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Effects of diuretics on GABA-gated chloride current in frog isolated sensory neurones

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1 Effects of three diuretics (furosemide, amiloride and α-human atrial natriuretic polypeptide (α-hANP)) on GABA-activated chloride current (I_{Cl}) were investigated in frog isolated sensory neurones, following suppression of Na^+, K^+ and Ca^{2+} currents, by use of a 'concentration-clamp' technique.

2 Furosemide inhibited the GABA-activated I_{Cl} in a non-competitive manner and facilitated the inactivation phase, while amiloride inhibited the GABA response in a competitive manner, both inhibitions being concentration-dependent. α-hANP had no effects on the GABA-induced I_{Cl}.

3 The reversal potential of GABA-activated I_{Cl} (E_{GABA}) was not shifted in the presence of amiloride or furosemide.

4 The results suggest that amiloride may act at the GABA binding site while furosemide may act on the GABA-gated chloride channel.

Introduction

Furosemide, one of the loop diuretics, blocks Cl^- transport in human red blood cells (Brazy & Gunn, 1976) and in guinea-pig hippocampal neurones (Misgeld *et al.*, 1986). Amiloride, another type of diuretic, is reported to inhibit Na^+ transport and Na^+-Ca^{2+} exchange in rat synaptosomal membrane (Schellenberg *et al.*, 1985). Also, α-human atrial natriuretic polypeptide (α-hANP) has a potent diuretic action (Kangawa & Matsuo, 1984), and atriopeptin II, an analogue of α-hANP, can directly inhibit amiloride-sensitive Na^+ transport in renal epithelial cells, probably through its stimulation of cyclic GMP (Cantiello & Ausiello, 1986). It has been demonstrated that ANP-like immunoreactivity exists in the spinal cord (Skofitsch *et al.*, 1985), in the brain, e.g. hypothalamus (Tanaka *et al.*, 1984; Morii *et al.*, 1985; Saper *et al.*, 1985) and in the peripheral autonomic nervous system (Debinski *et al.*, 1986). Since furosemide may affect Cl^- transport in neurones, the possibility that it might exert a direct action upon the GABA-gated Cl^- current of sensory neurones was investigated. The effects of furosemide were compared with those of two other diuretics, amiloride and α-hANP, which interfere with Na^+ transport.

GABA action on cell bodies of dorsal root ganglia may be considered as a model for GABA-induced presynaptic inhibition at the intraspinal nerve terminals (Feltz & Rasminsky, 1974; Gallagher *et al*, 1978), since GABA acts in a similar way on the soma membrane as it does on the nerve terminal (Levy, 1977; Padjen & Hashiguichi, 1983). Recently, we have recorded the GABA-gated I_{Cl} in frog isolated sensory neurones using a 'concentration-clamp' technique under voltage-clamp conditions and studied the pharmacological and electrical properties of the GABA response (Hattori *et al.*, 1984; Ishizuka *et al.*, 1984; Akaike *et al.*, 1985; 1986; Inoue *et al.*, 1986). The newly developed 'concentration-clamp' technique combines internal perfusion with a modified suction pipette technique (Hattori *et al.*, 1984; Ishizuka *et al.*, 1984); rapid solution change within a few ms in the cell-attached condition provides a reliable and efficient means of studying the kinetics of agonist-induced ionic currents even in the activation phase (Akaike *et al.*, 1986). The effects of furosemide, amiloride and α-hANP on GABA-activated I_{Cl} in the frog sensory soma membrane were studied by use of the 'concentration-clamp' technique.

Methods

Dorsal root ganglia dissected from the decapitated frog (*Rana catesbiana*) were used. The thick connective

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tissue surrounding the ganglia was carefully stripped off with micro-forceps and the capsules enveloping the ganglion masses were digested in 10 ml normal Ringer solution containing 0.3% (w/v) collagenase and 0.05% (w/v) trypsin (pH 7.4, 18 min at 37°C) (Akaike *et al.*, 1985). During the enzyme treatment, the preparation was agitated by bubbling 99.9% O_2 through the solution. Thereafter, single cells were isolated mechanically from the ganglion mass with finely polished pins under binocular observation, and left overnight in a culture medium consisting of equal parts of Ringer solution and Eagle MEM (Nissui, Japan) at about 10°C.

The isolated neurone was perfused externally and internally with Na^+-, K^+- and Ca^{2+}-free test solutions. The Cl^- concentrations in both extracellular and intracellular solutions were kept at 120 mM. The ionic compositions of the solutions were (mM): internal, CsCl 95, Cs-aspartate 10, TEA-Cl 25, EGTA 2.5; external, Tris-Cl 83, CsCl 2, $MgCl_2$ 5, TEA-Cl 25, 4-aminopyridine 3, glucose 5. The pH of all solutions was adjusted to 7.4 with Tris-base or N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES).

When GABA was applied to sensory neurones perfused with the internal test solution containing 120 mM Cl^- at a driving force (ΔV_H, the potential difference between holding membrane potential and Cl^- equilibrium potential, E_{Cl}) less than 10 to 20 mV, there were no Cl^- shifts during a continuous application of GABA. However, at a ΔV_H greater than 20 mV, a continuous application of GABA induced a time-dependent negative or positive shift of the reversal potential (E_{GABA}), depending upon the inward or outward currents. The results indicate that the decay of the GABA-induced current during GABA application at greater ΔV_H were the sum of 'true' receptor desensitization and Cl^- shifts (Akaike *et al.*, 1987a). We also found in the frog sensory neurones that the GABA-induced I_{Cl} was reduced in the presence of external Na^+ and that the inhibition was mediated by the uptake of GABA subserved by a Na-GABA co-transport mechanism (Akaike *et al.*, 1987b). To avoid such contribution of Cl^- shifts and GABA uptake in the presence of external Na^+ on the kinetic properties of the GABA-gated I_{Cl}, therefore, most of recordings of GABA-induced currents were made on neurones perfused with Na^+-, K^+- and Ca^{2+}-free external and internal solutions containing 120 mM Cl^- at ΔV_H less than 20 mV.

The suction-pipette technique was used for voltage clamp and internal perfusion of the neurones (Akaike *et al.*, 1985; 1986). The inner diameter of the finely-polished tip of the suction pipette was about 8 μm and the electrode resistance was about 200–300 kΩ. The membrane potential was controlled with the single-electrode voltage-clamp amplifier of the sample-and-hold type (Ishizuka *et al.*, 1984).

The drugs used were γ-aminobutyric acid (GABA, Tokyo Kasei), amiloride (Sigma), furosemide (Hoechst) and α-human atrial natriuretic polypeptide (α-hANP, Suntory).

All experiments were carried out at room temperature (20–23°C). Both voltage and current were monitored on an oscilloscope (National, type VP-5730A) and were simultaneously recorded on an ink-writing recorder (Rikadenki, type R-22) and stored on an FM data recorder (TEAC, type R-22) for later analysis.

Results

In our previous experiments, the EC_{50} in the GABA dose-response curve was determined to be 1.3×10^{-5} M (Akaike *et al.*, 1986). Thus, the effects of

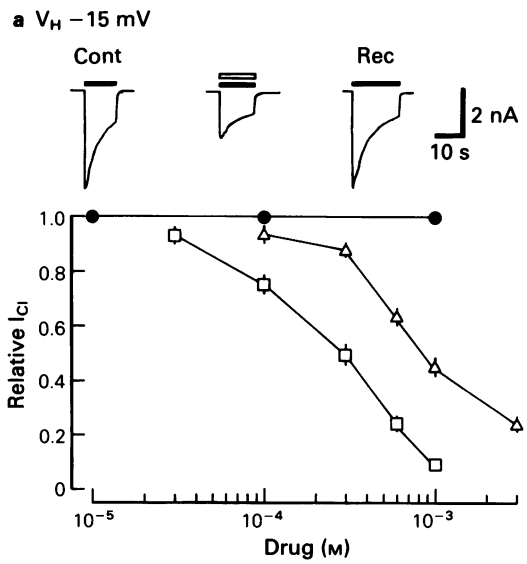

Figure 1 Effects of amiloride, furosemide and α-human atrial natriuretic polypeptide (α-hANP) on the peak inward Cl^- current (I_{Cl}) induced by 10^{-5} M GABA in voltage-clamped frog sensory cell body. Holding potential (V_H) was −15 mV. All recordings were obtained from the cells perfused with the internal and external solutions containing 120 mM Cl^-: (a) shows typical original I_{Cl}-traces induced by 10^{-5} GABA (solid bar) in the absence (left) and in the presence of 3×10^{-4} M amiloride (open bar) (centre), and the recovery (right). In (b) are shown GABA-induced I_{Cl} in the presence of diuretics at various concentrations: (□) amiloride; (△) furosemide; (●) α-hANP. In all experiments, preparations were pretreated with diuretics for 1 min and GABA was applied in the presence of diuretic. Each point and vertical bar gives mean ± s.e.mean ($n = 5$). All responses were normalized for the peak I_{Cl} induced by 10^{-5} M GABA alone.

amiloride, furosemide and α-hANP on the peak inward I_{Cl} induced by 10^{-5} M GABA at a holding potential (V_H) of −15 mV were examined. Simultaneous application of amiloride or furosemide with GABA depressed I_{Cl} in a concentration-dependent manner ($n = 5$) (Figure 1). The concentrations at which I_{Cl} was blocked by 50% were 8×10^{-4} M for furosemide and 3×10^{-4} M for amiloride, respectively. α-hANP (10^{-5} to 10^{-3} M) had no effect on I_{Cl} induced by 10^{-5} M GABA ($n = 5$) (Figure 1).

The voltage-dependency of the peak I_{Cl} induced by 10^{-5} M GABA, in the absence and presence of furosemide or amiloride, was studied in neurones held at various membrane potentials. Both 3×10^{-4} M amiloride and 10^{-3} M furosemide depressed GABA-induced peak I_{Cl} by about 55 and 65% at every membrane potential, respectively, and the current-voltage (I-V) relationships obtained in the presence of amiloride or furosemide were linear (Figure 2). The results suggest that the blockade of the GABA response with amiloride and furosemide is not voltage-dependent. When the reversal potential of GABA-induced I_{Cl} (E_{GABA}) was estimated from the intercepts with the voltage axis of the I-V relationship, E_{GABA} was $+4.1 \pm 0.8$ mV ($n = 8$). This is close to the Cl^- equilibrium potential (E_{Cl}) of about +4 mV calculated from the Nernst equation, knowing the intra- and extracellular Cl^- activity coefficients (Akaike *et al.*, 1986). Both furosemide and amiloride did not induce any shift of E_{GABA} (Figure 2, b).

The effects of 10^{-3} M furosemide or 3×10^{-4} M amiloride on the GABA concentration-response curve were investigated quantitatively. The results are shown in Figure 3b, in which amiloride caused a parallel shift of the curve to the right without affecting the maximum GABA response. The half-maximum dose (EC_{50}) estimated from concentration-response curves changed from 1.3×10^{-5} M (control) to 2.2×10^{-5} M after adding amiloride ($n = 4$). This indicates that amiloride decreases GABA-induced I_{Cl} in a competitive manner. On the other hand, furosemide decreased the maximum current with no or little shift of the GABA dose-response curve, i.e. the EC_{50} in the presence of furosemide was 1.5×10^{-5} M.

The GABA dose-response relationship accorded with the conventional expression:

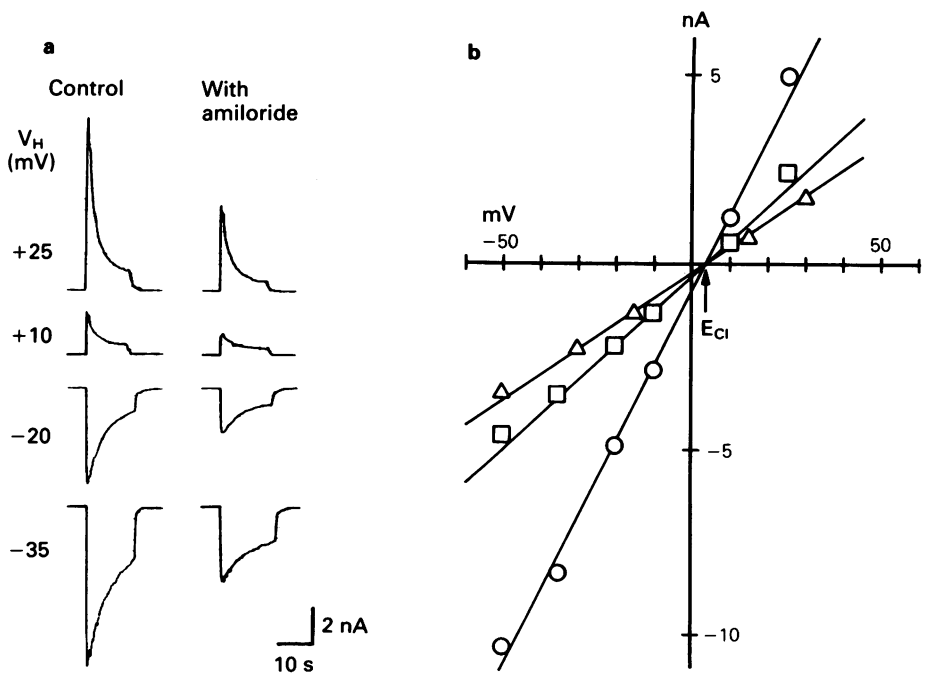

Figure 2 Effects of amiloride and furosemide on 10^{-5} M GABA-induced I_{Cl} and the I-V relationships. In (a) the original I_{Cl} traces in the absence and presence of 3×10^{-4} M amiloride at various V_H are shown. I-V relationships with and without diuretics are shown in (b). Note that GABA-induced I_{Cl} reverses direction at about +4 mV regardless of the presence of amiloride or furosemide: (○) GABA 10^{-5} M; (□) amiloride 3×10^{-4} M; (△) furosemide 10^{-3} M. All data were obtained from the same cell. Similar results were obtained from three other neurones.

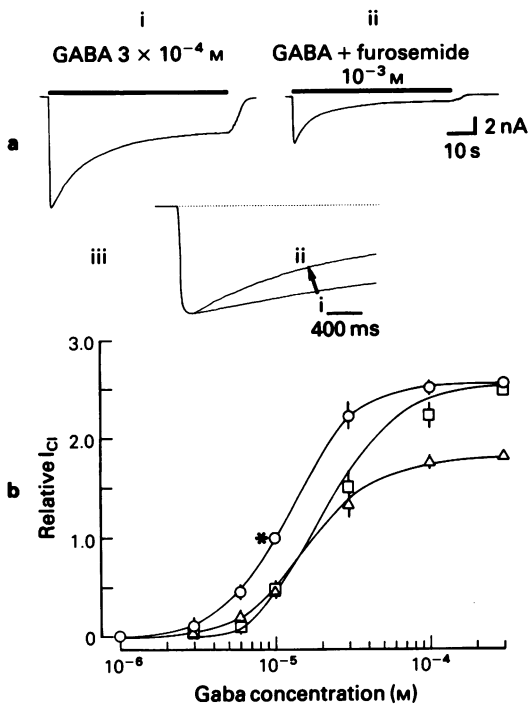

Figure 3 (a) GABA (3×10^{-4} M) -induced I_{Cl} in the absence (i) and presence (ii) of 10^{-3} M furosemide. The current traces are normalized (iii). V_H was -15 mV. Note a facilitation of the I_{Cl} inactivation process by furosemide. All recordings were obtained from the same neurone. (b) Concentration-response curves of GABA-induced I_{Cl} with and without 3×10^{-4} M amiloride (□) or 10^{-3} M furosemide (Δ); (○) = control. All responses were normalized for the peak current (*) induced by 10^{-5} M GABA alone. Theoretical curves show $EC_{50} = 1.3 \times 10^{-5}$ M for control, 2.2×10^{-5} M for amiloride and 1.5×10^{-5} for furosemide. Each point gives mean value from eight neurones and s.e.mean shown by a vertical line.

$$I = I_{max} \frac{C^n}{C^n + EC_{50}^n} \quad (1)$$

Here, I is the observed GABA-induced I_{Cl}, I_{max} the maximum value of I_{Cl}, C the GABA concentration, EC_{50} a constant, and n the Hill coefficient. Thus, continuous lines in Figures 3b were drawn according to eqn. (1) using $n = 2$, EC_{50} (1.3×10^{-5} for control, 1.5×10^{-5} M for furosemide, 2.2×10^{-5} M for amiloride) and I_{max} (2.56 for both control and amiloride, 1.8 for furosemide). The calculated theoretical curves agree well with the experimental points, as shown in Figure 3b. In addition, inactivation of GABA-gated I_{Cl} was facilitated in the presence of 10^{-3} M furosemide (Figure 3a).

Discussion

Misgeld *et al.*(1986) reported that the decrease in the hyperpolarizing and depolarizing responses to GABA by furosemide was due to a shift in E_{GABA} as a consequence of blocking Cl^- transport. Their observations on pyramidal cells in the CA_3 region of guinea-pig hippocampus indicated that furosemide did not reduce the GABA-induced conductance increase. The present results on internally perfused neurones show that furosemide inhibited dose-dependently the GABA-induced I_{Cl} in a non-competitive manner without changing E_{GABA}, and also facilitated the inactivation phase of I_{Cl}. The reason for this discrepancy between the results is not clear at the moment and further studies are necessary. In addition, the facilitation of inactivation by furosemide may be attributed to open channel blockade of GABA-activated Cl^- channels. Overall, the results show clearly that furosemide could suppress not only ionic transport systems but also agonist-gated I_{Cl}.

Amiloride is known to inhibit a number of Na^+ transport systems, including the tight epithelial Na^+ channel (Hamilton & Eaton, 1985), Na^+-H^+ exchange system (Kinsella & Aronson, 1980), the Na^+-Ca^{2+} exchange system (Schellenberg *et al.*, 1983) and Na^+/K^+-ATPase (Soltoff & Mandel, 1983). In the present experiments we found that amiloride blocked GABA-induced I_{Cl} in a competitive manner, indicating the direct action of the drug on GABA-binding sites. This suggests that amiloride has multiple actions on ion transport and ion channels in a variety of cell membranes. We conclude from the experiments described above that furosemide and amiloride have different modes of blocking action on GABA-activated I_{Cl} and that α-hANP, unlike the former two diuretics, has no effect on GABA-induced I_{Cl}.

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