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Cl^- transporter block enhances GABAergic spontaneous activity in rat hippocampal CA3 cells

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CHLORIDE–cation Transporter Blockers (CTBs) furosemide and ammonium were used to test the effects of changes in the internal [Cl^-] on the spontaneous and miniature GABAergic post-synaptic currents (PSCs) of CA3 pyramidal cells of rat hippocampal slices with the whole-cell patch technique. Application of CTBs in the presence of kynurenic acid raised (65 ± 21%) the frequency of GABAergic spontaneous PSCs leaving unchanged the miniature frequency, indicating that the increase in synaptic activity was caused by interneurone firing. Partial removal of external chloride yielded the same effect, suggesting that E_{Cl} contributes to the resting potential of interneurones. PSC rise times were prolonged and their mean amplitude was lowered by furosemide as well as the response to exogenous muscimol, confirming that furosemide exerts some $GABA_A$ receptor antagonism.

Key words: Hippocampal slice; CA3; Interneurones; Synaptic activity; GABA; Chloride transport; Patch-clamp

Introduction

Pyramidal cells in the hippocampal CA3 region of neonatal rats receive a major input directly from local GABAergic interneurones.[1] The interneurones themselves also receive a large GABAergic afference.[2] Since chloride is the main charge carrier for the ionotropic GABA receptor, its intracellular concentration ($[Cl^-]_i$) plays a fundamental role in the regulation of cellular excitability. The transmembrane chloride gradient is regulated by several mechanisms which can produce opposite effects on cells on the same tissue.[3,4] This explains why in adult pyramidal cells GABA has the usual inhibitory effect, while in neonatal pyramidal cells or in adult dentate gyrus GABA elicits membrane depolarization.[5–7] Development changes in the [Cl^-] gradient have been particularly studied in pyramidal cells of the neonatal hippocampus CA1 regions.[8] Little is known about the role of $[Cl^-]_i$ on interneurone activity. The purpose of the present study was to investigate in which way $[Cl^-]_i$ influences the spontaneous GABAergic activity generated by interneurones in the CA3 region of neonatal hippocampus.

In the rat hippocampus $[Cl^-]_i$ is controlled by chloride transporters among which the most relevant seems to be the so-called chloride–cation transporter (CCT), which also involves Na^+ and K^+.[9] Furosemide and ammonium ions were used in order to change $[Cl^-]_i$ since they are known to block the activity of CCT in thick hippocampal slices,[4] in hippocampal organotypic cultures[10] and in other preparations.[4,11–13] In order to perform frequency, amplitude and rise time analysis of spontaneous synaptic currents (sPSCs) with minimal noise the whole-cell patch-clamp technique was used.

Materials and Methods

Wistar rats 6–12 days old were anaesthetized with 0.2–0.3 ml 5% urethane solution and decapitated. After decerebration the brain was immersed in oxygenated (95% O_2, 5% CO_2) Krebs medium with the following composition (mM): NaCl 126, KCl 3.5, $NaH_2PO_4 \cdot H_2O$ 1.2, $MgCl_2 \cdot 6H_2O$ 1.3, $CaCl_2 \cdot 2H_2O$ 2, $NaHCO_3$ 25, glucose 11. Slices of nominal thickness of 300 μm were cut at 2–4°C and kept at 32°C until transfer to the recording chamber at room temperature (20–22°C). The slices were continuously perfused at a rate of 2–3 ml min^{-1}. Patch electrodes (1.5 or 2 mm o.d. borosilicate capillaries) had a resistance of 3–5 MΩ. The composition of the intracellular solution was (mM): CsCl 146, Na_2ATP 1.5, HEPES 10, EGTA 11, $CaCl_2$ 1. pH was adjusted to physiological values with KOH. Cells having series resistance (Ω_s) > 30 MΩ were discarded. The average Ω_s value was 23 ± 4 MΩ. An average input resistance of 202 ± 16 MΩ was measured in control conditions. A holding potential of −70 mV was used in all experiments. Kynurenic acid (1 mM) was used in all experiments to block glutamatergic inputs. pH did not significantly vary with the drug application. The signal was amplified, recorded on a videotape, filtered off-line at 2 kHz and digitized at 5 kHz with the Digidata-1200 acquisition board driven by the pCLAMP acquisition software. Frequency, amplitude and rise time analysis was performed with the program N05 generously supplied by Dr Stephen Traynelis. Only events not apparently overlapping were selected for analysis. Data are reported as mean ± s.e.m. Since a large variability was found from cell to cell, particularly in the mean frequencies, the Student's *t*-test on the differences was

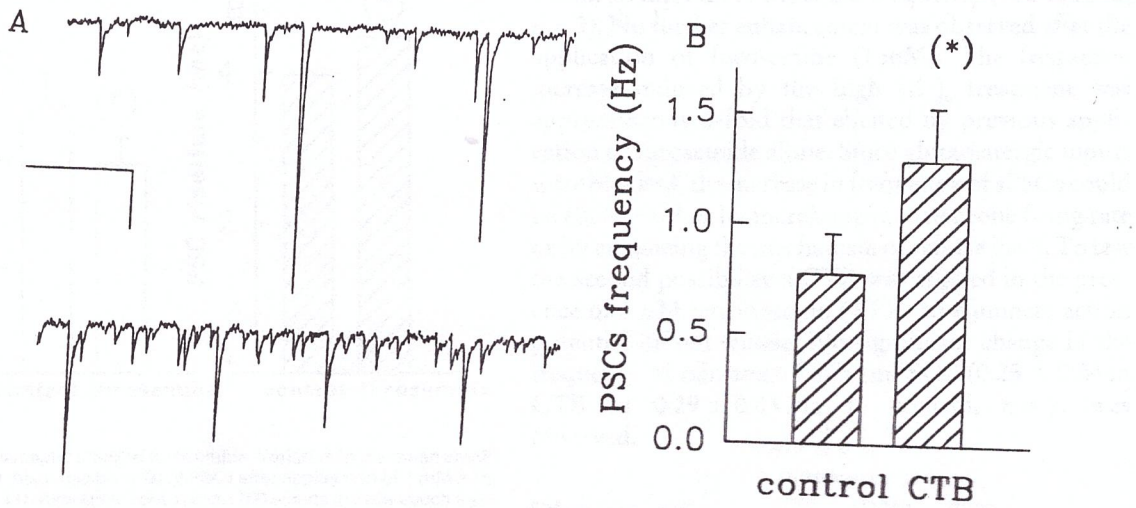

FIG. 1. Effect of chloride transport blockers on PSC frequency. (**A**) above: control recording, below: recording after 150 s application of 1 mM furosemide. Calibration bar: 2 s, 40 pA. (**B**) frequency variation after treatment with CTBs: control condition *vs* CTB treatment ($n = 22$). The asterisk indicates differences from control values which are significant at $p < 5\%$. The frequencies were calculated over time spans of 2–5 min in order to have at least 100 events per epoch. F

used to assess the statistical significance with t values corresponding to $p < 5\%$ being accepted as significative.

Results

Spontaneous PSCs had an average frequency of 0.86 ± 0.30 Hz and a mean amplitude of 18.1 ± 4.7 pA ($n = 27$). These were $GABA_A$ mediated currents since were abolished by 2 μM bicuculline methiodide, 50 μM picrotoxin, or 2 mM penicillin G, and their reversal potential was about 0 mV, close to the Nernst potential for chloride. After application of 1 mM furosemide the frequency of sPSCs increased from 0.97 ± 0.20 to 1.67 ± 0.38 Hz ($n = 11$, Fig. 1A). In some experiments this effect was reproduced up to 6 times in the same cell. The effect was reversible and dose dependent, with a dissociation constant K_d of 0.87 ± 0.08 mM (Fig. 2). The cumulative distribution of inter-event intervals is shown in Figure 2A for a typical cell in control conditions and after furosemide treatment. When furosemide was applied for 2–4 min complete recovery was usually obtained after 10 min. Furosemide lowered sPSC amplitudes (34 ± 5%,

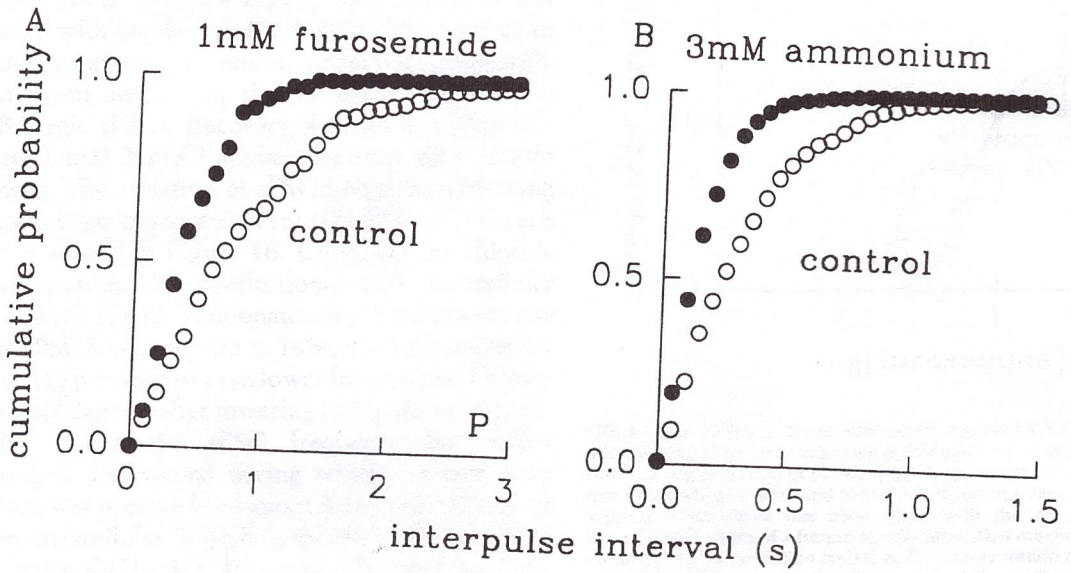

FIG. 2. (**A**) and (**B**) Effect of furosemide and ammonium. Cumulative frequency histograms before (●) and after (○) CTB treatment for 1 mM furosemide and 3 mM NH_3Cl, respectively, in single, representative cells.

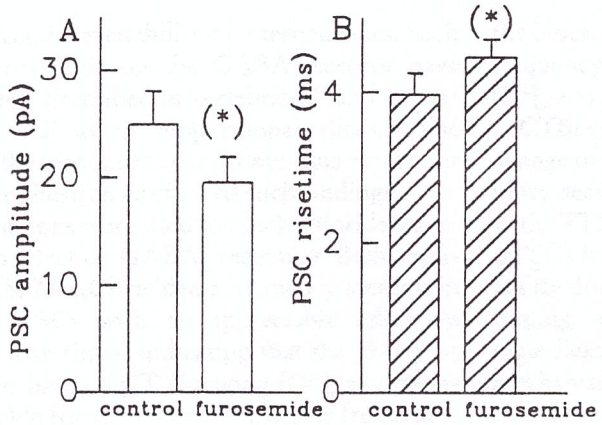

FIG. 3. Post-synaptic effect of furosemide. Variation in the mean amplitude (**A**) and mean rise time (**B**) of PSCs after application of 1 mM furosemide ($n = 11$). Averages from at least 100 events in each epoch were considered as above. The average amplitude decreases while the mean rise time increases. The asterisk indicates differences compared with control values that are statistically significant at $p < 5\%$.

$n = 11$, $p < 0.05$) and increased the rise time values (28 ± 8%, Fig. 3) with no significant change in the decay times (12 ± 14%). The presence of a post-synaptic effect of furosemide was tested with applications of the $GABA_A$ agonist muscimol. Bath-applied muscimol (100 nM) elicited a sustained inward current (95 ± 12 pA, $n = 3$) and an increase in cellular conductance (24 ± 11%, $n = 3$). Furosemide application (3 min before muscimol) produced a statistically significant ($p < 0.05$) reduction (16 ± 5%) in the inward current and a diminution of the conductance increase (11 ± 6%) induced by muscimol. Full recovery was obtained after 10 min washout.

Ammonium was also used as chloride transport blocker. Two to three minutes after application of 3 nM NH_3Cl an increase in the frequency of sPSCs was detected (58 ± 19%, $n = 11$, Fig. 2B), similar to that obtained with furosemide (72 ± 23%). The increase in frequency induced by ammonium did not significantly affect mean amplitude, rise or decay time of the GABAergic sPSCs. Recovery was not complete following 3 mM NH_3Cl application even after 20 min washout. The variation of sPSC frequency following the application of furosemide or NH_3Cl ($n = 11$ in each case) is shown in Figure 1B. Changing the chloride reversal potential by substitution of 50% extracellular chloride $[Cl^-]_o$ with isethionate elicited a transient rise in the sPSC frequency (73 ± 16%, $n = 3$), followed by a return to previous or even lower frequencies. Furosemide applications after lowering $[Cl^-]_o$ did not significantly change the sPSC frequency but rather prolonged the interval during which the frequency increase was present from about 3 min to 6–10 min. A rise in extracellular $[K^+]$ ($[K^+]_o$ should impair Cl^- transport since CCTs are driven by the $[K^+]$ gradient. Changing $[K^+]_o$ from 3.5 to 10 mM elicited, after about 3 min, an increase in the sPSC frequency (118 ± 83%, $n = 3$). No further enhancement was observed after the application of furosemide (1 mM). The frequency increase induced by the high $[K^+]_o$ treatment was approximately 3-fold that elicited by previous application of furosemide alone. Since glutamatergic inputs were blocked, the increase in frequency of sPSCs could be elicited either by increasing interneurone firing rate or by enhancing the mechanism of release itself. To test the second possibility a CTB was applied in the presence of 1 μM tetrodotoxin (TTX) to eliminate action potential driven release. No significant change in the frequency of miniature PSCs (mPSCs) (0.25 ± 0.04 in CTB *vs* 0.29 ± 0.04 Hz in control, $n = 5$) was observed.

Discussion

Bath application of furosemide reversibly enhanced the sPSCs mean frequency. The K_d value ($K_d = 0.67 \pm 0.08$, Fig. 4) was similar to that found for a low affinity binding site for furosemide in the rat brain synaptosome preparation[15] ($K_d = 0.68$ mM). Since the block of glutamate receptors by kynurenate eliminated the main excitatory input, the frequency increase induced by furosemide on sPSCs was likely due to an enhancement in the activity of the interneurones directly impinging on the pyramidal cells. Mean amplitude and rise times of the GABAergic sPSCs also changed significantly after furosemide application. While the frequency variation was certainly a presynaptic effect, the variation in the mean amplitude and rise time may be explained by a partial blockade by furosemide of the response to GABA, which could also

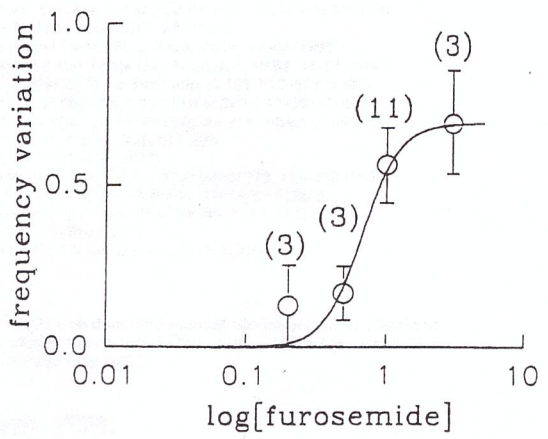

FIG. 4. Dose–response curve. Each point represents the average variation of frequency for n (indicated above each point) cells *vs* the logarithm of concentration of furosemide. Frequencies were calculated 3 min after the application and over 2 min recording. Mean ± s.e.m. are reported. The curve has been fitted with the equation $\Delta f = \Delta f_0 [1 + (K_d/C)^n]^{-1}$, where Δf = frequency variation, Δf_0 = maximal obtainable variation, K_d = dissociation constant, C = concentration of furosemide and = Hill coefficient, yielding the values of $K_d = 0.67 \pm 0.08$ mM and $n = 3.3 \pm 1.0$.

indirectly affect the excitability of interneurones. Such effects of furosemide on the GABA-receptor have been previously described in vertebrates[13] and invertebrates[16] as well as in hippocampal slices.[17] The reduction of the response to exogenous muscimol after furosemide application confirmed such findings.

Ammonium ions were used to block chloride transport, with no effect on $GABA_A$ receptors. Bath application of 3 mM NH_3Cl yielded a frequency increase in GABAergic sPSCs with no appreciable effect on amplitude or rise times, indicating that the ability of furosemide to block CCT changing $[Cl^-]_i$ was probably responsible for the increase in sPSCs frequency. Two mechanisms determine $[Cl^-]_i$ passive Cl^- flow (by leak through Cl^- selective pores and GABA channels opened by the sPSCs) and Cl^- transport via CCT.[8] The former regulates $[Cl^-]_i$ in the first postnatal week (PN 2–5) whereas the latter prevails in the adult animal (PN 15–20). The present data are comparable with those obtained by Zhang *et al*[8] for the intermediate pool of cells at PN 8–13. At this developmental stage both mechanisms seem to determine $[Cl^-]_i$: the presence of a Cl^- passive conductance leads the Cl^- reversal potential (E_{cl}^-) to contribute to testing potential (V_r) proportionally to the cell Cl^- conductance itself whereas CCTs decrease interneuronal excitability using the $[K^+]$ gradient generated by the N_a^+, K^+-exchanger. A reason for the frequency enhancement could be the fall of the chloride gradient induced by CTBs, which decreased the effectiveness of a tonic, chloride-mediated inhibition onto the presynaptic interneurones[2] and thus lowered their firing threshold. Such an interpretation would be in agreement with studies on the cat motor cortex.[18] Another possibility that does not exclude the previous one is that the variations in the chloride reversal potential induced by CTBs directly affected the interneurone V_r. V_r is in fact determined by the resting permeability to the different ions, including chloride. This view was confirmed by the fact that a positive shift of chloride reversal potential by partial substitution of extracellular chloride with isethionate elicited a transient increase in sPSC frequency. The short duration of the activity enhancement elicited by lowering $[Cl^-]_o$ was probably due to a redistribution of $[Cl^-]_i$ due to passive and active transport. Furosemide application after low $[Cl^-]_o$ treatment did not increase sPSC frequency but prolonged the interval during which sPSC frequency was enhanced, as expected in the case that CCTs contributed to E_r in a hyperpolarizing direction. The increase in the frequency of sPSCs following the elevation of $[K^+]_o$ was mostly due to membrane depolarization, but was also partly due to the block of CCTs, since a further increase in the sPSC frequency following application of furosemide in high $[K^+]_o$ was not observed.

CTBs could affect directly GABA release, through a change of $[Cl^-]_i$ inside the axonal terminals. This possibility seems unlikely since block of action potentials with TTX prevented a CTB-induced increase of mPSCs frequency. The rise in the interneurone excitability does not put forward the idea of a reverse functioning of their chloride–cation co-transporter, regardless the details of the frequency enhancement mechanisms.

Conclusions

Block of chloride transport with furosemide or ammonium induced a large increase in the frequency of the spontaneous GABAergic sPSCs in CA3 pyramidal cells obtained from neonatal rats, suggesting that chloride transport plays an important role in excitability of the CA3 pyramidal cell neurones, acting not only through direct control of the pyramidal cell Cl^- reversal potential but also modulating the firing rate of GABAergic presynaptic interneurones. Enhancement of the sPSC frequency suggests that the block of chloride transport in rat hippocampal interneurones shifts their chloride reversal potential towards more depolarizing values, lowering their firing threshold. A degree of antagonism by furosemide on the $GABA_A$ receptor was confirmed.

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