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The Role of Chloride Transport in Postsynaptic Inhibition of Hippocampal Neurons

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Hippocampal inhibitory postsynaptic potentials are depolarizing in granule cells but hyperpolarizing in CA3 neurons because the reversal potentials and membrane potentials of these cells differ. Here the hippocampal slice preparation was used to investigate the role of chloride transport in these inhibitory responses. In both cell types, increasing the intracellular chloride concentration by injection shifted the reversal potential of these responses in a positive direction, and blocking the outward transport of chloride with furosemide slowed their recovery from the injection. In addition, hyperpolarizing and depolarizing inhibitory responses and the hyperpolarizing and depolarizing responses to the inhibitory neurotransmitter γ-aminobutyric acid decreased in the presence of furosemide. These effects of furosemide suggest that the internal chloride activity of an individual hippocampal neuron is regulated by two transport processes, one that accumulates chloride and one that extrudes chloride.

HYPERPOLARIZING INHIBITORY postsynaptic potentials (IPSP's) of cat motoneurons (*1*) and crayfish stretch receptor neurons (*2*) are generated by the influx of chloride ions down a gradient that is maintained by an outwardly directed chloride transport mechanism. Mammalian hippocampal neurons in vitro exhibit both hyperpolarizing and depolarizing responses to presynaptic stimulation (*3*) as well as to the inhibitory neurotransmitter γ-aminobutyric acid (GABA) (*4*). We have used furosemide (*5*), which blocks chloride transport, to investigate the role of transport in the inhibitory responses of pyramidal cells in the CA3 region of the hippocampus and of dentate granule cells (GC).

We used guinea pig hippocampal slices (*6*) to record intracellularly from pyramidal cells in the CA3 region with electrodes containing K_2SO_4. Orthodromic and antidromic (*6*) stimulation always elicited a hyperpolarizing IPSP (*7*) that had a reversal potential (E_{IPSP}) of -70 ± 8.4 mV (mean ± SD, $n = 11$). The resting membrane potential (E_M) of these cells was -59 ± 6.9 mV. However, when we used KCl-containing electrodes, stimulation elicited a purely depolarizing response in most CA3 cells, although E_M of the cells was unchanged. A new E_{IPSP} for the response (-55 ± 7.5 mV, $n = 7$) was attained within about 5 minutes. Because the KCl electrode raised the chloride ion concentration in the cells, this result confirms the well-established role for chloride ions in the IPSP's of pyramidal neurons (*8*).

Granule cells in the dentate gyrus had a higher E_M (*9*) than CA3 neurons (-67 ± 5.9 mV) measured with both K_2SO_4- ($n = 24$) and KCl-filled ($n = 9$) electrodes. In contrast to CA3 cells, orthodromic and antidromic stimulation elicited purely depolarizing postsynaptic potentials in 21 of 24 neurons, even with K_2SO_4-filled electrodes. The apparent E_{IPSP} was -62 ± 8.4 mV. In addition, impalement of GC with KCl-filled electrodes did not depolarize the E_{IPSP} (-64 ± 8.8 mV, $n = 9$). The opposite directions of the driving forces for the IPSP's ($E_{IPSP} - E_M$) of CA3 pyramidal cells and GC paralleled the opposite responses to somatic GABA application (*10*); the response of CA3 cells to GABA was predominantly hyperpolarizing, that of GC was depolarizing.

Bath application of 0.5 to 2 m*M* furosemide (*11*) to GC or CA3 cells shifted the apparent E_{IPSP} in a depolarizing direction (Fig. 1A) and increased the amplitude of the depolarizing postsynaptic response (Fig. 1B) when we used a KCl-filled electrode. Depolarizing responses to GABA were also larger, which indicated that the observed increase in the postsynaptic response was due to a depolarizing action on the GABA-dependent inhibitory component. A new steady state was usually attained within about 20 minutes after furosemide application. The effect was reversible after a wash of about 1 hour (Fig. 1B, upper left and lower left). Furosemide enhanced the depolarizing effect of Cl^- injections (1 nA, 5 to 10 minutes) on the postsynaptic response (Fig. 1B). Under control conditions, the effect was short, with half-times of decay between 20 and 30 seconds ($n = 6$). Furosemide slowed the recovery by a factor of 20 to 30 (Fig. 1C). During washing, the time of recovery returned to control (Fig. 1C).

Furosemide might have impeded the recovery by reducing Cl^- permeability or blocking Cl^- transport, but several experiments indicated that the effect on Cl^- permeability was small. There was a slight increase in resting membrane resistance (control, 40.5 ± 18.4 megohms, furosemide, 49.8 ± 22.9 megohms, $n = 9$). Inward rectification was enhanced by furosemide, but the change in rectification ratio (*12*) was also small (control, 1.21 ± 0.43; furosemide, 1.65 ± 0.6). To test further for effects of furosemide on passive Cl^- permeability, we measured changes of E_{IPSP} in-

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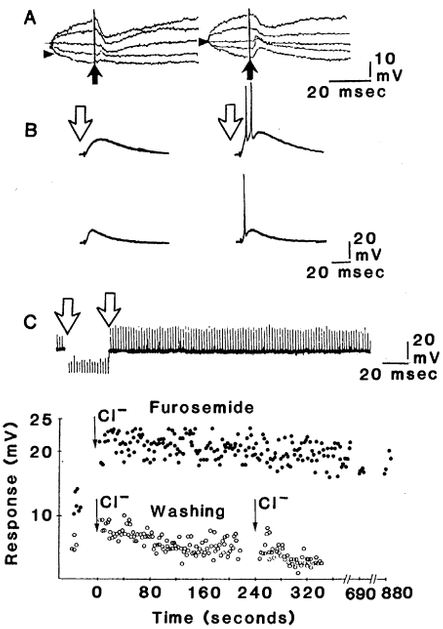

Fig. 1 (A) E_{IPSP} of a CA3 neuron (E_M = −55 mV) monitored with a KCl electrode. IPSP's were elicited by orthodromic stimulation (arrow) at different membrane potentials in control (left) and in the presence of 2 m*M* furosemide (right). Membrane potential was altered by ±0.1- to ±0.3-nA current steps (200 msec). Arrowheads indicate E_{IPSP}. (B) Postsynaptic potentials recorded from a GC (E_M = −67 mV) with a KCl electrode in the presence of furosemide (2 m*M*) before (upper left) and after (upper right) injection of Cl^- (−1 nA, 5 minutes) and before (lower left) and after (lower right) cessation of a corresponding Cl^- injection during washing. Stimulus intensity to activate the GC orthodromically was constant. The action potentials are truncated. (C) Pen recording from the same GC as (B) showing the increase in amplitude after Cl^- injection. Stimulation frequency was 0.5 Hz. Open arrows indicate last response before (B, upper left) and first response after (B, upper right) Cl^- injection. The intensity of the trace has been adjusted during photographic reproduction to eliminate action potentials. (D) Amplitude of the postsynaptic response of the GC in (B) and (C) (25 msec after stimulation) plotted against time. Closed circles represent the changes caused by Cl^- injection in the presence of furosemide. Open circles are the changes induced by Cl^- injections during washing with control solution.

duced by altering membrane potential. When E_M was decreased by about 15 mV for 5 minutes by a steady current injection from a K_2SO_4 electrode, which allowed Cl^- entry, the E_{IPSP} was unaltered in control conditions. With furosemide, however, the E_{IPSP} followed the imposed changes of E_M, an effect opposite that expected if furosemide had only reduced resting membrane Cl^- permeability. This established that furosemide blocks Cl^- transport in hippocampal neurons as in other tissues (*5*).

Furosemide slowed the IPSP recovery from intracellular Cl^- injections in both CA3 cells and GC, suggesting that both have an outward Cl^- transport. This, however, does not explain the difference in IPSP driving forces. To test whether an inward Cl^- transport was also involved, we applied furosemide to CA3 cells and GC and monitored them with K_2SO_4-filled electrodes. In all CA3 cells, furosemide (2 m*M*) decreased the E_{IPSP} in the direction of the resting potential about 7 mV (Fig. 2A), reducing the E_{IPSP} to a value of 62.3 ± 3 mV (n = 6). The decline of E_{IPSP} decreased the IPSP driving force by about 60 percent, close to the value found in crayfish stretch receptor neurons (*2*). In GC, however, furosemide application produced a negative shift of the E_{IPSP} toward E_M. The effect was detectable with 0.5 m*M* furosemide; 2 m*M* furosemide decreased the IPSP driving force by about 40 percent (n = 6).

Regardless of the stimulation site activated, the Cl^--dependent IPSP is contaminated somewhat by an EPSP and possibly by a late K^+-dependent hyperpolarization (*13*). Because the contribution of these other responses is presumably constant for the tests used here, the validity of conclusions about the Cl^- dependence of changes in the E_{IPSP} is not affected.

To test further the validity of our conclusions, we determined the effect of furosemide on Cl^--dependent GABA responses. In CA3 cells, GABA elicited a biphasic potential consisting of a hyperpolarizing and a depolarizing component. The hyperpolarizing response reversed near −70 mV, that is, at the E_{IPSP}. The depolarizing response reversed near −55 mV. Both are Cl^--dependent $GABA_A$ responses because, as in CA1 neurons (*14*), they were blocked by bicuculline and picrotoxin. Further, Cl^- injection reversed the hyperpolarizing component and increased the amplitude of the depolarizing component. As in CA1 cells (*4*), the response depended upon the location of the GABA-containing pipette. Application in stratum pyramidale produced a hyperpolarization with a depolarization interspersed. When GABA was applied above the mossy fiber region, a depolarization preceded the hyperpolarization (Fig. 2B). Application of furosemide caused the biphasic response to become monophasic (Fig. 2B). Although the resulting reversal potential was negative to E_M, it was positive to that of the hyperpolarizing response in control (Fig. 2C) and corresponded to the E_{IPSP}. Furosemide did not reduce the GABA-induced conductance increase. Responses of GC to GABA applied in the cell layer were depolarizing (Fig. 2D). Furosemide shifted the reversal potential of this response in a hyperpolarizing direction (Fig. 2, D and E).

The decrease of the E_{IPSP} of CA3 cells monitored with K_2SO_4-filled electrodes and

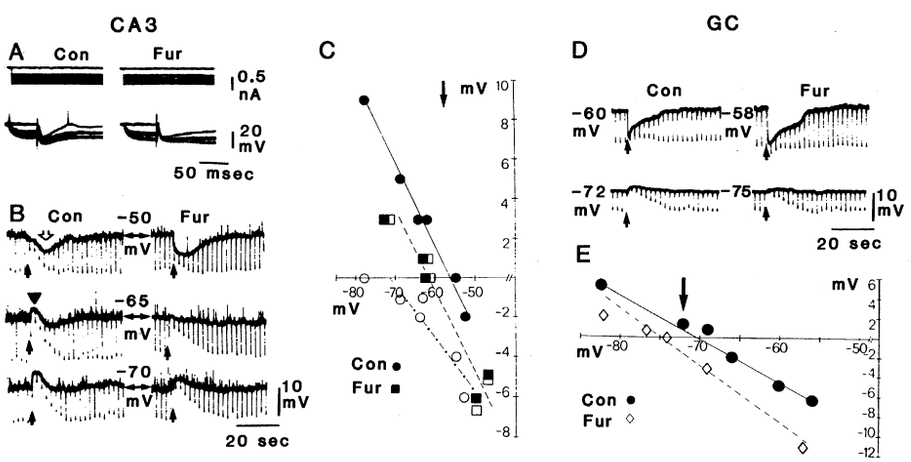

Fig. 2. (A) Hyperpolarizing IPSP of a CA3 neuron monitored with a K_2SO_4 electrode (E_M = −57 mV). Current steps of increasing amplitude (upper traces) were injected to alter membrane potential (lower traces) during orthodromic stimulation in control solution (Con) and in the presence of 2 m*M* furosemide (Fur). (B) Dendritic application of GABA (closed arrow, 100-msec pressure pulse) to a CA3 neuron (E_M = −55 mV) in control solution (Con) and in the presence of 2 m*M* furosemide (Fur). Various membrane potentials were obtained by direct-current injection. A constant hyperpolarizing current pulse (50 msec, 0.6 nA, 0.5 Hz) was also applied to monitor input resistance. (C) Plot of GABA responses (ordinate) against membrane potential (abscissa). GABA responses [as in (B)] were measured at two time points. Closed circles are values obtained at the peak depolarization [arrowhead in (B)]. Open circles are values at the peak hyperpolarization [open arrow in (B)]. At the same time points, GABA responses (closed and open squares, respectively) obtained in presence of furosemide (2 m*M*) were measured. Lines were calculated by linear regression. Arrow indicates E_M. (D and E) Examples and plots of responses to somatic GABA application (arrow, 2-second pressure pulse) of a GC (E_M = −72 mV). Same protocol as in (B) and (C) except that the current pulse for input resistance measurement was 0.4 nA and only one time point (3 seconds after onset of GABA application) was measured.

the slowing of the recovery following Cl^- injection in the presence of furosemide suggest a furosemide-sensitive net outward transport of Cl^-. In GC, the E_{IPSP} was fairly independent of whether 1*M* KCl or 0.6*M* K_2SO_4 was present in the recording electrode. Yet, the results of Cl^- injections indicated that the IPSP was dependent on Cl^- and that, because of rapid recovery, an outward pump must be present in GC. However, a furosemide-sensitive outward pump seems difficult to reconcile with the usually depolarizing IPSP of GC. Because the depolarizing IPSP is diminished in the presence of furosemide, GC must have an additional inward pump mechanism. Yet only an outwardly directed Cl^- transport mechanism has been described in mammalian central neurons (*1*). However, there is an inward pump sensitive to ammonium (*15*) or furosemide in frog motoneurons (*16*) and rat sympathetic neurons (*17*). The furosemide-induced shifts of the apparent E_{IPSP} and of the GABA reversal potential observed in this study suggest that the internal Cl^- activity of GC and CA3 cells is regulated by inward and outward Cl^- transport processes. A homogeneous distribution of inward and outward Cl^- pumps in GC and a dendritic and somatic localization of these pumps in CA3 cells (*18*) accounts for both the changes in E_{IPSP} and the observed shifts in the multiphasic GABA responses.

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15. There are contradictory reports concerning ammonium sensitivity of outward transport in hippocampal CA1 neurons. Chloride transport in rat CA1 neurons was found to be insensitive to ammonium [B. E. Alger and R. A. Nicoll, *J. Neurophysiol.* **49**, 1381 (1983)], but was ammonium-sensitive in guinea pig CA1 neurons because ammonium induced a positive shift in the E_{IPSP} after intracellular iontophoresis of Cl^- [J. R. Hotson and D. A. Prince, *J. Neurophysiol.* **43**, 409 (1980)]. This corresponds to our own observations. Whereas 0.5 m*M* furosemide was sufficient to induce a positive shift in E_{IPSP} of CA3 neurons after intracellular Cl^- iontophoresis, only 2 m*M* furosemide induced a clear shift in E_{IPSP} of CA3 neurons at normal intracellular Cl^- concentration.
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18. GABA can hyperpolarize as well as depolarize CA1 pyramidal cell dendrites [B. E. Alger and R. A. Nicoll, *ibid.* **328**, 125 (1982); R. K. S. Wong and D. J. Watkins, *J. Neurophysiol.* **48**, 938 (1982)]. The explanation that these effects are mediated by two different receptors is contradicted by our conclusion indicating two spatially separated inward and outward Cl^- pumps. The two-receptor hypothesis depends on the assumption that GABA applied to the dendrites does not reach somatic receptors.

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Identification of a Missense Mutation in the Factor VIII Gene of a Mild Hemophiliac

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DNA probes derived from the cloned factor VIII gene can be used to detect mutations in the factor VIII gene of hemophiliacs. DNA hybridization analysis led to the identification of two contrasting point mutations in the same codon. In a severe hemophiliac with no detectable factor VIII activity, the normal arginine codon (number 2307) is converted to a stop codon, while in a mild hemophiliac with 10 percent of normal activity, this same codon is converted to glutamine.

THE GENETIC DISEASE HEMOPHILIA A (classic hemophilia) is caused by a defect in the blood coagulation protein, factor VIII. From the cloning of the human factor VIII gene (*1–4*), the sequence of the 2332–amino acid protein has been determined. This sequence data provides the basis for understanding the mechanism of action of factor VIII, and the cloned gene has been instrumental in elucidating the molecular basis of hemophilia. We have previously reported the use of cloned DNA probes to demonstrate examples of both deletion and point mutations in the factor VIII gene of several patients with severe hemophilia (*5*). All of the point mutations in these patients resulted in stop codons (nonsense mutations). We now describe an amino acid substitution due to a missense mutation that occurs in a mild hemophiliac. This amino acid change is in the same codon in which a nonsense mutation led to severe disease.

In our initial survey of hemophilia DNA samples (*5*), the enzyme Taq I proved to be particularly useful for detecting mutations in the factor VIII gene. The recognition sequence for Taq I (TCGA) contains the dinucleotide CpG, the most common site of methylation in human DNA and a possible site of increased mutation due to deamination of cytosine leading to C to T transitions (*6*). In two surveys of hemophilia DNA's for changes in the Taq I sites within the factor VIII gene, the expected mutation was identified in three of the seven Taq I sites occurring in the protein-coding regions (*5, 7*). One example we have described is the case of a severe hemophiliac, H22, having no detectable factor VIII activity, in which a Taq I site normally present in the coding region of exon 26 (the 3′-most exon) is missing (*5*). Cloning and sequencing of the H22 DNA in this region demonstrated the expected C to T change in the CpG dinucleotide of the Taq I site. This mutation converts a CGA (arginine) codon to a TGA (stop) codon 26 amino acids before the normal chain termination site (Fig. 1).

The same Taq I site is also missing in the DNA of another patient, H104, who is a mild hemophiliac (9 percent factor VIII activity). To account for the phenotypic difference between these two individuals, we

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