BJP BRITISH JOURNAL OF PHARMACOLOGY

BPS BRITISH PHARMACOLOGICAL SOCIETY

British Journal of Pharmacology (2009), 157, 1514–1522

0007-1188/09
www.brjpharmacol.org

RESEARCH PAPER

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

Y-G Lu[1,2], Z-Q Tang[2], Z-Y Ye[1], H-T Wang[2], Y-N Huang[1,2], K-Q Zhou[1], M Zhang[2], T-L Xu[3] and L Chen[1,2]

[1]*Hefei National Laboratory for Physical Sciences at Microscale and School of Life Sciences, University of Science and Technology of China, Hefei, China and* [2]*Auditory Research Laboratory, University of Science and Technology of China, Hefei, China and* [3]*Institute of Neuroscience, Chinese Academy of Sciences, Shanghai, China*

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 EC_{50} 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.
British Journal of Pharmacology (2009) **157,** 1514–1522; doi:10.1111/j.1476-5381.2009.00321.x; published online 7 July 2009

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

Abbreviations: EC_{50}, half-maximal concentration; HEK, human embryonic kidney; IC, inferior colliculus; I_{Gly}, glycine-induced current; TM, transmembrane segment; V_H, 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

Correspondence: Dr Lin Chen, Auditory Research Laboratory, University of Science and Technology of China, Hefei 230027, China. E-mail: linchen@ustc.edu.cn
Received 26 March 2009; accepted 30 March 2009

system. Besides gastrointestinal toxicity (Wallace, 1997), aspirin or salicylate at a high dose can cause toxic symptoms such as seizures (Temple, 1981) and tinnitus (Putterman and Ben-Chetrit, 1990; Cazals, 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 $GABA_A$ 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 $GABA_A$ 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 (α1, α2, α3 and α4) 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 *BJP'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 (1.5×10^6 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-5′-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_2 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_2 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). 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 3.0×10^5 cells planted 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; $MgCl_2$ 1; $CaCl_2$ 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; $MgCl_2$ 1; $CaCl_2$ 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_{Gly}) was examined, voltage-activated Na^+, K^+ and Ca^{2+} channels were blocked by adding 0.3 μM tetrodotoxin (Hebei Fisheries Research Institute, China) and 0.2 mM $CdCl_2$ in the standard external solution and replacing K^+ with Cs^+ in the pipette solution.

Glycine, tetrodotoxin and $CdCl_2$ 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 (OriginLab Corporation, USA). The membrane potential recordings were corrected for junction potentials with Clampex (Version 9.2, Axon Instruments, USA) based on ionic mobility, ionic activity and ionic strength of the bath and pipette solutions. The membrane conductance was calculated from the linear portion of the linear current–voltage relationship of the I_{Gly} under voltage-clamp mode at –60 or +60 mV. All of the data are represented as the mean ± standard error of the mean (SEM) with statistical significance assessed with one-way analysis of variance (ANOVA) using SigmaStat (Version 2.03, SPSS, USA). If the null hypothesis for ANOVA was rejected, a *post hoc* Bonferroni test would then be performed to determine which specific groups differed in means. Statistically significant difference was assumed as $P < 0.05$, $P < 0.01$ or $P < 0.001$ for all the data. The level of significance and the number of neurones are represented by P and n respectively.

Results

Salicylate inhibited the maximal I_{Gly} without significantly altering the EC_{50} value and the Hill coefficient

The application of 100 μM glycine elicited an inward current at a holding potential (V_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_{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_{Gly} amplitude when it was co-applied with glycine (Figure 1A). In addition, the time course of inhibition by salicylate on the I_{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_{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_{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_{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_{Gly} in a non-competitive manner.

Intracellular dialysis with sodium salicylate had no effects on the inhibition of the I_{Gly} by salicylate

In order to determine whether there exists an intracellular modulating site for salicylate to inhibit the I_{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_{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_{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.

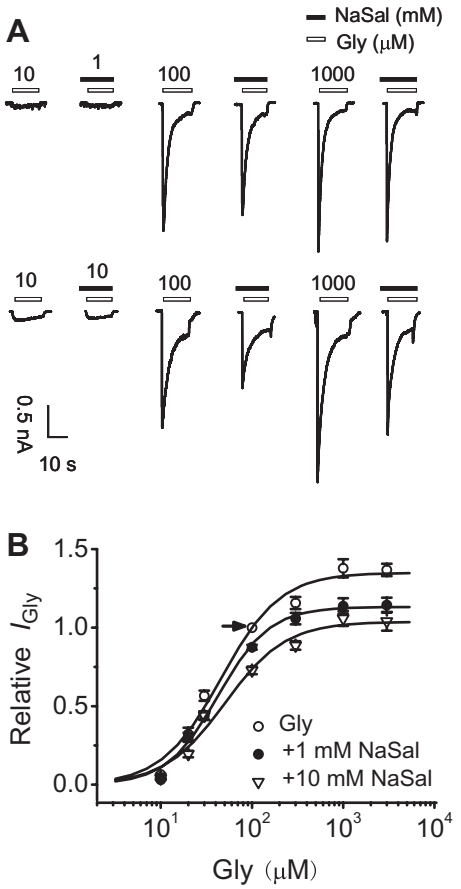

Figure 1 Salicylate (NaSal) inhibited the maximal I_{Gly} without significantly altering the EC_{50} value and the Hill coefficient. (A) Sample currents recorded from one neurone of rat inferior colliculus induced by glycine (Gly) at 10, 100 and 1000 μM in the absence and presence of 1 mM salicylate (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_{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_{Gly} without significantly altering the EC_{50} (48.5 ± 7.6 μM in the absence of salicylate, 42.5 ± 4.3 μM in the presence of 1 mM salicylate and 49.0 ± 8.8 μM in the presence of 10 mM salicylate) and the Hill coefficient (1.48 ± 0.26 in the absence of salicylate, 1.54 ± 0.19 in the presence of 1 mM salicylate and 1.40 ± 0.28 in the presence of 10 mM salicylate) ($P > 0.05$, one-way ANOVA). Also note that salicylate effectively inhibited the maximal current induced by glycine at a high concentration (3 mM) ($P < 0.05$, one-way ANOVA). Arrowhead indicates that all the data are normalized to the current induced by 100 μM glycine. Each point represents the averaged value from 5 to 14 neurones. Vertical bars represent ±SEM.

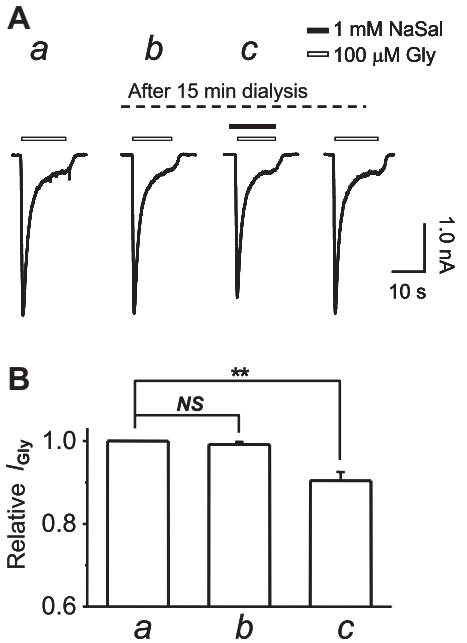

Figure 2 Intracellular salicylate (NaSal) dialysis had no effects on the inhibition of the I_{Gly} by salicylate. (A) Sample recordings of the I_{Gly} with a micropipette containing 1 mM salicylate under conditions *a* (before the dialysis), *b* (following intracellular salicylate dialysis for 15 min) and *c* (in the presence of extracellular 1 mM salicylate following the dialysis). (B) Summary data for the I_{Gly} recorded from five neurones under conditions *a*, *b* and *c*. Note the I_{Gly} was not changed by the dialysis (condition *b*) but changed by extracellular application of salicylate following the dialysis (condition *c*). The I_{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 depressed the I_{Gly} only when applied simultaneously with glycine

To determine whether the antagonistic action of salicylate on glycine receptors is state-dependent, we used three different drug application protocols to examine the inhibitory effect of salicylate on the I_{Gly} (Figure 3A). In protocol *a*, neurones were pretreated with salicylate (1 mM) for about 15 s and then exposed to glycine (100 μM) and salicylate simultaneously. In protocol *b*, neurones were exposed to glycine alone after pretreatment with salicylate for about 15 s. In protocol *c*, neurones were exposed to glycine and salicylate simultaneously without pretreatment of any drugs. We found that the I_{Gly} was significantly depressed by application of salicylate with protocols *a* and *c* ($P < 0.01$, $n = 6$), rather than with protocol *b* ($P > 0.05$, $n = 6$) (Figure 3B). In other words, salicylate depressed the I_{Gly} only when applied with glycine simultaneously. Sequential application of salicylate and glycine (protocol *b*) did not decrease the I_{Gly}. This result indicates that salicylate exerts an antagonistic effect on glycine receptors only in the open state.

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

To determine whether or not the inhibitory effect of salicylate on the I_{Gly} is membrane potential-dependent, we examined the current–voltage relationship of the I_{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_{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

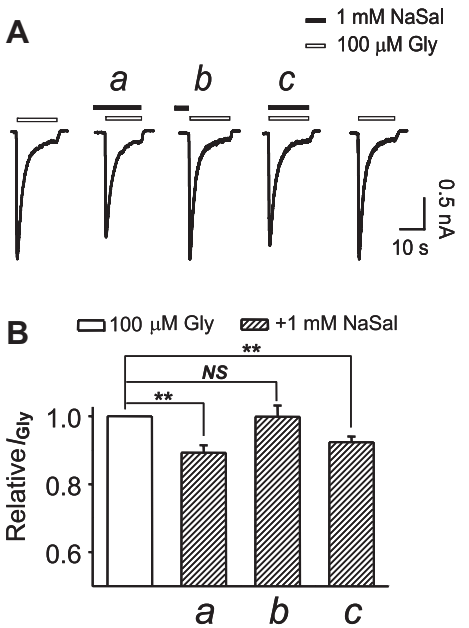

Figure 3 Salicylate (NaSal) depressed the I_{Gly} only when applied simultaneously with glycine (Gly). (A) Sample traces of the I_{Gly} recorded with three different drug application protocols. In protocol *a*, the neurone was pretreated with salicylate for 15 s and then exposed to salicylate and glycine simultaneously; in protocol *b*, the neurone was exposed to glycine alone immediately after 15-s pretreatment of salicylate; in protocol *c*, the neurone was exposed to salicylate and glycine simultaneously without pretreatment of any drugs. (B) Summary data for the I_{Gly} recorded from six neurones with the three application protocols. Note that salicylate depressed the I_{Gly} only when applied with glycine simultaneously (with protocols *a* and *c*). The I_{Gly} is normalized to the response induced by 100 μM glycine alone. Vertical bars represent SEM. *NS* indicates no significant difference. ** indicates $P < 0.01$.

current–voltage relationship of the I_{Gly}. We found that salicylate decreased the membrane conductance induced by 100 μM glycine to a larger extent when the membrane potential was held at +60 mV (decreased from 25.29 ± 2.40 to 16.98 ± 2.42 nS, $P < 0.01$, $n = 9$) than at –60 mV (decreased from 27.95 ± 2.15 to 23.20 ± 1.93 nS, $P < 0.01$, $n = 9$) (Figure 4C). This result indicates that the inhibition of the I_{Gly} by salicylate is voltage-dependent, being greater at positive membrane potentials than at negative potentials.

Salicylate specifically inhibited the current mediated by glycine receptors containing α1-subunits

To assess whether there are specific subunits responsible for the inhibition of the I_{Gly} by salicylate, we expressed homomeric α1-, α2- and α3-glycine receptors as well as heteromeric α1β-, α2β- and α3β-glycine receptors in HEK293T cells. The cells with dominant expression of heteromeric αβ-glycine receptors were distinguished from those with dominant expression of homomeric α-glycine receptors with 10 μM picrotoxin, to which heteromeric αβ-glycine receptors were not sensitive but homomeric α-glycine receptors were (Pribilla *et al*., 1992). We found that salicylate reversibly reduced the I_{Gly} only in the cells transfected with either α1- or α1β-glycine receptors ($P < 0.05$, $n = 5$–8), but not in the cells transfected

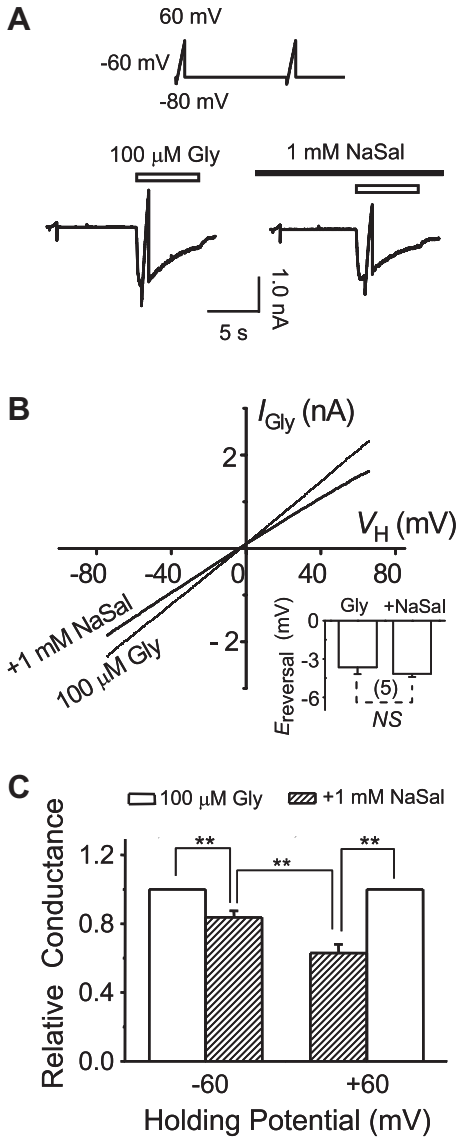

Figure 4 Salicylate (NaSal)-induced decrease in glycine-activated membrane conductance was voltage-dependent. (A) Voltage-ramp protocol used to derive the current–voltage relationship. In this protocol, the first ramp occurred in the absence of drug and the second ramp in the presence of drug. Current–voltage relationship was derived by subtracting the current trace produced by the first ramp from that produced by the second ramp. (B) Example of derived current–voltage relationship for a neurone in the absence and presence of 1 mM salicylate. The relationship was corrected for junction potentials. The reversal potential in this example was –2.89 mV and –3.60 mV in the absence and the presence of 1 mM salicylate respectively. Inset shows the averaged reversal potentials ($E_{reversal}$) from five neurones. *NS* indicates no significant difference. (C) Summary data of glycine-activated membrane conductance in the presence of 1 mM salicylate at a negative (–60 mV) and a positive (+60 mV) holding potentials ($n = 9$). Note that salicylate decreased the conductance to a larger extent at the positive potentials than at the negative potentials. The conductance is normalized to the response in the absence of salicylate. Vertical bars represent SEM. ** indicates $P < 0.01$.

with either of α2-, α3-, α2β- or α3β-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 α1-subunits.

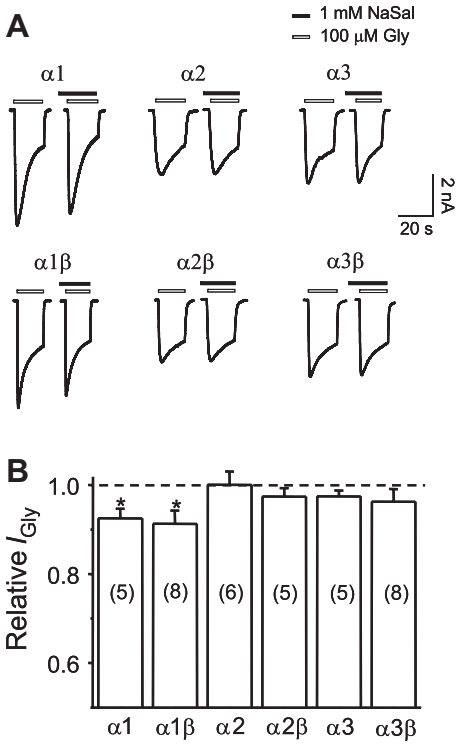

Figure 5 Salicylate (NaSal) specifically inhibited the current mediated by glycine receptors containing α1-subunits expressed in HEK293T cells. (A) Sample traces of the current mediated by different recombinant glycine receptors in the absence and presence of 1 mM salicylate. (B) Summary data showing the effects of 1 mM salicylate on the I_{Gly}. Note that salicylate only inhibited the current mediated by recombinant α1- or α1β-glycine receptors. The I_{Gly} 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 $P < 0.05$.

A single site mutation of isoleucine to valine at position 240 in the α1-subunit abolished action of salicylate on 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 I_{Gly} 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

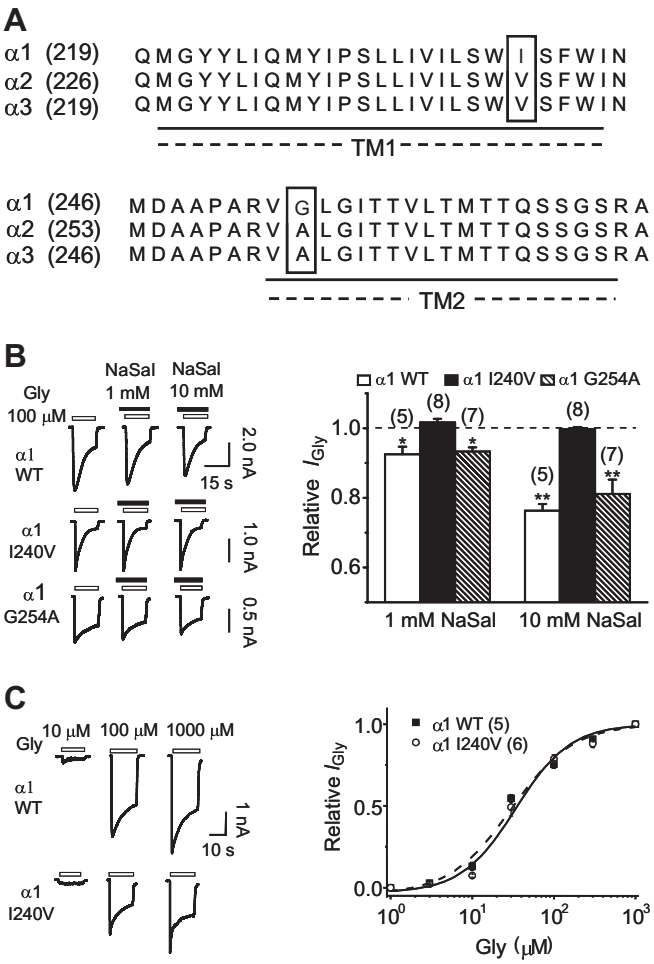

Figure 6 A single site mutation of isoleucine to valine at position 240 in the α1-subunit abolished action of salicylate on α1-glycine receptors expressed in HEK293T cells. (A) Aligned sequences showing that near the pore-forming region, the α1-subunit is different from the α2-/α3-subunit only in residues at positions 240 in TM1 and 254 in TM2. (B) Representative traces (left panel) and summary data (right panel) showing the effects of salicylate (1 and 10 mM) on the currents mediated by α1-glycine receptors of 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_{50} 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.

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 curve and the EC_{50} value of mutant I240V α1-glycine receptors in the presence of glycine only were not different from those of wild-type α1-glycine receptors (34.3 ± 5.5 μM vs. 30.0 ±

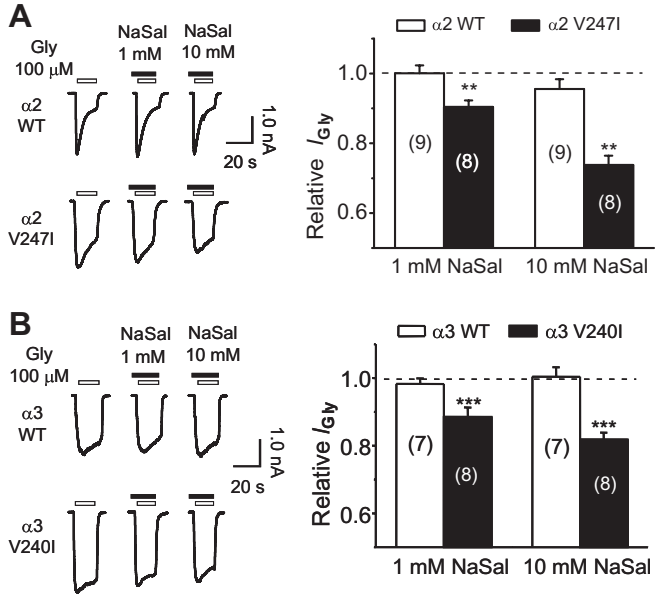

Figure 7 A single site mutation of V247I in the α2-glycine receptors and V240I in α3-glycine receptors made the receptor become sensitive to salicylate. (A) Representative traces (left panel) and summary data (right panel) showing the effects of salicylate (1 and 10 mM) on the currents mediated by wild-type (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 and 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 $P < 0.001$.

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.

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 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 EC_{50} 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 I_{Gly} (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 I_{Gly}. 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 I_{Gly} (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

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 I_{Gly} 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 previous study in animals (Jastreboff *et al.*, 1986). Thus, the 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 (Putterman 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.

Acknowledgements

We thank Dr Xiao-Bing Zhang and Mr Hui Fang for their technical assistance. We thank Dr Peng Jiang and Dr Zhi Zhang for their constructive comments on the manuscript. This work was supported by the National Natural Science Foundation of China (Grants 30470560 and 30730041), the National Basic Research Program of China (Grant 2007CB512306) and the CAS Knowledge Innovation Project (Grant KSCX1-YW-R-36).

Conflict of interest

The authors state no conflict of interest.

References

Alexander SP, Mathie A, Peters JA (2008). Guide to Receptors and Channels (GRAC), 3rd edition. *Br J Pharmacol* **153** (Suppl. 2): S1–209.

Argence M, Saez I, Sassu R, Vassias I, Vidal PP, de Waele C (2006). Modulation of inhibitory and excitatory synaptic transmission in rat inferior colliculus after unilateral cochleectomy: an in situ and immunofluorescence study. *Neuroscience* **141**: 1193–1207.

Betz H (1991). Glycine receptors: heterogeneous and widespread in the mammalian brain. *Trends Neurosci* **14**: 458–461.

Bormann J, Rundstrom N, Betz H, Langosch D (1993). Residues within transmembrane segment M2 determine chloride conductance of glycine receptor homo- and hetero-oligomers. *EMBO J* **12**: 3729–3737.

Cazals Y (2000). Auditory sensori-neural alterations induced by salicylate. *Prog Neurobiol* **62**: 583–631.

Connolly CN, Wafford KA (2004). The Cys-loop superfamily of ligand-gated ion channels: the impact of receptor structure on function. *Biochem Soc Trans* **32**: 529–534.

Dieffenbach CW, Dveksler GS (1995). *PCR Primer: A Laboratory Manual: Plainview*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Gabriel SE, Fehring RA (1992). Trends in the utilization of non-steroidal anti-inflammatory drugs in the United States, 1986–1990. *J Clin Epidemiol* **45**: 1041–1044.

Gignoux M, Martin H, Cajgfinger H (1966). [Cochleo-vestibular complications after attempted suicide with aspirin]. *J Fr Otorhinolaryngol Chir Maxillofac* **15**: 631–635.

Gong N, Zhang M, Zhang XB, Chen L, Sun GC, Xu TL (2008). The aspirin metabolite salicylate enhances neuronal excitation in rat hippocampal CA1 area through reducing GABAergic inhibition. *Neuropharmacology* **54**: 454–463.

Grudzinska J, Schemm R, Haeger S, Nicke A, Schmalzing G, Betz H *et al.* (2005). The beta subunit determines the ligand binding properties of synaptic glycine receptors. *Neuron* **45**: 727–739.

Insel PA (1996). *The Pharmacological Basis of Therapeutics*. McGraw-Hill, New York.

Jastreboff PJ, Hansen R, Sasaki PG, Sasaki CT (1986). Differential uptake of salicylate in serum, cerebrospinal fluid, and perilymph. *Arch Otolaryngol Head Neck Surg* **112**: 1050–1053.

Jiang P, Yang CX, Wang YT, Xu TL (2006). Mechanisms of modulation of pregnanolone on glycinergic response in cultured spinal dorsal horn neurons of rat. *Neuroscience* **141**: 2041–2050.

Kopp E, Ghosh S (1994). Inhibition of NF-kappa B by sodium salicylate and aspirin. *Science* **265**: 956–959.

Laube B, Maksay G, Schemm R, Betz H (2002). Modulation of glycine receptor function: a novel approach for therapeutic intervention at inhibitory synapses? *Trends Pharmacol Sci* **23**: 519–527.

Lipton RB, Goldstein J, Baggish JS, Yataco AR, Sorrentino JV, Quiring JN (2005). Aspirin is efficacious for the treatment of acute migraine. *Headache* **45**: 283–292.

Liu Y, Li X (2004a). Effects of salicylate on transient outward and delayed rectifier potassium channels in rat inferior colliculus neurons. *Neurosci Lett* **369**: 115–120.

Liu Y, Li X (2004b). Effects of salicylate on voltage-gated sodium channels in rat inferior colliculus neurons. *Hear Res* **193**: 68–74.

Liu Y, Li X, Ma C, Liu J, Lu H (2005). Salicylate blocks L-type calcium channels in rat inferior colliculus neurons. *Hear Res* **205**: 271–276.

Lynch JW (2004). Molecular structure and function of the glycine receptor chloride channel. *Physiol Rev* **84**: 1051–1095.

Malosio ML, Marqueze-Pouey B, Kuhse J, Betz H (1991). Widespread expression of glycine receptor subunit mRNAs in the adult and developing rat brain. *EMBO J* **10**: 2401–2409.

Murase K, Ryu PD, Randic M (1989). Excitatory and inhibitory amino acids and peptide-induced responses in acutely isolated rat spinal dorsal horn neurons. *Neurosci Lett* **103**: 56–63.

Patrono C (1994). Aspirin as an antiplatelet drug. *N Engl J Med* **330**: 1287–1294.

Peng BG, Chen S, Lin X (2003). Aspirin selectively augmented N-methyl-D-aspartate types of glutamate responses in cultured spiral ganglion neurons of mice. *Neurosci Lett* **343**: 21–24.

Piechotta K, Weth F, Harvey RJ, Friauf E (2001). Localization of rat glycine receptor alpha1 and alpha2 subunit transcripts in the developing auditory brainstem. *J Comp Neurol* **438**: 336–352.

Pribilla I, Takagi T, Langosch D, Bormann J, Betz H (1992). The atypical M2 segment of the beta subunit confers picrotoxinin resistance to inhibitory glycine receptor channels. *EMBO J* **11**: 4305–4311.

Pusch M, Neher E (1988). Rates of diffusional exchange between small cells and a measuring patch pipette. *Pflugers Arch* **411**: 204–211.

Putterman C, Ben-Chetrit E (1990). Tinnitus due to low-dose aural aspirin therapy. *N Engl J Med* **323**: 1846.

Qiao L, Hanif R, Sphicas E, Shiff SJ, Rigas B (1998). Effect of aspirin on induction of apoptosis in HT-29 human colon adenocarcinoma cells. *Biochem Pharmacol* **55**: 53–64.

Rich JB, Rasmusson DX, Folstein MF, Carson KA, Kawas C, Brandt J (1995). Nonsteroidal anti-inflammatory drugs in Alzheimer's disease. *Neurology* **45**: 51–55.

Roth GJ, Stanford N, Majerus PW (1975). Acetylation of prostaglandin synthase by aspirin. *Proc Natl Acad Sci USA* **72**: 3073–3076.

Ruel J, Chabbert C, Nouvian R, Bendris R, Eybalin M, Leger CL *et al*. (2008). Salicylate enables cochlear arachidonic-acid-sensitive NMDA receptor responses. *J Neurosci* **28**: 7313–7323.

Smith JB, Willis AL (1971). Aspirin selectively inhibits prostaglandin production in human platelets. *Nat New Biol* **231**: 235–237.

Steinbach JH, Bracamontes J, Yu L, Zhang P, Covey DF (2000). Subunit-specific action of an anticonvulsant thiobutyrolactone on recombinant glycine receptors involves a residue in the M2 membrane-spanning region. *Mol Pharmacol* **58**: 11–17.

Stewart WF, Kawas C, Corrada M, Metter EJ (1997). Risk of Alzheimer's disease and duration of NSAID use. *Neurology* **48**: 626–632.

Tang ZQ, Lu YG, Zhou KQ, Xu TL, Chen L (2006). Amiloride attenuates glycine-induced currents in cultured neurons of rat inferior colliculus. *Biochem Biophys Res Commun* **350**: 900–904.

Temple AR (1981). Acute and chronic effects of aspirin toxicity and their treatment. *Arch Intern Med* **141**: 364–369.

Vane JR (1971). Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. *Nat New Biol* **231**: 232–235.

Wallace JL (1997). Nonsteroidal anti-inflammatory drugs and gastroenteropathy: the second hundred years. *Gastroenterology* **112**: 1000–1016.

Wang HT, Luo B, Zhou KQ, Xu TL, Chen L (2006). Sodium salicylate reduces inhibitory postsynaptic currents in neurons of rat auditory cortex. *Hear Res* **215**: 77–83.

Wang HT, Luo B, Huang YN, Zhou KQ, Chen L (2008). Sodium salicylate suppresses serotonin-induced enhancement of GABAergic spontaneous inhibitory postsynaptic currents in rat inferior colliculus in vitro. *Hear Res* **236**: 42–51.

Weissmann G (1991). Aspirin. *Sci Am* **264**: 84–90.

Wong BC, Zhu GH, Lam SK (1999). Aspirin induced apoptosis in gastric cancer cells. *Biomed Pharmacother* **53**: 315–318.

Xu H, Gong N, Chen L, Xu TL (2005). Sodium salicylate reduces gamma aminobutyric acid-induced current in rat spinal dorsal horn neurons. *Neuroreport* **16**: 813–816.

Ye ZY, Lu YG, Sun H, Cheng XP, Xu TL, Zhou JN (2008). Fluoxetine inhibition of glycine receptor activity in rat hippocampal neurons. *Brain Res*.

Yin MJ, Yamamoto Y, Gaynor RB (1998). The anti-inflammatory agents aspirin and salicylate inhibit the activity of I(kappa)B kinase-beta. *Nature* **396**: 77–80.