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# Mechanisms of $GABA_A$ receptor blockade by millimolar concentrations of furosemide in isolated rat Purkinje cells

Sergey N. Kolbaev *, Irina N. Sharonova, Vladimir S. Vorobjev, Vladimir G. Skrebitsky

*Brain Research Institute, Russian Academy of Medical Sciences, Moscow 103064, Russia*

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**Abstract**

The action of diuretic furosemide on the $GABA_A$ receptor was studied in acutely isolated Purkinje cells using the whole-cell recording and fast application system. Furosemide blocked stationary component of GABA-activated currents in a concentration-dependent manner with $IC_{50}$ value $> 5$ mM at $-70$ mV. The inhibition was rapid in the onset, fully reversible and did not require drug pre-perfusion. The termination of GABA and furosemide co-application was followed by transient increase in the inward current 'tail' current, which was not observed when furosemide was continuously present in the solution. The degree of furosemide block did not depend on GABA concentration. Furosemide block increased with membrane depolarization. Five millimolar furosemide depressed GABA currents by $32.4\pm1.3\%$ at –70 mV and by $76.7\pm5.0\%$ at +70 mV. Analysis of the voltage dependence of the block suggests that furosemide binds at the site located within $GABA_A$ channel pore with a dissociation constant of $5.3\pm0.5$ mM at 0 mV and electric distance of 0.27. Our results provide evidence that furosemide interacts with Purkinje cell $GABA_A$ receptors (most probably composed of $\alpha1\beta2/3\gamma2$ subunits) through a low affinity site located in channel pore and suggest that furosemide acts as a sequential open channel blocker, which prevents the dissociation of agonist while the channel is blocked. 

*Keywords:* GABA; Cerebellum; Furosemide; Patch-clamp; Open channel block

## 1. Introduction

$GABA_A$ receptors mediate the majority of fast inhibitory neurotransmission in the mammalian brain. Structurally, $GABA_A$ receptors are presumably heteropentameric complexes and are assembled by combining homologous subunits which enclose an integral anion channel (Nayeem et al., 1994; Tretter et al., 1997). Seven different $GABA_A$ receptor subunit families have been identified in mammals ($\alpha$, $\beta$, $\gamma$, $\delta$, $\varepsilon$, $\pi$ and $\theta$) [for review, see Kardos (1999); Sieghart et al. (1999)]. Several of the subunit families have multiple subtypes ($\alpha1$–6, $\beta1$–3, $\gamma1$–3). The majority of native receptors are formed by combination of $\alpha\beta\gamma$ or $\alpha\beta\delta$ subunits (McKernan and Whiting, 1996). The particular subunits of which receptors are composed determine the pharmacological characteristics of different $GABA_A$ receptor subtypes (Sieghart, 1995). The subunits have similar transmembrane topology with an ~200 amino acid $NH_2$-terminal extracellular domain, four closely spaced membrane-spanning domains (TM1–TM4), a long and sequence-variable intracellular loop between TM3 and TM4 and a short extracellular COOH terminus. $GABA_A$ receptors are regulated by several classes of modulatory compounds one of the most receptor-subtype specific of which is furosemide. Furosemide is a loop diuretic exerting its action by interfering with the $Na^+/K^+/2Cl^-$ co-transport system in the lumen membrane (Greger and Wangemann, 1987). This diuretic compound, has been also shown to be an antagonist at $GABA_A$ receptors, eliciting approximately 100-fold greater sensitivity for $\alpha6\beta2\gamma2$ and for $\alpha4\beta2\gamma2$ receptors than for $\alpha1\beta2\gamma2$ receptors (Knoflach et al., 1996; Korpi et al., 1995; Wafford et al., 1996). The domain required for the action of furosemide was described to residue amino-terminal to TM1 of the $\alpha$ 6 subunit (Fisher et al., 1997). By making $\alpha1/\alpha6$ chimeras Thompson et al. (1999) identified a transmem-

* Corresponding author. Tel.: +7-95-917-84-52; fax: +7-95-917-23-82..

*E-mail address:* shar@cc.nifhi.ac.ru (S.N. Kolbaev).

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brane region (209–279) responsible for the high furosemide sensitivity of α6β3γ2 receptors. Furosemide appears to reduce $GABA_A$ receptor channel current in a non-competitive manner (Inomata et al., 1988), but the mechanisms by which furosemide suppresses GABA-evoked current is unknown. Some studies indicate that furosemide acts as ion channel blocker (Inomata et al., 1988). Other data support its allosteric regulation of the $GABA_A$ receptors (Korpi and Luddens, 1997). It is possible that mode of furosemide interaction with $GABA_A$ receptor depends on subunit composition.

The aim of the present study was to investigate the mechanism of furosemide interaction with native $GABA_A$ receptors containing neither α6, nor α4 subunits and having low sensitivity to this drug. We have applied patch-clamp technique to study the effect of furosemide on $GABA_A$ receptors in freshly dissociated cerebellar Purkinje cells. Our results indicate that furosemide interacts directly with the $GABA_A$ receptor pore and acts as a sequential open channel blocker, which prevents the dissociation of agonist when the channel is blocked.

## 2. Materials and methods

### 2.1. Preparation of Purkinje cells

All experiments were performed in compliance with standards for use of laboratory animals and were approved by the USSR Academy Commission on Laboratory Animals usage Control. Neurons were dissociated acutely from cerebella of 2–3 week-old male Wistar rats as was described previously (Vorobjev et al., 1996). Briefly, sagittal slices of cerebellum were incubated at room temperature for 1–6 h on a mesh near the bottom of a beaker. The incubation solution had the following composition (in mM): NaCl 125, KCl 5, $CaCl_2$ 1.5, $MgCl_2$ 1.5, $NaH_2PO_4$ 1.28, $NaHCO_3$ 25, glucose 10, Phenol Red 0.01%, it was continuously bubbled with carbogene (5% $CO_2$+95% $O_2$). One at a time, slices were transferred to the recording chamber and neurons were isolated by vertical vibration of a glass sphere, 0.7 mm in diameter, placed close to the surface of the slice (Vorobjev, 1991). Manipulation and cell identification was perfomed using an inverted microscope. Isolated Purkinje cells were distinguished from other cerebellar cells based on their large cell bodies (ca. 20 μm) and characteristic pear shape attributable to the stump of the apical dendrite. The solution for dissociation and recording had the following composition: NaCl 150, KCl 5, $CaCl_2$ 2.7, $MgCl_2$ 2.0, HEPES 10, pH adjusted to 7.4 with NaOH.

### 2.2. Whole-cell recording

Voltage-clamp recording was obtained using the whole-cell configuration of the patch-clamp technique (Hamill et al., 1981). Glass recording patch pipettes were prepared from filament-containing borosilicat tubes using a two-stage puller. The electrodes, having resistance of 2–3 MOhm, were filled with recording solution of the following composition (in mM): 140 CsCl, 0.5 $CaCl_2$, 4 $MgCl_2$, 10 HEPES, 5 EGTA, 4 ATP-Na (pH adjusted to 7.2 with CsOH). Recordings were carried out at room temperature (20–23°C) using an EPC 7 patch-clamp amplifier. Currents were filtered at 10 kHz, sampled at 500 Hz, and stored on a computer disk.

Cells were held at a membrane potential of −70 mV, and *I–V* relationships were generated with test potentials from −70 mV to +70 mV by 20 mV intervals.

### 2.3. Drug solutions and drug application

A fast perfusion technique was used for application of agonist-containing solutions (Vorobjev et al., 1996). Isolated Purkinje cells were first patch clamped and then lifted into the application system, where it was continuously perfused with control bath solution. The substances were applied through two different glass capillaries, ~0.2 mm in diameter. The delivery ports of the capillaries were positioned within 0.2 mm from the cell under the study. For exposure the application system was moved so as to place the cell in the solution stream leaving one of the application capillaries. Exchange time was measured at the open electrode tip by switching between solutions of different osmolarities. Rise time in these experiments measured as the time elapsed from 10 to 90% of the peak amplitude of the response was 20±3 ms ($n$=6). The flow through each tube was gravity-driven. For activation of $GABA_A$ channels, in most experiments GABA was applied for periods of 1 s, at 30–40 s intervals. Solutions were dissolved in extracellular buffer to the desired concentration from the following stock solutions: 100 mM GABA in $H_2O$ and 200 mM furosemide in 200 mM NaOH. Furosemide was dissolved further in extracellular buffer and pH of final solution was controlled. All reagents were obtained from Sigma.

### 2.4. Data analysis

Whole-cell records were analysed off-line using original homemade software or being exported as text files to Prism (GraphPad Software, San Diego, CA) for further analysis. Agonist concentration–response curve were fit by the least-square method to:

$$\frac{I}{I_{max}} = \frac{([A]/EC_{50})^{n_H}}{1 + ([A]/EC_{50})^{n_H}}, \quad (1)$$

where $I$ is the peak current evoked by agonist concentration [A], $I_{max}$ is the peak current evoked by a maximal agonist concentration (100 μM GABA), $EC_{50}$ is the con-

centration giving half the maximal current, and $n_H$ is the Hill coefficient. The curve fit was performed on an IBM PC-compatible computer using the program PRISM (GRAPHPAD Software). Data values are presented as mean±s.e.m.

We have made every effort to minimize animal discomfort in our experimental procedures, and we have used as few animals as necessary for this in vitro study.

## 3. Results

### 3.1. Effects of furosemide on $GABA_A$ currents

Rapid application of GABA (2 μM) alone or together with furosemide induced in isolated Purkinje cells membrane currents which were essentially completely blocked by bicuculline (20 μM, not shown), identifying them as $GABA_A$ receptor-mediated $Cl^-$ currents (Konnerth et al., 1990). Furosemide (1–5 mM) applied together with GABA (2 μM), at −70 mV suppressed the responses to GABA, measured at steady state, in a concentration-dependent manner. Representative superpositions of the currents induced by 2 μM GABA (control) or by GABA and furosemide co-applied at different concentrations are shown in Fig. 1A. The greatest inhibition observed was 32.4±1.3% at 5 mM furosemide. Due to low solubility of furosemide full concentration-response curve could not be constructed. By itself, furosemide (up to 5 mM) had no action on resting currents (data not shown).

The effect of furosemide developed quickly, and was easily reversible. In order to test whether the blocking action of furosemide required activation of ion channel gating by agonist, furosemide was applied for 20–30 s prior to a concentration-jump application of GABA in the continued presence of furosemide. Records on Fig. 2 demonstrate that GABA currents evoked in the continuous presence of 5 mM furosemide were indistinguishable from those in co-application experiment except for the kinetics of the recovery of responses. Pretreatment of the neuron with furosemide (up to 5 mM) did not affect the response to GABA when the agonist was applied immediately after termination of washout of the antagonist, suggesting that furosemide effect does not involve closed channel block.

Simultaneous application of furosemide with GABA (2 μM) caused a decrease in the rate of GABA receptor activation. Under control conditions, 2 μM GABA induced a reproducible non-decreasing GABA response. The onset of this response was fitted by one exponential with a time constant ($\tau_{on}$) of 120±8 ms ($n$=6). Furosemide (2–5 mM) slowed the activation time of GABA (2 μM) response. This effect was concentration-dependent. The time course of the rising phase of currents during co-application of GABA and blocker could be characterized by single exponential time constants of 180±12 ms and 252±24 ms for 2 and 5 mM furosemide, respectively ($n$=6). The relationship between the relative time of activation ($\tau_{on(blocked)}/\ \tau_{on(control)}$) and concentration of furosemide is shown on Fig. 1C.

The time course of furosemide action is demonstrated in Fig. 2C, where the degree of block was measured with intervals of 96 ms during co-application of GABA (2 μM) and furosemide (5 mM). One can see that the degree of block is maximal at the beginning of co-application and reaches the equilibrium at the end of the 1-s application.

The termination of GABA and furosemide co-application was followed by transient increase in the inward current ('tail' current) (Fig. 1A). The onset of this current coincided with the initiation of perfusion with normal extracellular solution. The amplitude and kinetics of the tail current were dependent on concentration of blocker and holding potential (Figs. 1 and 4). In the continuous presence of furosemide the tail current was not recorded (Fig. 2).

### 3.2. Blocking action of furosemide at different agonist concentrations

To determine whether inhibition by furosemide involved a competitive interaction with the channel activation by agonist, we examined its effect on the concentration-response relationship for GABA (Fig. 3).

The percent of block did not depend on the GABA concentration (Fig. 3B). Furosemide (5 mM) inhibited $GABA_A$ receptor current by 32.4±1.3% ($n$=6), 27.8±3.2% ($n$=4), 36.5±1.5% ($n$=4) and 28.6±3.0% ($n$=4) at GABA concentrations of 2, 5, 10 and 50 μM, respectively. Representative superpositions of the currents elicited by GABA alone used at different concentrations (control) or by GABA co-applied with 5 mM furosemide are illustrated in Fig. 3A. Furosemide does not significantly modify the $EC_{50}$ value of GABA, as illustrated in Fig. 3C; the GABA concentration-response curve performed in the presence of 5 mM furosemide gives an $EC_{50}$ value of 2.9±0.1 μM with Hill coefficient of 2±0.1 ($n$=4), which is close to the values determined in the absence of furosemide ($EC_{50}$ 2.8±0.1 μM and Hill coefficient 1.8±0.1, $n$=4) ($p$ values were 0.99 for $EC_{50}$ and 0.82 for Hill coefficient).

### 3.3. Furosemide block at different holding potentials

To determine the voltage dependence of furosemide antagonism, block of the response to GABA was investigated at several different membrane potentials. Fig. 4A illustrates the degree of block produced by 5 mM furosemide on the current elicited by 2 μM GABA at −70, −50, −30, −10, +10, +30, +50 and +70 mV. The GABA current–voltage relationship was roughly linear

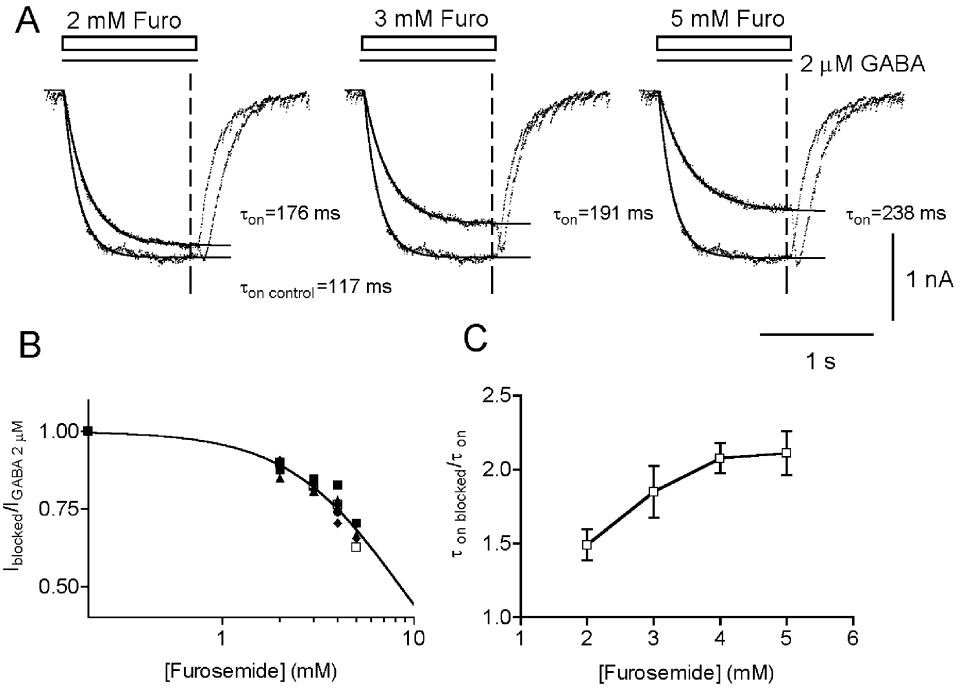


Fig. 1. Furosemide blocks GABA-mediated currents in a dose-dependent manner. (A) Representative currents in acutely isolated cerebellar Purkinje cell induced by 2 μM GABA. The control current (lower trace) is superposed with the current induced by GABA co-application with different concentrations of furosemide (2, 3 and 5 mM). GABA applications are marked by the line and furosemide by the open bars above the current traces. The rise phase of response was adequately described by a single exponential function, and the *smooth lines* superimposed on the experimental points represent an exponential function fit to the data. The time constants ($\tau_{on}$ ) are shown to the right of the curves. (B) Concentration–response analysis for block of GABA-activated currents by furosemide. Data points represent individual values from six cells normalized with respect to the current obtained in control condition (2 μM GABA, −70 mV); solid line, the average data fitted by the equation: $I_{blocked}/I_{non\text{-}blocked} = 1/(1 + ([FURO]/IC_{50})^{n_H})$, were $I_{blocked}$ is the current induced by co-application of GABA (2 μM) and furosemide at a given concentration and $I_{non\text{-}blocked}$ is the amplitude of steady-state current evoked by GABA (2 μM), $IC_{50}$ is the concentration of the furosemide producing a half-maximal block of GABA-mediated responses, and $n_H$ is the Hill coefficient. Fitting the data with this logistic equation, where maximal percent of block was set at 100% produced an apparent $IC_{50}$ of 8.9±0.7 mM and Hill slope of 1.5±0.1. Current amplitude was measured at the end of application at the moment marked by a dashed line. (C) The relationship between the relative activation time ($\tau_{on\text{-}blocked}/\tau_{on\text{-}control}$) and concentration of furosemide.

in the absence of furosemide, but showed significant inward rectification in the presence of furosemide (Fig. 4B). Thus, the block of GABA-induced currents was greatest at the most positive holding potentials. At 5 mM, furosemide depressed stationary GABA current by 32.4±1.3% at −70 mV and by 76.7±5.0% at +70 mV ($p<0.001$).

At all membrane potentials the co-application of GABA and furosemide was associated with an appearance of the tail current following cessation of the application. The maximal relative amplitude of the tail current measured from the level of the steady-state current was observed when the degree of block was maximal — at furosemide concentration of 5 mM and holding potential +70 mV (Fig. 4).

For estimation of voltage-dependence of tail current the absolute amplitude (from baseline) was measured and normalized to GABA response (Fig. 4B). This normalized current-voltage relationship was similar to that obtained for GABA alone. Reversal potential of tail current was close to zero. Its absolute amplitude was smaller than the amplitude of control steady-state current ($I_{tail}/I_{control}$=0.85, $p<0.001$).

### 3.4. Woodhull analysis of furosemide block

The voltage dependence of block was further analysed according to the method of Woodhull (1973) which provides a means of calculating the fraction of the transmembrane field sensed by charged blocking ligand at its acceptor site. Furosemide contains a carboxyl group which has a negative charge. Therefore, the molecule would be expected to carry a single negative charge at physiological pH. According to Woodhull's model, two sets of GABA currents (steady-state currents in the absence and presence of furosemide) were measured in the same cell at different membrane potential. Then the relation of the $B$ value ($B = I_{blocked}/I_{control}$) and membrane potential ($V$) were approximated by:

$$B = \frac{1}{1 + ([\text{Furosemide}]/K_D(0)) * \exp(z\delta FV/RT)}, \quad (2)$$

where $K_D(0)$ is the dissociation constant of the furosemide-binding site on the $GABA_A$ complex at a transmembrane potential of 0 mV, $\delta$ is the measureless factor which reflects the fraction of the total electric field sensed at the binding site, $z$ is the charge of furosemide

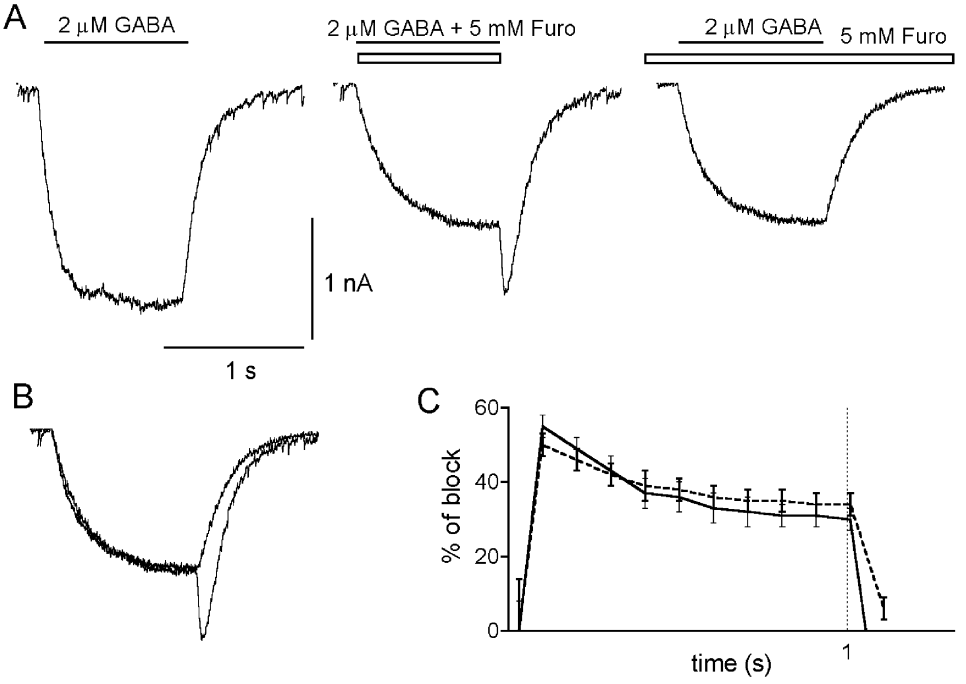


Fig. 2. The development of furosemied effects does not require pre-equilibration. The continuous presence of furosemide prevents the appearance of the tail current. (A) Traces show the effect of 5 mM furosemide on currents produced by 2 μM GABA. When furosemide remained after GABA application was stopped (trace 3) there was no tail current. (B) Superposition of the second and third traces presented on A. (C) The time course of development of furosemide block. The relative degree of block (percentage of block) during co-application with GABA is marked by a solid line, percentage of furosemide block in the continuous presence of blocker is marked by dashed line. Mean values from four cells.

(=−1), $F$ is the Faraday constant, $R$ is the universal gas constant and $T$ is the ambient absolute temperature [$F/(RT) = 0.03972\ \mathrm{mV}^{-1}$]. [Furosemide]−furosemide concentration ($5\times10^{-3}$ M). The values of $K_D(0)$ and $\delta$ were determined as a result of nonlinear regression. The results of fitting and experimental data are shown on Fig. 4C. The best fitted values of $K_D(0)$ and $\delta$ were 5.3±0.3 mM and 0.27±0.03, respectively. The $K_D$ (−70 mV) was evaluated using equation (Woodhull, 1973):

$$K_D(V) = K_D(0)*\exp\left(\frac{z\delta FV}{RT}\right). \quad (3)$$

The derived value (10 mM) is in agreement with the value obtained from the concentration-block experiment shown in Fig. 1, which was obtained at a holding potential of –70 mV (> 5 mM). The results of such analysis provide evidence for binding of furosemide to a site within the membrane electrical field.

## 4. Discussion

### 4.1. Blocking action of furosemide

In the present study we have characterized the effects of furosemide on native $GABA_A$ receptor channels in cerebellar Purkinje cells. Furosemide was reported to be a subtype selective, non-competitive inhibitor of $GABA_A$ receptors. It displays a high affinity for inhibition of currents elicited by GABA in recombinant receptors containing α4 or α6 subunit and very low affinity in α1β2γ2 receptors (the order of magnitudes of $IC_{50}$ is ~100 μM for α4- or α6-contanig receptors versus ~10 mM for α1, α2-, α3- and α5-contaning receptors) (Knoflach et al., 1996; Korpi et al., 1995; Wafford et al., 1996). In situ hybridization studies have show strong expression of the α1, β2, β3 and γ2 mRNA in rat cerebellar Purkinje cells (Laurie et al., 1992). The functional properties of $GABA_A$ receptors of Purkinje cells also suggest the presence of α1, β2, β3 and γ2 subunits. These include the high sensitivity to zolpidem (Itier et al., 1996; our unpublished observation), indicating the presence of α1 and γ2 subunits, the high potency of loreclezole (our unpublished data), suggesting the presence of β2/3 subunits, and the relatively low sensitivity to $Zn^{2+}$ (Sharonova et al., 2000), reflecting the presence of γ subunits. All these facts imply that the majority of functional $GABA_A$ receptors in cerebellar Purkinje cells most probably have α1β2/3γ2 subunit composition.

As expected, these receptors display a very low affinity for furosemide. The direct measurement of the $IC_{50}$ value for furosemide inhibition was hampered by its solubility limit. However, estimation of an $IC_{50}$ gives the value >5 mM, which is in agreement with $IC_{50}$ values measured by other authors on recombinant α1β2/3γ2 receptors expressed in different heterologous systems

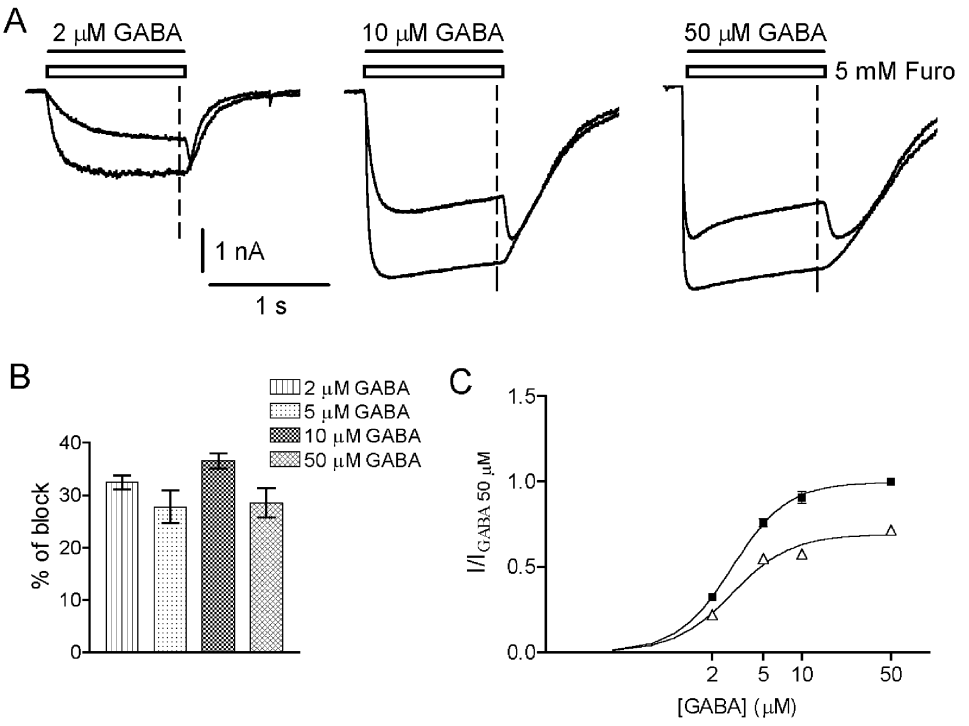


Fig. 3. The degree of furosemide-induced block does not depend on GABA concentration. (A) Responses to 2, 10 and 50 μM GABA co-applied with 5 mM furosemide (smaller responses) and corresponding control responses. All responses are from a single cell. GABA application is marked by a line and furosemide exposure by an open bar above the traces. Current amplitude was measured at the end of application at the moment marked by a dashed line. (B) Percent of block by furosemide (5 mM) does not dependent on GABA concentration. (C) Furosemide does not modify the $EC_{50}$ value of GABA. Concentration-response curve to GABA obtained in control (squares) or presence of 5 mM furosemide (triangles). Data points represent averages from four cells. Data are normalized with respect to the response to 50 μM GABA in the absence of furosemide. The data are fit with eq. (1): control: $EC_{50}$=2.8±0.1 μM, $n_H$=1.8±0.1 and at 5 mM furosemide: $EC_{50}$=2.9±0.1 μM, $n_H$=2±0.1.

(Fisher et al., 1997; Korpi et al., 1995). Thus, furosemide interacts with low-affinity site/sites on $GABA_A$ receptors in Purkinje cells.

We have demonstrated that furosemide induces concentration and voltage-dependent suppression of GABA-activated $Cl^-$currents. Under experimental conditions used (co-application with GABA during 1 s) furosemide effects were mediated by its direct interaction with $GABA_A$ receptor. This statement is supported by the following observations:

1. no current was evoked by isolated 1–30 s application of furosemide;
2. co-application of GABA and furosemide in the presence of bicuculline did not elicit any response; and
3. furosemide did not change the reversal potential of GABA responses.

Therefore, the effect of furosemide in the present experiments is neither mediated by the changes of membrane properties due to non-specific binding nor by the shift in reversal potential, as would result from an altered distribution of chloride.

The results of the present study support the suggestion that furosemide is a non-competitive $GABA_A$ receptor antagonist (Inomata et al., 1988; Kumamoto and Murata, 1997). The non-competitive nature of furosemide antagonism is proved by concentration–response data. We have not observed the decrease in the percentage of furosemide antagonism when the concentration of GABA was raised. The $EC_{50}$ value of GABA seems not to be modified by the presence of furosemide also implying a non-competitive mechanism of blockade.

Analysis of blocking action of furosemide on $GABA_A$ receptors in Purkinje cells has led us to two main conclusions:

1. furosemide is an open channel blocker of these $GABA_A$ receptors;
2. channel block by furosemide prevents dissociation of agonist from $GABA_A$ receptors.

### *4.2. Furosemide is an open-channel blocker*

The mechanism of furosemide-induced suppression of GABA-gated chloride conductance is largely unknown. We provide evidence that furosemide inhibits GABA currents in cerebellar Purkinje cells by a mechanism of open channel block. The following observations support this statement:

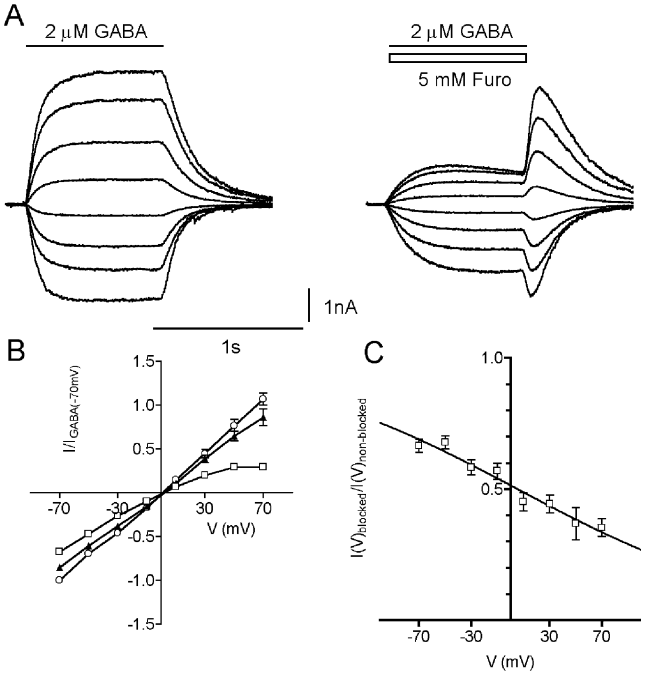


Fig. 4. Block of GABA induced currents by furosemide is voltage-dependent. (A) Responses to 2 μM GABA at holding potentials −70, −50, −30, −10, 10, 30, 50 and 70 mV in control (left) and during co-application with 5 mM furosemide (right). Holding potential was adjusted 10 s before GABA exposures during time indicated by lines above the traces. Offset current in the traces is subtracted. (B) The current-voltage relationships for the control (2 μM GABA) currents (circles), the currents blocked by 5 mM furosemide (squares) and for the tail current (triangles). The $I-V$ curves constructed from measurements in four cells. Responses obtained at different voltages were normalized to the response at −70 mV in the absence of furosemide. (C) Woodhull analysis of furosemide block. Normalized block is plotted versus for the holding potential. The data are fitted using eq. (2) after normalizing. The fraction of the membrane field sensed by furosemide is 0.27±0.03 with $IC_{50}$ (0 mV) equal to 5.3±0.3 mM.

1. no inhibitory action of furosemide on closed channel was observed; and
2. furosemide block of GABA-induced current was voltage-dependent.

Our data show that furosemide binds rapidly to the open, but not to the closed (unliganded) state of $GABA_A$ receptor. Pre-incubation in furosemide prior to co-application of agonist and furosemide did not influence either the onset kinetics of GABA response or the block amplitude, suggesting essentially no blocking action of furosemide application on closed channel. Thus, the results of our experiments indicate that block of $GABA_A$ channels by furosemide is use-dependent. This is typical for the action of drugs that need to access the channel pore in order to exert their blocking action. Open channel blockers generally are known to exhibit a marked voltage-dependent inhibition. Voltage dependency suggests that the drug binds to a site sensitive to electric field across the membrane bilayer, i.e. a site within the transmembrane region of the channel. The present study has demonstrated that the inhibition of $GABA_A$-receptor-mediated whole-cell currents by furosemide was voltage dependent. It means that the weak block occurring at negative potentials is significantly increased when the cell is brought to depolarized potentials. Analysis of the steady-state fractional block values for furosemide according to the method of Woodhull provided an estimated electrical depth of the binding site of 0.27. This parameter represents the fraction of the membrane electrical field that is sensed by furosemide at its binding site. The value of 0.27 suggests that the putative channel binding site for these molecules must be located in the first third of the field across the ionic pore (assuming that electrical field across the lipid bilayer is constant).

Thus, our results suggest that furosemide antagonizes responses to GABA in Purkinje cells by entering and blocking the ion channel activated by the $GABA_A$ receptors. This $GABA_A$ antagonism by high furosemide concentrations differs from that of α6-containig receptor since the interaction of furosemide with the high affinity binding site is voltage-independent (Korpi et al., 1995), suggesting an allosteric mechanism of inhibition. The low affinity site is unlikely to coincide with the picrotoxin-sensitive binding site since the latter is located much deeper into the channel pore (Xu et al., 1995; Zhorov and Bregestovski, 2000).

### *4.3. Furosemide prevents the $GABA_A$ channel from closing*

Two main mechanisms of the open channel block have been described for ligand-gated ion channels: 'trapping' and sequential block. Trapping channel blocker permits agonist dissociation and channel closure while the blocker is bound in the activated channel. It results in trapping of the blocker in the channel (Blanpied et al., 1997; MacDonald et al., 1987, 1991; Samoilova et al., 1999). When the blocker is trapped in the channel pore, the recovery occurs only in the presence of agonist. In the sequential scheme, the channel cannot close while blocked (Adams, 1976; Antonov and Johnson, 1996; Benveniste and Mayer, 1995; Neher and Steinbach, 1978; Neher, 1983; Sobolevsky et al., 1999).

The kinetic scheme for sequential channel-blocking model implies that agonist (A) cannot dissociate from its binding site on a receptor (R) when the ion channel is in the open state:

$$R \leftrightarrow AR \leftrightarrow \ldots \leftrightarrow A_nR \leftrightarrow A_nR_{open} \rightarrow A_nR_{blocked/open}.$$

This scheme also implies that prior to dissociation of the agonist, channels which are blocked must return to the closed state via the open state. In this case one may expect the appearance a tail currents developing upon removal of agonist and blocker. Tail currents of that type were observed after termination of the co-application of

NMDA or aspartate together with a number of NMDA receptor channel blockers: tacrine (Vorobjev and Sharonova, 1994), 9-aminoacridine (Benveniste and Mayer, 1995; Costa and Albuquerque, 1994), tetrabutylammonium (Koshelev and Khodorov, 1995).

Our results provide evidence that similar mechanisms may underlie furosemide action on GABA currents:

1. the recovery of $GABA_A$ receptor from furosemide block was fast and did not require the presence of an agonist; and
2. furosemide induced the appearance of a tail current after termination of its co-application with the agonist.

The amplitude of the tail current was dependent on membrane potential and blocker concentration. The tail current was not observed when furosemide perfusion was continued after GABA application was stopped. The above mentioned properties of furosemide-induced inhibition are consistent with the sequential model of open channel block.

We have observed that furosemide slowed the onset of GABA-induced current in a concentration-dependent manner. This phenomenon also could be explained in the framework of the sequential model. This model predicts that total open time/burst should be unchanged in the presence of a blocking drug (Neher and Steinbach, 1978). After dissociation of a drug, the channel passes back through the open state before closing. We can suppose that at the beginning of application furosemide induces the fast blockade of high proportion of open GABA channels. Subsequent partial rescue from the block may result from the prolongation of bursts and reopening of channels after dissociation of the blocker. Another possible explanation for the described effect is that the blocker prohibits not only the channel closure, but also does not allow the receptor to desensitize. A similar explanation has been proposed to explain the identical effect of tetrapentylammonium on NMDA channels (Sobolevsky et al., 1999). Further kinetic study of this effect and simulation experiments are needed to clarify this issue.

Kinetic studies on nicotinic (Neher, 1983) and NMDA receptors (Benveniste and Mayer, 1995) suggest that sequential channel block prevents escape of agonist from its binding site. Similar mechanism seems to occur in the case of block of $GABA_A$ channels by furosemide. The molecular mechanisms underlying agonist trapping during open channel block are not yet understood, but this effect suggests a close coupling between conformational changes in receptor during channel opening/block and agonist dissociation. Experiments with the channel blockers that prevent channel closure could be useful for giving insight into the gating mechanism of ligand-gated ion channels, including $GABA_A$ receptors.

In conclusion, the results of our experiments strongly suggest that the voltage-dependent block produced by furosemide occurs by an open channel blocking mechanism and conclusively demonstrate that it is a sequential blocker that prevents the closure of the channel. Therefore it is reasonable to use furosemide and its derivatives to study the relationship between agonist binding and channel gating.

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