Inhibition of glycine response by amiloride in rat spinal neurons

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Abstract

The modulatory effect of amiloride on glycine-activated current (I_Gly) was investigated in acutely dissociated rat spinal dorsal horn neurons using the whole-cell patch clamp technique. Amiloride inhibited I_Gly reversibly in a concentration-dependent manner. It shifted the concentration-response relationship to the right without altering the maximum response and Hill coefficient of I_Gly. Amiloride did not change the ion selectivity of glycine receptor either. In addition, Na^+ or Ca^{2+}-free extracellular solutions and intracellular application of amiloride did not alter the amiloride inhibition of I_Gly. These results indicate that amiloride directly inhibited the glycine receptor response by decreasing the affinity of glycine to its receptor.

The diuretic amiloride is well known for its inhibitory action on Ca^{2+} channel [19], Na^+ channel [9] and transporters including the Na^+/H^+ antiporter and Na^+/Ca^{2+} exchanger [4,11], and it is clinically used for treatment of hypertension and renal failure [16,17]. Amiloride has also been shown to modulate the activity of several G-protein coupled receptors, including the adenosine receptor [5], adrenergic receptor [7] and dopamine receptor [6]. Recently, Fisher [3] investigated the amiloride modulation of recombinant neurotransmitter receptor, GABA_A receptor (GABA_AR) in detail, confirming an earlier report with GABA AR in frog sensory neurons [8]. Like GABA_AR, glycine receptor (GlyR) is a major inhibitory receptor and plays important roles in modulating neuronal excitability and signal transmission in the adult mammalian CNS [1,12,13]. Our recent study demonstrates that GABA_AR and GlyR cross-inhibit each other [14,21], suggesting that drugs acting at one receptor might also affect the function of the other receptor. This encourages us to examine the possible modulation of GlyR by amiloride in acutely dissociated rat spinal dorsal horn neurons.

The care and use of animals for these experiments followed the guidelines and protocols approved by our institutional Animal Care and Use Committee. Two-week-old Wistar rats were anesthetized with pentobarbital sodium (45–50 mg/kg, i.p.) and a segment of the lumbosacral (L5–S3) spinal cord was quickly dissected out and immersed into freezing incubation solution. Then 400 μm transverse slices were sectioned with a vibratome tissue slicer (VT1000S, Leica Instruments Ltd., Wetzlar, Germany). A vibration-isolation system [10,22] was then used to mechanically dissociate the spinal dorsal horn neurons. Briefly, a fire-polished glass pipette mounted on a vibrator touched lightly and vibrated horizontally at about 5–10 Hz on the surface of the spinal dorsal horn of the slice under the control of a pulse generator. The vibration-dissociation lasted for about 3 min and then the slices were removed from the dish. Within 20 min of dissociation, isolated neurons attached to the bottom of the culture dish and were ready for electrophysiological experiments. Whole-cell voltage-clamp recordings were performed using an Axopatch 200B patch-clamp amplifier which was connected to a Pentium III computer equipped with Digidata 1320A. The recording solutions were as described [15]. Clampexp and Clampfit software were used for data acquisition and analysis. In most experiments, 70–90% series resistance compensation was applied. Unless noted, the membrane potential was held at −50 mV in the voltage-clamp studies. All the experiments were performed at room temperature.
(22–25 °C). All the data were shown as the mean ± SEM, with statistical significance assessed by Student’s t-test. Statistically significant differences were assumed as P < 0.05 for all data. P and n represented the value of significance and the number of neurons, respectively.

Under the present experimental conditions, application of 30 μM glycine elicited inward currents at a holding potential of −50 mV on nearly all acutely dissociated dorsal horn neurons tested and these currents could be inhibited by 1 μM strychnine completely, the selective inhibitor of GlyR (data not shown). Amiloride, which is used clinically as a K⁺-sparing diuretic and the antagonist of acid-sensing ion channels [16], induced no detectable current when applied alone whereas it decreased glycine-activated current (IGly) reversibly in a concentration-dependent manner when co-applied with glycine (Fig. 1A). The averaged IC₅₀ for amiloride inhibition of IGly was 237.8 μM (n = 5) (Fig. 1B).

To examine the mechanism by which amiloride inhibited IGly, the concentration-response relationships of IGly with or without amiloride (300 μM) were examined. As shown in Fig. 2A,B, amiloride shifted the concentration-response curve to the right and increased the EC₅₀ value of IGly from 48.9 ± 5.4 to 137.6 ± 18.9 μM while it did not affect the Hill coefficient (1.07 ± 0.13 and 1.06 ± 0.12 with or without amiloride, respectively) or maximal value. A similar result was observed for taurine, the low affinity agonist of GlyR. At a taurine concentration near the EC₅₀ (100 μM) [20], amiloride (100 μM) significantly decreased taurine-activated current (ITau), whereas it had no obvious effect on current induced by saturating taurine concen-
tation (2 mM) [20] (Fig. 2C,D). These results suggest that amiloride inhibited \( I_{\text{Gly}} \) by decreasing the affinity of glycine to GlyR. Similar to the amiloride modulation of \( I_{\text{Gly}} \) and \( I_{\text{Tau}} \), amiloride dose-dependently inhibited GABA-activated current (\( I_{\text{GABA}} \)). Amiloride (300 \( \mu \)M) inhibited 30 \( \mu \)M \( I_{\text{GABA}} \) to 49.7 \( \pm \) 4.9% of control (\( P < 0.001, n = 9 \)) whereas it has no significant effect on the saturating concentration of 1 mM \( I_{\text{GABA}} \) (96.8 \( \pm \) 6.3% of control, \( P > 0.2, n = 6 \)), suggesting a competitive inhibition of GABA\( \alpha R \) by amiloride [3] in the present preparation.

The possible interaction of amiloride and strychnine, the selective antagonist of GlyR, was investigated. The application of 300 \( \mu \)M amiloride further inhibited 100 \( \mu \)M \( I_{\text{Gly}} \) in the presence of 0.1 \( \mu \)M strychnine (36.8 \( \pm \) 7.0% and 26.7 \( \pm \) 5.8% of control response without or with amiloride, respectively; \( P < 0.001, n = 8 \)), indicating that amiloride and strychnine might act on different binding sites of GlyR.

We next examined the inhibitory effect of 300 \( \mu \)M amiloride on 30 \( \mu \)M \( I_{\text{Gly}} \) at different holding potentials. The current–voltage relationships of \( I_{\text{Gly}} \) were studied in the absence or presence of amiloride, respectively (Fig. 3). The inhibitory effect of amiloride showed no voltage dependence over a range of membrane voltage from –110 to +30 mV. In addition, the reversal potential of \( I_{\text{Gly}} \) remained close to the theoretic equilibrium potential for chloride ions (\( \text{Cl}^- \)), indicating that amiloride did not change the ion selectivity of the GlyR.

Since the diuretic amiloride is best known for its inhibitory action on \( \text{Na}^+ \), \( \text{Ca}^{2+} \) channels [9,19] and transporters including the \( \text{Na}^+/\text{H}^+ \) transporter and \( \text{Na}^+/-\text{Ca}^{2+} \) exchanger [4,11], the possible involvement of \( \text{Na}^+ \) and \( \text{Ca}^{2+} \) in the amiloride-induced depression of \( I_{\text{Gly}} \) was investigated. The removal of extracellular \( \text{Ca}^{2+} \) or replacement of \( \text{Na}^+ \) with \( N \)-methyl-D-glucamine did not alter the inhibition of \( I_{\text{Gly}} \) by amiloride (data not shown). The question as to whether the site of amiloride modulation is intracellularly or extracellularly located was also explored with amiloride-containing microelectrodes. After 20 min of cell dialysis with 1 mM amiloride, the amplitude of \( I_{\text{Gly}} \) did not significantly differ from that of initial recordings. However, an additional extracellular application of 300 \( \mu \)M amiloride to the amiloride-containing neurons decreased \( I_{\text{Gly}} \) to 45.2 \( \pm \) 3.1% (\( n = 6 \)), an amount similar to the inhibition rate observed in neurons recorded with the standard pipette solution (Fig. 1B).

We have demonstrated that the diuretic amiloride concentration-dependently inhibited \( I_{\text{Gly}} \) in acutely dissociated rat spinal dorsal horn neurons for the first time. Amiloride seemed to inhibit \( I_{\text{Gly}} \) directly by decreasing the affinity of glycine to GlyR for the following reasons. Firstly, amiloride shifted the \( I_{\text{Gly}} \) concentration-response curve parallel to the right without altering the maximum \( I_{\text{Gly}} \) response and Hill coefficient. Secondly, amiloride did not change the ion selectivity of the GlyR (Fig. 3). Thirdly, intracellular application of amiloride produced no effect on the \( I_{\text{Gly}} \). Finally, removal of extracellular \( \text{Ca}^{2+} \) or replacement of \( \text{Na}^+ \) with NMDG did not alter the inhibition of \( I_{\text{Gly}} \) by amiloride, indicating that the inhibition was not due to the effect of amiloride on \( \text{Na}^+ \) and \( \text{Ca}^{2+} \) channels. Therefore amiloride is most likely to act directly on GlyR.

Glycine and GABA are the two most important fast inhibitory neurotransmitters in mammalian CNS. Recently, Fisher [3] demonstrated that amiloride produced an apparent rebound current as well as competitively inhibited the amplitude of \( I_{\text{GABA}} \) in transfected L929 cells. Similarly, amiloride competitively inhibited \( I_{\text{Gly}} \) and \( I_{\text{GABA}} \) in the present study. Taurine is a low affinity agonist for GlyR and is known to gate a \( \text{Cl}^- \) current in the same preparation [20]. The similarity of amiloride inhibition on the \( I_{\text{Gly}} \), \( I_{\text{GABA}} \) and \( I_{\text{Tau}} \) indicates that amiloride could act as a non-specific \( \text{Cl}^- \) channel inhibitor. Amiloride further inhibited \( I_{\text{Gly}} \), even in the presence of strychnine, the selective GlyR antagonist, which further supports this conclusion. However, in our preparation amiloride did not induce the rebound response

![Fig. 3](image-url)
after washout of agonist [3]. This difference may be attributed to the structural and pharmacological distinctions of GlyR and GABA<sub>A</sub>R though they are both ligand-gated Cl<sup>-</sup> channels [13]. In addition, in contrast to the transfected L929 cells used in Fisher’s study [3], acutely dissociated neurons were used in the present experiment, which can better reflect the in vivo physiological conditions.

Amiloride is used clinically as a K<sup>+</sup>-sparing diuretic through its inhibitory action on renal Na<sup>+</sup> channels [16,17]. Although we showed that amiloride inhibited both GlyR and GABA<sub>A</sub>R in vitro, in general, its in vivo effect on CNS neurons may only be pronounced in conditions of high amiloride concentration or defective blood–brain barrier because amiloride and its derivatives could not pass the blood–brain barrier [18]. However, amiloride was recently used to treat some CNS disorders such as brain tumors by direct application to the cerebrospinal fluid [2,18]. The present result indicates that much attention should be paid to its CNS side effects when amiloride is applied intracerephally. Moreover, since GlyR plays important roles in many physiological functions such as modulation of neuronal excitability and nociceptive transmission, the research of amiloride modulation of GlyR might provide valuable evidence for the exploration of new clinical functions of amiloride.

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References