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RESEARCH PAPER

Flurazepam effect on GABAergic currents depends on extracellular pH

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Background and purpose: Benzodiazepines (BDZs) are widely used in clinical practice and are known as positive modulators of GABAergic currents. BDZs increase binding affinity and recently they were found to affect $GABA_A$ receptor gating, including desensitization. Binding and desensitization are also strongly modulated by extracellular pH, a factor that may be severely altered in a pathological brain. It is thus of interest to examine the combined action of BDZ and protons.
Experimental approach: Pharmacokinetic analysis was based on patch clamp recordings of miniature IPSCs (mIPSCs) and current responses to GABA applications in rat cultured hippocampal neurons. High temporal resolution of currents evoked by exogenous GABA was achieved by using an ultrafast perfusion system (exchange time ca. 80 μs).
Key results: At acidic pH, flurazepam produced a stronger enhancement of mIPSC amplitudes than at physiological pH. At low GABA concentrations, flurazepam markedly enhanced current amplitudes both at normal and acidic pH, but at the latter, the relative effect was larger. In contrast, at saturating GABA concentrations, flurazepam reduced current amplitudes at both pH 7.2 and 6.0. The slowing of deactivation kinetics by flurazepam decreased with GABA concentration, but at pH 6.0, this trend was shifted toward a higher GABA concentration.
Conclusions and implications: Acidification of extracellular medium may significantly affect the susceptibility of phasic and tonic components of GABAergic currents to modulation by BDZs. Quantitative analysis and model simulations indicate that protons and flurazepam additively affect binding and desensitization of $GABA_A$ receptors.
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Keywords: flurazepam; proton; $GABA_A$ receptor; desensitization; binding rate; mIPSC; synaptic transient; patch clamp; ultrafast perfusion

Abbreviations: [GABA], GABA concentration; mIPSC, miniature inhibitory postsynaptic current

Introduction

GABA is known to play a crucial role in mediating inhibition in the adult mammalian CNS. Most of GABA-induced inhibition is achieved by the activation of ionotropic $GABA_A$ receptors that mediate both phasic and tonic currents (Farrant and Nusser, 2005). So far, as many as 20 subunits of $GABA_A$ receptor (α1–6, β1–4, γ1–3, δ, ρ1–3, ε, π and θ) have been cloned (Cherubini and Conti, 2001; Fritschy and Brunig, 2003), suggesting enormous heterogeneity. However, typically, $GABA_A$ receptors consist of two α, two β and one γ or δ subunit (Whiting, 2003; Wafford, 2005).

Benzodiazepine (BDZ) receptor agonists are widely used in clinical practice. These compounds exert an upmodulation of specific $GABA_A$ receptors containing a γ subunit (Rudolph and Mohler, 2004, 2006; Wafford, 2005). Moreover, BDZs were commonly found to enhance the amplitude of IPSCs in synapses showing incomplete receptor occupancy and prolong the decaying phase of these currents (Frerking *et al.*, 1995; Nusser *et al.*, 1997; Perrais and Ropert, 1999, 2000; Hajos *et al.*, 2000; Mozrzymas *et al.*, 2007). Several lines of evidence indicate that BDZs increase the binding affinity of $GABA_A$ receptors (Lavoie and Twyman, 1996; Krampfl *et al.*, 1998; Mozrzymas *et al.*, 2007). However, more recently, it has been suggested that these drugs also potently interfere with $GABA_A$ receptor gating properties, including receptor efficacy (Downing *et al.*, 2005; Rusch and Forman 2005; Campo-Soria *et al.*, 2006) and desensitization (Mercik *et al.*, 2007; Mozrzymas *et al.*, 2007). Interestingly, binding affinity and desensitization of $GABA_A$ receptors are also subjected to profound modulation by extracellular pH (Mozrzymas *et al.*, 2003a). Most importantly, several brain disorders, such as hypoxia, ischemia and hypoglycemia, are associated with severe acidosis (lowered pH) of the extracellular fluid in the brain tissue (Kraig *et al.*, 1986; Bengtsson *et al.*, 1990; Katsura *et al.*, 1993; Katsura and Siesjo, 1998). Thus, it is likely that the modulatory effects of BDZs on $GABA_A$ receptors in a

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pathological brain might be altered by acidosis. In particular, a decrease in binding affinity and desensitization at low pH (Mozrzymas *et al*., 2003a) is expected to counterbalance the effects induced by BDZs. However, it is not known whether the effects of these modulators (BDZ and low pH) on $GABA_A$ receptors are additive or they allosterically affect the action of each other. Moreover, the possibility that extreme non-equilibrium conditions for activation of $GABA_A$ receptors during IPSCs (Clements, 1996; Overstreet *et al*., 2002; Mozrzymas, 2004) render the synaptic currents particularly sensitive to the modulators affecting agonist binding needs to be taken into account (Mozrzymas *et al*., 1999, 2003a, b, 2007; Mozrzymas, 2004). To our knowledge, a combined action of BDZs and protons on IPSCs and on $GABA_A$ receptor properties has, so far, not been systematically studied. In the present study, we have investigated the impact of extracellular pH on miniature IPSC (mIPSC) modulation by a BDZ, flurazepam, and found that acidic pH enhances the effect of this BDZ on mIPSCs. Recordings of current responses to ultrafast GABA applications indicated that the effects of flurazepam and protons are additive. We conclude that changes in extracellular pH not only affect the amplitude and time course of mIPSCs but also alter their susceptibility to modulation by BDZ receptor agonists.

Methods

Neuronal primary cell culture

Primary hippocampal cell culture was prepared as described previously (Andjus *et al*., 1997). Briefly, P2–P4-old Wistar rats were killed by decapitation. This procedure is in agreement with the Polish Animal Welfare Act and has been approved by a local ethical commission. Hippocampi were dissected, sliced, treated with trypsin, mechanically dissociated and centrifuged twice at 40 *g*, plated in Petri dishes and cultured. Experiments were performed on cells between 7 and 16 days in culture.

Electrophysiological recordings

Currents were recorded at −70 mV using the patch clamp technique (Axopatch 200B amplifier; Molecular Device Corporation, Sunnyvale, CA, USA) from the outside-out patches excised from visually distinguished pyramidal neurons. The intrapipette solution contained (mM) 137 CsCl, 1 $CaCl_2$, 2 $MgCl_2$, 11 BAPTA (tetracesium salt), 2 ATP and 10 HEPES (pH 7.2) with CsOH. The external solution contained (mM) 137 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 20 glucose and 10 HEPES (pH 7.2) with NaOH. Signals recorded in the whole-cell configuration in the free-run mode (mIPSC recordings) were low-pass filtered with Butterworth filter at 3 kHz and sampled at 10 kHz using the analogue-to-digital converter Digidata 1322A (Molecular Device Corporation). The current responses to rapid GABA applications (especially at high [GABA]) required a higher time resolution and were filtered at 10 kHz and sampled at 50–100 kHz. For acquisition and data analysis, pClamp 9.2 software was used (Molecular Device Corporation). Miniature IPSCs were recorded in the whole-cell configuration of the patch clamp technique in the presence of tetrodotoxin (1 μM) and kynurenic acid (1 mM). Solutions were provided by a glass tube (i.d. 0.5 mm) directly onto the recording area with a flux of ca. 1 mL min^{-1}. This system allowed for reliable control of the pH value and drug concentrations in the surrounding of neurons from which the recordings were made. Recordings in the presence and absence of flurazepam and at different pH values were performed from the same cell. After application of flurazepam, cells were washed for at least 4 min. Cells exhibiting rundown of the mIPSC amplitude greater than 20% during the entire recording period were excluded from the statistics. Access resistance was monitored and compensation at 30–80% was applied. Cells exhibiting access resistance greater than 15 MΩ (after compensation) were rejected. Current responses to 1 μM GABA were barely detectable in the excised patch configuration and for this reason were recorded in the whole-cell mode using a multibarrel system (RSC-200, exchange time ca. 15–30 ms; Bio-Logic, Grenoble, France). Since acidic pH is known to reduce $GABA_A$ receptor affinity, for experiments performed at pH 6.0, 30 mM GABA was used to assure conditions of saturation (Mozrzymas *et al*., 2003a).

Rapid drug application

GABA was applied to excised patches using an ultrafast perfusion system based on a piezoelectric-driven theta-glass application pipette (Jonas, 1995). The piezoelectric translator was from Physik Instrumente (preloaded HVPZT translator 80 μm; Waldbronn, Germany) and theta-glass tubing was from Hilgenberg (Malsfeld, Germany). The open-tip recordings of the liquid junction potentials revealed that 10–90% exchange of solution occurred within 50–80 μs. In experiments in which the effect of flurazepam was examined, the drug was present at the same concentration in solutions supplied by both channels (wash and GABA-containing solution) of the theta-glass capillary. Before the agonist was applied (in the presence or absence of flurazepam), the patch was exposed to the washing solution for at least 2 min.

Materials

All the chemicals used were from Sigma-Aldrich (Steinheim, Germany) except tetrodotoxin (Latoxan, Valence, France), BAPTA (Merck, Warsaw, Poland) and HEPES and NaOH (Carl Roth, Karlsruhe, Germany).

Data analysis

The kinetics of the current-rising phase was quantified as 10–90% rise-time. Deactivation current was fitted with a sum of two exponential functions: $y(t) = A_1 \exp(-t/\tau_{fast}) + A_2 \exp(-t/\tau_{slow})$, where A_1 and A_2 are the percentages and τ_{fast} and τ_{slow} are the time constants. For normalized currents, $A_1 + A_2 = 1$. The mean deactivation time constant was calculated as $\tau_{mean} = A_1\tau_{fast} + A_2\tau_{slow}$. The desensitization kinetics were described by a sum of one exponential function and a constant value representing the steady-state current. The effect of flurazepam on mIPSCs or on current responses was assessed from the comparison between control and test recordings obtained from the same cell (or excised patch).

For this reason, all the results are presented as relative values normalized to the respective controls.

The kinetic modeling was performed using the Channel-Lab 2.0 software (developed by S. Traynelis for Synaptosoft, Decatour, GA, USA). This software converted the considered kinetic model (based on the frame of the scheme by Jones and Westbrook (1995)) into a set of differential equations and solved them, numerically assuming, as the initial condition, that at $t=0$ no bound or open receptors were present. The solution of such equations predicted the time courses of occupancies of all the states included in the model. The current time course was modeled as the time evolution of the sum of open-state occupancies.

Data are expressed as mean ± s.e. and Student's *t*-test was used for comparison of data. Unless otherwise stated, the paired *t*-test was applied. Each time this test was used to compare two sets of absolute values (control and test) and each data pair was obtained from a different cell. Since in the normalized graphs, the effects of modulators were not obscured by the cell-to-cell variability in measured characteristics (for example, amplitudes), the impact of modulators was quantified by normalizing the measured test values by respective control values. The Kolmogorov–Smirnov test was used to assess the significance of shifts in cumulative distributions (Figure 1f).

All experiments were performed at room temperature (22–24 °C).

Results

The effect of flurazepam on mIPSC amplitude and the decaying phase depends on pH

Miniature IPSCs were recorded in the whole-cell configuration at −70 mV in the presence of 1 μM tetrodotoxin. Typical current traces recorded at different pH values and in the

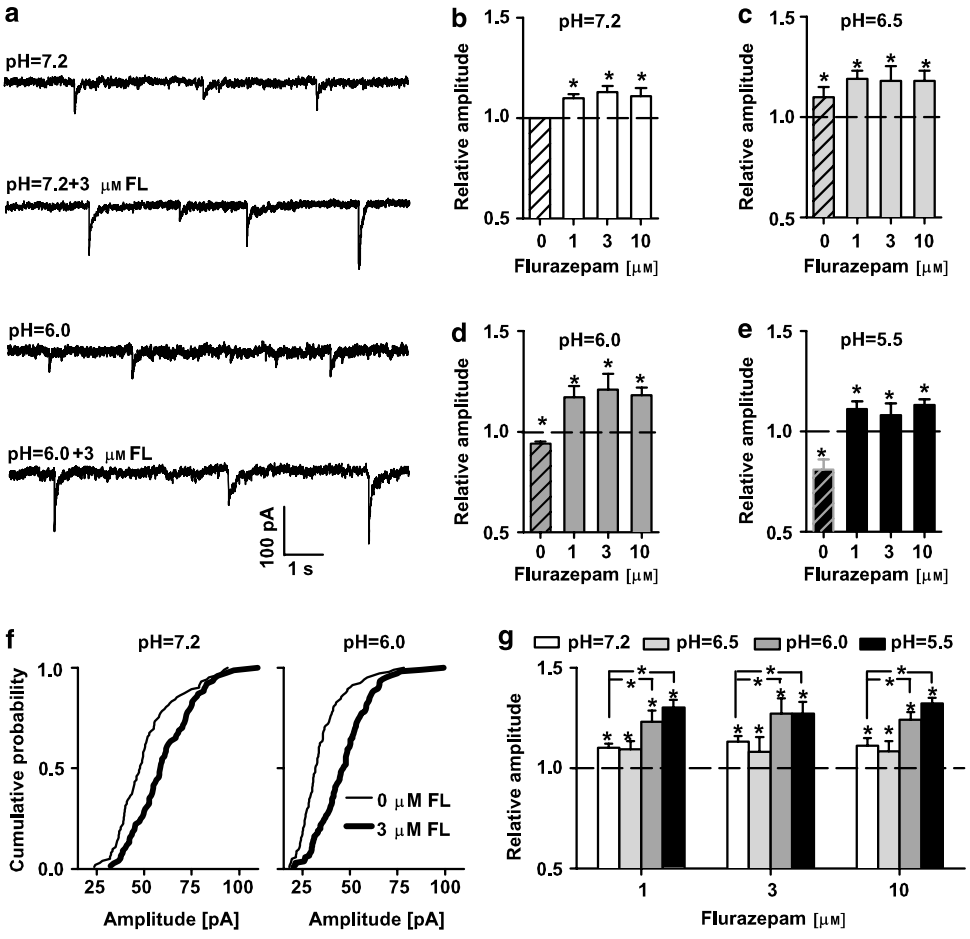

Figure 1 Low pH increases mIPSC susceptibility to modulation by flurazepam. (**a**) Examples of mIPSCs recorded at −70 mV in control conditions (upper trace) and in the presence of modulators as indicated above the traces that are below the control trace. (**b**–**e**) Statistics of flurazepam effect on mIPSC amplitude at pH 7.2 (**b**), 6.5 (**c**), 6.0 (**d**) and 5.5 (**e**). The dashed lines indicate the normalized value of mIPSC amplitude at pH 7.2. *Significant difference between either of the control values and values from the other group indicated in the histograms. (**f**) Typical examples of cumulative histograms obtained for mIPSCs recorded in the absence (thin line) and presence (thick line) of flurazepam at pH 7.2 (left panel) and pH 6.0 (right panel). In both cases, flurazepam induced a significant shift in the cumulative distributions (Kolmogorov–Smirnov test). (**g**) Statistics of flurazepam effect measured with respect to control (no flurazepam) measured at a given pH value. Note that the relative enhancement of mIPSC amplitude increased with solution acidification. *Significant effect of flurazepam with respect to the amplitude value measured from the same cell at the same pH and in the absence of flurazepam (paired *t*-test) (as explained in Results). Comparisons between values represented by adjacent columns (any given flurazepam concentration and different pH values) were made using the unpaired *t*-test (data at different pH values were collected from different cells). The averaged values were obtained from at least $n=6$ cells. FL, flurazepam; mIPSC, miniature IPSC.

presence or absence of flurazepam are presented in Figure 1a. The average amplitude of mIPSCs in control conditions (pH 7.2) was -49.8 ± 1.85 pA ($n=30$). When the pH of the extracellular solution was reduced to 6.5, an increase in the average amplitude was observed (relative amplitude: 1.10 ± 0.05, $n=8$, $P<0.05$; Figure 1c), but a further acidification to pH 6.0 and 5.5 resulted in a significant reduction of these values (relative amplitudes: 0.94 ± 0.01, $n=8$ and 0.81 ± 0.05, $n=6$, respectively; $P<0.05$; Figures 1d and e). These data qualitatively reproduce our previous results (Mozrzymas *et al.*, 2003a). As expected, addition of flurazepam (at the concentration range 1–10 μM) to the control extracellular solution (pH 7.2) resulted in a significant increase in the mean amplitude of mIPSCs (1.10 ± 0.02, $n=6$; 1.13 ± 0.03, $n=13$ and 1.11 ± 0.04, $n=7$ for 1, 3 and 10 μM flurazepam, respectively; $P<0.05$; Figure 1b). To check whether protons affected the modulation of mIPSCs by flurazepam, analogous recordings, such as those depicted in Figure 1b, were performed at pH values of 6.5, 6.0 and 5.5. To extract the precise values of the relative changes in mIPSC characteristics induced by either flurazepam or protons (or both), all test recordings were alternated by mIPSC measurements in control conditions (pH 7.2, no flurazepam). Figures 1b–e present the effects induced by flurazepam and by changes in pH on mIPSC averaged amplitudes, relative to control values determined from the same cell. As shown in Figure 1c, an increase in mIPSC amplitude due to a reduction of pH to 6.5 was further enhanced by flurazepam, reaching values considerably larger than in control conditions (Figures 1b and c). Interestingly, although at pH 6.0 and 5.5, mIPSCs amplitude was significantly reduced with respect to control, addition of flurazepam enhanced the amplitudes to a greater (at pH 6.0) or similar (pH 5.5) extent as in control conditions (Figures 1d and e). This trend is seen also as a larger shift in the amplitude cumulative histogram at pH 6.0 with respect to that at pH 7.2 (Figure 1f). In these distributions, 3 μM flurazepam produced a nearly twofold greater shift (measured as *D*—statistic defined as a maximum vertical deviation between the two compared cumulative distributions) at pH 6.0 with respect to that at pH 7.2 (at pH 7.2, $D=0.14$ and at pH 6.0, $D=0.27$).

Analyses of the relative changes of mIPSC amplitudes induced by flurazepam and changes in pH indicate that mIPSC sensitivity to this BDZ at low pH values (Figures 1d and e) is more than at a pH closer to the control values (Figures 1b and c). To visualize this trend better, we calculated the relative amplitudes with respect to the amplitude values measured at a given pH in the absence of flurazepam (Figure 1g). Since at any considered pH value in Figure 1g, recordings in the presence and absence of flurazepam were made from the same cell, an asterisk above each column indicates significance assessed using the paired *t*-test. However, because of the limited stability of recordings, it was not possible to collect recordings for each group (pH values and flurazepam concentrations) considered in Figure 1g from a single cell. For this reason, data for a given flurazepam concentration but at different pH values were

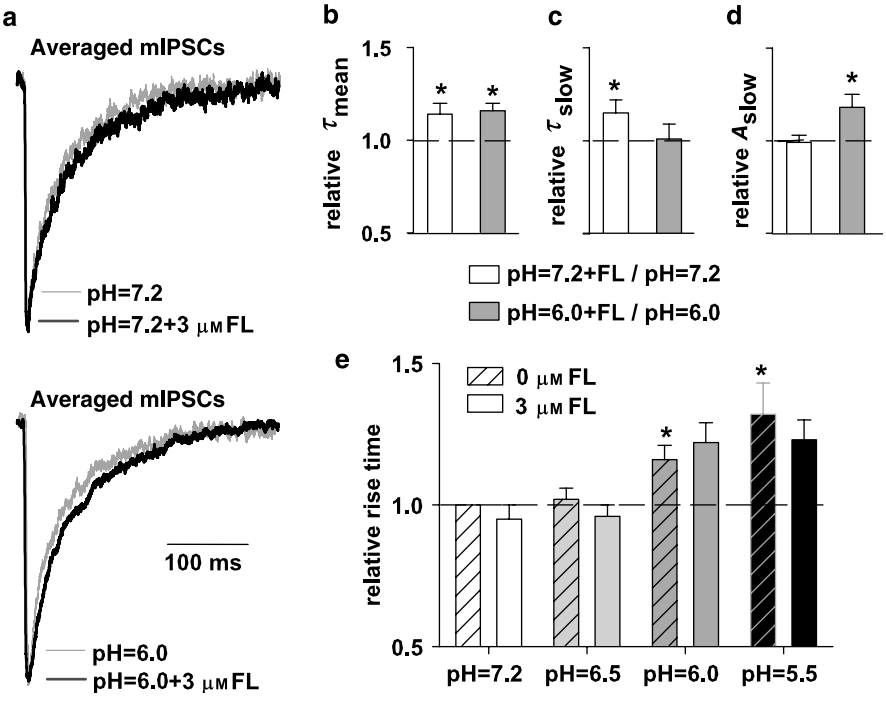

Figure 2 Flurazepam prolongs the decaying phase of mIPSCs both at physiological (7.2) and acidic pH (6.0) but does not change the rise-time kinetics. (**a**) Typical averaged and superimposed mipscs recorded in control conditions (thin line) and in the presence of 3 μM flurazepam (thick line) at pH 7.2 (upper panel) and pH 6.0 (lower panel). (**b**) The effect of flurazepam on the mean decay time constant (τ_{mean}) at pH 7.2 and at pH 6.0. (**c, d**) The effect of flurazepam on (**c**) the slow decay component (τ_{slow}) and (**d**) the percentage of this component (A_{slow}) at pH 7.2 and 6.0. (**e**) The effect of flurazepam on the rise-time of mIPSCs recorded at different pH. Note that flurazepam does not influence mIPSC rise-time at any of the pH values studied. *Significant difference with respect to the control values. The averaged values were obtained from at least $n=6$ cells. FL, flurazepam; mIPSC, miniature IPSC.

obtained from different cells and comparisons between normalized values for these groups were performed using the unpaired *t*-test. As shown in Figures 1b, c and g, for the whole range of flurazepam concentrations investigated, a relative increase in mIPSC was very similar at pH 7.2 and 6.5. However, at lower pH values (6.0 and 5.5), the relative increase in amplitude induced by flurazepam was significantly larger than at pH 7.2 (Figures 1d, e and g). These findings provide evidence that the susceptibility of mIPSCs to modulation by flurazepam depends on pH and increases on acidification of extracellular medium.

Flurazepam-induced increase in mIPSC amplitude was accompanied by a change in current kinetics. In control conditions, the decay could be well fitted with a sum of two exponentials ($\tau_{fast} = 8.36 \pm 0.62$ ms, $\tau_{slow} = 65.64 \pm 2.01$ ms, $A_{slow} = 0.56 \pm 0.02$, $n = 30$) and the mean decay time constant was $\tau_{mean} = 34.37 \pm 1.23$ ms ($n = 30$). The effect of solution acidification on mIPSC decaying phase has been extensively described elsewhere (Mozrzymas *et al*., 2003a). In the present study, strong acidification (pH 5.5) markedly reduced cell viability making several test recordings alternated by controls very difficult. Moreover, at pH 5.5, the baseline noise increased substantially and therefore the biexponential fitting of mIPSC decaying phase was not reliable. For these reasons, we have focused on investigating the impact of 3 μM flurazepam on mIPSC decay in control conditions (pH 7.2) and at pH 6.0. As shown in Figure 2a and b, flurazepam induced a similar increase in τ_{mean} at pH 7.2 as at 6.0 (relative τ_{mean} were 1.14 ± 0.06, $n = 10$ and 1.16 ± 0.05, $n = 11$, respectively; $P < 0.05$). This effect was associated with a significant increase in τ_{slow} at pH 7.2 with no effect on A_{slow}, and at pH 6.0, τ_{slow} was unaffected whereas A_{slow} was increased (Figures 2c and d).

The frequency of mIPSCs at pH 7.2 was 0.23 ± 0.035 ($n = 28$) and it was not affected by flurazepam (1–3 μM; data not shown). Acidification of extracellular medium to pH 6.0 did not affect the mIPSC frequency significantly (data not shown) similar to findings from our previous study (Mozrzymas *et al*., 2003a). However, lowering the pH value to 5.5 resulted in a reduction of mIPSCs to 0.53 ± 0.1 ($n = 8$). Flurazepam (3 μM) had no effect on mIPSC frequency for pH values ranging between 6.0 and 7.2. However, at pH 5.5, addition of this BDZ at the same concentration increased the mIPSC frequency by a factor of 1.61 ± 0.1 ms (relative value, $n = 7$, $P < 0.05$). The reduction of mIPSC frequency at pH 5.5 might be due to a combination of increased baseline noise commonly observed at low pH (see for example, Figure 1a) and reduced mIPSC amplitude, whereas the frequency increase in the presence of flurazepam at this pH could result from a marked enhancement of mIPSC amplitude by this BDZ.

In control conditions (pH 7.2), the onset of mIPSCs was characterized by a 10–90% rise-time of 0.85 ± 0.1 ms ($n = 28$). Acidification of extracellular solution slowed down the mIPSC rising phase and this effect became significant at pH 6.0 (Figure 2e), qualitatively similar to our previous observations (Mozrzymas *et al*., 2003a). Interestingly, addition of 3 μM flurazepam had no effect on mIPSC onset kinetics for the entire pH range investigated (5.5–7.2; Figure 2e).

The effect of flurazepam on the amplitude of current responses to low and high [GABA] depends on pH

Results presented in Figures 1 and 2 show that the effect of flurazepam on mIPSC amplitudes and decaying phases is modified by acidification of the extracellular medium, indicating that both these factors strongly influence GABAergic synaptic transmission. However, the analysis of synaptic currents is insufficient to fully explore the mechanisms of such a combined action of these two modulators. In particular, it is not clear whether the effects of flurazepam and protons are additive and how their actions combine in specific conditions of very brief synaptic agonist application. To elucidate these points, the effects of flurazepam and protons were tested on current responses to rapid applications of exogenous GABA.

At low [GABA], the amplitudes of current responses critically depend on the binding step and, therefore, they are expected to be strongly sensitive to modulators affecting the receptor affinity. Since experiments with rapid perfusion system are very difficult and time consuming, we have restricted the range of flurazepam concentrations examined to subsaturating and saturating concentrations. At 1 μM GABA, 3 and 10 μM flurazepam produced the same enhancement of responses, indicating full saturation of the BDZ effect (data not shown), whereas 1 μM flurazepam produced $77 \pm 5.4\%$ ($n = 7$) of saturation. Acidification of the extracellular solution to pH 6.0 resulted in a significant reduction of the amplitude of currents evoked by 1 μM GABA (relative amplitude 0.68 ± 0.02, $n = 3$, $P < 0.05$; Figures 3a and b) that is consistent with a decrease in the binding rate by acidic pH (Mozrzymas *et al*., 2003a). As expected, 3 μM flurazepam markedly enhanced the responses to 1 μM GABA in control conditions (pH 7.2; relative amplitude 1.96 ± 0.16, $n = 13$, $P < 0.05$; Figures 3c and d). As presented in Figures 3c and d, 3 μM flurazepam also strongly increased the current response to 1 μM GABA at pH 6.0 (relative amplitude 2.86 ± 0.32, $n = 9$, $P < 0.05$) and, interestingly, this effect at pH 6.0 was significantly larger than that at pH 7.2.

To pursue this issue further, we studied the combined action of flurazepam and protons on current responses to larger [GABA]. In a recent study (Mozrzymas *et al*., 2007), we found that at higher [GABA] (ca. 30 μM), the effect of flurazepam on current amplitudes became negligible, whereas at subsaturating and saturating [GABA], this BDZ induced a significant decrease in the amplitude. As shown in Figures 3e and f, an acidic pH clearly enhanced the current response to 30 μM GABA. However, both at pH 6.0 and 7.2, flurazepam did not significantly affect the amplitude (relative amplitude: 1.09 ± 0.09, $n = 5$, $P > 0.05$; 1.04 ± 0.04, $n = 14$, $P > 0.05$ for pH 6.0 and 7.2, respectively; Figures 3g and h). These findings demonstrate that at both pH 6.0 and 7.2, the effect of flurazepam on current amplitudes tends to disappear at this range of [GABA].

As already mentioned, at saturating [GABA] and pH 7.2, flurazepam reduced the amplitudes of current responses (Mozrzymas *et al*., 2007). It is thus of interest to find out whether this effect is maintained at acidic pH. Since low pH reduces receptor affinity (Mozrzymas *et al*., 2003a), 30 mM GABA was applied to assure saturation conditions at pH 6.0. As expected, a reduction of pH from 7.2 to 6.0 resulted in a

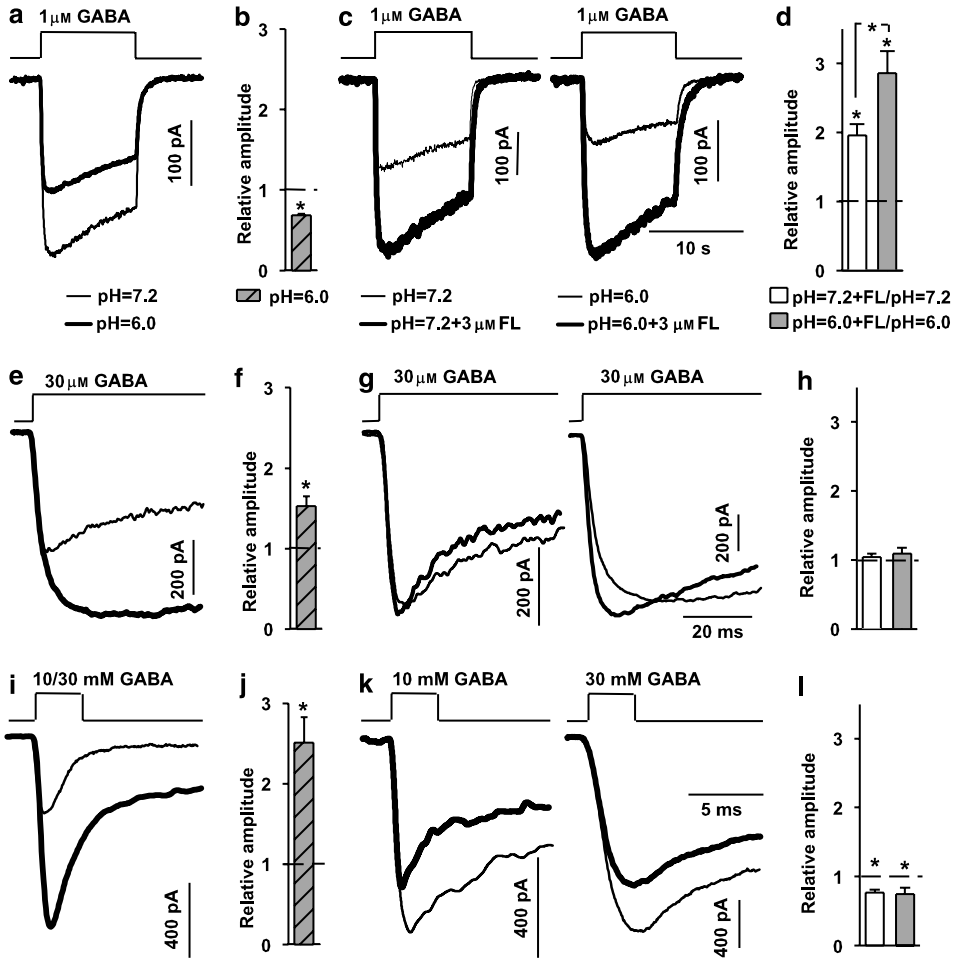

Figure 3 The effects of flurazepam on the amplitudes of current responses at pH 7.2 and 6.0 are qualitatively similar. The left column shows examples of current responses evoked in control conditions (pH 7.2, thin lines) and at pH 6.0 (thick line), whereas the middle column with current traces shows examples of currents recorded at pH 7.2 in the absence (thin line) or presence (thick line) of 3 μM flurazepam. The traces shown in the right column represent currents recorded at pH 6.0 in the absence (thin line) and in the presence (thick lines) of 3 μM flurazepam. (**a**) Examples of currents evoked by 1 μM GABA at normal (thin line) and acidic (thick line) pH. Note that a decrease in the pH reduces the current amplitude. (**b**) A summary of the effect of acidic pH on amplitude of currents elicited by 1 μM GABA. (**c**) Examples of currents evoked by 1 μM GABA in the absence (thin lines) and presence (thick lines) of 3 μM flurazepam at control pH (left panel) and pH 6.0 (right panel). Note that the relative effect of flurazepam in control conditions was smaller than that at pH 6.0. (**d**) A summary of flurazepam effects on current responses elicited by 1 μM GABA at pH 7.2 and 6.0. Columns represent the relative flurazepam effect on current amplitudes with respect to controls recorded at a given pH value. (**e**) Examples of currents evoked by 30 μM GABA at normal and acidic pH. Note that a decrease in pH significantly enhanced the current amplitude (**f**). (**g**) Examples of current traces evoked by 30 μM GABA. (**h**) A summary of flurazepam effects on current responses elicited by 30 μM GABA at pH 7.2 and 6.0. (**i**) Examples of currents evoked by saturating [GABA]. Note that acidic pH markedly enhanced the current amplitude (**j**). (**k**) Examples of currents evoked by saturating [GABA]. (**l**) A summary of flurazepam effects on current responses elicited by saturating [GABA] at pH 7.2 and 6.0. *Significant difference between either of the control values and values from the other groups indicated in the histograms. The averaged values were obtained from at least $n=3$ cells. FL, flurazepam; [GABA], GABA concentration.

robust increase in current amplitude (Figures 3i and j; Mozrzymas *et al*., 2003a). Most interestingly, flurazepam (3 μM) induced a similar reduction of current amplitudes at both pH values (relative amplitudes: 0.75 ± 0.09, $n=12$, $P<0.05$ and 0.77 ± 0.04, $n=7$, $P<0.05$, at pH 6.0 and 7.2, respectively; Figures 3k and l).

Combined effect of flurazepam and protons on the time course of current responses

In our recent study (Mozrzymas *et al*., 2007), we found that at pH 7.2, flurazepam strongly slowed down the deactivation kinetics (current relaxation following agonist removal) of current responses to low [GABA], but this effect tended to disappear with increasing agonist concentration, becoming negligible at ca. 30 μM GABA. It is thus of interest to check whether acidic pH affects this pattern of modulation of the deactivation process. GABA-evoked responses were elicited by agonist applications of sufficient duration for the current to reach the maximum or plateau value. At a [GABA] of 1 μM, flurazepam (3 μM) prolonged the deactivation kinetics to a similar extent at pH 7.2 and 6.0 (relative τ_{mean}: 2.39 ± 0.21, $n=6$ and 2.19 ± 0.23, $n=5$, $P<0.05$, respectively; Figures 4a and b). Interestingly, for current responses to 30 μM GABA, flurazepam did not affect the deactivation time course at pH 7.2 (Mozrzymas *et al*., 2007), but at pH 6.0, it induced a

significant prolongation of the decaying phase (relative τ_{mean}: 1.04 ± 0.04, $n=6$, $P>0.05$ and 1.23 ± 0.06, $n=5$, $P<0.05$ for pH 7.2 and 6.0, respectively; Figures 4c and d). Moreover, at saturating [GABA], no significant effect of flurazepam on the deactivation time course was observed either at pH 7.2 or 6.0 (relative τ_{mean}: 0.99 ± 0.06, $n=16$, $P>0.05$ and 0.99 ± 0.10, $n=15$, $P>0.05$ for pH 7.2 and 6.0, respectively; Figures 4e and f).

At [GABA] comparable to EC_{50}, the onset kinetics of current responses is expected to strongly depend on the binding rate. At 30 μM GABA, the 10–90% rise-time was clearly shortened in the presence of flurazepam, but this effect was significantly larger at pH 6.0 (relative rise-time: 0.77 ± 0.03, $n=6$, $P<0.05$ and 0.55 ± 0.03, $n=5$, $P<0.05$ for pH 7.2 and 6.0, respectively; Figures 4g and h).

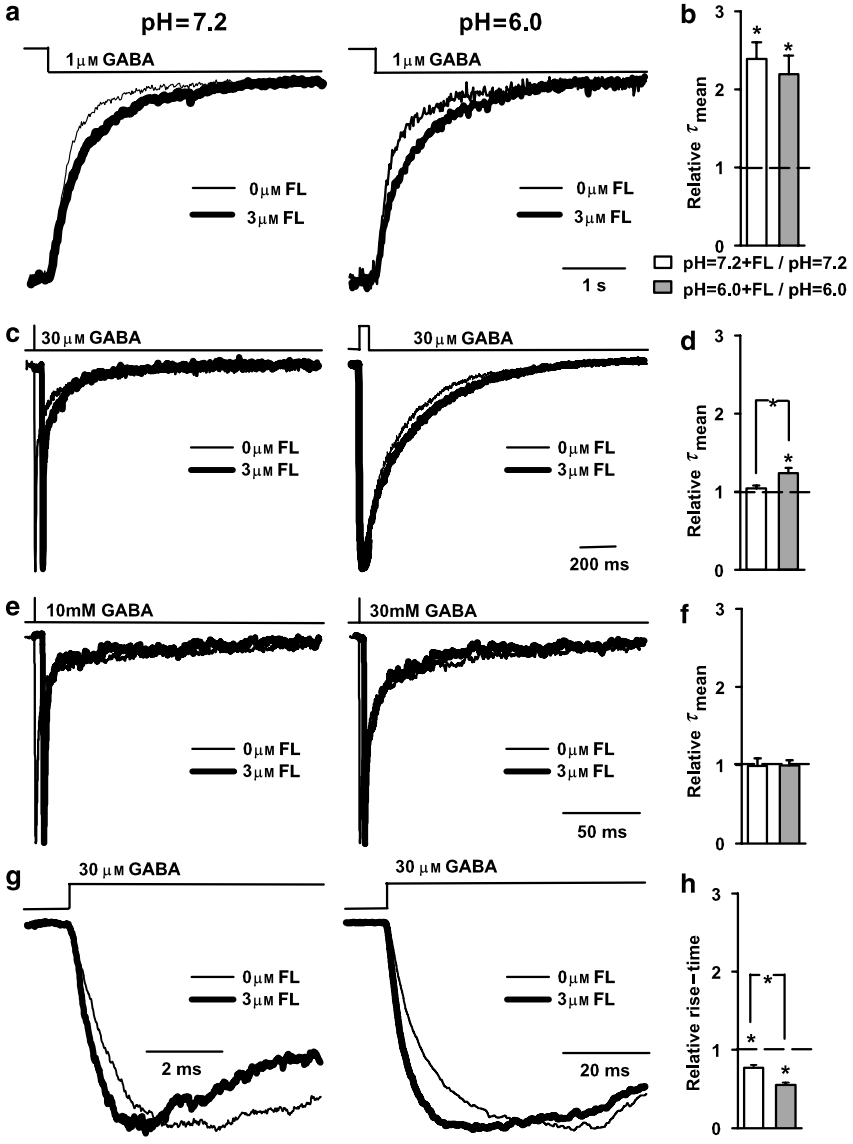

Figure 4 Effect of flurazepam on the deactivation kinetics is dependent on the extracellular pH. The left panel shows current traces recorded at pH 7.2 in the absence (thin line) and presence (thick line) of 3 μM flurazepam. The traces in the right panel are analogous to those presented in the left panel but recorded at pH 6.0. (**a**) Examples of normalized and superimposed deactivation currents recorded after the removal of 1 μM GABA in the absence and presence of 3 μM flurazepam (**b**) Summary of flurazepam effect on the mean deactivation time constant (τ_{mean}) measured for responses to 1 μM GABA recorded at pH 7.2 and 6.0. (**c**) Examples of normalized and superimposed current responses evoked by 30 μM GABA in the absence and presence of 3 μM flurazepam. (**d**) Summary of flurazepam effect on the mean deactivation time constant (τ_{mean}) measured for currents elicited by 30 μM GABA and recorded at pH 7.2 and 6.0. Note that the effect of flurazepam on τ_{mean} was significantly more marked at pH 6.0. (**e**) Examples of normalized and superimposed current responses evoked by saturating [GABA] in the absence and presence of 3 μM flurazepam. (**f**) Summary of flurazepam effects on the mean deactivation time constant (τ_{mean}) measured for current responses evoked by saturating [GABA] and recorded at pH 7.2 and 6.0. Note that at saturating [GABA] flurazepam did not affect the deactivation kinetics either at pH 7.2 or pH 6.0. (**g**) Examples of the normalized and superimposed rising phases of current responses evoked by a subsaturating concentration of GABA (30 μM) in the absence and presence of 3 μM flurazepam. (**h**) Summary of flurazepam effect on the 10–90% rise-time of currents evoked by 30 μM GABA. Note that flurazepam shortened the rise-time both at pH 7.2 and 6.0, but for the latter, this effect was significantly more marked. *Significant difference between either of the control values and values from the other groups indicated in the histograms. The averaged values were obtained from at least $n=5$ cells. FL, flurazepam; [GABA], GABA concentration.

Since it is known that desensitization critically shapes the GABAergic currents, we have additionally considered the effect of flurazepam and protons on this process. To this end, prolonged (60 ms) applications of saturating [GABA] were applied. In control conditions, the desensitization time constant was $\tau_{des} = 2.06 \pm 0.15$ ms ($n = 34$) and the steady-state/peak (ss/peak) was 0.23 ± 0.028 ($n = 34$). First, we examined the effect of protons alone on desensitization and confirmed that acidification of the external solution to pH 6.0 decreased both the rate and extent of desensitization (relative values to those obtained at pH 7.2: relative τ_{des} 2.14 ± 0.33, $n = 33$, $P < 0.05$; relative ss/peak 1.64 ± 0.03, $n = 33$, $P < 0.05$), similar to our previous data (Mozrzymas *et al*., 2003a). At pH 7.2, flurazepam did not affect either the desensitization time constant or the ss/peak value (Mozrzymas *et al*., 2007). However, at pH 6.0, this BDZ significantly reduced the ss/peak ratio (relative ss/peak: 0.83 ± 0.36, $n = 18$, $P < 0.05$) without affecting the desensitization time constant (data not shown).

Model simulations

Our experimental evidence, especially the one based on the analysis of current responses to exogenous GABA applications, suggests that the effects of flurazepam and protons might be additive. To examine this possibility further, model simulations were considered. For this purpose, we have used a minimum requirement model based on the scheme by Jones and Westbrook (1995) (Figure 5a). As discussed in detail recently (Mozrzymas *et al*., 2007), the action of flurazepam was qualitatively reproduced by enhancing the binding (k_{on}) and desensitization (d_2) rates. The major simplification of this model is on the assumption that there is only one set of fully bound states (open, closed and desensitized). In particular, it is known that besides the fast desensitized state included in this model, there could be several slower desensitization components, which might additionally shape the current responses, especially those elicited by long GABA pulses. In our previous study (Mozrzymas *et al*., 2003a), we showed that protons modulate $GABA_A$ receptors mainly by reducing the binding rate as well as the rate and extent of desensitization. Thus, the major effect of both modulators concerns agonist binding and desensitization. The effect of acidic pH was modeled as a reduction of k_{on} and d_2 to qualitatively reproduce (formal rate constant optimization was not applied) our experimental observations at pH 6.0 (k_{on} reduced from 8.0 to 4.5 ms^{-1} and d_2 reduced from 12 to 5 ms^{-1}). The effect of BDZ (at pH 7.2) was modeled by enhancement of binding and desensitization rates (k_{on} increased from 8.0 to 12.0 ms^{-1} and d_2 increased from 12 to 15 ms^{-1}) to qualitatively reproduce our data obtained for 3 μM flurazepam. Additive effects of BDZ and protons were simulated as a proportional change in the binding and desensitization rates induced by the two factors ($k_{on} = 7$ ms^{-1}, $d_2 = 6.5$ ms^{-1}). In the present simulations that included combined action of protons and BDZ, a better reproduction of our experimental data was obtained when it was assumed that BDZ increases the desensitization rate d_2 from 12 to 15 ms^{-1} rather than to 17 ms^{-1}, as proposed in our recent study (Mozrzymas *et al*., 2007).

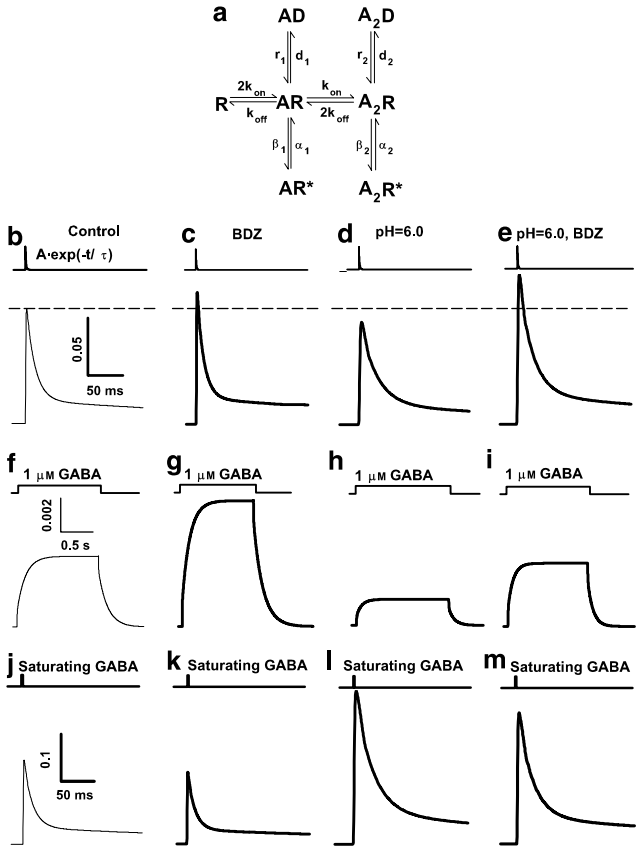

Figure 5 Model simulations of the effects of flurazepam and protons on GABAergic currents. The frame of the model derived by Jones and Westbrook (1995) was used (**a**) with rate constants (obtained by us in a recent study (Mozrzymas *et al*., 2007)): $k_{on} = 8.0$ ms^{-1} mM^{-1}, $k_{off} = 1$ ms^{-1}, $d_2 = 12$ ms^{-1}, $r_2 = 0.07$ ms^{-1}, $\beta_2 = 3.0$ ms^{-1}, $\alpha_2 = 0.4$ ms^{-1}, $d_1 = 0.045$ ms^{-1}, $r_1 = 0.014$ ms^{-1}, $\beta_1 = 0.15$ ms^{-1}, $\alpha_1 = 1.5$ ms^{-1}. The effect of BDZ was modeled by increasing k_{on} to 12.0 ms^{-1} mM^{-1} and d_2 to 15 ms^{-1}. The effect of acidic pH (6.0) was modeled as a reduction of k_{on} and d_2 to 4.5 and 5 ms^{-1}, respectively. Additive effects of BDZ and protons were simulated as a proportional change in the binding and desensitization rates induced by the two factors ($k_{on} = 7$ ms^{-1}, $d_2 = 6.5$ ms^{-1}). The IPSCs were modeled as responses to GABA transient described by exponentially decaying function: $A\exp(-t/\tau)$, where $A = 1.0$ mM and $\tau = 0.1$ ms. (**b**–**e**) Modeled synaptic currents in control conditions (**b**; pH 7.2 no BDZ), at control pH and in the presence of BDZ (**c**), at acidic pH (**d**) and in the presence of BDZ and at acidic pH (**e**). The dashed line indicates the amplitude of the IPSC in control conditions. Note that the BDZ-induced increase in IPSC amplitude is larger at acidic pH than that at normal pH (compare **c** and **e**), whereas acidic pH alone reduces IPSC amplitude. (**f**–**i**) Simulated current responses to 1 μM GABA. BDZ clearly increases current amplitude at pH 7.2 (**f**, **g**). A decrease in pH reduces the response to 1 μM GABA (**f**, **h**). At acidic pH, BDZ induces a larger relative increase in current amplitude than in control conditions (**i** compared with **f** and **g**). (**j**–**m**) Simulated current responses to brief (2 ms) applications of saturating [GABA]. Note that flurazepam reduces the current amplitude at pH 7.2 (**j**, **k**), whereas a decrease in pH enhances the amplitude of the current response to saturating [GABA] (**l**). At acidic pH, BDZ reduces the amplitude of the response to saturating [GABA] to a similar extent as at pH 7.2 (compare **j**, **k** with **l**, **m**). Note that the model simulations qualitatively reproduce the experimental results depicted in Figures 1–3. BDZ, benzodiazepine; [GABA], GABA concentration.

Synaptic currents were simulated as responses to the exponentially decaying agonist waveform ($y(t) = A\exp(-t/\tau_{transient})$, where $A = 1.0$ mM and $\tau_{transient} = 0.1$ ms). As

shown in Figures 5b–e, such simulations of IPSCs correctly reproduced the increase in amplitude by BDZ (Figure 5c), whereas the opposite effect was predicted for acidic pH (Figure 5d). Importantly, in agreement with the experimental results, the combined effect of protons and BDZ resulted in a more substantial increase in IPSC amplitude than in the case of BDZ alone (compare Figures 5c and e). At first glance, the fact that the combined action of protons (reduction of IPSC amplitude) and BDZ (enhancement of IPSC) results in a larger upregulation of IPSCs than in the case of BDZ alone might be regarded as contradictory for an additive action of these compounds. However, the finding that the synaptic agonist transient is very brief (Clements, 1996; Overstreet *et al*., 2002; Mozrzymas, 2004) needs to be borne in mind, and for this reason, modulation of mIPSCs may differ from that observed for current responses elicited by much longer agonist pulses. In particular, in spite of relatively high synaptic peak [GABA] (commonly believed to reach millimolar concentrations (Clements, 1996; Overstreet *et al*., 2002; Mozrzymas, 2004)), the response of postsynaptic $GABA_A$ receptors is not close to saturation. In these conditions, BDZ-induced enhancement of the binding rate gives rise to a substantial increase in the recruitment of receptors into bound conformations. Although a larger proportion of receptors desensitize due to an increased desensitization rate (d_2) in the presence of BDZ, the net effect of increased k_{on} and d_2 is to increase the occupancy of open receptors and, therefore, the enhancement of IPSC amplitude (Figure 5c). In contrast, a reduction of k_{on} at acidic pH strongly reduces the percentage of receptors that get bound during the brief agonist transient and, in spite of a reduced d_2, IPSC amplitude is reduced (Figure 5d). When the pH is acidic and BDZ is present, a reduction of k_{on} by protons is largely compensated for by BDZ. Since the effect of protons on desensitization is markedly stronger than that of BDZ (Mozrzymas *et al*., 2003a, 2007), the net effect is to reduce d_2 substantially with respect to the control value. In this situation, the synaptic transient recruits a similar proportion of bound receptors as in control conditions but, due to the markedly smaller desensitization rate d_2, a larger proportion of receptors enter into the bound open state. Thus, an examination of the additive actions of protons and BDZ together with specific synaptic conditions of $GABA_A$ receptor activation is sufficient to reproduce all the major regulatory effects of these compounds (Figures 5b–e).

At low [GABA], the amplitude of the current responses critically depends on the binding rate k_{on} and, therefore, it is expected to be particularly sensitive to modulators affecting this rate constant. As expected, our simulations demonstrated that BDZ enhanced responses to 1 μM GABA both in control conditions (pH 7.2) and at acidic pH (Figures 5f–i). Interestingly, the BDZ-induced enhancement of current amplitude is larger at pH 6.0 than that at pH 7.2, qualitatively similar to our experimental observations (Figure 3). Thus, a relatively larger flurazepam effect at an acidic pH need not indicate a stronger action of this BDZ on k_{on} at low pH. Since protons reduce $GABA_A$ receptor affinity (Mozrzymas *et al*., 2003a), the current evoked by 1 μM GABA at pH 6.0 is markedly smaller (Figures 3a and b) than that at pH 7.2 and, therefore, the relative increase in current amplitude in the presence of flurazepam is larger. Put simply, the larger the distance from saturation, the greater the relative effect of drug enhancing the receptor affinity.

At saturating [GABA], the current amplitude strongly depends on the balance between open and desensitization rates (Jones and Westbrook, 1995; Mozrzymas *et al*., 2003a). Thus, compounds affecting the desensitization rate are expected to have a strong impact on the amplitudes of these responses. The fact that acidic pH has a larger impact on amplitudes of currents elicited by saturating [GABA] than BDZ (Figures 3e and f) further confirms that the former modulator affects desensitization to a greater extent than the latter one. As shown in Figures 5j–m, our model simulations adequately reproduce moderate (and comparable) relative reductions of current amplitudes by BDZ in control conditions and at acidic pH, whereas acidification of the extracellular medium strongly enhances the amplitude of current elicited by saturating [GABA].

Altogether, model simulations presented above further indicate that the modulator effects of BDZ and protons are additive.

Discussion

The major finding of the present study is that acidic pH enhances the susceptibility of mIPSCs to modulation by BDZ flurazepam. Interestingly, as described in Model simulations, the greater sensitivity of mIPSC to BDZ at acidic pH appears to result from additive actions of flurazepam and protons on $GABA_A$ receptor properties in combination with specific non-equilibrium conditions of synaptic receptor activation. From the point of view of the receptor structure, the additive action of these compounds is not surprising as modulation by BDZs and protons have distinct molecular effects on the $GABA_A$ receptor macromolecule (Krishek *et al*., 1996; Wilkins *et al*., 2002; Huang *et al*., 2004; Rudolph and Mohler, 2004). However, distinct structural localization of the binding sites does not exclude mutual allosteric interactions that could give rise to a functional interference between these two modulators. To our knowledge, the present study is the first to provide evidence that the action of flurazepam and protons is functionally additive, even if they affect the same receptor characteristics (binding and desensitization). An additional issue that needs to be examined whenever two (or more) modulators are investigated is their possible interaction. In particular, changing pH over a wide range could affect ionization of flurazepam molecules. However, the pK values for flurazepam are 1.9 and 8.2 and, therefore, a change in flurazepam ionization within the considered pH range (5.5–7.2) is negligible.

The results presented here appear particularly interesting as several brain disorders, such as hypoxia, ischemia or hypoglycemia, may induce acidosis of the extracellular fluid in the brain tissue (Kraig *et al*., 1986; Bengtsson *et al*., 1990; Katsura *et al*., 1993; Katsura and Siesjo, 1998). Our data indicate that a marked change in mIPSC modulation by BDZ can be observed only at a strong acidosis in the range of one pH unit. Although in most cases a smaller reduction of pH is observed in pathological brains (Katsura and Siesjo, 1998),

such a strong acidosis can occur (Anderson and Sundt, 1983) especially when different disorders combine with each other (for example, ischemia and hypo- or hyperglycemia (Kraig *et al*., 1986; Smith *et al*., 1986)).

Inhibition mediated by ionotropic $GABA_A$ receptors is known to consist of phasic (synaptic) and tonic components (Farrant and Nusser, 2005). The latter results from activation of $GABA_A$ receptors by ambient GABA that is present in the extracellular fluids at submicromolar concentrations (Farrant and Nusser, 2005). Since protons have been shown to reduce the binding rate markedly (Mozrzymas *et al*., 2003a), it is expected that acidosis would reduce the tonic inhibition. However, our data show that the relative enhancement of such tonic-like current by BDZ at acidic pH is larger than at physiological pH (Figure 3). Thus, our results suggest that in conditions of lowered pH, the relative impact of BDZs on phasic and tonic inhibition is larger than that at normal pH. However, the possibility that tonic conductance is the result of the activity of different $GABA_A$ receptor subtypes needs to be taken into account. In particular, several studies have provided evidence that a considerable component of tonic inhibition is mediated by $GABA_A$ receptors with a high agonist affinity and a weak, if any, BDZ sensitivity (Farrant and Nusser, 2005). Conversely, in our recent study (Mozrzymas *et al*., 2007), we have demonstrated that the whole-cell current evoked by micromolar [GABA] shows a similar BDZ sensitivity as that mediated by $\alpha1\beta2\gamma2$ receptors, indicating that, at least in our model, a considerable component of tonic inhibition is mediated by $GABA_A$ receptors sensitive to BDZs. In addition, in a model of hippocampal pyramidal neurons, results have been obtained indicating that the BDZ-sensitive component of tonic current involves, in addition to $\alpha1\beta2\gamma2$ receptors, those containing an $\alpha5$ subunit (Yeung *et al*., 2003; Caraiscos *et al*., 2004).

As already mentioned in Model simulations, although an additive effect of flurazepam and protons is not apparent when their effects are examined on the amplitudes of mIPSCs (Figure 1), the analysis of current responses (Figures 3 and 4) and model simulations consistently support such a mechanism. However, as shown in Figures 4c and d, flurazepam had no effect on deactivation kinetics for responses elicited by 30 μM GABA at pH 7.2, but at pH 6.0, this drug induced a significant prolongation of the decay time course at the same [GABA]. This different effect of flurazepam does not necessarily argue against additive modulation. In our recent study (Mozrzymas *et al*., 2007), we demonstrated that flurazepam markedly slowed down deactivation kinetics only for current responses evoked by low [GABA] and this effect tended to disappear at [GABA] close to 30 μM. However, acidic pH markedly decreases binding affinity (Mozrzymas *et al*., 2003a). Thus, at pH 6.0, the effect of flurazepam on deactivation kinetics (at 30 μM GABA) would be expected to follow the pattern observed at lower [GABA] at normal pH, that is, conditions in which flurazepam does affect the deactivation time course (Mozrzymas *et al*., 2007). The analysis of the rising-phase kinetics of currents evoked by 30 μM GABA revealed that flurazepam accelerated the current onset both at pH 7.2 and at pH 6.0, but this effect was significantly stronger at the acidic pH (Figures 4g and h). Again, this difference might result from the fact that a decrease in pH makes the responses to this [GABA] more distant from saturation and, therefore, the effect of flurazepam on the rising phase is more prominent than at control pH. The analysis of desensitization kinetics revealed that although at pH 7.2 flurazepam had no effect on either the rate or extent of desensitization, at pH 6.0, this BDZ significantly affected the ss/peak ratio. The reason for this difference is not clear as recordings at both pH values were performed at saturating [GABA]. We speculate that, as the ss/peak was small (0.23 ± 0.028) at pH 7.2, a further significant reduction of this value was beyond the resolution of our recordings, whereas at pH 6.0, ss/peak was larger and its significant decrease in the presence of flurazepam was detectable.

As presented in Model simulations, all effects of flurazepam or protons (or both) can be qualitatively reproduced for both current responses and IPSCs (simulated here as currents elicited by quickly decaying GABA transient; Figure 5). However, as already mentioned, the model is oversimplified and leads to some predictions that were not observed in the experiment. For instance, an intrinsic feature of the applied scheme of Jones and Westbrook (1995) is that an increase in d_2 would lead to the prolongation of deactivation kinetics. Thus, an increase in this rate constant by BDZ would be expected to prolong the slow deactivation component of currents elicited by saturating [GABA], an effect that was not observed. The reason for this discrepancy is not clear but it is speculated that different desensitized states (for example, those not included in the model) could be endowed with a different BDZ sensitivity. A more complete discussion of this and additional possible shortcomings of this model are presented in Mozrzymas *et al*. (2007).

In the Model simulations, we have assumed, as a minimum requirement, that both flurazepam and protons affect only the binding and desensitization rates (k_{on} and d_2). Although these parsimonious assumptions proved sufficient to obtain a reasonable reproduction of modulatory actions of these two compounds, we cannot exclude the possibility that BDZ might affect other rate constants. For instance, recently Rusch and Forman (2005) as well as Downing *et al*. (2005) have proposed that in a spontaneously active $GABA_A$ receptor mutant, the primary action of BDZ is to enhance the efficacy by BDZs. More recently, Campo-Soria *et al*. (2006) observed that in oocytes expressing ultrahigh levels of wild-type $GABA_A$ receptors, diazepam alone induced a small but detectable current mediated by $GABA_A$ receptors. These authors interpreted such a diazepam-induced current as an evidence for enhancement of $GABA_A$ receptor efficacy by this compound. Although we do not exclude an effect of BDZ on the opening/closing mechanism, our data do not support the notion that such a mechanism predominantly accounts for our results (see reference Mozrzymas *et al*., 2007 for a more extensive discussion on this point). In addition, a substantial effect of BDZ on opening/closing kinetics would be expected to alter the single-channel lifetimes, an effect that was not observed by Twyman *et al*. (1989); Rogers *et al*. (1994). Although our present results indicate that the mechanisms involved are predominantly postsynaptic, a presynaptic effect of protons and flurazepam cannot be completely ruled out. The finding that for pH values within

6.0 and 7.2, neither protons nor flurazepam (or both) affected mIPSC frequency seems to support this view. However, at a strongly acidic pH of 5.5, mIPSC frequency was decreased, but flurazepam reversed this effect. As already mentioned, this could be due to large baseline noise values at strongly acidic pH values that would obscure a decrease in mIPSCs, whereas a relatively strong effect of flurazepam at this pH would uncover these events. Although such an explanation appears plausible, the possibility that such a large proton concentration could induce some presynaptic alterations that, at least partially, contribute to the observed changes in mIPSC frequency at pH 5.5 cannot be excluded.

Yet another possibility is that the modulators studied could alter the agonist release pattern giving rise to a change in the synaptic GABA transient. However, the reduction in the rising rate of the mIPSC induced by protons (Figure 2e; see also reference Mozrzymas *et al*., 2003a) most probably represents an effect of protons on the $GABA_A$ receptor kinetics (Mozrzymas *et al*., 2003a). Notably, over the entire pH range studied, flurazepam had no effect on the mIPSC rise-time indicating further, although indirectly, that this BDZ does not interfere with the synaptic GABA transient.

The effect of flurazepam on mIPSC amplitude described here is slightly smaller than that observed recently by Mozrzymas *et al*. (2007). This discrepancy reflects the fact that our present and the previous studies were performed on different preparations, because all the effects of the modulators studied had to be quantified as relative values with respect to controls obtained from the same cells. The reason why the effect of flurazepam was slightly weaker than in our more recent preparations is not clear, but it is possible that it could result from, for example, differences in culture conditions (for example, batch of serum).

In conclusion, we provide evidence that acidosis affects the susceptibility of phasic and tonic GABAergic currents to modulation by BDZs. Importantly, this pattern of combined effects of flurazepam and protons results from a functionally additive action of these modulators.

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Conflict of interest

The authors state no conflict of interest.

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