Korean J Physiol Pharmacol
Vol 15: 195−201, August, 2011
http://dx.doi.org/10.4196/kjpp.2011.15.4.195

Inhibitory Effects of Quercetin on Muscle-type of Nicotinic Acetylcholine Receptor-Mediated Ion Currents Expressed in *Xenopus* Oocytes

Byung-Hwan Lee[1], Tae-Joon Shin[1], Sung-Hee Hwang[1], Sun-Hye Choi[1], Jiyeon Kang[1], Hyeon-Joong Kim[1], Chan-Woo Park[1], Soo-Han Lee[2], and Seung-Yeol Nah[1]

[1]Department of Physiology, College of Veterinary Medicine and Bio/Molecular Informatics Center, Konkuk University, Seoul 143-701, [2]Department of Clinical Pharmacology and Therapeutics, University of Ulsan College of Medicine, Seoul 138-736, Korea

The flavonoid quercetin is a low molecular weight compound generally found in apple, gingko, tomato, onion and other red-colored fruits and vegetables. Like other flavonoids, quercetin has diverse pharmacological actions. However, relatively little is known about the influence of quercetin effects in the regulation of ligand-gated ion channels. Previously, we reported that quercetin regulates subsets of nicotinic acetylcholine receptors such as $\alpha 3\beta 4$, $\alpha 7$ and $\alpha 9\alpha 10$. Presently, we investigated the effects of quercetin on muscle-type of nicotinic acetylcholine receptor channel activity expressed in *Xenopus* oocytes after injection of cRNA encoding human fetal or adult muscle-type of nicotinic acetylcholine receptor subunits. Acetylcholine treatment elicited an inward peak current (I_{ACh}) in oocytes expressing both muscle-type of nicotinic acetylcholine receptors and co-treatment of quercetin with acetylcholine inhibited I_{ACh}. Pre-treatment of quercetin further inhibited I_{ACh} in oocytes expressing adult and fetal muscle-type nicotinic acetylcholine receptors. The inhibition of I_{ACh} by quercetin was reversible and concentration-dependent. The IC_{50} of quercetin was 18.9±1.2 μM in oocytes expressing adult muscle-type nicotinic acetylcholine receptor. The inhibition of I_{ACh} by quercetin was voltage-independent and non-competitive. These results indicate that quercetin might regulate human muscle-type nicotinic acetylcholine receptor channel activity and that quercetin-mediated regulation of muscle-type nicotinic acetylcholine receptor might be coupled to regulation of neuromuscular junction activity.

Key Words: Flavonoids, Quercetin, Muscle-type nicotinic acetylcholine receptors, *Xenopus* oocyte

INTRODUCTION

Nicotinic acetylcholine receptors are members of the Cys-loop family of ligand-gated ion channels, which also contains 5-hydroxytryptamine 3 (5-HT_3), glycine receptors and γ-aminobutyric acid receptors [1]. Seventeen different nicotinic acetylcholine receptor subunits are known to date and various subunits of nicotinic acetylcholine receptors are α ($\alpha 1\sim 10$), β ($\beta 1\sim 4$), γ, δ and ε have been identified [2]. The $\alpha 2$-6 neuronal nicotinic acetylcholine receptors are usually expressed as a heteropentamer in combination with $\beta 2$-4 subunits and their activations are mainly involved in rapid synaptic transmissions in the central and peripheral nervous systems [3-6]. For example, the $\alpha 3$ and $\beta 4$ subunits can form heteromeric receptors [7] and the $\alpha 7$ and $\alpha 9$ subunits can express homopentameric receptors [5,8-10]. The muscle-type nicotinic acetylcholine receptor consists of $\alpha 1\beta 1\delta\gamma$ in fetal tissue, while, in adult tissue, the γ subunit is replaced by ε [11]. Although many nicotinic acetylcholine receptor subunits are expressed in the central and peripheral nervous systems, the distributions of muscle-type of nicotinic acetylcholine receptor are mainly restricted to the neuromuscular junction and activation of muscle-type nicotinic acetylcholine receptor initiates contraction of skeletal muscle fibers by triggering endplate potentials at neuromuscular junctions. In the muscle-type nicotinic acetylcholine receptor, two molecules of acetylcholine bind at interfaces between the $\alpha 1$ and δ or γ subunits [12].

The flavonoid quercetin is a low molecular weight compound generally found in apple, gingko, tomato, onion and other red-colored fruits and vegetables [13] (Fig. 1). Flavonoids derived from plants or tea extracts affect acetylcholine release, muscle contraction or neuromuscular junction activity [14-18]. Especially, quercetin inhibits end-plate currents at the mouse neuromuscular junction [19]. However, the underlying mechanisms of flavonoids in general and quercetin in particular on the regulation of

Received June 3, 2011, Revised July 20, 2011,
Accepted August 2, 2011

Corresponding to: Seung-Yeol Nah, Department of Physiology, College of Veterinary Medicine and Bio/Molecular Informatics Center, Konkuk University, 1, Hwayang-dong, Gwangjin-gu, Seoul 143-701, Korea. (Tel) 82-2-450-4154, (Fax) 82-2-450-3037, (E-mail) synah@konkuk.ac.kr

ABBREVIATIONS: ACh, acetylcholine; 5-HT3A, 5-hydroxytryptamine 3A; Que, quercetin.

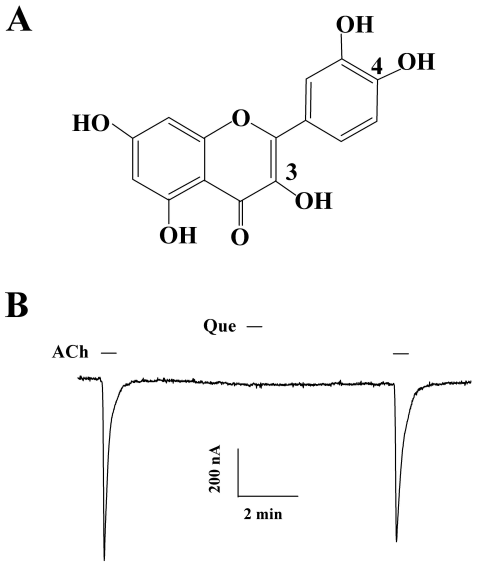

Fig. 1. Chemical structure of quercetin (A) and its effect in oocytes expressing $\alpha 1\beta 1\delta\varepsilon$ nicotinic acetylcholine receptors (B). Quercetin had no effect on I_{ACh} in oocytes expressing $\alpha 1\beta 1\delta\varepsilon$ nicotinic acetylcholine receptors.

muscle contraction or neuromuscular junction activity are unclear, especially concerning the regulation of muscle-type nicotinic acetylcholine receptor channel activity involved in neuromuscular junctions.

In previous reports, we have shown that quercetin regulates the Cys-loop family of ligand-gated ion channels, such as 5-HT_{3A}, human glycine $\alpha 1$, $\alpha 7$, $\alpha 9\alpha 10$ and $\alpha 3\beta 4$ nicotine acetylcholine receptors [20-24]. However, relatively little is known about the effects of quercetin on the muscle-type nicotinic acetylcholine receptor channel activity. In this study, we investigated the effects of quercetin on the muscle-type nicotinic acetylcholine receptor channel activity. We expressed human muscle-type nicotinic acetylcholine receptor cRNAs in *Xenopus* oocytes and studied the effect of quercetin on acetylcholine-elicited inward currents (I_{ACh}). Co- or pre-treatment of quercetin with acetylcholine reduced I_{ACh}. Inhibition of I_{ACh} by quercetin was concentration-dependent, reversible and voltage-independent. Moreover, the inhibition of quercetin on I_{ACh} was non-competitive with acetylcholine. The results indicate the potential of quercetin as a novel agent for the regulation of muscle-type of nicotinic acetylcholine receptor and further indicate that quercetin might play an important role for the regulation of neuromuscular junction activity.

METHODS

Materials

Human $\alpha 1$, $\beta 1$, δ, γ and ε muscle nicotine acetylcholine receptor subunit cDNAs were kindly provided by Dr. Jon Lindstrom (University of Pennsylvania Medical School, Philadelphia, USA). Quercetin (Fig. 1A) and all other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). Quercetin was dissolved in dimethyl sulfoxide (DMSO) as a stock solution and was diluted with bath medium before use.

Preparation of Xenopus laevis oocytes and microinjection

X. laevis frogs were purchased from Xenopus I (Ann Arbour, MI, USA). Animal care and handling were in accordance with the highest standards of Konkuk University 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 3~5 days after oocyte isolation. For muscle nicotine acetylcholine receptor experiments, $\alpha 1$, $\beta 1$, δ, γ and ε nicotine acetylcholine receptor subunit encoding cRNAs (40 nl) were co-injected into the animal or vegetal pole of each oocyte 1 day after isolation using a 10 μl microdispenser (VWR Scientific, West Chester, PA, USA) fitted with a 15~20 μm-diameter tapered glass pipette tip [20].

cRNA preparation of $\alpha 1\beta 1\delta\gamma$ and $\alpha 1\beta 1\delta\varepsilon$ nicotinic acetylcholine receptors

The cDNA constructs were linearized at the 3' ends by digestion with *Not*I and run-off transcripts were prepared using the methylated cap analogue $m^7G(5')ppp(5')G$. The cRNAs were prepared using a mMessage mMachine transcription kit (Ambion, Austin, TX, USA) with T7 RNA polymerase. The absence of degraded RNA was confirmed by denaturing agarose gel electrophoresis followed by ethidium bromide staining. The final cRNA products were re-suspended at a concentration of 1 μg/μl in RNase-free water and stored at −80°C [20].

Data recording

A custom-made Plexiglas net chamber was used for two-electrode voltage-clamp recordings, as previously reported [20]. A single oocyte was constantly superfused with a recording solution (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, and 10 mM HEPES, pH 7.5) in the absence or presence of glutamate 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 a model OC-725C Oocyte Clamp (Warner Instruments, Hamden, CT, USA) and were digitised using a Digidata 1200A apparatus (Molecular Devices, Sunnyvale, CA, USA). Stimulation and data acquisition were controlled using pClamp 8 software (Molecular Devices). For most electrophysiological data, the oocytes were clamped at a holding potential of −80 mV. For current and voltage (I-V) relationship, voltage ramps were applied from −100 to +60 mV for 300 ms. In the different membrane-holding potential experiments, the oocytes were clamped at the indicated holding potentials. Linear leak and capacitance currents were corrected by

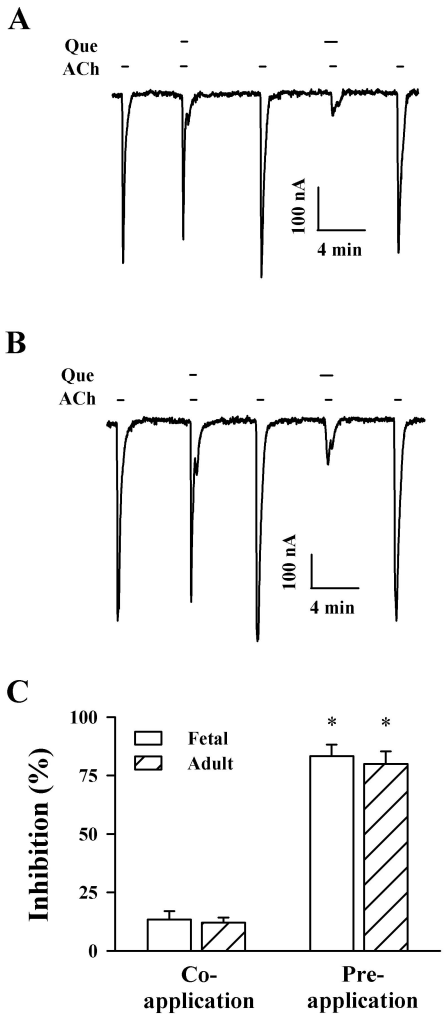

Fig. 2. Effect of quercetin (Que) on I_{ACh} in oocytes expressing $\alpha1\beta1\delta\gamma$ and $\alpha1\beta1\delta\varepsilon$ muscle-type nicotinic acetylcholine receptors. (A) Acetylcholine (100 μM) was first applied and then acetylcholine was co- or pre-treated with quercetin (100 μM). Thus, co-treatment of quercetin with acetylcholine and pre-treatment of quercetin before acetylcholine application inhibited I_{ACh} in oocytes expressing $\alpha1\beta1\delta\gamma$ nicotinic acetylcholine receptors. (B) Acetylcholine (100 μM) was first applied and then acetylcholine was co- or pre-treated with quercetin (100 μM). Thus, co-treatment of quercetin with acetylcholine and pre-treatment of quercetin before acetylcholine application inhibited I_{ACh} in oocytes expressing $\alpha1\beta1\delta\varepsilon$ nicotinic acetylcholine receptors. The resting membrane potential of oocytes was about −35 mV and oocytes were voltage-clamped at a holding potential of −80 mV prior to drug application. Traces are representative of six separate oocytes from three different frogs. (C) Summary of % inhibition by quercetin of I_{ACh} was calculated from the average of the peak inward current elicited by acetylcholine alone before quercetin and the peak inward current elicited by acetylcholine alone after co- and pre-treatment of quercetin with acetylcholine. Each point represents the mean±S.E.M. (n=9∼12 from three different frogs).

means of the leak subtraction procedure.

Data analysis

To obtain the concentration-response curve for the effect of quercetin on the inward peak I_{ACh} mediated by the $\alpha1\beta1\delta\gamma$ and $\alpha1\beta1\delta\varepsilon$ nicotinic acetylcholine receptors, the I_{ACh} peak was plotted at different concentrations of quercetin. Origin software (OriginLab, Northampton, MA, USA) was used to fit the plot to the Hill equation: $I/I_{max}=1/[1+(IC_{50}/[A])^{nH}]$, where I_{max} is maximal current obtained from each ED_{50} value of acetylcholine in wild-type receptors, IC_{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 represent the mean±S.E.M. The differences between the means of control and treatment data were determined using the unpaired Student's t-test. A value of $p<0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

Effect of quercetin on I_{ACh} in oocytes expressing fetal $\alpha1\beta1\delta\gamma$ and adult $\alpha1\beta1\delta\varepsilon$ nicotinic acetylcholine receptors

The addition of acetylcholine to the bathing solution induced a large inward current in oocytes injected with fetal $\alpha1\beta1\delta\gamma$ and adult $\alpha1\beta1\delta\varepsilon$ nicotinic acetylcholine receptors, indicating the expression of these nicotinic acetylcholine receptors in this system (Fig. 1B). Quercetin itself had no effect in oocytes expressing the $\alpha1\beta1\delta\gamma$ and $\alpha1\beta1\delta\varepsilon$ nicotinic acetylcholine receptors at a holding potential of −80 mV (Fig. 1B). However, the co-treatment of quercetin (100 μM) with acetylcholine (100 μM) for 30 s slightly inhibited I_{ACh} by 13.4±3.5 and 12.1±2.1% in oocytes expressing the $\alpha1\beta1\delta\gamma$ and $\alpha1\beta1\delta\varepsilon$ nicotinic acetylcholine receptors, respectively (Fig. 2, n=10∼13 from three different frogs). Interestingly, the pre-treatment of quercetin (100 μM) induced a much larger inhibition of I_{ACh}. Thus, quercetin inhibited I_{ACh} by 83.3±5.0 and 80.1±5.3% for the $\alpha1\beta1\delta\gamma$ and $\alpha1\beta1\delta\varepsilon$ nicotinic acetylcholine receptors, respectively (Fig. 2, $^*p<0.005$, compared to co-treatment of quercetin). Since pre-treatment of quercetin induced further inhibition of I_{ACh}, we first treated quercetin for 30 s and followed by 30 s of acetylcholine alone for next 30 s to make sure whether there are any inhibitory effects of quercetin by this protocol. We found that quercetin alone treatment did not show any effects on the followed I_{ACh} (data not shown). The inhibition of I_{ACh} by quercetin in oocytes expressing the $\alpha1\beta1\delta\gamma$ and $\alpha1\beta1\delta\varepsilon$ nicotinic acetylcholine receptors was reversible with a negligible desensitization (Fig. 2A, B). Thus, these results showed that quercetin could also regulate heteromeric as well as homomeric nicotinic acetylcholine receptor channel activities. In addition, we did not observe any difference in quercetin-mediated regulations of fetal and adult form of muscle-type of nicotinic acetylcholine receptor channel activity.

Concentration-dependent effect of quercetin on I_{ACh} in oocytes expressing the $\alpha1\beta1\delta\varepsilon$ nicotinic acetylcholine receptor

Since pre-treatment of quercetin induced further inhibition on I_{ACh} in oocytes expressing the muscle-type of nicotinic acetylcholine receptor, compared to co-treatment, in the next experiments we examined the effects of quercetin on I_{ACh} after pre-treatment of quercetin in the adult form of the receptor. In concentration-response experiments, pre-treatment of quercetin for 30s inhibited I_{ACh} in a con-

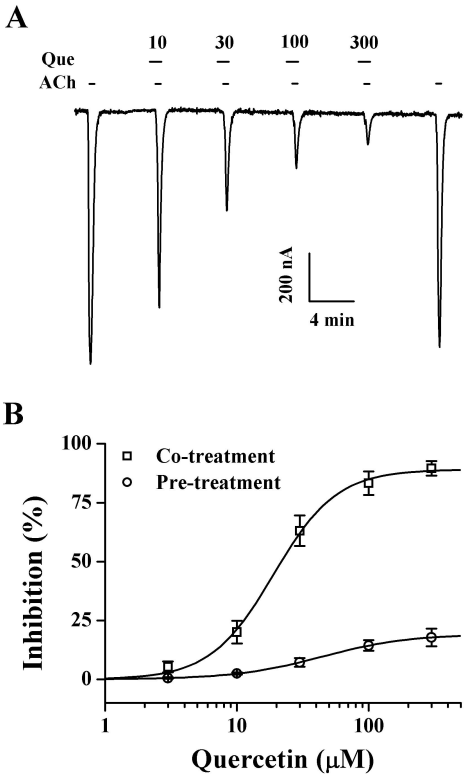

Fig. 3. Dose-dependent effect of quercetin on I_{ACh} in oocytes expressing $\alpha 1\beta 1\delta\varepsilon$ nicotinic acetylcholine receptors. (A) Acetylcholine (100 μM) was first applied and then acetylcholine was pre-treated with different concentration of quercetin. Thus, co-treatment of quercetin with acetylcholine and pre-treatment of quercetin before acetylcholine application inhibited I_{ACh}. The resting membrane potential of oocytes was about −35 mV and oocytes were voltage-clamped at a holding potential of −80 mV prior to drug application. Traces are representative of six separate oocytes from three different frogs. (A) I_{ACh} in oocytes expressing $\alpha 1\beta 1\delta\varepsilon$ nicotinic acetylcholine receptors was elicited at −80 mV holding potential with indicated time in the presence of 100 μM acetylcholine and then the indicated concentration of quercetin was pre-treated for 30 s before acetylcholine. Traces are representative of 6∼9 separate oocytes from three different frogs. (B) Percent inhibition by quercetin of I_{ACh} was calculated from the average of the peak inward current elicited by acetylcholine alone before quercetin and the peak inward current elicited by acetylcholine alone after co-treatment of quercetin with acetylcholine and pre-treatment of quercetin before acetylcholine application. The continuous line shows the curve fitted according to the equation. $y/y_{max}=[Quercetin]/([Quercetin]+K_{1/2})$, where y_{max}, the maximum inhibition (89.1±2.2% and 19.3±1.3 from pre-treatment and co-treatment, respectively, mean±S.E.M.) and $K_{1/2}$ is the concentration for half-maximum inhibition (18.9±1.3 and 44.3±1.1 μM from pre-treatment and co-treatment, respectively, mean±S.E.M.), and [Quercetin] is the concentration of quercetin. Each point represents the mean±S.E.M. (n=9∼12 from three different frogs).

centration-dependent manner in oocytes expressing the $\alpha 1\beta 1\delta\varepsilon$ nicotinic acetylcholine receptor (Fig. 3). Pre-treatment of quercetin inhibited I_{ACh} by 5.3±2.4, 20.1±4.8, 163.1±6.5, 83.3±4.9 and 89.6±3.1% at 3, 10, 30, 100 and 300 μM, respectively, in oocytes expressing the $\alpha 1\beta 1\delta\varepsilon$ nicotinic acetylcholine receptor. The IC_{50} of I_{ACh} was 18.9±1.2 μM for pre-treatment in oocytes expressing the $\alpha 1\beta 1\delta\varepsilon$ nicotinic acetylcholine receptor (n=10 or 11, from three different frogs).

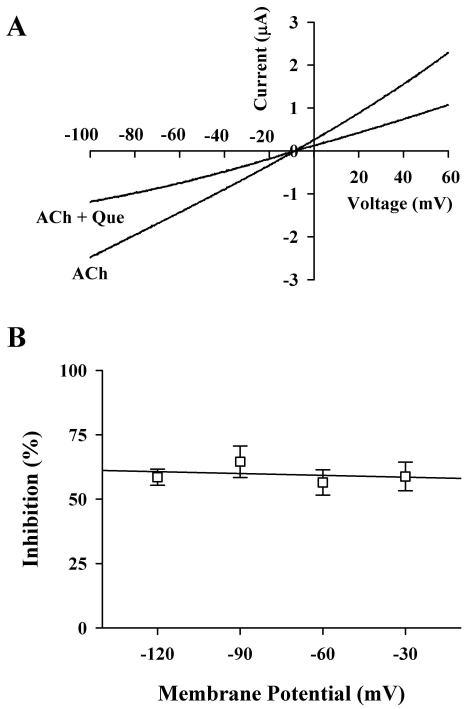

Fig. 4. Current-voltage relationship and voltage-independent inhibition by quercetin. (A) Current-voltage relationships of I_{ACh} inhibition by quercetin in $\alpha 1\beta 1\delta\varepsilon$ nicotinic acetylcholine receptors. Representative current-voltage relationships were obtained using voltage ramps of −100 to +60 mV for 300 ms at a holding potential of −80 mV. Voltage steps were applied before and after application of 100 μM acetylcholine in the absence or presence of 20 μM quercetin. (B) Voltage-independent inhibition of I_{ACh} in the $\alpha 1\beta 1\delta\varepsilon$ nicotinic acetylcholine receptors by quercetin. The values were obtained from the receptors in the absence or presence of 20 μM quercetin at the indicated membrane holding potentials.

Current-voltage relationship and voltage-independent inhibition in oocytes expressing $\alpha 1\beta 1\delta\varepsilon$ nicotinic acetylcholine receptors by quercetin

In experiments exploring the current-voltage (I-V) relationship, the membrane potential was held at −80 mV, and a voltage ramp was applied from −100 to +60 mV for 300 ms. Leakage correction was executed by subtraction of the I-V curve obtained by the same voltage protocol before the application of acetylcholine. The application of acetylcholine to the bathing medium induced a mainly inward current at negative voltages and an outward current at positive voltages (Fig. 4A). Pre-treatment of quercetin before acetylcholine decreased both inward and outward currents. The reversal potentials were −8.8±1.3 mV and −7.5±2.3 mV with application of acetylcholine alone and pre-treatment of quercetin with acetylcholine in oocytes expressing the $\alpha 1\beta 1\delta\varepsilon$ nicotinic acetylcholine receptor. The pre- treatment of quercetin with acetylcholine did not affect $\alpha 1\beta 1\delta\varepsilon$ nicotinic acetylcholine receptor channel properties; quercetin did not alter the reversal potential of the $\alpha 1\beta 1\delta\varepsilon$ nicotinic acetylcholine receptor (Fig. 4A). In addition, the inhibitory effect of

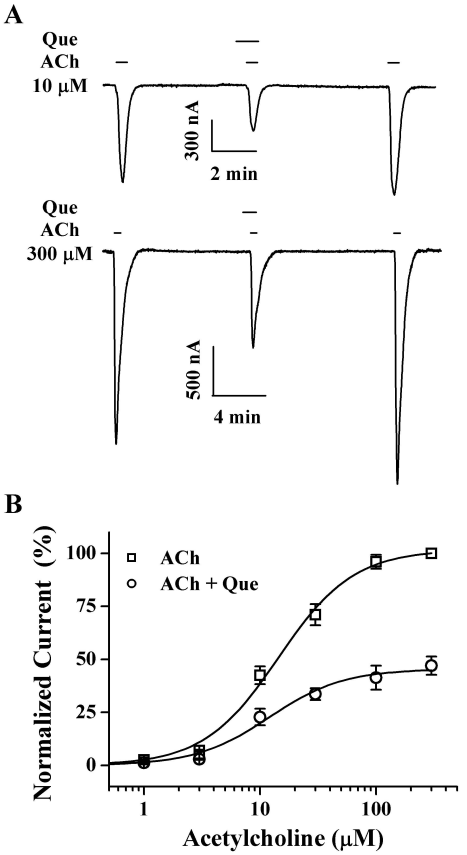

Fig. 5. Concentration-dependent effects of acetylcholine on quercetin-mediated inhibition of I_{ACh}. (A) The representative traces were obtained from $\alpha 1\beta 1\delta\varepsilon$ nicotinic acetylcholine receptors expressed in oocytes. I_{ACh} of the upper and lower panels were elicited from concentration of 10 μM and 300 μM acetylcholine at a holding potential of −80 mV, respectively. (B) Concentration-response relationships for acetylcholine in the $\alpha 1\beta 1\delta\varepsilon$ nicotinic acetylcholine receptors applied with acetylcholine (1∼300 μM) alone or with acetylcholine plus pre-application of 20 μM quercetin. The I_{ACh} of oocytes expressing the $\alpha 1\beta 1\delta\varepsilon$ nicotinic acetylcholine receptors was measured using the indicated concentration of acetylcholine in the absence (□) or presence (○) of 20 μM quercetin. Oocytes were exposed to ACh alone or to ACh with quercetin. Oocytes were voltage-clamped at a holding potential of −80 mV. Each point represents the mean±S.E.M. (n=8∼12/group).

quercetin (20 μM) on I_{ACh} was independent of the membrane-holding potential (Fig. 4B). Quercetin inhibited acetylcholine (100 μM)-mediated inward current by 58.6±3.2, 64.6±6.2, 56.5±4.9 and 58.8±5.4% at membrane-holding potentials of −120, −90, −60 and −30 mV, respectively, in oocytes expressing the $\alpha 1\beta 1\delta\varepsilon$ nicotinic acetylcholine receptor (n=8∼11, from three different frogs). We also examined quercetin effects on different concentration of acetylcholine (10 or 30 μM)-mediated inward current at various membrane-holding potentials. We found that the inhibitory effects of quercetin on I_{ACh} were not significantly changed in these concentrations of acetylcholine (data not shown).

Non-competitive inhibition of $\alpha 1\beta 1\delta\varepsilon$ nicotinic acetylcholine receptor channel currents by quercetin

To study further the mechanism by which quercetin inhibits I_{ACh} in oocytes expressing $\alpha 1\beta 1\delta\varepsilon$ nicotinic acetylcholine receptors, we analyzed the effect of 20 μM quercetin on I_{ACh} evoked by different acetylcholine concentrations in oocytes expressing $\alpha 1\beta 1\delta\varepsilon$ nicotinic acetylcholine receptors (Fig. 5). Pre-treatment of quercetin with different concentrations of acetylcholine did not shift the dose-response curve of acetylcholine to the right (ED_{50}, from 14.4±1.7 to 12.1±2.5 μM and Hill coefficient, from 1.34 to 1.36) in oocytes expressing $\alpha 1\beta 1\delta\varepsilon$ nicotinic acetylcholine receptors, indicating that quercetin regulates $\alpha 1\beta 1\delta\varepsilon$ nicotinic acetylcholine receptor channel activity in a non-competitive manner (n=8∼12 from three different frogs) (Fig. 5).

The axon terminus of motor neurons of the spinal cord forms a kind of synapse with motor end plate, which is called a neuromuscular junction. The neuromuscular junction is a highly-excitable region of muscle fiber plasma membrane responsible for initiation of action potential across the muscle's surface, resulting in the muscle contraction. In vertebrates, acetylcholine released from the axon terminus of a motor neuron binds to the muscle-type nicotinic acetylcholine receptors in the motor end plate to initiate fast signals for muscle contraction. Thus, since muscle-type nicotinic acetylcholine receptors play an important role for skeletal and respiration-related muscle contraction and relaxation, these receptors are one of main targets of drugs and toxins. In previous reports, flavonoids derived from plants or tea extracts were demonstrated to affect acetylcholine release, muscle contraction and neuromuscular junction activity [14-18]. However, relatively little is known how flavonoids including quercetin regulate muscle-type nicotinic acetylcholine receptor channel activity.

In the present study, we investigated the effects of quercetin on fetal and adult muscle-type of nicotinic acetylcholine receptor channel activity. Four major findings are reported. First, pre-treatment rather than co-treatment of quercetin induced much larger inhibitions of I_{ACh} in both receptors. Second, there was no difference between fetal and adult muscle-type of nicotinic acetylcholine receptors in quercetin-mediated inhibition of I_{ACh}. Third, pre-treatment of quercetin inhibited I_{ACh} in oocytes expressing muscle nicotinic acetylcholine receptor in reversible and concentration-dependent manner. Fourth, the inhibition of I_{ACh} by pre-treatment of quercetin occurred in a voltage-independent and non-competitive manner in oocytes expressing $\alpha 1\beta 1\delta\varepsilon$ nicotinic acetylcholine receptors, indicating that quercetin could also regulate the muscle-type nicotinic acetylcholine receptor channel activity.

In previous studies, we demonstrated that quercetin regulates $\alpha 3\beta 4$, $\alpha 7$ or $\alpha 9\alpha 10$ nicotinic acetylcholine receptor-gated ion currents [22-24]. For example, quercetin inhibited heteromeric $\alpha 3\beta 4$ and $\alpha 9\alpha 10$ nicotinic acetylcholine receptor channel activities, whereas quercetin enhanced homomeric $\alpha 7$ nicotinic acetylcholine receptors, indicating a differential effect of quercetin on nicotinic acetylcholine receptor regulations. In the present study, we found that quercetin inhibited I_{ACh} in *Xenopus* oocytes expressing fetal and adult muscle-type nicotinic acetylcholine receptors. Interestingly, we found that quercetin exhibited a distinguishing action in the inhibition muscle-type of nic-

otinic acetylcholine receptors. Quercetin showed much larger inhibition of I_{ACh} after pre-treatment of quercetin in fetal and adult nicotinic acetylcholine receptors compared to other subtypes of nicotinic acetylcholine receptors examined (Fig. 2, 3), indicating that quercetin-mediated regulation of muscle-type nicotinic acetylcholine receptor might be indirectly achieved through phosphorylation or dephosphorylation of the muscle-type nicotinic acetylcholine receptor. Since quercetin did not show any significant differences in the inhibition of I_{ACh} between the adult and fetal type receptors, quercetin does not interact with the γ or ε subunits to inhibit I_{ACh}.

From the present results, however, it is unclear exactly how quercetin acts to inhibit I_{ACh} in oocytes expressing muscle-type nicotinic acetylcholine receptors. One possible mechanism is that quercetin may act as open channel blocker of muscle type nicotinic acetylcholine receptors. However, the inhibitory effect of quercetin on I_{ACh} in oocytes expressing $\alpha 1\beta 1\delta\varepsilon$ nicotinic acetylcholine receptors was not voltage-dependent (Fig. 4). These results suggest that quercetin may be not an open channel blocker because common open channel blockers are strongly voltage dependent [25-27].

Another possibility is that quercetin may act as a competitive inhibitor by inhibiting acetylcholine binding to its binding site(s) in muscle-type nicotinic acetylcholine receptors. In competition experiments, we observed that the inhibitory effect of quercetin on I_{ACh} was not affected by increasing concentrations of acetylcholine in oocytes expressing adult $\alpha 1\beta 1\delta\varepsilon$ nicotinic acetylcholine receptors without changing the Hill coefficient (Fig. 5). Thus, these results indicate that quercetin interaction site(s) with adult $\alpha 1\beta 1\delta\varepsilon$ nicotinic acetylcholine receptor might not be related to acetylcholine binding sites and γ or ε subunit as a non-competitive inhibitor, which is similar to results obtained from $\alpha 3\beta 4$ and $\alpha 9\alpha 10$ nicotinic acetylcholine receptors [23,24].

The flavonoid genistein inhibits $\alpha 7$ nicotinic acetylcholine receptor channel activity in oocytes expressing $\alpha 7$ nicotinic acetylcholine receptor [28]. The flavone nobiletin inhibits catecholamine release by acetylcholine, but stimulates catecholamine release via activation of Ca^{2+} channels or Na^{+}/Ca^{2+} exchangers [29]. In addition, another study reported the quercetin-mediated inhibition of endplate currents at the mouse neuromuscular junction [19]. Presently, we also found that quercetin inhibited muscle-type nicotinic acetylcholine receptor channel currents. Taken together, the data are consistent with the idea that flavonoids including quercetin might have regulatory effects on subsets of nicotinic acetylcholine receptors, which are in central or peripheral nervous systems.

In summary, we found that quercetin inhibited I_{ACh} in oocytes expressing human muscle-type of nicotinic acetylcholine receptors. Since muscle-type of nicotinic acetylcholine receptors are closely related with functions of neuromuscular junction such as muscle contraction, the inhibitory effects of quercetin on I_{ACh} in oocytes expressing human muscle-type of nicotinic acetylcholine receptor might provide a molecular basis for explanation of the neuropharmacological effects of quercetin.

ACKNOWLEDGEMENTS

This work was supported by the Priority Research Centers Program through the National Research Foundation of Korea (NRF), funded by the Ministry of Education, Science and Technology (2009-0093824) (KRF-2006-005-J03403) and by the SMART Research Professor Program of Konkuk University.

REFERENCES

1. **Jensen ML, Schousboe A, Ahring PK.** Charge selectivity of the Cys-loop family of ligand-gated ion channels. *J Neurochem.* 2005;92:217-225.
2. **Dani JA, Bertrand D.** Nicotinic acetylcholine receptors and nicotinic cholinergic mechanisms of the central nervous system. *Annu Rev Pharmacol Toxicol.* 2007;47:699-729.
3. **Boulter J, Evans K, Goldman D, Martin G, Treco D, Heinemann S, Patrick J.** Isolation of a cDNA clone coding for a possible neural nicotinic acetylcholine receptor alpha-subunit. *Nature.* 1986;319:368-374.
4. **Boulter J, Connolly J, Deneris E, Goldman D, Heinemann S, Patrick J.** Functional expression of two neuronal nicotinic acetylcholine receptors from cDNA clones identifies a gene family. *Proc Natl Acad Sci USA.* 1987;84:7763-7767.
5. **Karlin A.** Emerging structure of the nicotinic acetylcholine receptors. *Nat Rev Neurosci.* 2002;3:102-114.
6. **Gotti C, Clementi F.** Neuronal nicotinic receptors: from structure to pathology. *Prog Neurobiol.* 2004;74:363-396.
7. **Boorman JP, Groot-Kormelink PJ, Sivilotti LG.** Stoichiometry of human recombinant neuronal nicotinic receptors containing the b3 subunit expressed in Xenopus oocytes. *J Physiol.* 2000; 529:565-577.
8. **Couturier S, Bertrand D, Matter JM, Hernandez MC, Bertrand S, Millar N, Valera S, Barkas T, Ballivet M.** A neuronal nicotinic acetylcholine receptor subunit (alpha 7) is developmentally regulated and forms a homo-oligomeric channel blocked by alpha-BTX. *Neuron.* 1990;5:847-856.
9. **Elgoyhen AB, Johnson DS, Boulter J, Vetter DE, Heinemann S.** Alpha 9: an acetylcholine receptor with novel pharmacological properties expressed in rat cochlear hair cells. *Cell.* 1994;79:705-715.
10. **Gotti C, Hanke W, Maury K, Moretti M, Ballivet M, Clementi F, Bertrand D.** Pharmacology and biophysical properties of alpha 7 and alpha 7-alpha 8 alpha-bungarotoxin receptor subtypes immunopurified from the chick optic lobe. *Eur J Neurosci.* 1994;6:1281-1291.
11. **Mishina M, Takai T, Imoto K, Noda M, Takahashi T, Numa S, Methfessel C, Sakmann B.** Molecular distinction between fetal and adult forms of muscle acetylcholine receptor. *Nature.* 1986;321:406-411.
12. **Karlin A.** Emerging structure of the nicotinic acetylcholine receptors. *Nat Rev Neurosci.* 2002;3:102-114.
13. **Havsteen BH.** The biochemistry and medical significance of the flavonoids. *Pharmacol Ther.* 2002;96:67-202.
14. **Satoh E, Ishii T, Shimizu Y, Sawamura S, Nishimura M.** The mechanism underlying the protective effect of the thearubigin fraction of black tea (Camellia sinensis) extract against the neuromuscular blocking action of botulinum neurotoxins. *Pharmacol Toxicol.* 2002;90:199-202.
15. **Basu S, Chaudhuri T, Chauhan SP, Das Gupta AK, Chaudhury L, Vedasiromoni JR.** The theaflavin fraction is responsible for the facilitatory effect of black tea at the skeletal myoneural junction. *Life Sci.* 2005;76:3081-3088.
16. **Tong JJ.** Mitochondrial delivery is essential for synaptic potentiation. *Biol Bull.* 2007;212:169-175.
17. **Chiang HC, Iijima K, Hakker I, Zhong Y.** Distinctive roles of different beta-amyloid 42 aggregates in modulation of synaptic functions. *FASEB J.* 2009;23:1969-1977.
18. **Moustafa AM, Ahmed SH, Nabil ZI, Hussein AA, Omran MA.** Extraction and phytochemical investigation of Calotropis procera: effect of plant extracts on the activity of diverse muscles. *Pharm Biol.* 2010;48:1080-1190.
19. **Re L, Barocci S, Capitani C, Vivani C, Ricci M, Rinaldi L,**

Paolucci G, Scarpantonio A, León-Fernández OS, Morales MA. Effects of some natural extracts on the acetylcholine release at the mouse neuromuscular junction. *Pharmacol Res.* 1999; 39:239-245.

20. **Lee BH, Jeong SM, Lee JH, Kim JH, Yoon IS, Lee JH, Choi SH, Lee SM, Chang CG, Kim HC, Han Y, Paik HD, Kim Y, Nah SY.** Quercetin inhibits the 5-hydroxytryptamine type 3 receptor-mediated ion current by interacting with pre-transmembrane domain I. *Mol Cells.* 2005;20:69-73.
21. **Lee BH, Lee JH, Yoon IS, Lee JH, Choi SH, Pyo MK, Jeong SM, Choi WS, Shin TJ, Lee SM, Rhim H, Park YS, Han YS, Paik HD, Cho SG, Kim CH, Lim YH, Nah SY.** Human glycine alpha1 receptor inhibition by quercetin is abolished or inversed by alpha267 mutations in transmembrane domain 2. *Brain Res.* 2007;1161:1-10.
22. **Lee BH, Choi SH, Shin TJ, Pyo MK, Hwang SH, Kim BR, Lee SM, Lee JH, Kim HC, Park HY, Rhim H, Nah SY.** Quercetin enhances human α7 nicotinic acetylcholine receptor-mediated ion current through interactions with Ca(2+) binding sites. *Mol Cells.* 2010;30:245-253.
23. **Lee BH, Choi SH, Shin TJ, Pyo MK, Hwang SH, Lee SM, Paik HD, Kim HC, Nah SY.** Effects of quercetin on α9α10 nicotinic acetylcholine receptor-mediated ion currents. *Eur J Pharmacol.* 2011;650:79-85.
24. **Lee BH, Hwang SH, Choi SH, Shin TJ, Kang J, Lee SM, Nah SY.** Quercetin inhibits alpha3beta4 nicotinic acetylcholine receptor-mediated ion currents expressed in xenopus oocytes. *Korean J Physiol Pharmacol.* 2011;15:17-22.
25. **Sine SM, Taylor P.** Local anesthetics and histrionicotoxin are allosteric inhibitors of the acetylcholine receptor. Studies of clonal muscle cells. *J Biol Chem.* 1982;257:8106-8114.
26. **Heidmann T, Oswald RE, Changeux JP.** Multiple sites of action for noncompetitive blockers on acetylcholine receptor rich membrane fragments from torpedo marmorata. *Biochemistry.* 1983;22:3112-3127.
27. **Arias HR.** Luminal and non-luminal non-competitive inhibitor binding sites on the nicotinic acetylcholine receptor. *Mol Membr Biol.* 1996;13:1-17.
28. **Grønlien JH, Ween H, Thorin-Hagene K, Cassar S, Li J, Briggs CA, Gopalakrishnan M, Malysz J.** Importance of M2-M3 loop in governing properties of genistein at the α7 nicotinic acetylcholine receptor inferred from α7/5-HT3A chimera. *Eur J Pharmacol.* 2010;647:37-47.
29. **Zhang H, Toyohira Y, Ueno S, Shinohara Y, Itoh H, Furuno Y, Yamakuni T, Tsutsui M, Takahashi K, Yanagihara N.** Dual effects of nobiletin, a citrus polymethoxy flavone, on catecholamine secretion in cultured bovine adrenal medullary cells. *J Neurochem.* 2010;114:1030-1038.