QUERCETIN SUBUNIT SPECIFICALLY REDUCES GLYR-MEDIATED CURRENT IN RAT HIPPOCAMPAL NEURONS

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Abstract—Quercetin is a substance of low molecular weight found in vascular plants with a wide range of biological activities including antioxidative and anti-inflammatory activities. In the present study, the effects of quercetin on native glycine receptors (GlyRs) in cultured rat hippocampal neurons were investigated using a whole-cell patch–clamp technique. Quercetin reversibly and concentration-dependently depressed glycine-induced current (I_{Gly}), with an IC_{50} of 10.7 ± 0.24 μM and a Hill coefficient of 1.08 ± 0.12. Quercetin depressed maximum I_{Gly} and significantly changed the EC_{50} for glycine and the Hill coefficient. Kinetic analysis indicated that quercetin accelerated the rates of desensitization. Interestingly, after the end of glycine with quercetin coapplication, a transient rebound occurred. The quercetin effects also displayed voltage-dependence, being greater at positive membrane potentials. These effects suggested that quercetin may act as an open channel blocker. Furthermore, in the sequential application protocol, quercetin inhibited the peak amplitude of I_{Gly} to a macroscopic degree while slowing GlyR desensitization. These effects implied that quercetin has a depressant effect independent of GlyR channel’s opening, which maybe caused by an allosteric mechanism. Strikingly, quercetin inhibited the amplitude of recombinant-induced current mediated by α2α2β3- and α3β3- GlyRs but had no effects on α1- and αβ1-GlyRs that were expressed in HEK293T cells. We also investigated the effects of quercetin on I_{Gly} in spinal neurons during development in vitro. The extent of blockade by quercetin on I_{Gly} was slighter in spinal neurons than in hippocampal neurons in a development-dependent manner. Taken together, our results suggest that quercetin has possible effects in information processing within a neuronal network by inhibition of I_{Gly} and may be useful as a pharmacological probe for identifying the subunit types of GlyRs. © 2007 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: flavonoid, glycine receptor, whole-cell patch–clamp recording, desensitization, hippocampus.

Flavonoids are a class of natural polyphenolics that are ubiquitous in plants and therefore are ingested by humans and animals with their regular foods. Among the many different flavonoids present in plants, quercetin is the most abundant (Herrmann, 1988; Hertog et al., 1993; Goldbohm et al., 1996). Previous studies showed that quercetin exerts multiple pharmacological effects on mammalian cells and tissues, such as antioxidative capabilities, anti-inflammatory activities (Ross and Kasum, 2002), promoting vasodilatation and platelet disaggregation (Beretz et al., 1982), and blocking several key enzymes in vitro (Middleton and Kandaswami, 1993; Middleton and Kandaswami, 1994). In addition, they seem to possess anticarcinogenic properties (Ranelletti et al., 1992; Kuo, 1996).

In the CNS, the effects of quercetin and its related compounds on ion currents in neurons have been extensively studied. They have various neuropharmacological effects such as analgesia, effects on motility and sleep (Speroni and Minghetti, 1988; Picq et al., 1991), modulation of neuronal oxidative metabolism (Oyama et al., 1994), and proconvulsant, anticonvulsant, sedative and anxiolytic effects (Marder et al., 1995; Medina et al., 1997; Griebel et al., 1999). Recent electrophysiological studies demonstrate that quercetin antagonizes the responses mediated by GABA type A (GABA_{A}) and depresses GABA type C (GABA_{C}) receptors (Goutman et al., 2003) and depresses the ionic currents mediated by human homomeric GABA_{P} receptors expressed in Xenopus oocytes (Goutman and Calvo, 2004). In addition, the total flavone of Abelomoschus manihot L. medic can inhibit the N-methyl-D-aspartate receptor-mediated current in cultured rat hippocampal neurons (Cheng et al., 2006). Furthermore, genistein, another flavonoid, can directly block glycine receptors (GlyRs) of neurons isolated from the rat ventral tegmental area (Zhu et al., 2003).

Although electrophysiological results show that quercetin can regulate a number of ligand-gated ion channels including the nicotinic acetylcholine receptor, 5-HT receptors, glutamate kainate receptor, GABA_{A} and GABA_{C} receptors (Lee et al., 2002; Goutman et al., 2003; Goutman and Calvo, 2004), the mechanisms underlying the actions of quercetin on the glycine receptor were not studied before. Thus, in the present study, we decided to examine the modulatory effects of quercetin on native and recombinant GlyRs by using the whole-cell patch–clamp recording technique.

**EXPERIMENTAL PROCEDURES**

**Cell culture**

The use and care of animals in the present study followed the guidelines and protocols approved by the Institutional Animal Care and Use Committee of the University of Science and Technology
of China. All efforts were made to minimize the number of animals used and their suffering. The neurons used for cell culture were dissociated from the hippocampus and spinal cord of Wistar rats (postnatal day 0). The neonatal rat was deeply anesthetized with isoﬂurane, transferred to chilled Hank’s buffered salt solution and then decapitated. The whole brain or entire spinal cord was removed and placed in iced Hank’s solution. The hippocampus or spinal cord was collected under a dissection microscope and neurons were isolated by a standard enzyme treatment protocol. Briefly, the tissues were incubated in saline with 0.25% trypsin (Sigma Chemical Co., St Louis, MO, USA) for 15 min at 37 °C and mechanically dissociated by trituration with a pipette tip in Dulbecco’s modiﬁed Eagle’s medium (DMEM) (Gibco, Los Angeles, CA, USA). The tissue was then plated (1.5 × 10^6 cells/ml) on poly-L-lysine (Sigma)-coated cover glasses. The isolated neurons were grown in DMEM with L-glutamine plus 10% fetal bovine serum (FBS), 10% F-12 nutrient mixture, 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco) for 24 h. Then, neuron-basal medium (1.5 ml) with 2% B27 (Gibco) was replaced every 3–4 days. The cultures were treated with 5-ﬂuoro-5`-deoxyuridine (20 μg/ml, Sigma) on the 4th day after plating to block cell division of nonneuronal cells, which helped to stabilize the cell population. The cultures were maintained at 37 °C in a 5% CO2 humidified atmosphere. Cells were used for electrophysiological recordings 7–20 days after plating.

Transfection
All conﬁgurations were expressed in HEK293T cells. HEK293T cells were cultured at 37 °C in a humidified atmosphere of 5% CO2 and 95% air. The cells were maintained in DMEM (Gibco) supplemented with 2 mM L-glutamine, 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco). HEK293 cells were transfected by lipofection using 1 μg of cDNA and 2 μl of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) per 3.0 × 10^5 cells plated on 35-mm culture dishes. Cotransfection with a green ﬂuorescent protein expression vector, pEGFP, was used to enable identiﬁcation of transfected cells for patch clamping by monitoring the ﬂuorescence. When more than one of the glycine subunits was expressed, the multisubunits were cotransfected at a 1:1 ratio. Electrophysiological measurements were performed 16–48 h after transfection. The α2 subunit was kindly provided by Dr. Heinrich Betz (Department of Neurochemistry, Max-Planck-Institute for Brain Research, Frankfurt, Germany). The α3 subunit was donated by Dr. Jochen Meier (Department of Developmental Physiology, Johannes-Mueller Center of Physiology, Charité University Medicine, Berlin, Germany). The pEGFP was from Dr. Jian-hong Luo (Faculty of Medicine, Zhejiang University, Hangzhou, China).

Solutions and drugs
The standard external solution contained (in mM) 150 NaCl, 5 KCl, 1 MgCl2, 2 CaCl2, 10 glucose, and 10 Hepes. The pH was adjusted to 7.4 with Tris base. The osmolarity of the solutions was adjusted to 310–320 mosM/l with sucrose and a microosmometer (Advanced Instruments, Inc., Norwood, MA, USA; Model 3300). The pipette solution for whole-cell patch–clamp recording contained (in mM) 120 KCl, 30 NaCl, 1 MgCl2, 0.5 CaCl2, 5 EGTA, 2 Mg-ATP, and 10 Hepes. The pH of the internal solution was adjusted to 7.2 with Tris base. When the relationship between the effect of quercetin on the glycine-induced current (Igly) and the membrane potential was examined, voltage-activated Na+, Ca2+, and K+ channels were blocked by the addition of 0.3 μM tetrodotoxin and 0.2 mM CdCl2 to the standard external solution and by replacing K+ with Cs+ in the pipette solution. The drugs were predissolved in ion-free water or dimethylsulfoxide (DMSO) and then diluted to the ﬁnal concentrations in the standard external solution just before use. At the maximum concentration applied (0.1% (v/v)), DMSO neither induced nor blocked membrane currents and had no effect on the glycine response. Drugs were applied using a rapid application technique termed the “Y-tube” method throughout the experiments. This system allows a complete exchange of external solution surrounding a neuron within 20 ms.

Electrophysiological recordings
The electrophysiological recordings were performed in conventional whole-cell patch–recording conﬁguration under voltage-clamp mode. Patch pipettes were pulled from glass capillaries with an outer diameter of 1.5 mm on a two-stage puller (PP-830, Narishige, Tokyo, Japan). The resistance between the recording electrode ﬁlled with pipette solution and the reference electrode was 4–6 MΩ. Membrane currents were measured using a patch–clamp ampliﬁer (Axon 200B, Axon Instruments, Sunnyvale, CA, USA), sampled using a Digidata 1320A interface connected to a personal computer, and analyzed with Clampex and Clampfit software (Version 8.1, Axon Instruments). In most experiments, 70–90% series resistance was compensated. The membrane potential was held at −60 mV throughout the experiment. All experiments were carried out at room temperature (22–25 °C).

Data analysis
Clampﬁt software (Version 8.1, Axon Instruments) and Origin (Version 7.5, OriginLab, Natnick, MA, USA) were used for data analysis. The continuous theoretical curves for concentration–response relationships of glycine in the presence or absence of quercetin were drawn according to a modiﬁed Michaelis–Menten equation by the method of least-squares (the Newton–Raphson method) after the amplitude of the response was normalized,

\[ I = I_{\text{max}} \frac{C^h}{(C^h + EC_{50}^h)} \]

where \( I \) is the normalized value of the current, \( I_{\text{max}} \) is the maximal response, \( C \) is the drug concentration, \( EC_{50}^h \) is the concentration that induced the half-maximal response and \( h \) is the apparent Hill coefﬁcient. The curve for the effect of quercetin on \( I_{\text{gly}} \) was ﬁtted using the equation

\[ I = I_{\text{max}} \frac{(IC_{50}^h)^h}{(1 + IC_{50}^h)^h} \]

where \( IC_{50}^h \) represents the concentration that induced the half-maximal inhibitory effect and the others are the same as above. All data are represented as means ± S.E.M. Statistical signiﬁcance between two groups were determined with Student’s t test. One-way analysis of variance was employed for statistical signiﬁcance between multiple groups. Levels of statistical signiﬁcance were assumed as P<0.05. The \( I_{\text{gly}} \) desensitization kinetics was estimated as the 10–90% decay time (ms).

RESULTS
Quercetin inhibited glycine-induced current
Under the present experimental conditions, extracellular application of 100 μM glycine evoked an inward membrane current in nearly all cultured neurons of rat hippocampus at a holding potential (\( V_h \)) of −60 mV. This glycine–induced current was veriﬁed by application of 1 μM strychnine, a selective antagonist of GlyRs that completely abolished the \( I_{\text{gly}} \) (data not shown). Quercetin did not induce any detectable current in these neurons when the drug was applied alone. However, quercetin reversibly reduced the \( I_{\text{gly}} \) in a concentration-dependent manner when it was coapplied with glycine
following pretreatment of the drug for 30 s (Fig. 1A). The averaged IC50 for quercetin inhibition of I_Gly was 10.7 ± 0.24 μM (n=5–8; Fig. 1B), with Hill coefficient values of 1.08 ± 0.12.

**Quercetin inhibited the I_Gly in a noncompetitive manner**

To examine the mechanism by which quercetin inhibited I_Gly, we studied the concentration–response relationship of the I_Gly in the presence and absence of quercetin at a concentration near IC50 (10 μM). Figure 2A shows the sample traces of I_Gly induced by glycine with and without coapplication of 10 μM quercetin. Figure 2B shows that quercetin shifted the concentration–response curve of I_Gly to the right and significantly increased the EC50, which represents the glycine concentration that induced the half-maximal response, from 39.9 ± 5.3 to 118.2 ± 19.9 μM. Furthermore, quercetin both increased the Hill coefficient from 1.54 ± 0.22 to 1.97 ± 0.25 and significantly reduced the maximum response of the I_Gly (n=5–8). Thus, these data suggest that quercetin inhibits the I_Gly in a noncompetitive manner.
The inhibition of quercetin on $I_{\text{Gly}}$ was voltage-dependent

To investigate the relationship between the membrane potential and the inhibitory effect of quercetin on $I_{\text{Gly}}$, the current–voltage relationship of $I_{\text{Gly}}$ was examined using a voltage-ramp protocol in the absence and presence of quercetin (Fig. 3A). The inhibitory effect of quercetin (10 μM) showed obvious voltage dependence over a range of membrane potentials from −60 to +40 mV. Quercetin decreased $I_{\text{Gly}}$ to a significantly greater extent at positive membrane potentials. However, the reversal potentials of $I_{\text{Gly}}$, were not significantly changed by application of 10 μM quercetin (1 ± 1.58 mV vs. 1.3 ± 1.72 mV, n=5, $P>0.05$; Fig. 3B).

The inhibition of quercetin on $I_{\text{Gly}}$ depended on drug application mode

To further explore the mechanism by which quercetin inhibited $I_{\text{Gly}}$, we applied the drugs by three different proto-
cols to the same neurons. In the pretreatment protocol (protocol c), neurons were pretreated with quercetin for 30 s and then quercetin and glycine were applied together; in the sequential application protocol (protocol a), glycine was applied alone immediately after 30-s perfusion of quercetin; in the coapplication protocol (protocol b), quercetin and glycine were applied together without any pretreatment. As shown in Fig. 4A, the inhibition efficacy varied in three drug application protocols. With protocols a, b and c, the relative amplitude of $I_{Gly}$ was significantly reduced to 53±3.4% ($n$=5, $P<0.001$), 66.9±1.7% ($n$=5, $P<0.001$) and 38.4±2.6% ($n$=5, $P<0.001$), respectively (Fig. 4B). Furthermore, there was a significant difference in $I_{Gly}$ amplitudes between the different drug application protocols (protocol a vs. protocol c, $P<0.01$; protocol b versus protocol c, $P<0.001$; Fig. 4B). In addition, the termination of drug application was important for the appearance of the rebound current. When both quercetin and glycine were

![Fig. 3. Effects of quercetin on the current–voltage relationship of $I_{Gly}$. (A) Derived current–voltage relationships with a voltage-ramp protocol applied to $I_{Gly}$ (100 μM) in the absence and presence of 10 μM quercetin. Inhibition of $I_{Gly}$ by quercetin was membrane potential-dependent. (B) Voltage-ramp protocol (from −80 to +40mV, at rate of 120mV/700ms). (C) Histogram shows that the mean reversal potential of $I_{Gly}$ was not significantly changed by quercetin ($n$=5, $P>0.05$). NS indicates no statistical significance.](image-url)
terminated at the same time, a rebound current occurred (Fig. 4Ab, c). However, the rebound current did not appear in the sequential application mode (Fig. 4Aa).

**Quercetin altered GlyR desensitization**

We then studied the decay of $I_{\text{Gly}}$ in the absence and presence of quercetin. As shown in Fig. 5Aa and 5Ba, the current decay time constant ($\tau_{\text{des}10-90\%}$) of $I_{\text{Gly}}$ activated by 100 μM glycine was significantly reduced to $19\pm2\%$ of control in the presence of 10 μM quercetin with the preapplication model ($n=6, P<0.001$). Two mechanisms may underlie quercetin-induced increase of the $I_{\text{Gly}}$ decay rate. In the first, quercetin could act directly on the receptor channel to accelerate desensi-
tization; in the second mechanism quercetin could affect the apparent concentration of glycine and indirectly affect desensitization. To test the two hypotheses, we applied a saturating concentration of glycine (1 mM) and examined current decay with and without 10 μM quercetin (Fig. 5Ac). Using this concentration, quercetin would not significantly change the apparent concentration of glycine; any change of the current decay rate would result from a direct action of quercetin on the desensitization process. As illustrated by Fig. 5Bc1 and 5Bc2, the ratio of $\tau_{\text{des}}$ of $I_{\text{Gly}}$ activated by a saturating concentration of glycine (1 mM) was significantly decreased compared with that activated by glycine at 100 μM (n=6, $P<0.05$) and was further significantly decreased in the presence of 10 μM quercetin (n=6, $P<0.001$). Thus, these data indicate that quercetin has a huge effect on the desensitization process. However, with the sequential application of 10 μM quercetin (Fig. 5Ab, Bb), the $\tau_{\text{des}}$ increased (152±9% of control, n=6, $P<0.001$), indicating the possibility of quercetin slowing GlyR desensitization in the absence of the blockage effect.

Fig. 5. Effects of quercetin on GlyR desensitization. (A) Sample recordings showing the effects of quercetin on GlyR desensitization. (B) Mean ratios of the time constants of decay of $I_{\text{Gly}}$ in the absence and presence of quercetin with different application modes or different glycine concentrations (n=6). *** Significant difference from control, $P<0.001$ and $P<0.05$, respectively. ### $P<0.001$ versus between two bars. All data were normalized to the time constants of decay of control $I_{\text{Gly}}$ (100 μM).
The inhibition of quercetin on the $I_{\text{Gly}}$ was subunit-dependent

To determine the subunit responsible for quercetin-induced inhibition, we expressed homomeric $\alpha_1$, $\alpha_2$, $\alpha_3$- and heteromeric $\alpha_1\beta$, $\alpha_2\beta$, $\alpha_3\beta$-GlyR in HEK293T cells, which did not respond to glycine application before GlyR subunit transfection (data not shown). Meanwhile, the heteromeric $\alpha(1–3)\beta$-GlyR-mediated current was insensitive to $10\mu$M picrotoxin (data not shown). After the cells were preperfused with $10\mu$M quercetin for $30\,s$, $I_{\text{Gly}}$ was induced in the presence of quercetin (Fig. 6A). In agreement with the results in native GlyRs, quercetin reversibly inhibited the amplitude of $I_{\text{Gly}}$ mediated by $\alpha_2$, $\alpha_3$-, $\alpha_2\beta$- and $\alpha_3\beta$-GlyR channels to $34\pm4$, $50\pm4$, $29\pm6$ and $40\pm4\%$, respectively ($n=4–6$, $P<0.001$; Fig. 6B). Interestingly, quercetin had no significant effect on the amplitude of $I_{\text{Gly}}$. 

Fig. 6. Inhibition of $I_{\text{Gly}}$ by quercetin on recombinant GlyRs expressed in HEK293T cells. (A) Sample recording demonstrating the effects of $10\mu$M quercetin on homomeric $\alpha_1$, $\alpha_2$, $\alpha_3$- and heteromeric $\alpha_1\beta$, $\alpha_2\beta$, $\alpha_3\beta$-GlyRs. (B) Quercetin inhibited the peak amplitude of $I_{\text{Gly}}$ mediated by recombinant GlyRs. Each column represents the averaged value ($n=4–6$, ***$P<0.001$ compared with control).
mediated by α1- and α1β-GlyR channels (97±2% of control and 100±5% of control, respectively, n=5, P>0.05).

**Quercetin inhibited glycine-induced current in spinal neurons**

To further confirm the effects of quercetin on I_{Gly} observed above, the expression of functional GlyRs by neonatal rat spinal neurons during development in vitro was investigated. We studied I_{Gly} (100 μM) in the presence and absence of quercetin (10 μM) at 8 and 15 days in vitro (DIV). As illustrated in Fig. 7Aa and 7Ab, quercetin reversibly inhibited I_{Gly} in cultured spinal neurons. Compared with the effects of quercetin in hippocampal neurons (47±5% of control, n=5), the degree of quercetin inhibition of I_{Gly} was significantly weaker in spinal neurons. Furthermore, there was a significant difference between the inhibitory effects of quercetin on I_{Gly} in 8 DIV and 15 DIV and neurons (69±2% of control and 86±5% of control, respectively, n=8; Fig. 7B).

**DISCUSSION**

In the present research, we used the whole-cell patch-clamp technique to study the effects of quercetin on I_{Gly} in cultured

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Fig. 7. Inhibition of I_{Gly} by quercetin on GlyRs expressed in cultured spinal neurons. (A) Sample recording demonstrating the effects of quercetin in neurons at 8 and at 15 DIV (a and b, respectively). (B) Quercetin reduced the peak amplitude of I_{Gly} mediated by the GlyRs expressed in hippocampus neurons and in spinal neurons. Each column represents the averaged value (n=5–8). ** and *** Significant difference between two bars, P<0.05, P<0.01 and P<0.001, respectively.
hippocampal neurons. Our findings revealed that quercetin inhibited $I_{\text{Gly}}$ reversibly in a concentration-dependent manner. Quercetin $IC_{50}$ for GlyRs was about 10.7 $\mu$M, with Hill coefficient values of approximately 1.0 (Fig. 1B). In addition, quercetin produced a rightward shift with a depression of the maximal responses of the concentration–response curve for glycine. After quercetin treatment, significant increases in glycine $EC_{50}$ were observed (Fig. 2B). Consequently, the antagonism exerted by quercetin at GlyRs was noncompetitive.

The time course of quercetin inhibition of GlyRs was very rapid, and the response was completely reversed after washout for about 3 min. These effects are compatible with the fact that quercetin acts directly on GlyRs. The present results show that quercetin reduced the peak amplitude of $I_{\text{Gly}}$ and accelerated $I_{\text{Gly}}$, significant desensitization and that the extent of desensitization increased with the rise in quercetin concentrations (Fig. 1A).

Similar to the effect of genistein on GABA$_A$ and glycine receptor-mediated currents (Huang et al., 1999; Zhu et al., 2003), the presence of a tail is direct evidence of the open channel blocking effect (Fig. 2A). After the washout of quercetin and glycine, the quercetin molecule binding to the block site was removed and undesensitized GlyR might be reactivated by the agonist, which did not have sufficient time to unbind, thereby producing the rebound current. In support of this speculation, the rebound current was only induced by removal of glycine and quercetin (Fig. 4Ab, c), but did not occur in the sequential application mode (Fig. 4Aa). Moreover, the removal of glycine and quercetin at a low concentration ($<10$ $\mu$M) did not induce the rebound current (Fig. 1A), indicating that the quercetin blockage is state- and concentration-dependent. To further examine this possibility, we tested the effect of membrane voltage on the depressant action of quercetin on $I_{\text{Gly}}$. As illustrated in Fig. 3A, the quercetin effect was sensitive to membrane voltage, being greater at positive potential: the voltage on the depressant action of quercetin on $I_{\text{Gly}}$ was further examined on recombinant GlyRs. We expressed homomeric $\alpha_1$, $\alpha_2$, $\alpha_3$- and heteromeric $\alpha_1\beta$, $\alpha_2\beta$, $\alpha_3\beta$-GlyRs in HEK293T cells. The lack of sensitivity to picrotoxin presented in all heteromeric recombinant GlyRs. In the preapplication protocol, quercetin reversibly inhibited the $I_{\text{Gly}}$ mediated by homomeric $\alpha_2$, $\alpha_3$-GlyRs and heteromeric $\alpha_2\beta$, $\alpha_3\beta$-GlyRs, with the highest potency being found at the $\alpha_2\beta$-GlyRs. Corresponding to our result, in situ hybridization analysis shows that mRNAs for GlyR $\beta$, $\alpha_2$, and, to a lesser extent, $\alpha_3$ subunits are expressed in pyramidal and granular cell layers (Malosio et al., 1991; Sato et al., 1991, 1992; Racca et al., 1998). Surprisingly, in contrast with the above-mentioned configurations, $\alpha_1$ subunit containing GlyRs are resistant to quercetin, but the mechanism underlying the isofrom specificity is unclear. The GlyRs $\alpha$ subunits are highly homologous in their membrane-spanning domains and differ at only one position in the pore-forming region M2 ($\alpha_1^{G5254}$, $\alpha_2^{G5261}$, $\alpha_3^{G5254}$). Previous studies demonstrated that this position is critical in determining the ability of cyanotriphenylborate (CTB) to block GlyR currents (Rundstrom et al., 1994). CTB acts as a negatively charged open channel blocker of $\alpha_1$ receptors, and the block is reduced by the position mutation $\alpha_1^{G5254A}$. The equivalent position is of prime importance for other ligand-gated ion channels as well: this position corresponds to the most constricted site, the “central ring,” of the nicotinic acetylcholine channel (Villarroel et al., 1991), and mutating this position confers resistance of GABA$_A$ receptors (GABA$_A$Rs) to picrotoxin (French-Constant et al., 1993). Thus, in speculation, a large pharmacological difference between the $\alpha_1$ subunit containing GlyRs and the $\alpha_2/\alpha_3$ subunit containing GlyRs may account for a primary structure difference of the respective pore-forming region M2: glycine 254 present in $\alpha_1$ is exchanged for an alanine in the $\alpha_2$ and $\alpha_3$ subunits. This speculation is consistent with our mention above that the inhibition of quercetin on $I_{\text{Gly}}$ is mostly due to open-channel block. As previous studies demonstrated, picrotoxin allows homomeric receptors to be distinguished, because it reduces the rate of decay of the current activated by a submaximal and saturating concentration of glycine. Furthermore, in the presence of quercetin, the glycine concentration–response curve shifted to the right with a large change in $EC_{50}$. Thus, the effects of quercetin may be attributed to faster dissociation of glycine from its binding site, even though other possibilities are not excluded. On the other hand, quercetin might allosterically modulate the function of GlyRs, resulting in slower GlyR desensitization. The fact that preperfusion of quercetin prior to the application of glycine prolonged the time constant of $I_{\text{Gly}}$ decay indicates the possibility that quercetin slows GlyR desensitization.

**CONCLUSION**

In summary, the effects of quercetin may be caused by two mechanisms: the putative channel-blocking effect and allosteric modulation. To determine the isofrom specificity, the effect of quercetin on $I_{\text{Gly}}$ was further examined on recombinant GlyRs. We expressed homomeric $\alpha_1$, $\alpha_2$, $\alpha_3$- and heteromeric $\alpha_1\beta$, $\alpha_2\beta$, $\alpha_3\beta$-GlyRs in HEK293T cells. The lack of sensitivity to picrotoxin presented in all heteromeric recombinant GlyRs. In the preapplication protocol, quercetin reversibly inhibited the $I_{\text{Gly}}$ mediated by homomeric $\alpha_2$, $\alpha_3$-GlyRs and heteromeric $\alpha_2\beta$, $\alpha_3\beta$-GlyRs, with the highest potency being found at the $\alpha_2\beta$-GlyRs. Corresponding to our result, in situ hybridization analysis shows that mRNAs for GlyR $\beta$, $\alpha_2$, and, to a lesser extent, $\alpha_3$ subunits are expressed in pyramidal and granular cell layers (Malosio et al., 1991; Sato et al., 1991, 1992; Racca et al., 1998). Surprisingly, in contrast with the above-mentioned configurations, $\alpha_1$ subunit containing GlyRs are resistant to quercetin, but the mechanism underlying the isofrom specificity is unclear. The GlyRs $\alpha$ subunits are highly homologous in their membrane-spanning domains and differ at only one position in the pore-forming region M2 ($\alpha_1^{G5254}$, $\alpha_2^{G5261}$, $\alpha_3^{G5254}$). Previous studies demonstrated that this position is critical in determining the ability of cyanotriphenylborate (CTB) to block GlyR currents (Rundstrom et al., 1994). CTB acts as a negatively charged open channel blocker of $\alpha_1$ receptors, and the block is reduced by the position mutation $\alpha_1^{G5254A}$. The equivalent position is of prime importance for other ligand-gated ion channels as well: this position corresponds to the most constricted site, the “central ring,” of the nicotinic acetylcholine channel (Villarroel et al., 1991), and mutating this position confers resistance of GABA$_A$ receptors (GABA$_A$Rs) to picrotoxin (French-Constant et al., 1993). Thus, in speculation, a large pharmacological difference between the $\alpha_1$ subunit containing GlyRs and the $\alpha_2/\alpha_3$ subunit containing GlyRs may account for a primary structure difference of the respective pore-forming region M2: glycine 254 present in $\alpha_1$ is exchanged for an alanine in the $\alpha_2$ and $\alpha_3$ subunits. This speculation is consistent with our mention above that the inhibition of quercetin on $I_{\text{Gly}}$ is mostly due to open-channel block. As previous studies demonstrated, picrotoxin allows homomeric receptors to be distinguished, because it reduces the rate of decay of the current activated by a submaximal and saturating concentration of glycine. Furthermore, in the presence of quercetin, the glycine concentration–response curve shifted to the right with a large change in $EC_{50}$. Thus, the effects of quercetin may be attributed to faster dissociation of glycine from its binding site, even though other possibilities are not excluded. On the other hand, quercetin might allosterically modulate the function of GlyRs, resulting in slower GlyR desensitization. The fact that preperfusion of quercetin prior to the application of glycine prolonged the time constant of $I_{\text{Gly}}$ decay indicates the possibility that quercetin slows GlyR desensitization.
only the responses mediated by homomeric α receptors and has less effect for heteromeric αβ receptors. Ginkgolide B is a potent blocker of heteromeric αβ receptors and can be used to discriminate homomeric from heteromeric GlyRs (Kondratskaya et al., 2005). In addition, molecular modeling shows structural similarities between ginkgolide B and picrotoxin (Ivic et al., 2003). In contrast with these results, quercetin could not distinguish between two receptor configurations (homomeric and heteromeric ones), but could distinguish α1-containing GlyRs from α2/α3 subunit-containing GlyRs, indicating that quercetin is a subunit-selective inhibitor of GlyRs. That is, both α2-containing GlyRs and α3-containing GlyRs but not α1-containing GlyRs show a high sensitivity to quercetin.

The GlyR is best known for mediating inhibitory neurotransmission in the spinal cord, brainstem and cerebellum (Jonas et al., 1998; Chery and de Koninck, 1999; O’Brien and Berger, 1999), where they play a well-established role in the processing of motor and sensory information. The differential expression of subunits underlies diversity in GlyR pharmacology. The expression of subunits of GlyR in rat spinal neurons in vivo was developmentally regulated (Malosio et al., 1991; Watanabe and Akagi, 1995). A switch from α2 to α1 was completed by around postnatal day 20 in the rat, and α3 subunit was expressed at low levels and only in postnatal development (Malosio et al., 1991). On the other hand, many results suggest that hippocampal neurons express GlyRs, which are likely α2-containing receptors (Malosio et al., 1991; Sato et al., 1991, 1992; Racca et al., 1998). Consistent with these previous results and subunit studies above, our data showed that the inhibitory effect of quercetin on I_{gly} is weaker in cultured spinal neurons compared with that in cultured hippocampal neurons. Furthermore, in cultured spinal neurons, the inhibitory effect of quercetin on I_{gly} is significantly smaller at 15 DIV than at 8 DIV. The decrease in the degree of inhibition of quercetin on I_{gly} during development suggested that the change could have been due to both an increase in the density of α1-containing GlyRs and a decrease in the density of α2-containing GlyRs. Taken together, these results provide strong evidence that the inhibitory transmission mediated by GlyRs can be directly modulated by quercetin in a development-dependent manner.

Today, an inhibitory role for GlyRs in mature hippocampus is beginning to be appreciated. It is now clear that GlyRs are functionally expressed in hippocampus, established on electrophysiological (Chattipakorn and McMahon, 2002; Kondratskaya et al., 2004), immunocytochemical (Brackmann et al., 2004), and in situ hybridization (Malosio et al., 1991) levels. Interestingly, exogenous glycine application can depress seizure activity or hyperexcitability in the hippocampus (Cherubini et al., 1981; Seiler and Sarhan, 1984a,b; Chattipakorn and McMahon, 2003; Kirchner et al., 2003), whereas there are few reports suggesting that GlyRs may drive seizure-like rhythmic activities in the hippocampus and can be attenuated by cannabinoids (Lozovaya et al., 2005). In this respect, it may be of interest to study the effect of quercetin on epilepsy.

In summary, quercetin can directly depress GlyR-mediated currents in a noncompetitive and subunit-dependent manner, suggesting that quercetin may modulate glycineric transmission and hippocampal network activity. The isof orm specificity of quercetin may qualify this compound as a tool to probe the subunit composition of native GlyRs.

Acknowledgments—This work was supported by the National Key Project for Basic Science of China (No. 2006CB50705) and the Natural Science Foundation of China (30530310). We thank Mr. Ke-Qing Zhou, Mr. Yun-Gang Lu and Mr. Zheng-Quan Tang for assistance with technique.

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(Accepted 8 June 2007)
(Available online 30 July 2007)