at a holding potential of -80 mV for the indicated pre-application time of quercetin prior to drug application. (B) Quercetin-mediated enhancement on I_{ACh} was almost saturated after 60 s of pre-application. The resting membrane potentials of the oocytes were approximately -35 mV, and the oocytes were voltage-clamped at a holding potential of -80 mV. Each point represents the means ± S.E.M. (n = 9-12/group).

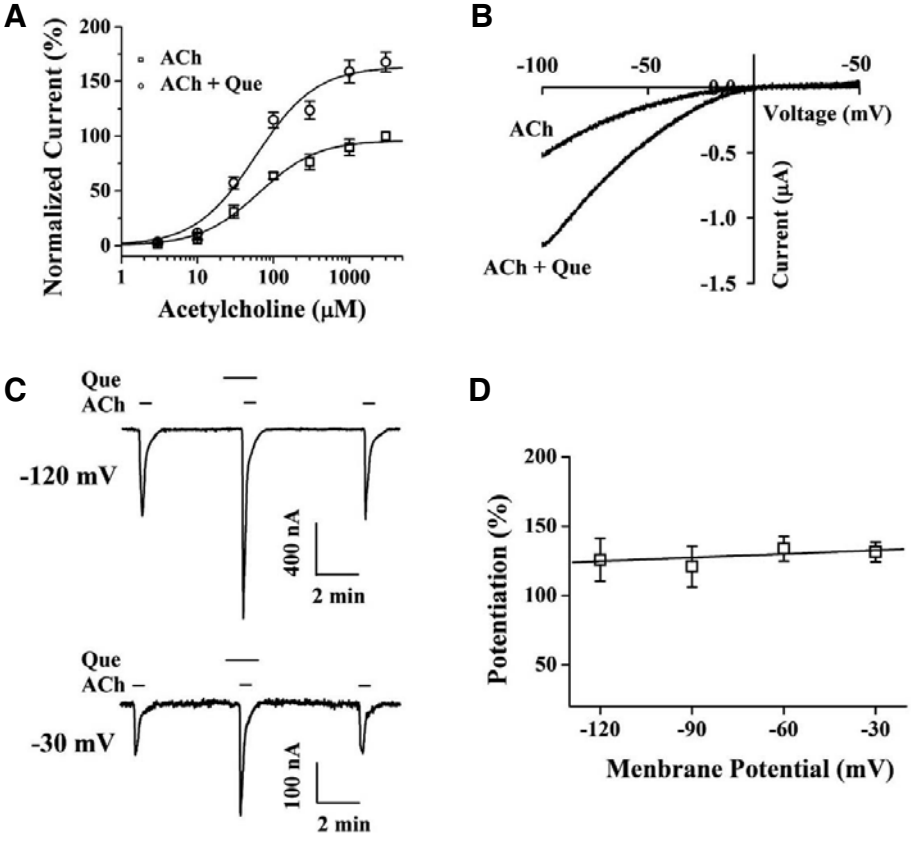


Fig. 4. Concentration-dependent effects of ACh and current-voltage relationship on quercetin-mediated enhancement of I_{ACh}. (A) Concentration-response relationships for ACh in the wild-type receptor treated with ACh (3-3000 μM) alone or with ACh plus 45 μM quercetin in oocytes expressing the wild-type receptor. The I_{ACh} of oocytes expressing the wild-type receptor was measured using the indicated concentration of ACh in the absence (□) or presence (○) of 45 μM quercetin (Que). Oocytes were exposed to ACh alone or to ACh with quercetin for 1 min before application. Oocytes were voltage-clamped at a holding potential of -80 mV. (B) Current-voltage relationships of I_{ACh} enhancement by quercetin in wild-type α7 nAChRs. Representative current-voltage relationships were obtained using voltage ramps of -100 to +50 mV for 300 ms at a holding potential of -80 mV. Voltage steps were applied before and after application of 200 μM ACh in the absence or presence of 100 μM quercetin (Que). The reversal potential for the receptor was -4.6 ± 1.3 mV and -3.4 ± 1.5 mV in the absence or presence of quercetin, respectively. Each point represents the mean ± S.E.M. (n = 7-9/group). (C) Voltage-independent enhancement of I_{ACh} in α7 nAChRs by quercetin. Left panel; The representative traces were obtained from wild-type receptors in the absence or presence of quercetin (Que) at different membrane holding potentials. Right panel; The summary of the percent potentiation induced by quercetin at the indicated membrane holding potentials in oocytes expressing the wild-type receptor. Each point represents the mean ± S.E.M. (n = 6-8/group).

effects were not affected by increasing concentrations of ACh in the range of 30 to 3000 μM ACh (Fig. 4A), suggesting that quercetin-mediated enhancement of I_{ACh} was not related to the ACh binding site.

In experiments examining the current-voltage relationship, the membrane potential was held at -80 mV, and a voltage ramp was applied from -100 to +50 mV for 300 ms. In the absence of ACh, the inward current at -100 mV was < 0.01 μA, and the outward current at +50 mV was near 0.1 μA (data not shown). The addition of ACh to the bathing medium induced a mainly inward current at negative voltages and an outward current at positive voltages. Pre-application of quercetin with

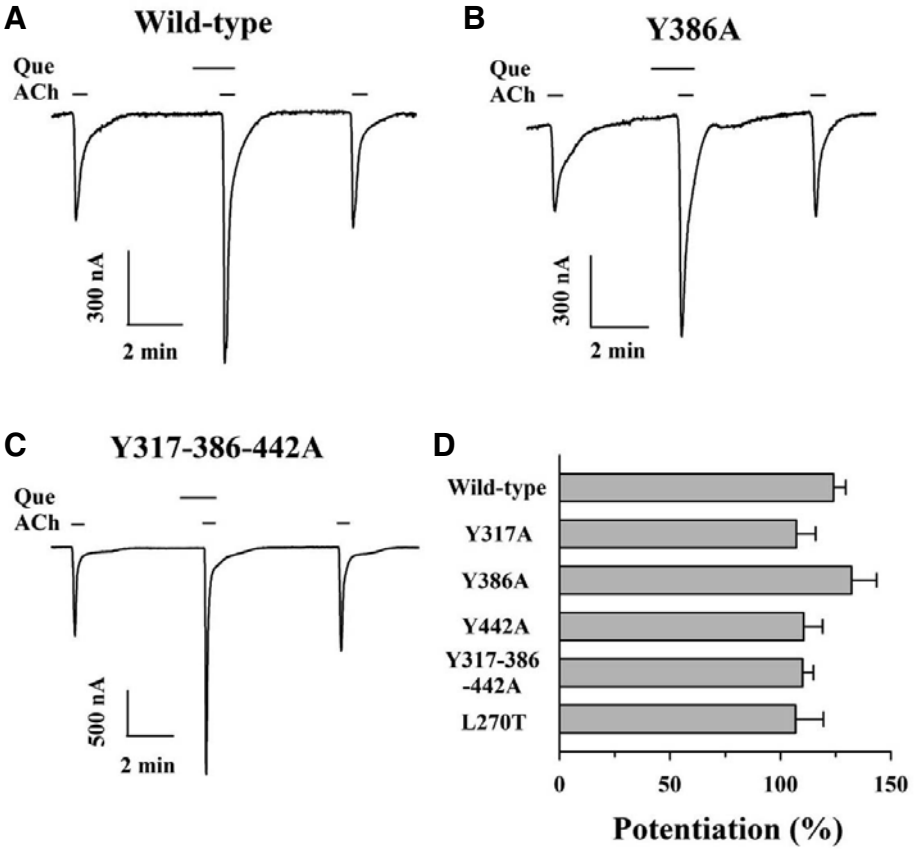


Fig. 5. Effects of quercetin on I_{ACh} in the wild-type, various mutant α7 nAChRs in tyrosine phosphorylation sites, and channel pores. I_{ACh} in oocytes expressing wild-type, L270T, Y317A, Y386A, Y442A or Y317-386-442A mutant receptors was elicited at a holding potential of -80 mV for the indicated time in the presence of ACh, followed by the pre-application of 100 μM quercetin (Que) together with 200 μM ACh. (A-C) Traces are representative of 8-12 separate oocytes from three different frogs. (D) Summary histograms of I_{ACh} enhancement by 100 μM quercetin in wild-type and mutant receptors in phosphorylation sites and channel pores. Each point represents the mean ± S.E.M (n = 8-12/group).

ACh increased both inward and outward current. The reversal potential was near 0 mV for both ACh alone and for ACh with quercetin. This indicates that ACh induces cation currents (Galzi et al., 1992; Revah et al., 1991). Also, the pre-application of quercetin with ACh further increased currents but did not affect the α7 nAChR channel property because quercetin did not change the reversal potential of α7 nAChR (Fig. 4B). In addition, the enhancing effect of quercetin on I_{ACh} was independent of the membrane holding potential (Fig. 4C). Quercetin increased I_{ACh} by 125.8 ±15.5, 120.9 ± 14.8, 133.9 ± 9.1, and 131.5 ± 7.2% at membrane holding potentials of -120, -90, -60, and -30 mV in oocytes expressing α7 nAChR, respectively (n = 10-12, from three different frogs; Fig. 4C).

Effects of quercetin on I_{ACh} in wild-types and in various mutant α7 nAChRs in tyrosine phosphorylation sites and channel pores

The above results indicate that quercetin may be a novel regulator of α7 nAChR channel activity. Furthermore, tyrosine phosphorylations or dephosphorylation of the intracellular cytoplasmic loop between the transmembrane domain TM3 and TM4 of α7 nAChR can be induced by genistein. As a flavonoid and a tyrosine kinase inhibitor, genistein regulates I_{ACh} through a nAChR (Charpantier et al, 2005; Cho et al., 2005). We investigated the effect of quercetin on peak I_{ACh} after site-directed mutations of tyrosine residues of α7 nAChR. To do this, we constructed four different kinds of mutant α7 nAChRs in cytoplasmic loop tyrosine residues such as Y317, Y386, and Y442 by replacing these residues with alanine (Y317A, Y386A, Y442A, and Y317A-Y386A-Y442A) (Cho et al., 2005). We chose these sites for site-directed mutations because the mutation of these residues of cytoplasmic loops affects genistein-mediated regulation of I_{ACh} (Charpantier et al, 2005). Figures 5A-5C show the representative traces in the absence or presence of quercetin in wild-type receptors, mutant receptors, or in a triple mutant receptor in the cytoplasmic loop between TM3 and TM4. Mutations of Y317A, Y386A, Y442A, and Y317A-Y386A-Y442A had no effects on the quercetin-induced enhancement of I_{ACh}. Figure 5D shows the summary histograms for I_{ACh} enhancement by quercetin in different mutants. The above results indicate that quercetin-mediated enhancement of I_{ACh} is not related to the phosphorylation state of tyrosine residues of the α7 nAChR cytoplasmic loop.

It has been reported that single point mutations in the highly conserved Leu270 to Thr270 in transmembrane domain 2 (TM2) of chick α7 AChR, which forms the channel pore region, creates gain-of-function alterations (i.e., increased acetylcholine affinity, slower desensitisation, and a linear current-voltage relationship) and alters pharmacological properties (i.e., conversion of various α7 AChR antagonists into agonists; Bertrand et al., 1992; Palma et al., 1996; Revah et al., 1991). In addition, the mutant receptor exhibits differential responses to divalent cations, including Ca^{2+} (Eddins et al., 2002). We also examined whether the enhancing effects of quercetin on I_{ACh} is affected by the mutation of Leu270, which is a homologous residue of chick α7 AChR, to Thr270. As shown in Fig. 5D, the quercetin action on I_{ACh} was not significantly different, indicating that quercetin does not act through the Leu270 residue to enhance I_{ACh}.

Effects of Ca^{2+} or Ba^{2+} on quercetin-mediated enhancement of I_{ACh}

Channels of α7 nAChR are known to be highly permeable to Ca^{2+} compared to other ligand-gated ion channels (Bertrand et al., 1993) and Ca^{2+} influx through this receptor is involved in a

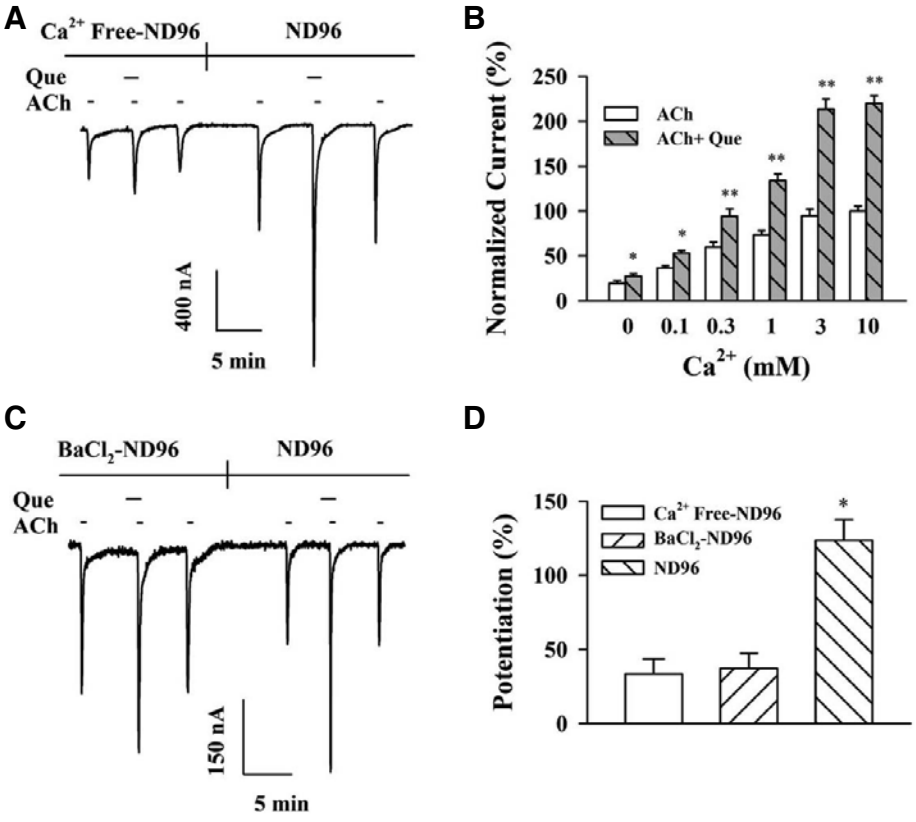


Fig. 6. Effects of Ca^{2+} or Ba^{2+} on quercetin-mediated enhancement of I_{ACh}. (A) The absence of extracellular free Ca^{2+} greatly attenuated the enhancement of I_{ACh} mediated by 100 μM quercetin. Traces representative of eight separate oocytes from three different frogs. (B) The histograms show that I_{ACh} is potentiated by increasing the extracellular Ca^{2+} concentration and that the presence of quercetin (100 μM) further enhances I_{ACh} compared to Ca^{2+} alone (*p < 0.01, **p < 0.001, compared to Ca^{2+} alone). (C) Ba^{2+} does not potentiate the quercetin-mediated enhancement of I_{ACh} as much as Ca^{2+}. Traces representative of ten separate oocytes from three different frogs. (D) The summary histograms of I_{ACh} enhancement by 100 μM quercetin in Ca^{2+}-free, Ba^{2+} or normal ND96 media (*p < 0.01, compared to Ca^{2+}-free and Ba^{2+}). Each point represents the mean ± S.E.M. (n = 8-10/group).

variety of Ca^{2+}-dependent cellular signal transduction pathways in central nervous systems (Brumwell et al., 2002; Klein et al., 2005). We examined whether or not quercetin-mediated enhancement of I_{ACh} is closely related to the Ca^{2+} ion. First, we examined the effects of Ca^{2+} ion on I_{ACh}. As shown in Fig. 6A and 6B, I_{ACh} was potentiated with extracellular Ca^{2+} in a concentration-dependent manner. We next examined the effects of quercetin in the absence of extracellular Ca^{2+} on I_{ACh}. As shown in Figs. 6A and 6B, the removal of extracellular Ca^{2+} from ND96 media in the presence of 0.1 mM EGTA greatly diminished the effects of quercetin on I_{ACh}. Thus, the presence of extracellular Ca^{2+} ions is required for further enhancement of I_{ACh} by quercetin. The effect of quercetin on I_{ACh} was dependent on extracellular Ca^{2+} concentrations. The quercetin effect was saturated at 3 mM of extracellular Ca^{2+} (Fig. 6B). Aside from the Ca^{2+} ion, we also examined the effect of Ba^{2+}, another divalent cation, on the quercetin-mediated enhancement of I_{ACh}. As shown in Fig. 6C, the addition of $BaCl_2$ rather than $CaCl_2$ in the ND96 media greatly reduced the I_{ACh} enhancement by quercetin in comparison to the ND96 media. This indicated that the Ca^{2+} ion was the main component of I_{ACh} enhanced by quercetin (Figs. 6C and 6D).

Quercetin on I_{ACh} in wild-type and mutant α7 nAChRs in relation to Ca^{2+} permeation and Ca^{2+} binding sites

The above results show the possibility that effects of quercetin might act by affecting the permeability of Ca^{2+}. We examined quercetin effects on I_{ACh} after the mutation of two amino acid residues that are known to play a key role in Ca^{2+} permeability (Bertrand et al., 1993). For this, we constructed two mutant α7 nAChRs, such as E260 and L277, that were related to Ca^{2+} permeability and that were homologous Ca^{2+} permeability-related residues of chick α7 nAChR. These mutant α7 nAChRs were created by replacing the E260 residue with alanine and the L277 residue with threonine (E260A and L277T; Bertrand et al., 1993). We chose this site for site-directed mutations, since mutations of these residues greatly reduced Ca^{2+} permeability in I_{ACh} (Bertrand et al., 1993). Mutations of E260A and L277T did not affect quercetin-induced enhancement of I_{ACh} compared to the wild type (Fig. 7D), indicating that quercetin-mediated enhancement of I_{ACh} might not be related to Ca^{2+} permeability.

We next examined whether or not the Ca^{2+} binding site of α7 nAChR is involved in quercetin action, since Ca^{2+} binding at Ca^{2+} binding sites in α7 nAChRs also allosterically potentiates I_{ACh} and the mutation of Ca^{2+} binding site completely diminished the Ca^{2+}-mediated potentiation of I_{ACh} (Galzi et al., 1996). For this, we again constructed mutant α7 nAChRs in Ca^{2+} binding site E195, which is homologous to the Ca^{2+}-binding residue of chick α7 nAChR, by replacing residue E195 with glutamine (E195Q; Galzi et al, 1996). Figures 7A and 7B show the representative traces in the absence or presence of quercetin in wild-type or mutant receptors at the Ca^{2+} binding site. Mutations of E195Q almost negated the quercetin-mediated enhancement of I_{ACh}, even at high concentrations of quercetin (Figs. 7B and 7C). Figure 7D shows the summary histograms for I_{ACh} enhancement by quercetin in wild-type and mutant receptors (*P < 0.001, compared to the wild-type receptor). The above results show that quercetin-mediated enhancement of I_{ACh} in α7 nAChR is achieved through interactions with the Ca^{2+} binding site.

DISCUSSION

Quercetin, a flavonoid, is a well-known anti-oxidant. For example, recent studies have shown that the application of quercetin protects central nervous systems against oxidative effects and

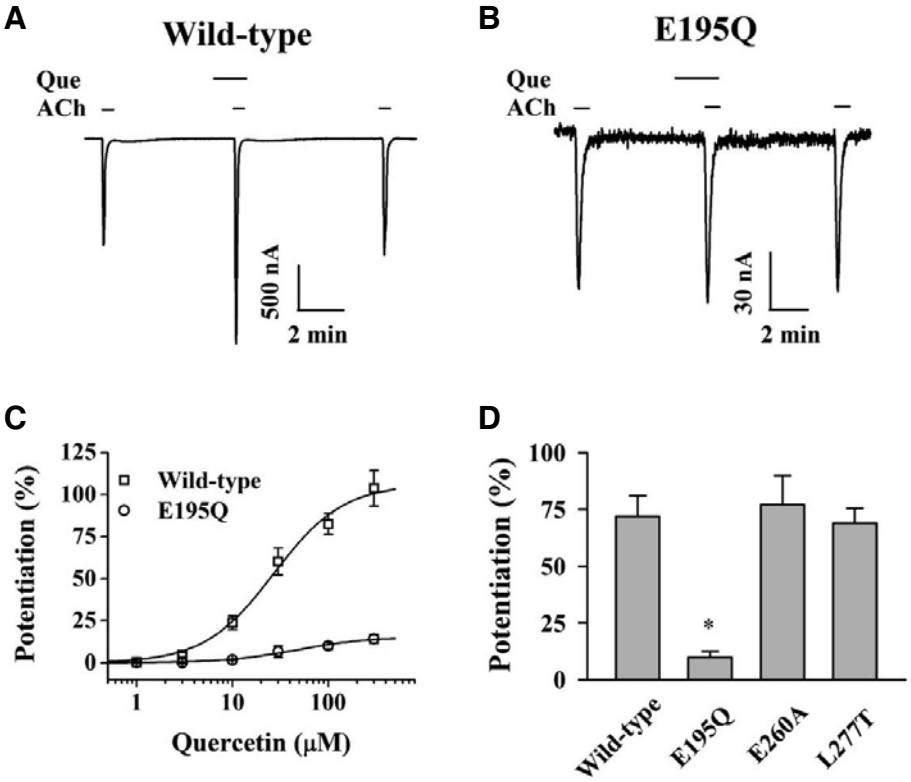


Fig. 7. Effects of quercetin on I_{ACh} in wild-type and mutant α7 nAChRs related to Ca^{2+} permeation and Ca^{2+} binding sites. (A) Representative traces on I_{ACh} enhancement by ACh (5 mM) alone or by pre-application of quercetin (Que; 100 μM) in wild-type receptors. Traces are representative of ten separate oocytes from three different frogs. (B) Representative traces of I_{ACh} enhancement by ACh (5 mM) alone or by pre-application of quercetin (Que; 100 μM) in receptors mutated at Ca^{2+} binding sites. Traces are representative of ten separate oocytes from three different frogs. (C) Concentration-dependent effects of the pre-application of quercetin on I_{ACh} in wild-type and E195Q mutant receptors. Qeurcetin enhances I_{ACh} in a concentration-dependent manner in wild-type receptors, whereas quercetin almost had no effects on I_{ACh} even in high concentrations in the E195Q mutant receptor. (D) A summary of I_{ACh} enhancement by pre-application of quercetin in wild-type, E195Q, E260A, and L277T mutant receptors (*p < 0.001, compared to wild-type). Each point represents the mean ± S.E.M. (n = 5/group).

decreases learning and memory damage as well as ischemic brain damage (Yao et al., 2010). However, the beneficial roles of quercetin in nervous systems are not fully understood except for their anti-oxidant effects. Furthermore, its molecular mechanisms and its ability to counteract various negative effects are relatively unknown. In previous studies, we have demonstrated that quercetin regulates ligand-gated ion channels such as 5-HT_3 and glycine receptors (Lee et al., 2005; 2007). However, little is known about the effects of quercetin on α7 nAChR, although α7 nAChR is widely expressed throughout the central nervous systems, including in the cortical and limbic areas. In addition, α7 nAChR plays an important role in normal brain function, as α7 nAChR dysfunction is associated with neurological disorders such as learning and memory loss, Alzheimer's disease, schizophrenia, and epilepsy (Changeux and Edelstein, 2001; Chini et al., 1994; Lena and Changeux, 1997; Weiland et al., 2000).

In the present study, we investigated the effects of quercetin on human α7 nAChR heterologously expressed in *Xenopus* oocytes. We found that: (1) pre- or co-application of quercetin with ACh induced a large enhancement of I_{ACh} in a reversible and concentration-dependent manner; (2) I_{ACh} enhancement by quercetin co- or pre-application with ACh was not dependent on the concentration of ACh or membrane voltage; (3) quercetin-mediated enhancement of I_{ACh} was Ca^{2+} specific, since quercetin acted on I_{ACh} and was greatly reduced by the substitution of Ca^{2+} with Ba^{2+} and was dependent on extracellular Ca^{2+} concentrations; and (4) the mutation of Glu195 to Gln195, a Ca^{2+} binding site, in α7 nAChR greatly attenuated the quercetin-mediated enhancement of I_{ACh}. These results indicate that the Ca^{2+} binding site of α7 nAChR plays an important role in quercetin-mediated I_{ACh} enhancement. In addition, we found that ED_{50} of quercetin on α7 nAChR activation is similar to IC_{50} values obtained from 5-HT_{3A} and glycine α1 receptors and voltage-dependent Na^+ channel in hippocampal CA1 neurons (Lee et al., 2005, 2007; Yao et al., 2010). Thus, these results again show that quercetin concentration for activations or inhibitions of ion channels and receptors examined are in similar range.

The activation of α7 nAChR is known to be linked to many physiological conditions (Gilbert et al., 2009; Gotti and Clementi, 2004; Khiroug et al., 2003). Several physiological roles of α7 nAChRs are derived from the high calcium permeability and the increase of $[Ca^{2+}]_i$ that result from the activation of α7 nAChR. These factors could be due to the effects of downstream pathways, such as the activation of the cAMP response element-binding protein, protein kinase A, and extracellular signal-regulated kinase 1/2 (Dajas-Bailador et al., 2002; 2004; Hu et al., 2002), or phosphatidylinositol 3-kinase, Akt, and Bcl-2 (Kihara et al., 2001). Thus, activations of Ca^{2+}-dependent diverse enzymes including kinases result in the expression of genes that are important to synaptic plasticity, such as cognitive processing or cell survival (Berg and Conroy, 2002; Dajas-Bailador and Wonnacott, 2004). In the present study, we found that quercetin enhanced I_{ACh} in *Xenopus* oocytes expressing human a7 nAChRs that were dependent on extracellular Ca^{2+} (Fig. 6). It was previously know that quercetin had anti-oxidative effects on neuroprotections which act against ischemic learning and memory damage (Yao et al., 2009). In addition, quercetin-induced I_{ACh} enhancement could be responsible for the actions of quercetin in nervous systems and could contribute to the facilitation of α7 nACh-mediated signal transductions. In addition, the findings in present study suggest that quercetin could be applied as a natural, though not synthetic, agent for the potentiation of α7 nAChR activity, although pre-clinical tests are required before clinical applications could be conducted.

It was unclear from the present results precisely how quercetin increases I_{ACh} in oocytes expressing α7 nAChR. One possibility is that quercetin might affect the tyrosine phosphorylation of the cytoplasmic loop of α7 nAChR, since the phosphorylation and dephosphorylation at the tyrosine residue of α7 nAChR regulates I_{ACh} (Charpantier et al., 2005). However, the enhancing effect of quercetin on I_{ACh} in oocytes expressing α7 nAChR was not affected by site-directed mutations of three tyrosine residues of the cytoplasmic loop of α7 nAChR (Fig. 5). The other possibility is that quercetin might affect ACh binding on α7 nAChR. When we examined quercetin actions on I_{ACh} by increasing the ACh concentration, we did not observe any significant changes in the actions of quercetin on I_{ACh} in oocytes expressing α7 nAChR (Fig. 4A). This suggests that the quercetin-mediated enhancement of I_{ACh} is independent of the ACh concentration and further shows that the quercetin interaction site(s) at α7 nAChR for I_{ACh} enhancement might be not related to the ACh binding sites.

It is known that α7 nAChRs exhibit a high Ca^{2+} permeability and that Ca^{2+} bindings at Ca^{2+}-binding sites of α7 nAChR potentiate I_{ACh} (Bertrand et al., 1993; Galzi et al., 1996). We also observed I_{ACh} potentiation by increasing extracellular Ca^{2+} concentrations. The presence of quercetin further enhanced I_{ACh}, and the main component of the additional I_{ACh} obtained by quercetin treatment was the Ca^{2+} ion (Fig. 6B). Thus, the third and final possibility is that quercetin could enhance I_{ACh} by changing the Ca^{2+} permeability or by affecting Ca^{2+} binding at Ca^{2+}-binding sites of α7 nAChR. It is unlikely that quercetin enhances I_{ACh} by increasing Ca^{2+} permeability, since the mutation of amino acid residues E260 or L277 into E260A or L277T involved in Ca^{2+} permeability did not affect the action of quercetin on I_{ACh} (Fig. 7D). However, the mutation of Ca^{2+}-binding site E195 to E195Q, which is homologous to the Ca^{2+}-binding residue of chick α7 nAChR, almost completely diminished the quercetin-mediated I_{ACh} enhancement. These results indicate that quercetin-induced enhancements of I_{ACh} are achieved through Ca^{2+}-binding sites of α7 nAChR. However, we could not exclude the possibility that quercetin could act through allosteric interactions with other unknown site(s) of α7 nAChR. In addition, we also could not exclude another possibility that quercetin could regulate α7 nAChR through direct hydrophobic interaction with Glu195. Further studies are required to elucidate how quercetin interactions with the Ca^{2+} binding site of α7 nAChR interact to enhance I_{ACh}.

In previous studies, we have demonstrated that quercetin inhibits 5-HT_3 receptor-gated ion currents through interactions with amino acid Arg222 residues in pre-transmembrane domain I. Quercetin inhibits or potentiates glycine receptors-gated ion currents through interactions with amino acid Ser256 residues at transmembrane domain II (Lee et al., 2005; 2007). In the present study, we found that quercetin enhanced α7 nAChR-gated ion currents by interacting with Glu195, a Ca^{2+}-binding site, at the N-terminal domain. Thus, although glycine, 5-HT_3, and α7 nAChR all form homomeric receptors and are in the Cys-loop family of ligand-gated ion channels, quercetin interaction sites in these homomeric receptors differs from one another.

In conclusion, we found that quercetin, a flavonoid, increased I_{ACh} in an extracellular Ca^{2+}-dependent manner in *Xenopus* oocytes expressing α7 nAChR and that the mutation of the Ca^{2+} binding site Glu195 to Gln195 almost completely diminished the effects of quercetin on I_{ACh}. The present results indicate that quercetin is a novel agent interacting with Ca^{2+}-binding sites to positively affect I_{ACh} enhancement. These results indicate that quercetin-mediated regulation of I_{ACh}, through interactions with the Ca^{2+}-binding site of α7 nAChR, could provide a molecular basis for the pharmacological actions of flavonoids in the nervous system.

ACKNOWLEDGMENTS

This work was supported by Basic Science Research Program through the National Research Foundation of Korea funded by the Ministry of Education, Science and Technology (R01-2008-000-10448-0), Priority Research Centers Program through the National Research Foundation of Korea funded by the Ministry of Education, Science and Technology (2009-0093824), and Brain Korea 21 to S. Y. Nah.

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