Salicylate, an aspirin metabolite, specifically inhibits the current mediated by glycine receptors containing α1-subunits

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Background and purpose: Aspirin or its metabolite sodium salicylate is widely prescribed and has many side effects. Previous studies suggest that targeting neuronal receptors/ion channels is one of the pathways by which salicylate causes side effects in the nervous system. The present study aimed to investigate the functional action of salicylate on glycine receptors at a molecular level.

Experimental approach: Whole-cell patch-clamp and site-directed mutagenesis were deployed to examine the effects of salicylate on the currents mediated by native glycine receptors in cultured neurones of rat inferior colliculus and by glycine receptors expressed in HEK293T cells.

Key results: Salicylate effectively inhibited the maximal current mediated by native glycine receptors without altering the EC50 and the Hill coefficient, demonstrating a non-competitive action of salicylate. Only when applied simultaneously with glycine and extracellularly, could salicylate produce this antagonism. In HEK293T cells transfected with either α1-, α2-, α3-, α1β-, α2β- or α3β-glycine receptors, salicylate only inhibited the current mediated by those receptors that contained the α1-subunit. A single site mutation of I240V in the α1-subunit abolished inhibition by salicylate.

Conclusions and implications: Salicylate is a non-competitive antagonist specifically on glycine receptors containing α1-subunits. This action critically involves the isoleucine-240 in the first transmembrane segment of the α1-subunit. Our findings may increase our understanding of the receptors involved in the side effects of salicylate on the central nervous system, such as seizures and tinnitus.


Keywords: salicylate; glycine receptor; whole-cell patch-clamp; transfection; cell culture; site-directed mutagenesis

Abbreviations: EC50, half-maximal concentration; HEK, human embryonic kidney; IC, inferior colliculus; IC50, glycine-induced current; TM, transmembrane segment; Vh, holding potential

Introduction

Aspirin or its metabolite sodium salicylate (salicylate) is perhaps the most widely used drug in the world. It is estimated that 20–30 billion aspirin tablets are consumed annually in the United States alone (Gabriel and Fehring, 1992). Aspirin or salicylate is prescribed for a number of medical purposes ranging from pain relief (Lipton et al., 2005) to stroke prevention (Patrono, 1994) due to its wide range of pharmacological actions (Weissmann, 1991). For example, the drug can mitigate symptoms and inflammatory processes such as rheumatoid arthritis and osteoarthritis partly by inhibiting cyclooxygenases (Roth et al., 1975; Smith and Willis, 1971; Vane, 1971) and nuclear factor-κB transcription factor (Kopp and Ghosh, 1994; Yin et al., 1998). The slower pathological progression in Alzheimer’s disease patients being medicated with aspirin is thought to result from the drug’s anti-inflammatory action (Rich et al., 1995; Stewart et al., 1997). Aspirin also induced apoptosis in cancer cells (Qiao et al., 1998; Wong et al., 1999).

While the therapeutic benefits of aspirin or salicylate are considerable, it also has other complex pharmacological effects, including unwanted side effects on the nervous system.
system. Besides gastrointestinal toxicity (Wallace, 1997), aspirin or salicylate at a high dose can cause toxic symptoms such as seizures (Temple, 1981) and tinnitus (Puttermann and Ben-Chetrit, 1990; Caazals, 2000). In a patient hospitalized for aspirin poisoning, the plasma salicylate level was 830 mg·L⁻¹ (5.19 mM) (Gignoux et al., 1966). In patients treated with aspirin or salicylate for chronic inflammatory diseases, the serum concentration of salicylate can range from 1 to 5 mM (Insel, 1996). In these concentrations, salicylate has a broad spectrum of pharmacological actions on neuronal receptors/ion channels such as sodium channels (Liu and Li, 2004b), calcium channels (Liu et al., 2005), potassium channels (Liu and Li, 2004a), N-methyl D-aspartate (NMDA) receptors (Ruel et al., 2008) and GABA<sub>A</sub> receptors (Xu et al., 2005). The targeting of neuronal receptors/ion channels has been proposed as being one of the pathways for salicylate to exert its side effects on the central nervous system (Wang et al., 2006; 2008; Gong et al., 2008). Currently, functional modulation of neuronal receptors/ion channels by salicylate has been reported by a number of studies (Peng et al., 2003; Liu and Li, 2004a,b; Xu et al., 2005; Wang et al., 2006); however, information regarding how salicylate acts on the glycine receptors has not been available.

Glycine receptors, along with GABA<sub>A</sub> receptors, are the main inhibitory neurotransmitter-gated chloride ion channels in the central nervous system, including the spinal cord and brain stem (Betz, 1991). The glycine receptor is a member of the cysteine loop family of ligand-gated ion channels (Connolly and Wafford, 2004). The receptor has a homomeric structure with five ligand-binding α-subunits (Lynch, 2004) or a heteromeric structure with two α-subunits and three β-subunits which do not bind to known ligands but have a role in determining the ligand binding properties (Grudzinska et al., 2005). So far, four types of α-subunits (α<sub>1</sub>, α<sub>2</sub>, α<sub>3</sub> and α<sub>4</sub>) have been identified for glycine receptors (Lynch, 2004). These homologous receptor proteins are comprised of a large N-terminal extracellular domain, four transmembrane segments (TM1–TM4), a long intracellular loop connecting TM3 and TM4 and a short extracellular C-terminus (Lynch, 2004). The purpose of the present study was to understand how salicylate functionally interacts with glycine receptors at a molecular level. We found that salicylate specifically inhibits the current mediated by glycine receptors containing α1-subunits in a non-competitive manner. We further demonstrate that this inhibitory action of salicylate is conferred by the isoleucine residue at position 240 in TM1 of the α1-subunit.

**Methods**

All the experimental procedures in the present study followed the guidelines and protocols approved by the Institutional Animal Care and Use Committee of University of Science and Technology of China. All efforts were made to minimize the number of animals used. The nomenclature for the drugs used in the present study as well as their molecular targets conforms with the BIP's Guide to Receptors and Channels (Alexander et al., 2008).

**Cell culture**

The neurones used for cell culture were dissociated from the inferior colliculus (IC) of Wistar rats (postnatal day 0) as previously described (Tang et al., 2006). In brief, the IC was dissected from the brainstem under a dissection microscope and then dissociated by 0.25% trypsin and plated (1.5 × 10<sup>5</sup> cell·mL⁻¹) on poly-L-lysine (Sigma, St. Louis, MO, USA)-coated cover glasses. The neurones were grown in Dulbecco’s modified Eagle’s medium (Gibco, Grand Island, NY, USA) with L-glutamine, 10% foetal bovine serum (Gibco) and 10% F-12 nutrient mixture (Gibco) for 24 h. Then, neurobasal medium (1.5 mL, Gibco) with 2% B27 (Gibco) was replaced every 3–4 days. Treatment with 5-fluoro-2′-deoxyuridine (20 μg/mL, Sigma) on the fourth day after plating was used to block the division of non-neuronal cells and stabilize the cell population. The cultures were maintained at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air. Cells were used for electrophysiological recordings 10–14 days after plating.

**Site-directed mutagenesis**

Mutations of receptor cDNA were constructed using the PCR method as described in a previous study (Dieffenbach and Dveksler, 1995) and with commercially synthesized mutagenic primers (Invitrogen Biotechnology, China). All mutants were verified with DNA sequencing analysis (Invitrogen Biotechnology, China).

**Transfection**

All constructs were expressed in HEK293T cells that were cultured at 37°C in a 5% CO<sub>2</sub>, humidified atmosphere. The cells were maintained in Dulbecco’s modified Eagle’s medium (Gibco) supplemented with 2 mM L-glutamine, 10% foetal bovine serum, and 100 units per mL penicillin/streptomycin (Gibco) and 1% sodium pyruvate (Gibco). The procedure of transient transfection was similar to that previously described (Ye et al., 2008) with a minor modification. Briefly, HEK293T cells were transfected with various subunit combinations by lipofection using 1 μg of cDNA and 2 μL of Lipofectamine 2000 (Invitrogen, USA) per 3.0 × 10<sup>5</sup> cells plated on 35-mm culture dishes. Co-transfection with a green fluorescent protein expression vector, pEGFP, was used to enable identification of transfected cells for patch clamping by monitoring its fluorescence. When more than one kind of GlyR subunit was expressed, the α- and β-subunits were co-transfected into HEK293T cells at the ratio of 1:2. Electrophysiological measurements were performed 24–48 h after transfection. The pEGFP was presented by Dr Jian-Hong Luo (School of Medicine, Zhejiang University, China). The donors of the subunit cDNA were the same as those acknowledged in the previous reports (Jiang et al., 2006; Ye et al., 2008).

**Solutions and chemicals**

The standard external solution used in this study contained (in mM): NaCl 150; KCl 5; MgCl<sub>2</sub> 1; CaCl<sub>2</sub> 2; glucose 10; and HEPES 10. The solution was adjusted to a pH of 7.4 with Tris base. The osmolarity of the solution was adjusted to 320–330 mOsm·L⁻¹ with sucrose and a micro-osmometer (Advanced Instruments, Model 3300, USA). The patch pipette
solution for whole-cell patch recording contained (in mM): KCl 150; MgCl₂ 1; CaCl₂ 0.5; EGTA 5; MgATP 2; and HEPES 10. The pH of the solution was adjusted to 7.2 with Tris base. When the voltage dependence of the effects of salicylate on the glycine-induced current (I\textsubscript{Gly}) was examined, voltage-activated Na⁺, K⁺ and Ca²⁺ channels were blocked by adding 0.3 μM tetrodotoxin (Hebei Fisheries Research Institute, China) and 0.2 mM CdCl₂ in the standard external solution and replacing K⁺ with Cs⁺ in the pipette solution.

Glycine, tetrodotoxin and CdCl₂ were first dissolved in ion-free water and then diluted to the desired concentrations in the standard external solution just before use. Sodium salicylate was first dissolved to 100 mM in the standard external solution or in the patch pipette solution and then diluted to the desired concentrations just before use. Drugs were applied with the so-called ‘Y-tube’ method, a rapid application technique that allows a complete exchange of external solution surrounding a neurone within 20 ms (Murase et al., 1989). All the drugs were purchased from Sigma, USA unless otherwise specified.

**Patch-clamp recordings**

The conventional whole-cell patch-clamp technique was employed in the present study. Membrane currents were measured with a patch-clamp amplifier (Axon 200B, Axon Instruments, USA), sampled with a Digidata 1320A interface (Axon Instruments) and analysed with a personal computer installed with software Clampex and Clampfit (Version 9.2, Axon Instruments, USA). Patch pipettes were pulled from glass capillaries with an outer diameter of 1.5 mm on a two-stage puller (PP-830, Narishige, Japan). The resistance between the recording electrode filled with pipette solution and the reference electrode was 4–6 MΩ. In the experiments, 70–90% series resistance was compensated. Unless otherwise specified, the membrane potential was held at −60 mV in cultured neurones or −50 mV in HEK293T cells respectively. All experiments were performed at room temperature (22–25°C).

**Data analysis**

The continuous theoretical curves for concentration–response relationships of glycine in the presence or absence of salicylate were fitted to sigmoidal curves using Origin 7.0 (Origin-Lab Corporation, USA). The membrane potential recordings were performed at room temperature (22–25°C). The application of 100 μM glycine elicited an inward current at a holding potential (V\textsubscript{H}) of −60 mV on all the tested IC neurones. This current could be completely abolished by 1 μM strychnine, a selective antagonist of glycine receptors (data not shown), confirming that the current was primarily mediated by glycine receptors in cultured IC neurones. The typical glycine concentration used in this study was 100 μM because glycine at this concentration elicits robust responses facilitating characterization of the I\textsubscript{Gly} (Tang et al., 2006). Salicylate did not induce any detectable current when it was applied alone at various concentrations. However, salicylate reduced the I\textsubscript{Gly} amplitude when it was co-applied with glycine (Figure 1A). In addition, the time course of inhibition by salicylate on the I\textsubscript{Gly} was rapid and the inhibitory action was completely reversed after washout for about 100 s.

To explore the mechanism underlying the inhibition of the I\textsubscript{Gly} by salicylate, we examined the currents induced by glycine at various concentrations in the presence and absence of 1 mM salicylate (Figure 1B). The result showed that 1 mM salicylate did not significantly alter the EC\textsubscript{50} value (48.5 ± 7.6 vs. 42.5 ± 4.3 μM) and the Hill coefficient (1.48 vs. 1.54). In addition, salicylate at 1 mM effectively inhibited the maximal current induced by glycine at a saturating concentration (3 mM) to the 89 ± 1% of the control (P < 0.01, n = 7). This pattern of inhibition suggests that salicylate exerts its inhibitory action on glycine receptors in a way that is non-competitive to glycine. To confirm this proposition, we applied salicylate at a higher concentration (10 mM) in a subsequent experiment. For salicylate at 10 mM, the EC\textsubscript{50} value was 49.0 ± 8.8 and the Hill coefficient was 1.40. Salicylate at 10 mM effectively inhibited the maximal current induced by 3 mM glycine to 80 ± 2% of the control (P < 0.001, n = 6) (Figure 1B), again suggesting that salicylate inhibits the I\textsubscript{Gly} in a non-competitive manner.

**Intracellular dialysis with sodium salicylate had no effects on the inhibition of the I\textsubscript{Gly} by salicylate**

In order to determine whether there exists an intracellular modulating site for salicylate to inhibit the I\textsubscript{Gly}, we performed intracellular dialysis with micropipettes containing sodium salicylate (Figure 2A). Using the method described by a previous study (Pusch and Neher, 1988), we calculated the intracellular diffusing time for 1 mM salicylate to be about 70–120 s. However, we allowed the intracellular dialysis to last for 15 min to make sure that 1 mM salicylate was thoroughly diffused into the neurones under test. We found that the I\textsubscript{Gly} remained unchanged following intracellular salicylate dialysis for 15 min (P > 0.05, n = 5) (Figure 2B), indicating that there is no intracellular site for salicylate to act on glycine receptors. However, extracellular application of 1 mM salicylate significantly decreased the I\textsubscript{Gly} following the intracellular dialysis with salicylate (P < 0.05, n = 5) (Figure 2B), confirming that the site of action for salicylate was not located inside the cell.
Salicylate (NaSal) inhibited the maximal $I_{\text{Gly}}$ without significantly altering the EC$_{50}$ value and the Hill coefficient. (A) Sample currents recorded from one neurone of rat inferior colliculus induced by glycine (Gly) at 10, 100 and 1000 μM in the absence and presence of 1 mM salicylate (upper panel) and those from the other neurone in the presence and absence of 10 mM salicylate (lower panel). (B) The concentration-response relationships of the glycine-induced current ($I_{\text{Gly}}$) in the absence and presence of 1 or 10 mM salicylate. The data were fitted to sigmoidal curve. Note that salicylate depressed the $I_{\text{Gly}}$ only when applied simultaneously with glycine. Each point represents the averaged value from 5 to 14 neurones. Vertical bars represent ± SEM.

**Figure 2** Intracellular salicylate (NaSal) dialysis had no effects on the inhibition of the $I_{\text{Gly}}$ by salicylate. (A) Sample recordings of the $I_{\text{Gly}}$ with a micropipette containing 1 mM salicylate under conditions $a$ (before the dialysis), $b$ (following intracellular salicylate dialysis for 15 min) and $c$ (in the presence of extracellular 1 mM salicylate following the dialysis). (B) Summary data for the $I_{\text{Gly}}$ recorded from five neurones under conditions $a$, $b$ and $c$. The $I_{\text{Gly}}$ is normalized to that recorded under condition $a$ (before the dialysis and in the absence of salicylate). Vertical bars represent SEM. NS indicates no significant difference. ** indicates $P < 0.01$.

**Salicylate-induced decrease in glycine-activated membrane conductance was voltage-dependent**

To determine whether or not the inhibitory effect of salicylate on the $I_{\text{Gly}}$ is membrane potential-dependent, we examined the current-voltage relationship of the $I_{\text{Gly}}$ in the absence and presence of 1 mM salicylate using a voltage-ramp protocol at different holding potentials ranging from −80 to 60 mV (Figure 4A). The reversal potentials of the $I_{\text{Gly}}$ recorded with this protocol in the absence and presence of salicylate (−3.67 ± 0.53 mV vs. −4.15 ± 0.58 mV, $P > 0.05$, $n = 5$) were close to the theoretical equilibrium potential (−1.3 mV) calculated from the given extra- and intracellular chloride concentrations with the Nernst equation (Figure 4B), indicating that salicylate did not change the ion selectivity of glycine receptors.

The membrane conductance activated by 100 μM glycine was then calculated from the linear portion of the
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To assess whether there are specific subunits responsible for the inhibition of the \( I_{\text{Gly}} \) by salicylate, we expressed homomorphic \( \alpha 1 \)-, \( \alpha 2 \)- and \( \alpha 3 \)-glycine receptors as well as heteromeric \( \alpha 1 \beta \), \( \alpha 2 \beta \) and \( \alpha 3 \beta \)-glycine receptors in HEK293T cells. The cells with dominant expression of heteromeric \( \alpha \beta \)-glycine receptors were distinguished from those with dominant expression of homomorphic \( \alpha \)-glycine receptors with 10 µM picrotoxin, to which heteromeric \( \alpha \beta \)-glycine receptors were not sensitive but homomorphic \( \alpha \)-glycine receptors were (Pribilla et al., 1992). We found that salicylate reversibly reduced the \( I_{\text{Gly}} \) only in the cells transfected with either \( \alpha 1 \)- or \( \alpha 1 \beta \)-glycine receptors (\( P < 0.05 \), \( n = 5–8 \)) but not in the cells transfected with either \( \alpha 2 \), \( \alpha 3 \), \( \alpha 2 \beta \) or \( \alpha 3 \beta \)-glycine receptors (\( P > 0.05 \), \( n = 5–8 \)) (Figure 5). This result indicates that salicylate specifically inhibits the current mediated by those glycine receptors that contained \( \alpha 1 \)-subunits.
A single site mutation of isoleucine to valine at position 240 in the α1-subunit abolished action of salicylate on α1-glycine receptors containing α1-subunits.

Sequence alignment reveals that the α1-subunit is only different from the α2-/α3-subunit in residues at positions 240 in TM1 (isoleucine vs. valine) (Figure 6A, upper panel) and 254 in TM2 (glycine vs. alanine) near the pore-forming region (Figure 6A, lower panel). If the residues at either of the two positions are responsible for the α1-subunit specificity of NaSal, then an exchange of these residues between the α1-subunit and the α2-/α3-subunit would transfer the sensitivity to salicylate from the α1-subunit to the α2-/α3-subunit and transfer the insensitivity to salicylate from the α2-/α3-subunit to the α1-subunit. To test this hypothesis, we first examined the effect of salicylate on the current mediated by the mutated forms of homomeric α1-glycine receptors with the isoleucine residue converted to valine at position 240 in TM1 (I240V) and the glycine residue to alanine at position 254 in TM2 (G254A) through site-directed mutagenesis (Figure 6A). We found that the I50 was not significantly depressed by salicylate at 1 mM or 10 mM in HEK293T cells with mutant I240V α1-glycine receptors, but was still significantly depressed by salicylate in the cells with mutant G254A α1-glycine receptors (Figure 6B). The I240V α1-glycine receptors behaved more like α2/3- than α1-glycine receptors of wild type in the presence of salicylate. The result clearly demonstrated that the mutation I240V in TM1, but not the mutation G254A in TM2, almost eliminated the receptor’s sensitivity to salicylate. In addition, the concentration–response relationships of the currents mediated by WT α1-glycine receptors and by I240V α1-glycine receptors were not significantly different (34.3 ± 5.5 μM vs. 30.0 ± 5.3 μM) (P > 0.05, one-way ANOVA). Sample sizes are indicated in parentheses. TM1, first transmembrane segment; TM2, second transmembrane segment; WT, wild type.

Figure 5 Salicylate (NaSal) specifically inhibited the current mediated by glycine receptors containing α1-subunits expressed in HEK293T cells. (A) Sample traces of the current mediated by different recombinant α1- or α1β-glycine receptors. The I50 is normalized to the response in the absence of salicylate. Sample sizes are indicated in parentheses. Vertical bars represent SEM. NS indicates no significant difference. * indicates P < 0.05.

Figure 6 A single site mutation of isoleucine to valine at position 240 in the α1-subunit abolished action of salicylate on α1-glycine receptors expressed in HEK293T cells. (A) Aligned sequences showing that near the pore-forming region, the α1-subunit is different from the α2-/α3-subunit only in residues at positions 240 in TM1 and 254 in TM2. (B) Representative traces (left panel) and summary data (right panel) for the currents mediated by glycine receptors containing WT, by I240V α1-glycine receptors or by G254A α1-glycine receptors. Note that the mutation I240V, rather than the mutation G254A, abolished action of salicylate on α1-glycine receptors. * indicates P < 0.05 and ** indicates P < 0.01. (C) Representative traces (left panel) of the currents mediated by WT α1-glycine receptors and by I240V α1-glycine receptors. Summary data (right panel) for the concentration–response relationships of the currents mediated by WT α1-glycine receptors and by I240V α1-glycine receptors. Data are normalized to the maximal response. Note that the EC<sub>50</sub> values for mutant I240V α1-glycine receptors and for WT α1-glycine receptors were not significantly different (34.3 ± 5.5 μM vs. 30.0 ± 5.3 μM) (P > 0.05, one-way ANOVA). Sample sizes are indicated in parentheses. TM1, first transmembrane segment; TM2, second transmembrane segment; WT, wild type.
by the isoleucine residue at position 240 in TM1 of the α1-subunit. Our findings provide useful insights into the underlying mechanisms for the pharmacological actions of salicylate on glycine receptors at a molecular level. We speculate that salicylate is most likely to serve as an allosteric modulator and putatively binds in the transmembrane region, although the pattern of the current–voltage curve (Figure 4) may suggest direct interference of salicylate with ion flow as a kind of negative amphiphile. Evidence from the present study supporting this speculation includes: (i) salicylate depressed the maximal current induced by glycine at a saturating concentration without significantly altering the EC50 value and the Hill coefficient (Figure 1), suggesting that salicylate does not compete with glycine for the binding site located in the extracellular interface; (ii) intracellular salicylate dialysis did not inhibit the IcGly (Figure 2), indicating that the site of action of salicylate on glycine receptors was not likely to be located inside the cell; (iii) salicylate was inhibitory only when applied simultaneously with glycine (Figure 3), raising a possibility that salicylate acts on the channel pore region in the open state; (iv) the mutation G254A in TM2 of the α1-subunit did not eliminate the receptor sensitivity to salicylate, but the mutation I240V in TM1 of the α1-subunit did (Figure 6B), suggesting an allosteric interaction of salicylate within the transmembrane region.

Sequence alignment shows that α1-, α2- and α3-subunits of glycine receptors are highly homologous near the pore-forming region and are only different in two positions, one in TM1 (α1 I240, α2 V247, α3 V240) and the other in TM2 (α1 G254, α2 A261, α3 A254) (Figure 6A). A couple of previous studies have demonstrated the importance of the residue at the second position (Bormann et al., 1993; Steinbach et al., 2000). For example, the residue at the second position (α3 A254), rather than the residue at the first position (α3 V240), is reported to confer the regulatory effect of αEMTBL, an anticonvulsant, on glycine receptors (Steinbach et al., 2000). Interestingly, our study shows that the residue at the first position (α1 I240), rather than the residue at the second position (α1 G254), confers the antagonist effect of salicylate when applied extracellularly (Figure 6B). The intracellular dialysis of salicylate (Figure 2) further confirms that salicylate does not bind at the site G254 in TM2, which is presumably located on the intracellular surface (Laube et al., 2002), and the dialysis would otherwise produce an effect on the IcGly. The site I240 is close to the intracellular end of TM1; however, the intracellular dialysis of salicylate did not produce an antagonistic effect on the IcGly (Figure 2), suggesting that the residue I240 in TM1 is critical for the conformational change induced by salicylate that binds at a site distant to the intracellular surface. Alternatively, the result obtained with the intracellular dialysis suggests that the site I240 is not on, although it is very close to, the intracellular surface (Laube et al., 2002) and is thus not accessible by salicylate for binding from the inside of the cell even when the channel is in the open state.

Our results show that salicylate can distinguish glycine receptors that contain α1-subunits from those that do not, although it cannot produce differential effects between homomeric α1-glycine receptors and heteromeric α1β-glycine receptors (Figure 5). The subunit specificity of salicylate may be used to probe the subtype of native glycine receptors in the

**Discussion**

The present study demonstrates that salicylate specifically inhibits the current mediated by glycine receptors containing α1-subunits in a non-competitive manner, which is conferred

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5.3 μM, P > 0.05, n = 5–6) (Figure 6C), suggesting that the mutation did not significantly alter the receptor response to the natural ligand. We then examined whether a reverse mutation in the α2-subunit and in the α3-subunit can make them become sensitive to salicylate. We converted the valine residue to isoleucine at position 247 in the α2-subunit to create a mutated form of homomeric V247I α2-glycine receptors and at position 240 in the α3-subunit to create a mutated form of homomeric V240I α3-glycine receptors. The points of mutation of V247I in α2-glycine receptors and V240I in α3-glycine receptors corresponded to the isoleucine at position 240 in the α1-subunit (Figure 6A). We found that salicylate (1 and 10 mM) significantly inhibited the current induced by 100 μM glycine in HEK293T cells expressed with mutated forms of V247I α2- and V240I α3-glycine receptors (Figure 7A and B), indicating that the α2-/α3-subunit with the reverse mutation becomes sensitive to salicylate. The results confirm that the isoleucine residue at position 240 in the α1-subunit confers the specific action of salicylate on glycine receptors containing α1-subunits.

**Figure 7** A single site mutation of V247I in the α2-glycine receptors and V240I in α3-glycine receptors made the receptor become sensitive to salicylate. (A) Representative traces (left panel) and summary data (right panel) showing the effects of salicylate (1 and 10 mM) on the currents mediated by wild-type (WT) α2-glycine receptors and V247I α2-glycine receptors. (B) Representative traces (left panel) and summary data (right panel) showing the effects of salicylate (1 and 10 mM) on the currents mediated by WT α3-glycine receptors and V240I α3-glycine receptors. The current is induced by 100 μM glycine normalized to the response in the absence of salicylate (dashed lines). Sample sizes are indicated in parentheses. Vertical bars represent SEM. ** indicates P < 0.01 and *** indicates P < 0.001.
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brain. Expression of subtypes of glycine receptors is both regionally and developmentally regulated in the central nervous system. During development, glycine receptor α2-subunit mRNA is accumulated prenatally and decreases after birth (Malosio et al., 1991). In adult animals, glycine receptor α1-subunit mRNA is abundant in the spinal cord but it is also found in a few brain areas. The present study shows that salicylate significantly reduces the k_{Gy} in cultured IC neurones (Figure 1), revealing the presence of glycine receptors that contain α1-subunits in IC neurones. This result is consistent with the previous findings that mRNAs for glycine receptors that contain α1-subunits, rather than α2/α3-subunits, are expressed predominantly in the IC (Piechotta et al., 2001; Argence et al., 2006).

Aspirin or salicylate is usually prescribed at a low dose; however, in some patients such as those receiving this drug for chronic inflammatory diseases, the serum concentration of salicylate can reach up to 5 mM (Insel, 1996). More importantly, the concentration of salicylate in the cerebrospinal fluid can reach one-third of that in the serum according to a fluid can reach one-third of that in the serum according to a typical concentration of salicylate (1 mM) used in the present study is clinically relevant and the findings may increase our understanding of the receptor basis for side effects of aspirin or salicylate on the nervous system. The targeting of neuronal receptors/ion channels is suggested as being one of the pathways by which salicylate causes side effects in the nervous system (Wang et al., 2006; 2008; Gong et al., 2008), but the exact underlying mechanism is not fully understood, partially because the pharmacological actions of salicylate are broad. For example, the drug has been shown to target a number of neuronal ion channels/receptors, such as sodium channels (Liu and Li, 2004b), calcium channels (Liu et al., 2005), potassium channels (Liu and Li, 2004a), NMDA receptors (Ruel et al., 2008) and GABA_a receptors (Xu et al., 2005). In particular, the inhibition of GABA_a receptors by salicylate is reported to cause hyperexcitation in hippocampal slices (Gong et al., 2008). The present study shows that salicylate inhibits glycine receptors to the same extent as it inhibits GABA_a receptors, suggesting that the targeting of glycine receptors by salicylate may also contribute to salicylate-induced symptoms such as seizures (Temple, 1981) and tinnitus (Puttermann and Ben-Chetrit, 1990; Cazals, 2000).

In conclusion, salicylate exerts an antagonistic action specifically on glycine receptors containing α1-subunits in a non-competitive manner, which is conferred by the isoleucine residue at position 240 in TM1 of the α1-subunit. We speculate that salicylate most likely serves as an allosteric modulator of glycine receptors and putatively binds near the channel pore region in the open state. The present study may help us to understand the receptor basis for the side effects of aspirin or salicylate on the nervous system.

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Conflict of interest

The authors state no conflict of interest.

References

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