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Local anesthetics reduce the inhibitory neurotransmitter-induced current in dissociated hippocampal neurons of the rat

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Abstract

The effects of local anesthetics on amino acid-induced currents were examined using the whole-cell configuration of the patch clamp technique in dissociated hippocampal pyramidal neurons of the rat. Lidocaine (3 mM) decreased the glycine-induced Cl^- current (Gly-I_{Cl}) more potently (to 46% of the control value) than the γ-aminobutyric acid-induced Cl^- current (GABA-I_{Cl}; to 75%), whereas the agent had little effect on the excitatory glutamate response. The reduction in the Gly-I_{Cl} was dose-dependent, with a dissociation constant (K_D) of 3 mM and a Hill coefficient of 0.96. A non-competitive inhibition was suggested by a double reciprocal plot of the effects of lidocaine on the concentration-response curve of the Gly-I_{Cl}. Benzocaine, a neutral local anesthetic at physiological pH, decreased the Gly-I_{Cl} more potently than lidocaine, while QX314, a permanently charged quaternary derivative of lidocaine, produced a much smaller inhibition, thereby indicating that the neutral form of local anesthetics is more effective in reducing the Gly-I_{Cl}. The depression of the Gly-I_{Cl} and GABA-I_{Cl} in central neurons may contribute to local anesthetic-induced convulsions.

Keywords: Local anesthetic; Hippocampal neuron; Chloride ion; Patch-clamp technique; GABA (γ-aminobutyric acid); Glycine

1. Introduction

Local anesthetics affect various types of ion channels in excitable membranes (Narahashi et al., 1970; Schwarz et al., 1977; Neher and Steinbach, 1978; Ogden et al., 1981; Oyama et al., 1988; Kaneda et al., 1989; Oda et al., 1992; Martin et al., 1993, Sugiyama and Muteki, 1994). When administered systemically at small doses, local anesthetics are known to have depressant effects on the central nervous system, which result in sedation or anesthesia. However, when a high concentration is achieved in blood, either by rapid absorption or by an accidental intravenous injection of large doses, local anesthetics have been reported to induce convulsions which can be prevented or treated with central nervous system depressants such as barbiturates and benzodiazepines (Koppanyi, 1962). Using various preparations, other investigators have carried out in vivo and in vitro studies to elucidate the actions of local anesthetics on transmission in the central nervous system. Frank and Sanders (1963) proposed the hypothesis of suppression of inhibitory neurons in the central nervous system. Tanaka and Yamasaki (1966) showed that lidocaine (7 mg/kg i.v) selectively blocked inhibitory synapses of the cortical neurons of unanesthetized rabbits. De Jong et al. (1969) suggested a preferential depression by lidocaine (5–25 mg/kg i.v.) of the segmental inhibitory transmission in the spinal cord of cats. Tsujimoto and Ikeda (1979) assessed presynaptic effects using rat brain synaptosomes and found that the Ca^{2+} uptake into the synaptosomes was depressed by local anesthetics (e.g. lidocaine 0.5–1 mM). They suggested that inhibition of γ-aminobutyric acid (GABA) release from the nerve endings might be involved in the mechanisms of the local anesthetic-induced convulsions.

In the present study, we examined the postsynaptic effects of the agents on excitatory and inhibitory amino acid-induced currents in dissociated hippocampal pyra-

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midal neurons of the rat and have found that local anesthetics decrease the inhibitory chloride currents, with a greater effect on the glycine (Gly)-evoked chloride current (Gly-I_{Cl}) than on the GABA-evoked chloride current (GABA-I_{Cl}).

2. Materials and methods

2.1. Preparation

The pyramidal neurons in the hippocampal CA1 region were acutely dissociated from 1- to 2-week-old Wistar rats by a technique developed by Kaneda et al. (1988) with some modification in enzymes used. In brief, the rats were anesthetized with diethyl ether and decapitated. The brain was rapidly dissected and sliced 400 μm thick with a microslicer (Dosaka DTK-1000, Kyoto, Japan) in an ice-cooled physiological salt solution (PSS, see below). After preincubation in the PSS for 50 min at room temperature, the slices were enzymatically treated in PSS containing 0.01% Pronase (protease, *Streptomyces griseus*, Calbiochem, La Jolla, CA, USA) then succeedingly 0.01% protease type X (Sigma, St. Louis, MO, USA) for 20 min each at 31°C. Solutions were constantly bubbled with pure oxygen throughout the procedures. The hippocampal CA1 region was micropunched out from the slices with a small needle. The neurons were mechanically dissociated in a culture dish filled with the PSS by gentle pipetting (namely, repeated uptake and expulsion of a cluster of neurons) with a fire-polished glass tube. The dissociated single cells adhered to the bottom of a dish within 30 min.

2.2. Electrical measurements

Ionic currents were recorded in the whole cell configuration of the patch clamp technique (Hamill et al., 1981). The resistance between the recording patch pipette and the reference electrode was 4–8 MΩ. The transmembrane currents were measured with a patch clamp amplifier (Axopatch-1D, Axon Instruments, Foster City, CA, USA). The current and voltage were monitored on both a storage oscilloscope (Textronix 5113, Beaverton, OR, USA) and a linear recorder (Graphtec WR3300, Tokyo, Japan), and stored on video tapes after digitization with a PCM processor (Sony PCM-501ES, Tokyo, Japan; 16-bit, bandwidth DC-20 KHz).

2.3. Application of drugs

Rapid application of the drugs was achieved by using a concentration-clamp technique termed 'Y-tube' method (Murase et al., 1989; Shirasaki et al., 1991). Using this technique, the external solution surrounding the neuron is completely exchanged within 10–20 ms so that the accurate peak value of the response is obtained before desensitization develops and hence makes the peak response oblique. An interval of 5 min was usually allowed between applications of drugs.

2.4. Solutions

The ionic composition of the external solution (PSS) was (in mM): NaCl 150, KCl 5, $CaCl_2$ 2, $MgCl_2$ 0.5, glucose 10, and Hepes 10. The intrapipette solution contained (in mM): KCl 150, $MgCl_2$ 2, Na_2ATP 2, EGTA 5, $CaCl_2$ 0.25, and Hepes 10. The pH of the external and internal solutions was adjusted to 7.4 and 7.2 with Trizma-base, respectively. In some experiments when a high concentration of drugs (10 mM) was used, the osmolarity of the control external solution was compensated with sucrose.

2.5. Drugs

Drugs used in the present study were lidocaine (Sigma), benzocaine, QX314, GABA, glycine and glutamate (Ishizu, Osaka, Japan). All drugs were directly dissolved in the PSS.

The pH was adjusted when lidocaine was dissolved, since the drug increased the pH values of the solution. Pre-application of the local anesthetics for 60s did not affect any of the neurotransmitter-induced responses in separate experiments, so that in succeeding experiments the local anesthetics were applied simultaneously with the amino acids.

All experiments were carried out at room temperature of around 22°C. Data are expressed as means ± S.D. Significance was assessed using the Student's *t*-test. The present study was approved by the Committee for Animal Experiments of the Faculty.

3. Results

3.1. Effects of lidocaine on the amino acid-induced currents

Glutamate is known as a major excitatory neurotransmitter in the mammalian central nervous system and in the present experiments glutamate evoked an inward current in dissociated hippocampal CA1 pyramidal neurons voltage-clamped at a holding potential of −60 mV. GABA and Gly are inhibitory neurotransmitters which activate chloride ionophores on the neuronal membrane, resulting in suppression of the neuronal activity. Under the present ionic conditions, the inhibitory transmitters induced an inward chloride cur-

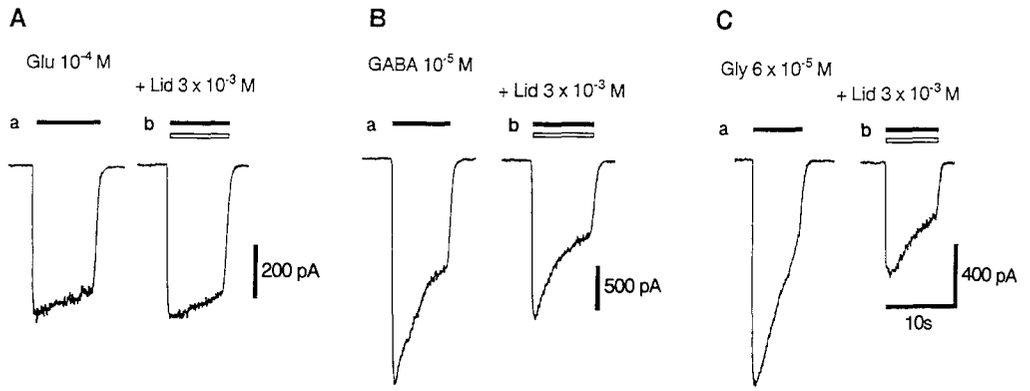

Fig. 1. Effects of lidocaine (Lid; 3×10^{-3} M) on the neurotransmitter-induced current in dissociated hippocampal pyramidal neurons of the rat. A: Glutamate (Glu; 10^{-4} M) evoked a slowly desensitizing inward current. Simultaneous application of lidocaine did not affect the glutamate response. B: γ-Aminobutyric aid (GABA; 10^{-5} M) evoked a desensitizing inward chloride current. Co-application of lidocaine decreased the GABA-I_{Cl}. C: Glycine (Gly; 6×10^{-5} M) evoked a desensitizing inward chloride current. Simultaneously applied lidocaine reduced the Gly-I_{Cl}. Note that lidocaine decreased the Gly-I_{Cl} more than the GABA-I_{Cl}. The holding potential was −60 mV. Each horizontal bar above the trace indicates the period of continuous drug application. Downward deflections reflect inward currents.

rent at −60 mV. Fig. 1A shows that the glutamate-induced excitatory response was not affected by lidocaine (3 mM). Lidocaine decreased the chloride current evoked by GABA or Gly, having a larger effect on the Gly-I_{Cl} than on the GABA-I_{Cl} (Fig. 1B and C). Fig. 2 illustrates the concentration-inhibition relationships of lidocaine on the currents induced by the three amino acid transmitters. Little effect was observed on the glutamate response at any of the concentrations tested. In contrast, both the GABA-I_{Cl} and the Gly-I_{Cl} were markedly reduced by lidocaine in a concentration-dependent manner. The Gly response, in particular, was decreased by 3 mM lidocaine to 46%.

3.2. Effects of three local anesthetics on the glycine-induced current

The effective form of local anesthetics (i.e. charged or neutral) is an important issue to understand how these compounds interact with the ion channels. Since local anesthetics dissociate according to their pK_a and the pH of the solution, the inhibitory effects on the Gly-I_{Cl} of two other agents were further examined: QX314, a permanently charged quaternary derivative of lidocaine, and benzocaine, a neutral local anesthetic at physiological pH. As shown in Fig. 3, QX314 (1 mM) had little effect on the Gly-I_{Cl}. In contrast, benzocaine (1 mM) reduced the Gly-I_{Cl} the most potently of the three local anesthetics. Fig. 4 depicts the concentration-inhibition relationships for the Gly-I_{Cl}, which shows that the three local anesthetics reduced the current in a concentration-dependent manner. The data points were fitted according to the equation:

$$I = I_{max} \cdot \{1 - C^n/(C^n + K_D^n)\} \quad (1)$$

where C = the concentration; n = the Hill coefficient; and K_D = the dissociation constant for the depressant effect of the local anesthetic. A least-squares fitting gave a K_D of 8.3 mM for the QX314 inhibition, 3 mM for lidocaine and 1.6 mM for benzocaine, and a Hill coefficient of 1.2, 0.96 and 1.3, respectively. The continuous lines were drawn using those values and an I_{max} of 1. The relative inhibitory potency was in the order of benzocaine > lidocaine > QX314. The results show that the neutral form of a local anesthetic is more potent than the charged form.

Fig. 5 depicts the current-voltage relationships for the Gly response in the presence and the absence of 3 mM lidocaine. The peak amplitude of the current was

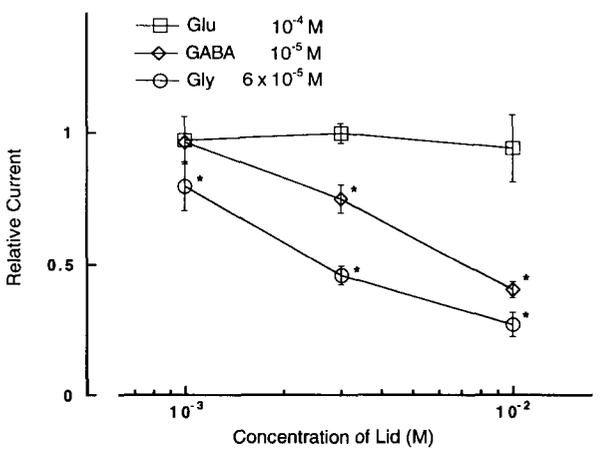

Fig. 2. The concentration-inhibition relationships of lidocaine (Lid) on the currents induced by Glu (10^{-4} M), GABA (10^{-5} M) and Gly (6×10^{-5} M). Note that lidocaine had little effect on the Glu response, whereas the local anesthetic markedly reduced the currents induced by GABA and Gly. The peak amplitude of the current was normalized to that of each control response. The holding potential was −60 mV. Each data point shows the average of 5–6 experiments and the vertical bar indicates standard deviation (S.D.). The asterisks show the significant decrease of the current ($P < 0.05$).

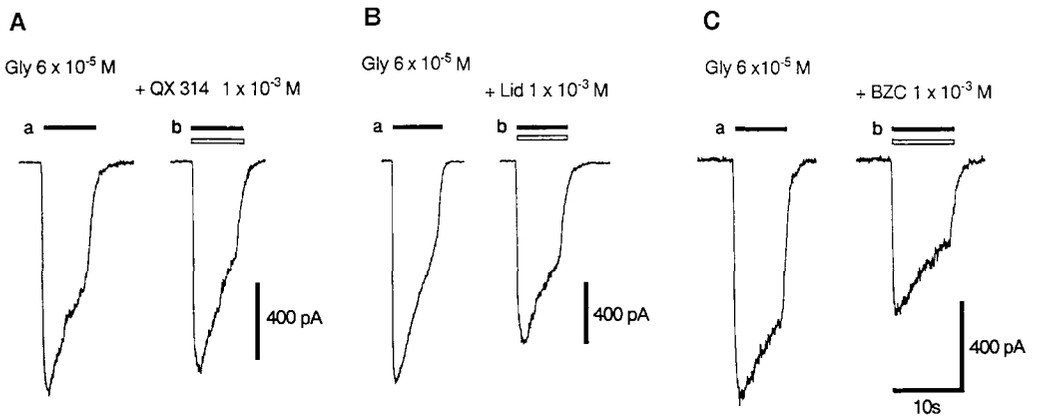

Fig. 3. Effects of three local anesthetics on the Gly-I_{Cl}. Gly (6×10^{-5} M) evoked an inward current in the dissociated neurons voltage-clamped at −60 mV (A-a, B-a and C-a). Simultaneously applied benzocaine (BZC; C-b) decreased the response more potently than QX314 (A-b) or lidocaine (Lid; B-b) did at the same concentration (10^{-3} M).

normalized to that elicited by 60 μM Gly at a holding potential of −60 mV and plotted against the potential. The current-voltage relations were almost linear and the Gly response reversed its polarity at about +6 mV, which is close to the equilibrium potential for chloride ions predicted by the Nernst equation in the present condition. The reversal potential for the Gly response was not affected by lidocaine. The results show that the inhibitory effect of lidocaine on the Gly response was not voltage-dependent.

3.3. Effects of lidocaine on the concentration-response relationship of the glycine response

The concentration-response relationships of the Gly-I_{Cl} in the absence and the presence of lidocaine (2 mM and 10 mM) are shown in Fig. 6A, where all responses are normalized to the peak amplitude of the current evoked by 60 μM of Gly. The concentration-response relations were in accord with the conventional expression:

$$I = I_{max} \cdot C^n / (C^n + K_D^n) \quad (2)$$

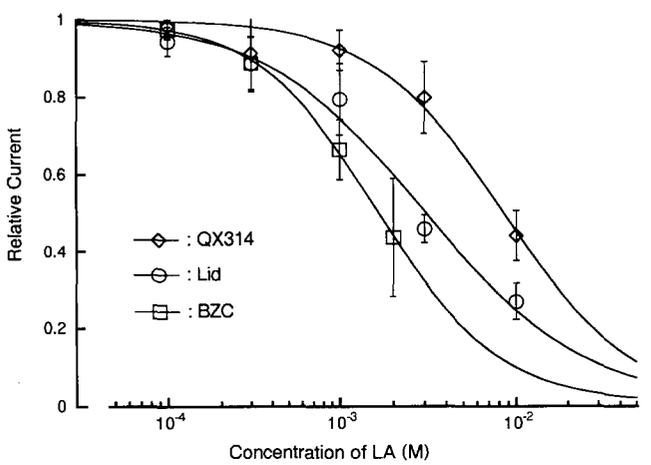

Fig. 4. The concentration-inhibition relationships of QX314, lidocaine (Lid) and benzocaine (BZC) on the Gly (6×10^{-5} M)-induced current. The ratio of inhibition by the three local anesthetics (LAs) is plotted against the concentration of the agents. All the local anesthetics decreased the Gly-I_{Cl} in a dose-dependent manner. A least-squares fit according to equation (1) in the text yielded a K_D of 8.3 mM for the QX314 inhibition, 3 mM for lidocaine and 1.6 mM for benzocaine, and the Hill coefficient was 1.2, 0.96 and 1.3, respectively. Each data point is the average of 5–6 experiments and the vertical bar indicates S.D.

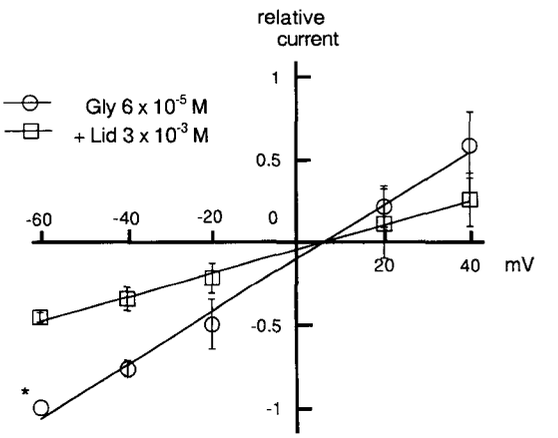

Fig. 5. The current-voltage relationships of the Gly-I_{Cl} in the absence and the presence of lidocaine (Lid; 3×10^{-3} M). The current-voltage relations were almost linear for the response induced by 6×10^{-5} M Gly either with or without lidocaine. The inhibitory effect of lidocaine was not affected by the holding potential. The Gly-I_{Cl} reversed its polarity at about +6 mV, which is close to the equilibrium potential for chloride ions in the present ionic condition. The reversal potential was not affected by lidocaine. All currents were normalized to that evoked by 6×10^{-5} M Gly at a holding potential of −60 mV(*). Each data point shows the average of 4–5 experiments and the vertical bar indicates S.D.

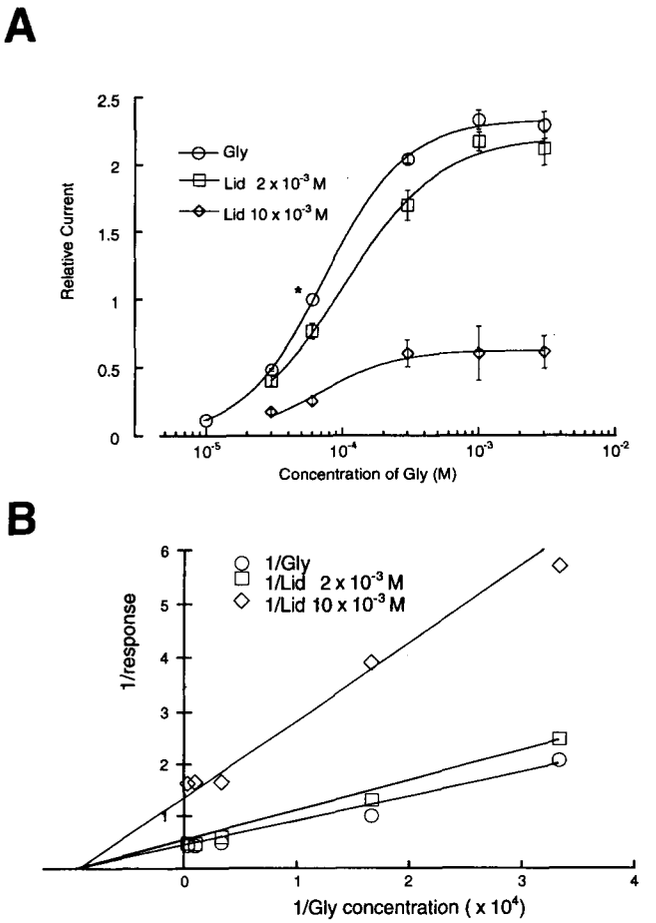

Fig. 6. The concentration-response relationships of the Gly-I_{Cl} in the absence and the presence of lidocaine (Lid; 2×10^{-3} M and 10^{-2} M). A: The decrease produced by 2×10^{-3} M lidocaine was not marked, while 10^{-2} M lidocaine markedly reduced the maximum value of the Gly-I_{Cl} without affecting the threshold. The peak amplitude of the current was normalized to that evoked by 6×10^{-5} M Gly (*). A least-squares fit was performed using equation (2) in the text and gave a K_D of 7.4×10^{-5} M and a Hill coefficient of 1.5 for the Gly response. The holding potential was −60 mV. Each data point shows the average of 4–7 experiments and the vertical bar indicates S.D. B: Double reciprocal plot of lidocaine inhibition on the Gly-I_{Cl}. The plot suggests that the inhibition is non-competitive.

where I = the observed (normalized) glycine-induced current; I_{max} = the maximum value of the current; C = the Gly concentration; K_D = the dissociation constant; and n = the Hill coefficient. A least-squares fitting gave a K_D of 74 μM and a Hill coefficient of 1.5 for the Gly response. The continuous lines were drawn using those values and I_{max} of 2.4. The inhibitory effect of 2 mM lidocaine was small. In contrast, a higher concentration of lidocaine (10 mM) markedly decreased the maximum value of the Gly-I_{Cl} without changing the threshold. A Lineweaver-Burk double reciprocal plot suggests a non-competitive inhibition (Fig. 6B).

4. Discussion

4.1. Effects of local anesthetics on the inhibitory neurotransmitter-induced current

GABA has long been known as a major inhibitory neurotransmitter in the mammalian central nervous system. Electrophysiological studies have shown that the $GABA_A$ receptor is coupled to the Cl^- channel, the conductance of which is increased by GABA binding to the receptor. The conductance increase leads to suppression of neuronal activities since the equilibrium potential for Cl^- is fairly negative due to low intracellular concentration of Cl^-. $GABA_A$ antagonists, bicuculline and picrotoxin, are potent convulsants, whereas conventional CNS depressants such as barbiturates and benzodiazepines have been reported to interact with the $GABA_A$ receptor and increase the total flux of Cl^- induced by GABA (Olsen and Tobin, 1990). Recent studies have shown that general anesthetics including volatile agents increase the GABA responses of the central neurons (Nakahiro et al., 1989; Wakamori et al., 1991; Hara et al., 1993, 1994). Gly is also a major inhibitory transmitter and is most abundantly found in the ventral quadrant of the spinal cord. Gly hyperpolarizes motoneurons by increasing the Cl^- conductance. A Gly antagonist, strychnine, is a well-known convulsant and CNS depressants (volatile anesthetics and an intravenous anesthetic) have been reported to enhance the Gly response in mammalian central neurons (Wakamori et al., 1991; Hales and Lambert, 1991).

The present study has shown that local anesthetics decrease the inhibitory neurotransmitter (GABA and Gly)-induced chloride currents in mammalian central neurons (Figs. 1–4) but have little effect on the excitatory glutamate response. An antagonist of Gly as well as those of GABA induce convulsions due to depression of the inhibitory pathways in the central nervous system. Our present findings, therefore, suggest that when the concentration of local anesthetics reaches a high level in the brain, the agents may suppress the inhibitory postsynaptic responses and make the central nervous system more excitable, which results in convulsions in animals.

It is of interest that the Gly-I_{Cl} was more sensitive to the local anesthetics than the GABA-I_{Cl}. GABA is known to be the major inhibitory neurotransmitter in the central nervous system, while Gly was considered to exert an inhibitory effect mainly in the spinal cord. Recently, however, immunological and molecular cloning studies have revealed that the Gly receptor is widely distributed not only in the spinal cord and brain stem but throughout the mammalian central nervous system (Betz, 1991). It is, therefore, likely that the reduction of both the Gly-I_{Cl} and GABA-I_{Cl} contributes to the local anesthetic-induced convulsions. A

double reciprocal plot of the effects of lidocaine on the concentration-response relationship of the Gly-I_{Cl} shows that the lidocaine inhibition was non-competitive (Fig. 6), thereby suggesting that lidocaine affects the Gly receptor-chloride ionophore complex by binding to a site distinct from that to which Gly binds.

4.2. Neutral form of local anesthetic is responsible

Benzocaine, a neutral local anesthetic at physiological pH, decreased the Gly-I_{Cl} more potently than lidocaine. QX314, a permanently charged quaternary derivative of lidocaine, produced a much smaller inhibition (Figs. 3 and 4). The lack of voltage dependence in the action of lidocaine also suggests that the charged molecules contribute much less to the reduction (Fig. 5). These findings indicate that the electrically neutral form of local anesthetics is more active in reducing the Gly-I_{Cl}. This mode of action of local anesthetics is somewhat different from the main effects of the agents on the sodium channel. In a series of studies, Narahashi and co-workers reported that the action potential was blocked by the charged form of local anesthetics inside the squid giant axon (Narahashi et al., 1970; Frazier et al., 1970, 1972). Hille (1977) and Hondeghem and Katzung (1977) proposed the modulated receptor model for the action of local anesthetics on the sodium channel: neutral and charged forms reach a hydrophobic pocket of the channel protein via hydrophobic (membrane phase) and aqueous (intracellular) pathways, respectively. The drugs are assumed to bind more tightly to the inactivated state than to the resting or activated form, which was deduced from the use-dependent block of the channel (Courtney, 1975).

Single-channel experiments showed that a Ca^{2+}-activated K^+ channel (big conductance K channel; BK channel) in the pyramidal neurons of the rat hippocampus was also blocked by the ionized form of local anesthetics from the cytoplasmic side. The BK channel was not affected by benzocaine at either side of the membrane (Oda et al., 1992). Further, the nicotinic acetylcholine channels of the skeletal muscle were reported to be blocked by both permanently charged quaternary derivatives of lidocaine (QX222 and QX314; Neher and Steinbach, 1978) and the uncharged local anesthetic, benzocaine (Ogden et al., 1981), which were applied on the external side of the cell membrane.

4.3. Central nervous system toxic concentrations of local anesthetics

The toxic concentration of lidocaine reportedly ranges from 5 to 14 μg/ml venous plasma (namely, 20–60 μM) (Foldes et al., 1960; Bromage and Robson, 1961). Arterial levels of the agent may be much higher (Moore et al., 1976) due to its rapid elimination from the blood through the liver (Katz, 1968; Stenson et al., 1971). Tissue distribution studies further showed that the accumulation of [^{3}H]lidocaine in the rat brain was 5 times higher than that in blood (Åkerman et al., 1966). Arai and Takasugi (1989) studied the onset of convulsive waves of the electroencephalogram in rabbits paralyzed with a muscle relaxant. They estimated the cerebral blood flow and injected lidocaine into a common carotid artery for one circulation time of the animal (20 s) so as to maintain the lidocaine concentration at a desired level in the cerebral blood stream. Even when the concentration was calculated to be as high as 300 μg/ml cerebral blood (1.3 mM), only five out of ten rabbits showed myoclonic brain waves, suggesting that the convulsion did not occur in all cases even with much higher concentrations of the anesthetic than the concentrations previously reported in humans.

Those arguments suggest that the seizure-inducing concentrations of local anesthetics in the brain of experimental animals may be in the order of millimolar, concentrations at which local anesthetics suppressed the Gly and GABA responses in the present study. The discrepancy with the concentration derived from human data may be due to differences in species and preparations. Experimental conditions including temperature may also be responsible for the discrepancy (Wamil and McLean, 1993).

4.4. Conclusions

The results obtained in the present study show that local anesthetics had little effect on the excitatory transmembrane current evoked by glutamate, whereas the agents decreased the inhibitory chloride currents evoked by Gly and GABA. The Gly-I_{Cl} was more sensitive than the GABA-I_{Cl} to the local anesthetics.

Acknowledgments

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