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Modulatory effects of γ-hydroxybutyric acid on a $GABA_A$ receptor from crayfish muscle

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Abstract

The effects of γ-hydroxybutyric acid (GHB) were evaluated with a γ-aminobutyric acid (GABA) activated Cl^- channel on crayfish deep extensor abdominal muscle. GABA and GHB were applied to outside-out patches using a fast application system. Application of GHB up to 10 mM did not result in detectable activation of the channel. After coapplication of GABA and GHB, a dose-dependent potentiation of the GABA-elicited current by GHB was observed. The maximal effect was obtained with 0.5–1 mM GHB, with which the amplitude was enhanced by about 50% with 0.4 or 1 mM GABA. Simultaneously with the potentiating effect, a decrease of the rise times was seen. Preapplication of GHB, prior to the GABA pulses, resulted in a reduction of the current amplitude elicited by GABA. This block was persistent throughout the application time of GABA. Therefore, two contrasting effects of GHB on this chloride channel, a potentiating one and a blocking one, seemed to occur simultaneously.

Keywords: Cl^- channel; Patch clamp; Kinetics; $GABA_A$ receptor; GHB (γ-hydroxybutyric acid)

1. Introduction

The structural analog of γ-aminobutyric acid (GABA), γ-hydroxybutyric acid (GHB) has been found in almost all brain regions of vertebrates (Vayer et al., 1988), often together with dopaminergic structures (Hechler et al., 1992) and also in non-neuronal tissue like brown fat, liver, heart and kidney (Nelson et al., 1981). It is enriched in the synaptosomal fraction of brain tissue preparations suggesting a possible role as a neurotransmitter or neuromodulator (Cash, 1994). The receptors for GHB, only found on neuronal cells so far, seem to be closely related to the $GABA_B$ receptors acting also via G-proteins to modulate the Ca^{2+} and K^+ permeabilities of the cell membranes. Other effects are the modulation of the dopaminergic and serotoninergic systems (see Maitre, 1997 for a brief description of all effects). GHB seems to bind to $GABA_B$ receptors selectively with weak affinity (Mathivet et al., 1997), but an action of GHB on $GABA_A$ receptors could not be demonstrated so far (Feigenbaum and Howard, 1996).

In vivo application of high doses of GHB, which penetrates the blood/brain barrier, results in rhythmic spike-and-wave discharges and behavioural changes similar to the human generalized non-convulsive epilepsy in animal models (Snead, 1988). Abuse of GHB can cause euphoria at low doses and sedation, anesthesia and coma at higher doses (Chin et al., 1992). From the clinical point of view, GHB can be used as a sedative, in the treatment of narcoleptic patients and to alleviate the withdrawal syndromes produced by alcohol or opiates.

We investigated the action of GHB on a $GABA_A$ receptor-mediating inhibition at the neuromuscular junction of the deep extensor abdominal muscle of crayfish. This channel shares the characteristics of central vertebrate $GABA_A$ receptors, like sensitivity to picrotoxin (Adelsberger et al., 1998a) and isoflurane (Adelsberger et al., 1998b), therefore being suitable as a model for the latter ones. The crayfish channel has been investigated in detail and a molecular reaction scheme has been proposed (Adelsberger et al., 1996; von Beckerath et al., 1995). It is characterized by five binding steps for GABA to the receptor in a positively cooperative way prior to opening. From the fully liganded receptor state, the two openings A_5O_f (f = fast) and A_5O_s (s = slow), clearly distinguish-

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able by their open time constants of about 0.5 and 5 ms, are branching. A further open state A_3O' originating from the receptor after binding of one molecule GABA carries no detectable current at the relatively high concentrations of agonist applied in the present study. Furthermore, a state A_4R', generated by isomerisation from the receptor that has bound four molecules of GABA, resulting in a slight depression of the dose–response relationship at intermediate GABA concentrations, does not seem also to be involved in the effects caused by GHB.

2. Materials and methods

2.1. Animal preparation

Crayfish (*Astacus astacus*) purchased from local suppliers were kept in tanks with running tap water at room temperature and fed with fish filet once a week. Animals between 5 and 10 cm were killed and the two lateral muscle bundles (L1 and L2) of the deep extensor abdominal muscle were prepared and pinned to the bottom of a sylgard chamber (Adelsberger and Dudel, 1996; Parnas and Atwood, 1966). The preparation was kept in a solution containing (in mM): 200 NaCl, 5.4 KCl, 13.5 $CaCl_2$, 2.5 $MgCl_2$, 10 Tris–maleate buffer, pH 7.4 adjusted with 5 M NaOH and 0.2 mg/ml collagenase type V (Sigma, Deisenhofen, Germany) for 1 h at room temperature.

2.2. Electrophysiological recordings

After the collagenase treatment, the muscle was superfused with the extracellular solution described above at room temperature. Patch pipettes were pulled using a pipette puller from borosilicate glass with an outer diameter of 1.5 mm (Clark Electromedical Instruments, Pangbourne, England) to a resistance of 8–12 MΩ and filled with an intracellular solution containing (in mM): 150 CsCl, 5 NaCl, 2 $MgCl_2$, 10 ethylenebis(oxonitrilo)tetraacetate (EGTA), and 10 Tris–maleate buffer, pH 7.4 adjusted with 5 N KOH. Patch clamp recordings were performed in the outside-out configuration of excised membrane patches at polarization values of -30 to -50 mV. For the single-channel recordings, patches were polarised to -100 mV in order to obtain a sufficient signal-to-noise ratio. GABA and GHB (Sigma, Deisenhofen, Germany) were diluted to 10 mM stock solutions every day using the extracellular solution. Drugs were applied to outside-out patches using the piezo-driven liquid filament switch. With this method, a continuous liquid filament is switched to the patch positioned about 10 μm above (Franke et al., 1987). Application and washout times of well-defined concentrations within 0.2 ms are possible, allowing more precise determination of rise- and off-times than by applying the drugs via bath superfusion.

2.3. Data analysis

Currents were recorded with an EPC-9 (Heka, Lamprecht, Germany) patch-clamp amplifier and stored on video tapes. Off-line analysis was performed on Pentium PC after low-pass filtering the data with an 8-pole Bessel-filter with cut-off frequencies f_c of 1–2 kHz. The digitization rate was 20 kHz.

The rise time of averaged current traces was defined as the time from 10 to 90% of the peak current. The off-time constants after rapid washout of the agonist representing the burst time of the channel was determined after logarithmation of the current. For evaluation of the single-channel kinetics, only measurements in which no simultaneous openings occurred were used. Currents reaching more than 66% of the maximal channel current amplitude were classified as openings, whereas declines to less than 50% of the maximal current were classified as closures. Significance of differences was considered with a P value < 0.01 using the Student's t-test. Open and closed times were fitted using the Marquardt–Levenberg–Routine.

3. Results

3.1. Dose–response relationship

The channel reached its maximal open probability of $P_{Omax} = 0.9$ with 10 mM GABA (von Beckerath et al., 1995). With 1 mM GABA, the open probability was $P_O = 0.6$ and with 0.4 mM GABA $P_O = 0.2$. Activation of the channel with 0.4 mM and 1 mM GABA led to slowly rising currents with rise times in the range of 30–50 ms. After reaching the maximum of the current amplitude, no or only negligible desensitization was observed, even at application times of GABA of more than 2000 ms. The patches contained between one and very rarely more than 10 channels resulting in current amplitudes, additionally dependent on the polarization of the patch, between 1 and 20 pA. Application of GHB alone with concentrations up to 10 mM to outside-out patches did not result in detectable activation of the channel. After coapplication of GHB together with GABA, an enhancement in the current amplitude, compared to the amplitude elicited with the same GABA concentration alone, was observed (Fig. 1). The effect increased with the GHB concentration and was maximal between 0.5 and 1 mM, by which the amplitude was potentiated by about 50%. Towards higher GHB concentrations, the potentiating effect decreased again and was about 40% with 10 mM GHB. The relative effect of GHB, evaluated setting the current amplitude achieved by GABA alone to one, was not significantly different in coapplications with 0.4 mM or 1 mM GABA.

Simultaneous with the increase of the amplitudes, a shortening of the rise times was observed with GHB (Fig. 2). The maximal effect was reached with 1 mM GHB,

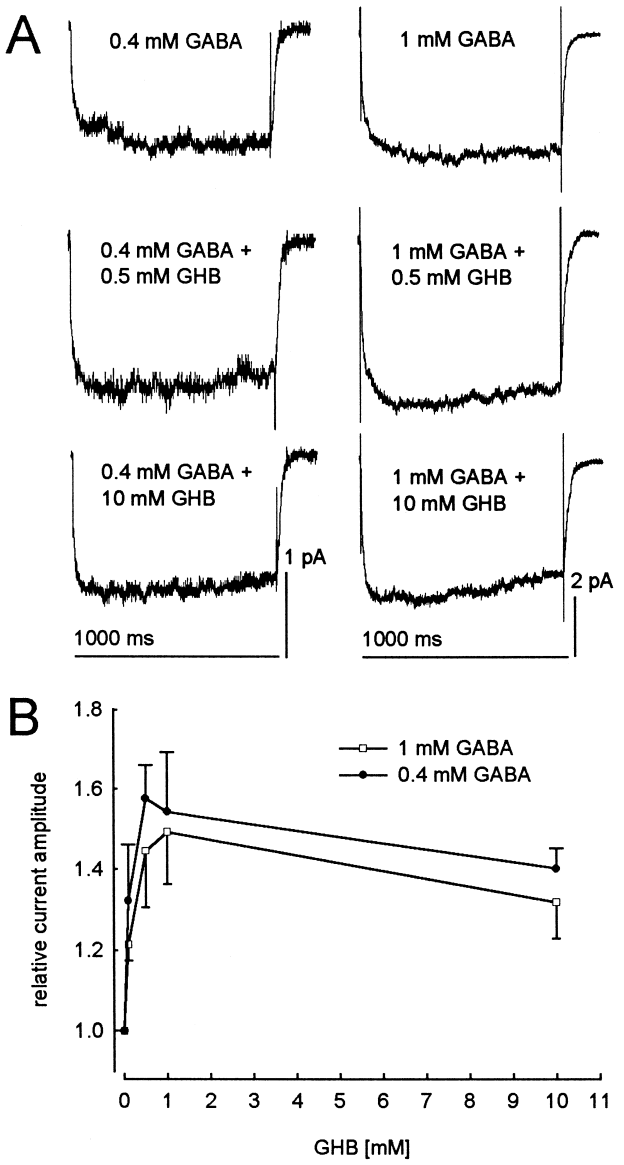


Fig. 1. (A) Averaged current traces (30–60 each) after application of 0.4 mM GABA and 0.4 mM GABA together with 0.5 mM and 10 mM GHB, and 1 mM GABA and 1 mM GABA together with the same GHB concentrations to the same outside-out patches. (B) Relative current amplitudes after application of 0.4 mM (dots) and 1 mM (squares) GABA alone and together with different GHB concentrations. The amplitudes achieved by application of GABA alone was set to one. The mean values ± S.E.M. are derived from at least 5 measurements with each concentration.

which shortened the rise time by about 50% on coapplication with 0.4 mM GABA, and by about 30% on coapplication with 1 mM GABA. A decrease of the effect at higher GHB concentrations, as observed for the current amplitudes, was not obvious for the rise times.

After coapplication of GHB together with GABA concentrations higher than 1 mM, the maximal potentiating effect was smaller due to the fact that the current amplitude reached the value of the maximal open probability of $P_{Omax} = 0.9$. Consequently, coapplication of GHB together with 10 mM GABA did not result in potentiation of the current amplitude, but still shortened its rise time (Fig. 3).

3.2. Single-channel kinetics

Single-channel measurements after coapplication of 1 mM GABA + 10 mM GHB were performed and compared to the data derived from recordings in which 1 mM GABA alone was applied. No influence of GHB on the single-channel conductance was observed. All open time histograms could be fitted with two time constants with

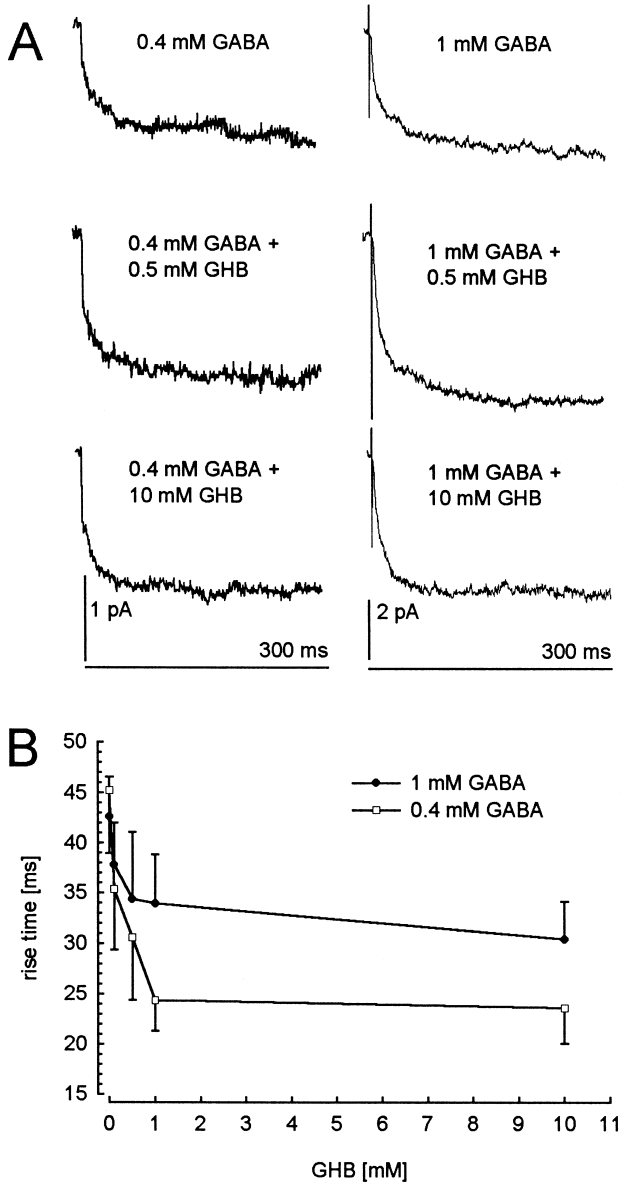


Fig. 2. (A) Averaged current traces from Fig. 1 in higher time resolution to demonstrate the decrease of the rise times after coapplication of GHB. (B) Rise times after application of 1 mM (dots) and 0.4 mM (squares) GABA alone and together with different GHB concentrations. The mean values ± S.E.M. are derived from at least 5 measurements with each concentration.

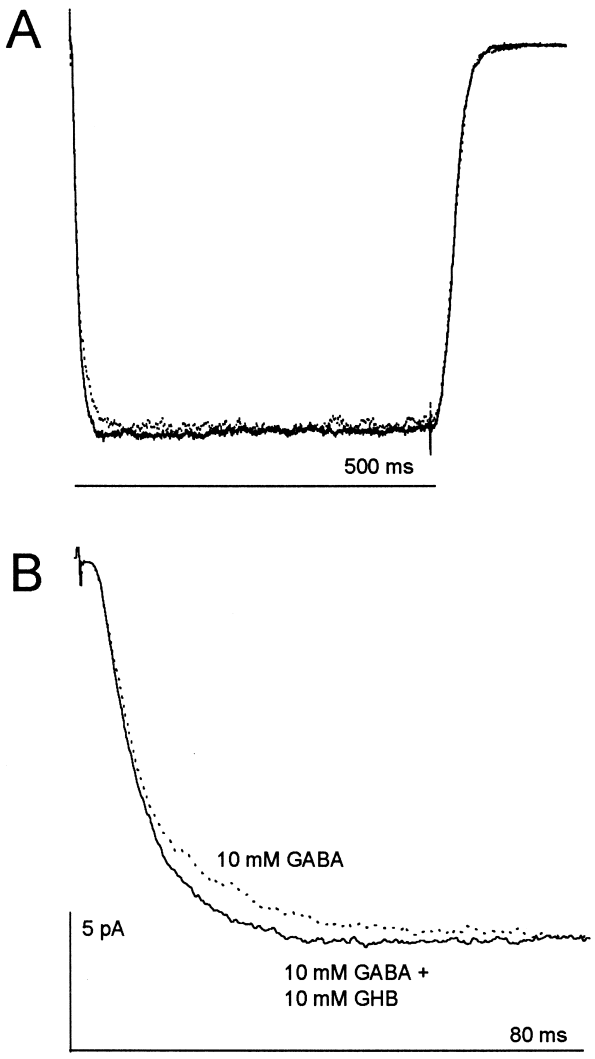


Fig. 3. (A) Averaged current traces (20–25 each) after activation of a outside-out patch with 10 mM GABA alone (dotted) and 10 mM GABA together with 10 mM GHB (solid) to demonstrate the decrease of the rise time in the presence of GHB. (B) Same traces as in (A) in higher time resolution.

values of $\tau_{o1} = 0.6 \pm 0.11$ ms (mean ± S.E.M.; $n = 6$) and $\tau_{o2} = 4.11 \pm 0.69$ ms (mean ± S.E.M.; $n = 6$), which were not significantly different from the time constants of $\tau_{o1} = 0.54 \pm 0.31$ ms (mean ± S.E.M.; $n = 4$) and $\tau_{o2} = 5.45 \pm 0.93$ ms (mean ± S.E.M.; $n = 4$) derived from recordings after application of 1 mM GABA alone (Fig. 4). Additional longer openings were artificially generated by the filter cut-off frequency due to unrecognized short closings as shown earlier (Adelsberger et al., 1996). The closed time histograms of the recordings in which 1 mM GABA and 10 mM GHB were applied shared all the common closed time constants $\tau_{c1} = 0.35 \pm 0.11$ ms (mean ± S.E.M.; $n = 6$) and $\tau_{c2} = 5.25 \pm 0.18$ ms (mean ± S.E.M.; $n = 6$). Again, no significant differences from the control measurements, which were fitted with the time constants $\tau_{c1} = 0.33 \pm 0.21$ ms (mean ± S.E.M.; $n = 4$) and $\tau_{c2} = 3.9 \pm 0.58$ ms (mean ± S.E.M.; $n = 4$) were obvious.

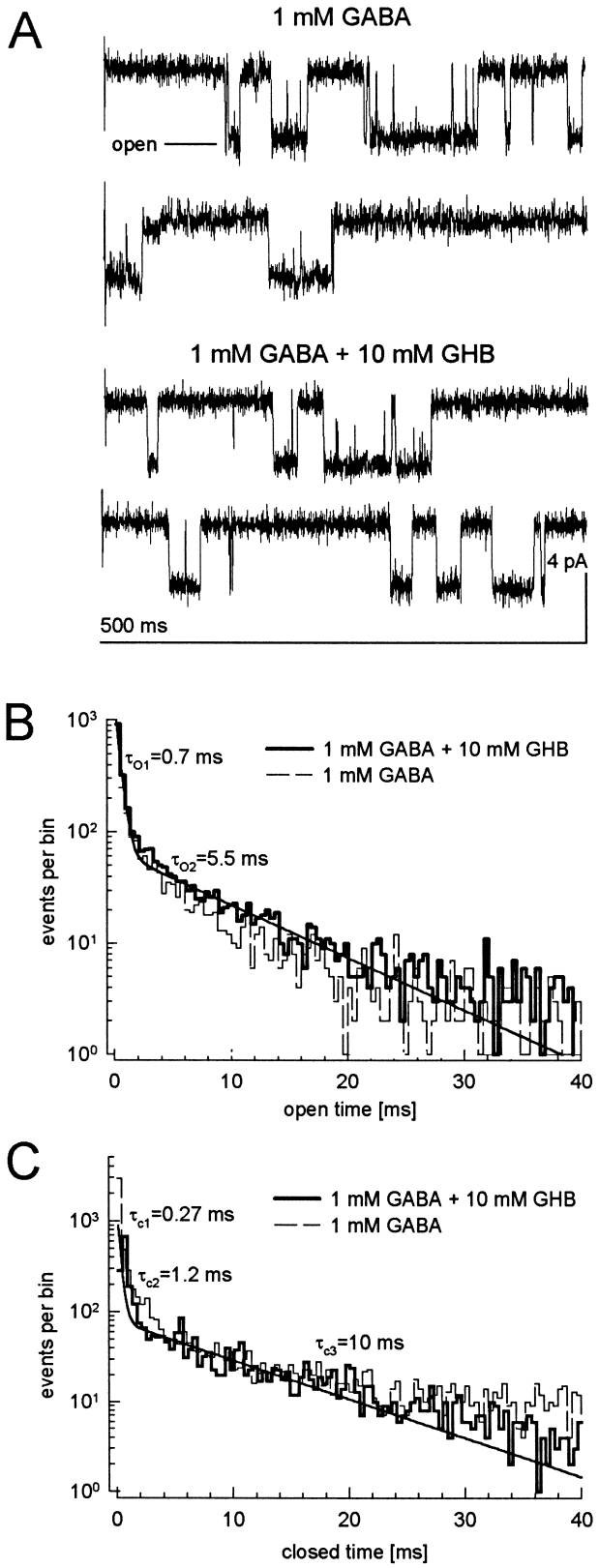
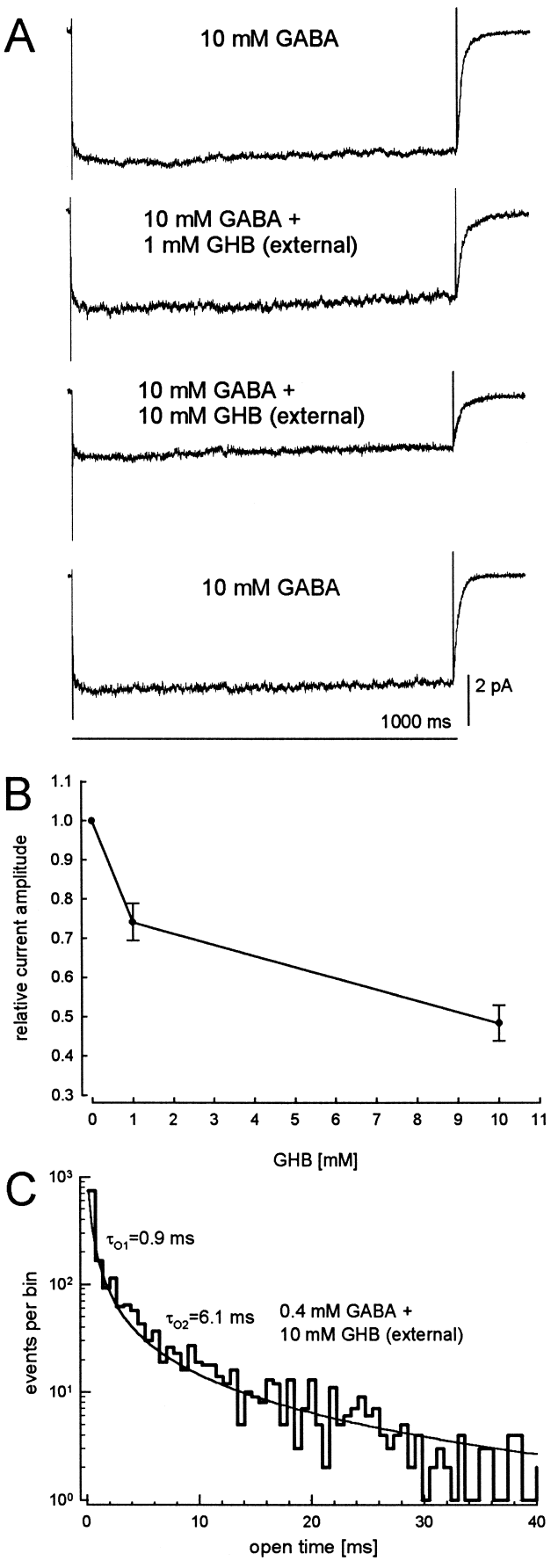


Fig. 4. Original current traces (A), open (B) and closed (C) time histograms of recordings after activation with 1 mM GABA alone (dashed) and 1 mM GABA together with 10 mM GHB (solid) to another patch. The values of the time constants correspond to the fits of the data derived after coapplication of GABA and GHB. Semi-logarithmic plots. Bin width = 0.4 ms.



Sometimes additional longer time constants were visible depending on the number of channels on the patch.

To determine the burst length of the channel, the decay time constants of averaged currents measured after rapid washout of the agonists were fitted. The time constant of decay $\tau_{decay} = 20.74 \pm 2.53$ ms (mean $\pm$ S.E.M.; $n = 6$) of recordings after application of 1 mM GABA alone was not different from the $\tau_{decay} = 21.72 \pm 1.53$ ms (mean $\pm$ S.E.M.; $n = 6$) determined from recordings after coapplication of 1 mM GABA and 10 mM GHB.

3.3. Block after preapplication of GHB

Preapplication of GHB in the external solution to outside-out patches prior to pulses containing exclusively GABA by switching between GHB- and GABA-containing solution, resulted in a reduction of the current amplitude achieved by the agonist (Fig. 5A,B). With 10 mM GHB in the external solution, the current amplitude reached only about 50% in the subsequent pulses with 10 mM GABA. Despite the fact that the pulses contained exclusively GABA, no increase of the current during the pulses, even with pulse lengths of 2000 ms, were observed. This indicated that GHB remained bound to the receptor during the presence of GABA or unbound very slowly. The durations of the intervals between the GABA pulses, when only GHB was present in the external solution, did not affect the degree of block. Binding of GHB to the receptor occurred during the change of the external solution to the GHB containing one that took a few seconds.

The off-times after rapid wash-out of GABA could be best fitted by one time constant of $\tau_{decay} = 23.15 \pm 4.1$ ms (mean $\pm$ S.E.M.; $n = 23$), indicating no changes of the burst length of the channel. The block after preapplication of GHB could be also observed at lower GABA concentrations. Single-channel data were recorded by activation with 0.4 mM GABA after preapplication of 10 mM GHB (Fig. 5C). The respective open time constants of $\tau_{o1} = 0.69 \pm 0.16$ ms (mean $\pm$ S.E.M.; $n = 3$) and $\tau_{o2} = 5.2 \pm 1.07$ ms (mean $\pm$ S.E.M.; $n = 3$) and closed time constants of $\tau_{c1} = 0.30 \pm 0.19$ ms (mean $\pm$ S.E.M.; $n = 3$) and $\tau_{c2} = 4.61 \pm 0.39$ ms (mean $\pm$ S.E.M.; $n = 3$) were not significantly different from the values derived from control measurements, indicating no influence of GHB on the single-channel kinetics during the block.

Fig. 5. (A) Averaged current traces (20–35 each) of a patch activated with 10 mM GABA after preapplication of 1 mM and 10 mM GHB in the external solution. The pulse interval was 2000 ms. (B) Relative current amplitudes after preapplication of 1 mM and 10 mM GHB. The current amplitudes achieved without preapplication of GHB were set to one. The mean values $\pm$ S.E.M. were derived from at least 28 recordings for each concentration. (C) Open time histogram after preapplication of 10 mM GABA and activation of the channels with 0.4 mM GABA.

4. Discussion

Despite the great structural similarities between GABA and GHB, no direct activation of the GABAergic channel with GHB concentrations up to 10 mM was observed. After coapplication, an activating effect of GHB on the GABA elicited current and a decrease of the current rise times was detected. The degree of the potentiation was independent from the different GABA concentrations applied and increased with the GHB concentration. Limitation of the potentiating effect was observed after reaching the maximal open probability of the channel. Therefore, coapplication of GHB together with 10 mM GABA did only result in shortening of the rise times. Obviously, GABA and GHB do not compete for binding sites. The affinity of the agonist is defined by the unbinding rate divided through the binding rate. GHB seems to bind to a specific site on the receptor resulting in a conformational change, which increases the binding rate for GABA to the receptor, i.e., a shift of the dose–response relationship towards lower GABA concentrations and a decrease of the current rise time. Since no influence of GHB on the single-channel kinetics was detected, an increase of the rates into the openings, resulting in a similar shift of the dose–response relationship, and a decrease of the rise times, could be excluded. An increasing effect of GHB on the rates for unbinding of GABA could also be excluded because this would result in shortening the rise times, but simultaneously decrease the current amplitudes. Furthermore, the burst length, which is defined by the ratio of the unbinding rate from the fully liganded receptor state to the rates into the open states, would be shortened. Changes in the burst length after coapplication of GABA and GHB have not been observed.

A blocking effect of GHB was observed after preapplication prior to the GABA pulse. During preapplication, GHB only could bind to the closed state of the receptor that is not liganded with GABA. The current amplitude elicited by the subsequent GABA pulse was decreased, suggesting that GHB isomerized the receptor, after binding to the unliganded state, to a blocked state. It seemed to be independent from the potentiating action of GHB because the block could also be observed at lower GABA concentrations in the preapplication experiments, and on the other hand, coapplication of GHB and 10 mM GABA did not result in blocking of the current (Fig. 3). Probably, unbinding of GHB was not possible as long as GABA was present or occurred with very low rates explaining the persistence during the GABA pulse. The rate into the block could be much lower than the one responsible for the potentiating effect of GHB, because the change of the background solution to the GHB-containing one took a few seconds, during which the block equilibrium was reached. Since the rate of unbinding of GHB is very low, also a low rate for binding has to be assumed in order to obtain the relatively small reduction of the current amplitude with high GHB concentrations. The observation that the potentiating effect of GHB is weaker with 10 mM compared to the lower concentrations may indicate the increasing influence of the block effect at this concentration, supporting the suggestion that binding of GHB to the closed receptor state occurred with a low rate.

The physiological consequences of these two opposing mechanisms would be a decrease of the inhibition by GABA when GHB is present before the release of the transmitter, and an increase of the inhibition by GABA when both substances are released simultaneously. Since GHB is enriched in the synaptosomal fraction of brain preparations, a neurotransmitter-like release of GHB could be possible, resulting in the described effects. GHB concentrations in the mM range were necessary to obtain the maximal effects in this study. These are higher concentrations than determined in different brain preparations in vivo (Maitre, 1997), but could be achieved locally after transmitter-like release. The lack of actions on vertebrate $GABA_A$ receptors described so far (Lloyd and Dreksler, 1979; Serra et al., 1991) could be due to the great diversity of this kind of ion channels resulting from the large number of different subunits forming receptors with different kinetics and pharmacology. Possibly also in vertebrates, GABAergic receptors with subunit compositions sensitive to GHB are present, but have not been found so far.

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