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Penicillin blocks human $\alpha_1\beta_1$ and $\alpha_1\beta_1\gamma_{2S}$ $GABA_A$ channels that open spontaneously

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Abstract

We used the open-channel blocker, penicillin (10 mM), as a tool to investigate if the human $\alpha_1\beta_1$ or $\alpha_1\beta_1\gamma_{2S}$ γ-aminobutyric acid type A ($GABA_A$) receptor channels opened in the absence of GABA. Application of penicillin to cells expressing the receptors resulted in a transient inward whole-cell current, the off-current, upon penicillin removal. The amplitude of the off-current was dependent on the duration of the penicillin application, it reversed in polarity at depolarized potentials and exhibited "run-down" similar to the GABA-activated currents. Bicuculline (100 μM) blocked the off-current response. Pentobarbital (50 μM) enhanced the peak off-current amplitude by 2.8 and 3.4 in $\alpha_1\beta_1$ and $\alpha_1\beta_1\gamma_{2S}$ receptors, respectively. Diazepam (1 μM) only enhanced the off-current peak response in $\alpha_1\beta_1\gamma_{2S}$ receptors (1.6) and induced the development of an inward current when applied alone. The results are consistent with that the $\alpha_1\beta_1$ or $\alpha_1\beta_1\gamma_{2S}$ $GABA_A$ receptors can open in the absence of GABA and raise the question of what role spontaneous channel openings have in the function of $GABA_A$ receptors.

Keywords: $GABA_A$ receptor; Benzodiazepine; Barbiturate; Inhibition; γ-aminobutyric acid; Spontaneous activity; Channel block; Tonic current

1. Introduction

Inhibitory neurotransmission in the brain is mostly mediated by γ-aminobutyric acid type A ($GABA_A$) receptors. These receptors are present both at synapses and outside of synapses on neurons, and when activated by GABA, excitation of the neurons is depressed. The receptor contains binding sites for GABA and modulatory drugs, such as the benzodiazepines, general anaesthetics and barbiturates, and is thought to be a hetero-pentamer containing a Cl^- channel. Activation of these channels is a common therapeutic strategy when treating diseases such as generalised anxiety disorders, muscle spasms and some forms of epilepsy.

To date, at least 19 different mammalian subunits have been cloned and they are grouped into α_{1-6}, β_{1-3}, γ_{1-3}, δ, ρ_{1-3}, π, θ and ε families based on subunit amino-acid sequence homology (Barnard et al., 1998). Considering the number of subunits that can form the receptors, variable functional characteristics can be expected.

Penicillin is an open-channel blocker at $GABA_A$ receptors (Twyman et al., 1992) and has been shown to exert a receptor specific inhibition in native neurons (Yeung et al., 2003). Penicillin also significantly reduces the leak current that is present in $GABA_A$ receptors when the 9′ leucine in the second transmembrane-spanning region is mutated (Tierney et al., 1996). We examined in wild-type recombinant $GABA_A$ receptors composed of commonly expressed subunits, $\alpha_1\beta_1$, $\alpha_1\beta_1\gamma_{2S}$ or $\alpha_1\beta_2\gamma_{2S}$, if we could detect $GABA_A$-specific current in the presence of penicillin but in the absence of GABA. An "off-current" was generated when the application of penicillin was rapidly terminated, and it was modulated by $GABA_A$ receptors' specific drugs such as bicuculline, pentobarbital or diazepam. The results demonstrate that $\alpha_1\beta_1$, $\alpha_1\beta_1\gamma_{2S}$ and $\alpha_1\beta_2\gamma_{2S}$ $GABA_A$ receptors can open in the absence of GABA. The generation of the off-current is consistent with a model where penicillin plugs the channel pore and holds the receptor in an open

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conformation but prevents ion conduction until it is removed. When penicillin is washed off, the collected channels are transiently open and unplugged and the off-current is recorded. The off-current provides a tool to study the current characteristics and pharmacological properties of spontaneously opening $GABA_A$ channels.

2. Material and methods

2.1. Construction and expression of receptors

The human $GABA_A$ α_1 and β_1 or β_2 cDNA sequences were subcloned into the dual promoter baculovirus transfer vector pAcUW31 (Clontech, Palo Alto, CA, USA) as described previously (Birnir et al., 1995). The γ_{2S} cDNA was subcloned into *Bam*H1/*Eco*R1 sites following the polyhedrin promoter in pBacPAK8 (Clontech). To allow expression of three subunits from one virus, a triple promoter plasmid pBAC3 was created by ligating a 2.25 kB *Bst*X1/*Hind*3 fragment, containing the triple promoter cassette from pAcAB3 (Pharmingen) into *Bst*X1/*Hin*d3 cut pBacPAK8. The result is a smaller triple promoter plasmid without a second unwanted *Eco*R1 site. To create the human β_2 amino acid sequence, the rat β_2 cDNA was mutated N323S, with the oligonucleotide GAGAAAGCTGCTAGCGCCAACAACGAG, using a USE mutagenesis kit (Pharmacia). This β_2 cDNA was subcloned into the *Bam*H1 site following a P10 promoter in pBAC3. The human α_1 cDNA was then subcloned into the *Bgl*II/*Eco*R1 sites following the other P10 promoter in β_2-pBAC3. Finally, the γ_{2S} cDNA was subcloned into the *Xba*I/*Stu*1 sites following the polyhedrin promoter in $\alpha_1\beta_2$-pBAC3, creating $\alpha_1\beta_2\gamma_{2S}$-pBAC3. Recombinant baculoviruses were generated from each transfer plasmid by homologous recombination as described previously (Birnir et al., 1995). Techniques for general handling of *Spodoptera frugiperda* (*Sf*)9 cells, production of high titer viral stock and infection procedures have been described (Birnir et al., 1995).

2.2. Electrophysiology

Cells were infected for an hour with the appropriate recombinant virus and then incubated at 25 ± 1 °C for 24 to 40 h before being used in electrophysiological experiments. Currents were recorded using standard whole-cell, tight-seal recording techniques (Hamill et al., 1981). Whole-cell currents were recorded from voltage-clamped cells at a pipette potential of −40 mV. At this potential, background-chloride current was minimized (Birnir et al., 1995). Patch electrodes were pulled from borosilicate glass capillaries (1.5 O.D. and 0.86 I.D., Harvard Apparatus, Edenbridge, UK). The electrodes were fire polished and had a resistance of 3 to 10 MΩ when filled with the pipette solution. All experiments were carried out at room temperature (20–22 °C). The bath solution consisted of (in mM): 180 NaCl, 1 $CaCl_2$, 1 $MgCl_2$ and 10 MES (2-(*N*-morpholino)ethanesulfonicacid) adjusted to pH 6.2 with NaOH. The pipette solution consisted of (in mM): 178 NaCl, 1 $CaCl_2$, 1 $MgCl_2$, 5 EGTA [ethylene glycol-bis (b-aminoethylether)tetraacetic acid) and 10 TES (*N*-Tris(hydroxymethyl)] adjusted to pH 7.2 with NaOH. ATP (4 mM) was included in some experiments but did not affect the results. GABA (γ-aminobutyric acid), penicillin, bicuculline and pentobarbital were dissolved in the bath solution whereas diazepam was first dissolved in dimethylsulfoxide (DMSO).

Currents were monitored with a current-to-voltage converter (Axopatch 200B, Axon Instruments, Foster City, CA, USA, or EPC7, HEKA Elektronik, Lambrecgt/pfalz, Germany). Data were digitized using an analog-to-digital converter (DigiData 1200, Axon Instruments) and a Pulse plus data acquisition program (HEKA Elektronik) and then analyzed using a Pulse fit analysis program (HEKA Elektronik).

2.3. Drug application

Drugs were applied by gravity feed via microperfusion tubes. All drugs were applied using this fast perfusion system as it has been shown that both high and low affinity ligands (Jones et al., 1998) bind rapidly to $GABA_A$ receptors. After establishing a whole-cell configuration, the cell was lifted off the bottom of the bath chamber and placed in front of the drug delivery tubes and the bath flow (>10 ml/min) was turned on. The rate of solution exchange using this method is fast. For an open-tip electrode, the solution can be changed in less than a millisecond (Birnir et al., 1995). Currents evoked by 100 μM GABA reach 80% of the peak-current value in 700 μs in outside-out patches from rat cultured hippocampal neurons (Birnir et al., 2000a) and in *Sf*9 cells expressing $\alpha_1\beta_1$ $GABA_A$ receptors; currents evoked by 10 mM GABA reach 90% of the whole-cell peak-current amplitude within 5 ms (Birnir et al., 1995). Each test drug concentration was bracketed with a control concentration in order to prevent differences in currents due to cellular "run-downs" to be interpreted as drug effects or a lack thereof (Dalziel et al., 2000).

3. Results

3.1. Off-current is associated with penicillin block of $GABA_A$ channels

Penicillin is an inhibitor of $GABA_A$ receptors (Twyman et al., 1992). Fig. 1 shows typical whole-cell current responses to 10 mM GABA (i) or 10 mM GABA plus 10 mM penicillin (ii) in cells expressing $\alpha_1\beta_1$ (Fig. 1A) or $\alpha_1\beta_1\gamma_2$ (Fig. 1B) receptors. When GABA alone was applied to cells expressing $\alpha_1\beta_1$ or $\alpha_1\beta_1\gamma_2$ receptors (Fig. 1Ai and Bi), the whole-cell current rose to a peak value and then decayed to a steady-state current level until GABA was

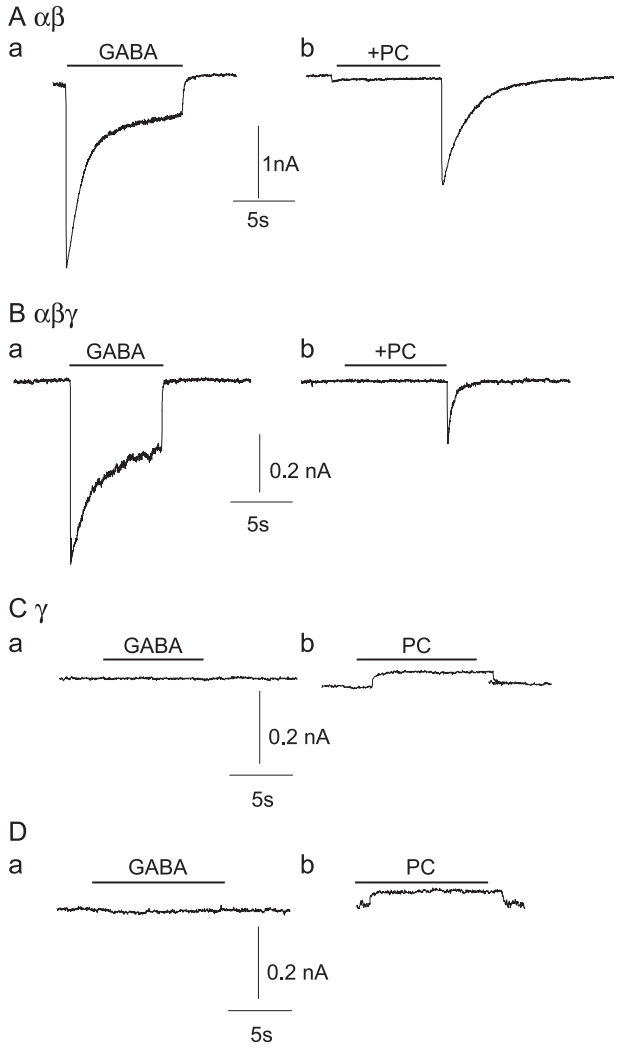

Fig. 1. Penicillin inhibition of GABA-activated currents in $\alpha_1\beta_1$ (A) and $\alpha_1\beta_1\gamma_{2S}$ (B) receptors. Currents evoked (a) by 10 mM GABA alone and then in the same cell (b) in the presence of 10 mM GABA plus 10 mM penicillin. No inward currents were evoked by 10 mM GABA in a cell infected with only the γ_{2S} baculovirus (Ca) or in a noninfected cell (Da) whereas 10 mM penicillin alone caused 45 pA (Cb) and 40 pA (Db) inhibition of the background current in the respective cells. The drugs were applied for the period indicated by the bars.

washed off. Application of 10 mM penicillin together with 10 mM GABA reduced the peak-current response to only 0.024 ± 0.012 ($n = 6$) and $0.0\ 4 \pm 0.004$ ($n = 3$) of the response evoked by 10 mM GABA alone, in cells expressing $\alpha_1\beta_1$ or $\alpha_1\beta_1\gamma_{2S}$ cells, respectively. Rapid removal of GABA plus 10 mM penicillin evoked an inward current (off-current) that rapidly peaked and then decayed to baseline. GABA-activated currents or currents activated upon removal of penicillin alone were never evoked in cells infected with the γ_{2S} subunit only ($n = 17$, Fig. 1C) or noninfected cells ($n = 13$, Fig. 1D).

When the drugs were applied for about 8 s, the peak off-current amplitude was half the amplitude of the saturating GABA current response in cells expressing $\alpha_1\beta_1$ (0.48 ± 0.05, $n = 6$; Fig. 1A) or $\alpha_1\beta_1\gamma_{2S}$ (0.50 ± 0.14, $n = 3$; Fig. 1B) receptors.

3.2. Penicillin alone evokes an off-current response in cells expressing $\alpha_1\beta_1$ or $\alpha_1\beta_1\gamma_{2s}$ receptors

Penicillin has been shown to block currents through mutated $GABA_A$ receptors (Tierney et al., 1996) in the absence of GABA. We examined whether a current could be detected when penicillin was applied to recombinant $\alpha_1\beta_1$ and $\alpha_1\beta_1\gamma_{2S}$ receptors in the absence of GABA. Off-currents in response to penicillin removal were recorded in 101 cells expressing the $\alpha_1\beta_1$ receptors and 96 cells expressing the $\alpha_1\beta_1\gamma_{2S}$ receptors. Provided that the removal of penicillin was rapid, most cells gave an off-current upon penicillin removal. However, in 22% and 20% of the $\alpha_1\beta_1$ and $\alpha_1\beta_1\gamma_{2S}$ receptors expressing cells, respectively, that responded to GABA, an off-current was not recorded when penicillin alone was removed. Whether the cause of this variability was associated with non-optimal drug removal or possibly different forms of, e.g., intracellular modification or cytoskeletal interactions of the receptors, was not explored in this study.

Fig. 2 shows the current time course when cells expressing the receptors are exposed to penicillin alone. Sustained application of 10 mM penicillin inhibited the background current slightly, but upon rapid removal of penicillin, an inward current was recorded that increased rapidly in amplitude and then decayed. The peak off-current amplitude varied from cell to cell but was on the average 331 ± 33 pA ($n = 32$) and 295 ± 47 pA ($n = 29$) for $\alpha_1\beta_1$ and $\alpha_1\beta_1\gamma_{2S}$ receptors, respectively, after 8 s of penicillin application. Examples of the larger currents recorded are shown in Fig. 2 and were 710 pA ($\alpha_1\beta_1$, Fig. 2A) and 846 pA ($\alpha_1\beta_1\gamma_{2S}$, Fig. 2B) in amplitude. Penicillin inhibition of the background current varied somewhat. It ranged from 0 to 277 pA and from 0 to 546 pA in $\alpha_1\beta_1$ and $\alpha_1\beta_1\gamma_{2S}$ cells, respectively, but was on the average 52 ± 7 pA ($n = 50$, $\alpha_1\beta_1$) and 83 ± 18 pA ($n = 50$, $\alpha_1\beta_1\gamma_{2S}$). Cells infected with the γ_{2S} recombinant virus alone do not express functional $GABA_A$ receptors (see Fig. 1C), but 10 mM penicillin still inhibited the background current. In these cells, the inhibition ranged from 0 to 255 pA and was on the average 72 ± 22 ($n = 17$). Ten millimolar penicillin inhibited the background current from 0 to 140 pA in noninfected cells and was on the average 52 ± 10 pA ($n = 13$). The inhibition of the background current by penicillin in γ_{2S} and noninfected cells suggests that penicillin inhibits some native or virus-induced channels in the *Sf*9 cells but probably by a different mechanism from the inhibition of the $GABA_A$ receptors as off-currents were not recorded in γ_{2S} or noninfected cells when the penicillin application was rapidly terminated.

The rise of the off-current for both receptor types was fast and well fitted with a monoexponential function (Fig. 2Aii $\alpha_1\beta_1$; Bii $\alpha_1\beta_1\gamma_2$). The time constants for the current rise and unblocking of the receptors (τ_{ub}) were 28 ± 3 ms

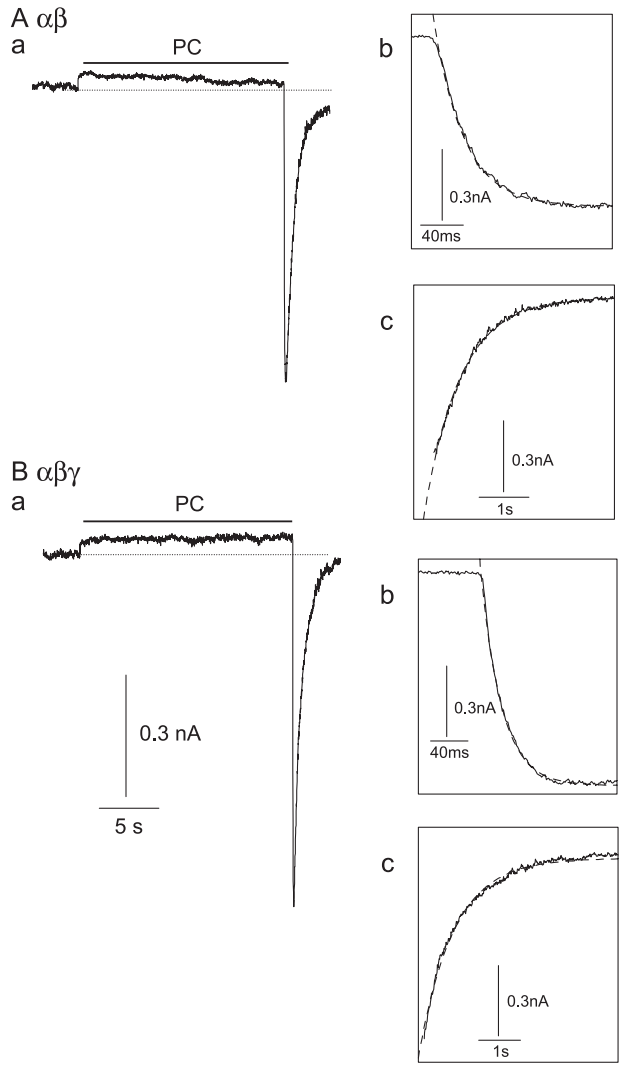

Fig. 2. Time course of the off-current response. (a) Penicillin (10 mM) alone was applied to a cell expressing $\alpha_1\beta_1$ (A) or $\alpha_1\beta_1\gamma_{2S}$ (B) receptors for the duration of the bars. An inward current developed when the penicillin application was stopped and penicillin was removed. (b) The time course of the upstroke of the inward current in (a) was fitted with a monoexponential function (τ_{ub}: 27 ms $\alpha_1\beta_1$; 19 ms $\alpha_1\beta_1\gamma_{2S}$). (c) The closure of the channel after the transient opening (a) followed a monoexponential time course (τ_c: 0.72 s $\alpha_1\beta_1$; 0.76 s $\alpha_1\beta_1\gamma_{2S}$). The scale bars in (Ba) apply to the current traces in (Aa). The drugs were applied for the period indicated by the bars.

($n=13$) and 23 ± 3 ms ($n=9$) for $\alpha_1\beta_1$ and $\alpha_1\beta_1\gamma_{2S}$ receptors, respectively. The decay of the off-current (Fig. 2Aiii $\alpha_1\beta_1$; Bc $\alpha_1\beta_1\gamma_2$), associated with closure of the channels after the transient opening, also followed a monoexponential function with the average time constants (τ_c) of 0.51 ± 0.07 s ($n=14$, $\alpha_1\beta_1$) and 0.53 ± 0.05 s ($n=10$, $\alpha_1\beta_1\gamma_{2S}$).

We examined if the off-current's peak amplitude changed depending on the length of time penicillin was applied for. Fig. 3 shows how the amplitude of the off-current varied with the time of exposure to 10 mM penicillin (Fig. 3A and B). The recordings are continuous for each receptor type and the penicillin application times were 2, 4, 8, 18 and 24 s.

In both types of receptors (Fig 3Ai $\alpha_1\beta_1$, $n=7$; Aii $\alpha_1\beta_1\gamma_{2S}$, $n=5$), the off-current amplitude increased as the time of exposure to penicillin was made longer but started to level off after about 8 s of application. In Fig. 3B, the average peak off-current value ($\alpha_1\beta_1$ triangles, $n=5$; $\alpha_1\beta_1\gamma_{2S}$ circles, $n=4$) is plotted as a function of the 10 mM penicillin application time. The data were fitted with a single exponential function with a time constant of 5.4 s and a *Y*-axis intercept of 0.3. For both receptor types, the peak off-current had attained about 80% of the maximal value after 8 s in 10 mM penicillin. The leveling off and the off-current's amplitude plateau level attained demonstrate the stability of the off-current responses (Fig. 3B). The *Y*-axis intercept of the fitted curve gives an estimate of the instantaneous reaction with penicillin and was about 25% of the maximal value for the off-current response. After 8 s of 10 mM penicillin application, the fractional off-current amplitudes evoked in $\alpha_1\beta_1$ and $\alpha_1\beta_1\gamma_{2S}$ receptors were 0.17 ± 0.02 ($n=18$) and 0.21 ± 0.04 ($n=25$) of the 10 mM GABA-activated peak-current amplitude, respectively. Using these values, we estimated the instantaneous effect of penicillin on the $\alpha_1\beta_1$ and $\alpha_1\beta_1\gamma_{2S}$ receptors to be about 6% of the saturating GABA-activated current response in the cells. Also, because the peak off-current amplitude levels off when in 10 mM penicillin for longer than 8 s, it is possible to estimate the size of the population of receptors that interact with 10 mM penicillin. This can be calculated from the fraction the maximal off-current response (Fig. 3B) is of the saturating GABA-activated current response. We estimated the population of receptors that responds to penicillin to be about 20% to 25% of all the receptors expressed in a cell.

Fig. 3C shows the effect of the holding potential on the peak off-current amplitude. For both receptor types, the currents were larger at depolarized 40 mV as compared to hyperpolarized 40 mV (Fig. 3Ci, $\alpha_1\beta_1$ $n=5$; $\alpha_1\beta_1\gamma_{2S}$ $n=4$) and showed outward rectification (Cb, $\alpha_1\beta_1$ triangles; $\alpha_1\beta_1\gamma_{2S}$ circles). The peak value of the off-current was also dependent on the penicillin concentration. In 5 mM penicillin (8 s), the fractional peak off-current amplitude decreased, as compared to values recorded in 10 mM penicillin (see above), to 0.10 ± 0.01 ($\alpha_1\beta_1$, $n=7$) and 0.09 ± 0.04 ($\alpha_1\beta_1\gamma_{2S}$, $n=6$) of the 10 mM GABA-activated peak-current amplitude.

We have reported previously that GABA-activated whole-cell currents often decrease in amplitude (''run-down'') when an *Sf*9 cell expressing recombinant $GABA_A$ receptors is repeatedly exposed to GABA (Dalziel et al., 2000). Similar observations have been made for the GABA-activated current response in neurons (Gyenes et al., 1988; Stelzer et al., 1988; Lim and Birnir, 2001). We examined in cells expressing the $\alpha_1\beta_1$ or $\alpha_1\beta_1\gamma_{2S}$ receptors where a ''run-down'' was recorded if a decrease in the current amplitude of the GABA-activated response was correlated with a decrease in the amplitude of the off-current. The current traces shown in Fig. 4 are from a cell expressing

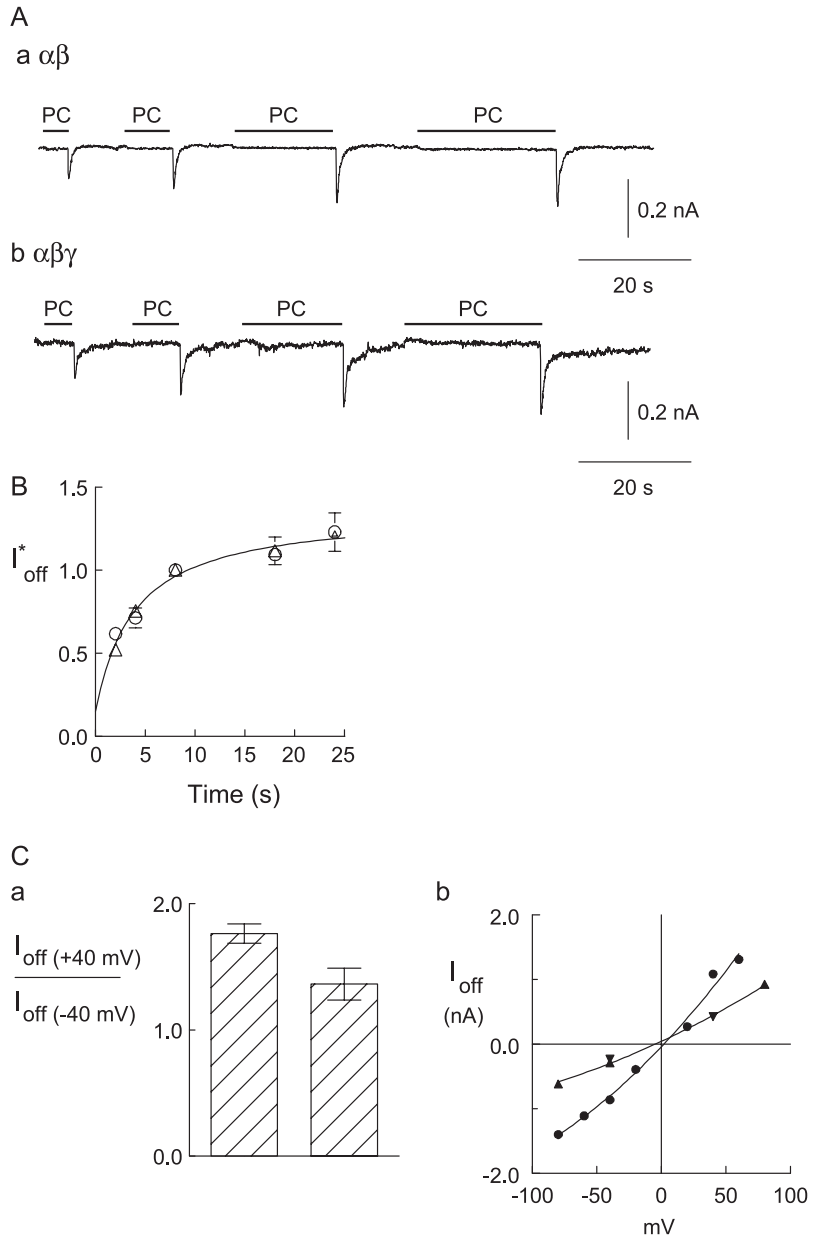

Fig. 3. The size of the off-currents in $\alpha_1\beta_1$ (Aa) and $\alpha_1\beta_1\gamma_{2S}$ (Ab) is related to the duration of the penicillin application. Penicillin (10 mM) was applied for ~ 4, ~ 8, ~ 18 and ~ 24 s as indicated by the bars. (B) Normalised peak off-current amplitudes (I^*_{off}) are plotted against time (s) in 10 mM penicillin. The currents were normalised to the average off-current value obtained after 8 s in 10 mM penicillin. $\alpha_1\beta_1$ (triangles, $n=5$), $\alpha_1\beta_1\gamma_{2S}$ (circles, $n=4$). All data points are the averages of three or more measurements apart from $\alpha_1\beta_1$ at 24 s that represents one measurement. S.E.M. is shown if larger than the symbol. The curve represents an exponential fit data. (C) Current–voltage relationship of the peak off-currents. The off-current in a cell was larger at depolarized 40 mV as compared to hyperpolarized 40 mV (Ca) in $\alpha_1\beta_1$ ($n=5$) and $\alpha_1\beta_1\gamma_{2S}$ ($n=5$) receptors. (Cb) An off-current–voltage relationship recorded in cells expressing $\alpha_1\beta_1$ (triangles, two different cells) or $\alpha_1\beta_1\gamma_{2S}$ (circles, one cell) receptors. The lines simply connect the data points and have no theoretical significance.

$\alpha_1\beta_1$ receptors (Fig. 4Ai–iv) and another cell expressing $\alpha_1\beta_1\gamma_{2S}$ receptors (Fig. 4Bi–iv). The current traces are in time sequence and show that a decrease in the peak off-current amplitude was correlated with a decrease in the peak amplitude of the GABA-activated current in the same cell. In contrast, the level of the background current that was inhibited by the penicillin did not decrease.

For the $\alpha_1\beta_1$ receptors, the current amplitudes evoked by the 1st and the 2nd application of 10 mM GABA were 1169 and 583 pA and 1st and 2nd off-current amplitudes were 494 and 61 pA. Similarly in $\alpha_1\beta_1\gamma_{2S}$ receptors, the current amplitudes evoked by the 1st and the 2nd application of 10 mM GABA were 2062 and 580 pA and 1st and 2nd off-current amplitudes were 1613 and 128 pA. The rate and extent of the decrease in current amplitude varied from cell to cell. In cells expressing $\alpha_1\beta_1$ receptors where run-down was recorded, the reduced GABA-activated peak current amplitude and the reduced off-current amplitude in the same

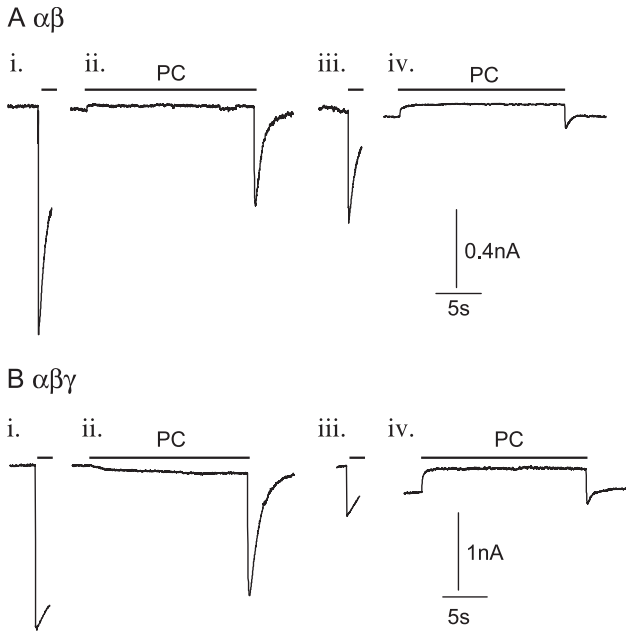

Fig. 4. Run-down of the GABA-activated and the off-current response. The peak amplitude of the GABA-activated whole-cell current and the peak amplitude of the off-current response decreased in a time-dependent manner. The traces (i–iv) were recorded in cells expressing $\alpha_1\beta_1$ (A) or $\alpha_1\beta_1\gamma_{2S}$ (B) receptors and are shown in time sequence. GABA (10 mM; i and iii) or penicillin (10 mM; ii and iv) applications are indicated by the bars.

cell were on the average 0.65 ± 0.05 ($n = 6$) and 0.43 ± 0.10 ($n = 6$), respectively, of the control response. For $\alpha_1\beta_1\gamma_{2S}$ receptors, the values were 0.54 ± 0.09 ($n = 7$) and 0.39 ± 0.07 ($n = 7$) for the GABA-activated and the off-current response, respectively, recorded in the same cell. In subsequent experiments (see below) where effects of drugs on the peak off-current amplitude were examined, each test concentration was bracketed with a control concentration in order to prevent differences in currents due to cellular "run-downs" to be interpreted as drug effects or a lack thereof.

To examine further the generality of the off-current response in recombinant receptors, we replaced the β_1 subunit with the β_2 subunit in $\alpha_1\beta\gamma_{2S}$ receptors and examined if an off-current was generated upon removal of penicillin alone. In cells expressing $\alpha_1\beta_2\gamma_{2S}$, inward off-currents were recorded when applications of 10 mM penicillin alone were terminated ($n = 5$), similar to what had been recorded for $\alpha_1\beta_1$ and $\alpha_1\beta_1\gamma_{2S}$ receptors (data not shown).

3.3. Pharmacology of the off-current response

As the penicillin off-current is associated with $GABA_A$ receptors, it might be expected to respond to drugs known to modulate GABA-activated currents. We therefore examined the effects on the off-current response of the antagonist bicuculline and the positive modulators pentobarbital and diazepam.

3.3.1. Antagonist

Bicuculline is a competitive as well as an allosteric inhibitor of $GABA_A$ receptors (Birnir et al., 2000a,b; Ueno et al., 1997). Bicuculline blocked the peak off-current amplitude and reduced the background current.

Fig. 5 shows the control response in 10 mM penicillin in cells expressing the $\alpha_1\beta_1$ (Fig. 5A) or the $\alpha_1\beta_1\gamma_{2S}$ (Fig. 5B)

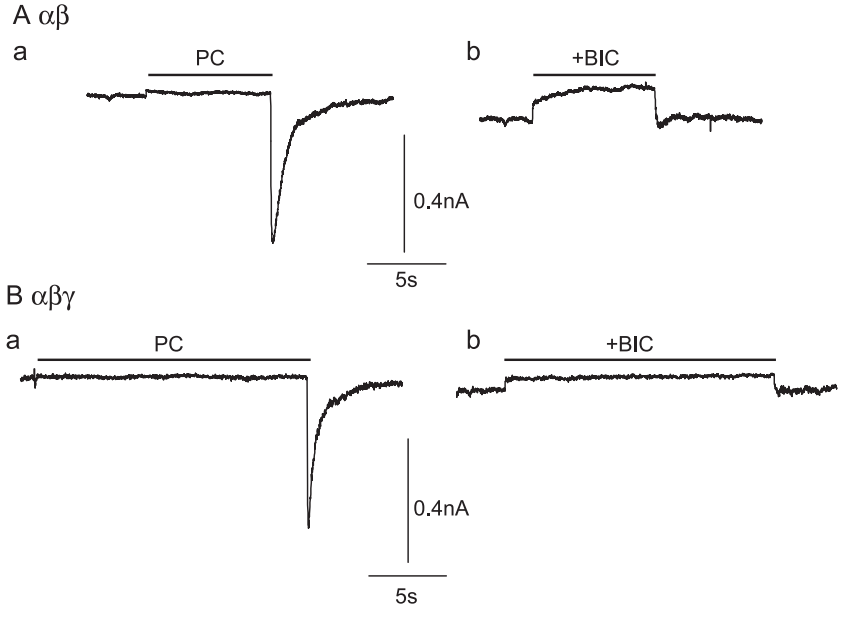

Fig. 5. Bicuculline blocks the off-current response in $\alpha_1\beta_1$ (A) and $\alpha_1\beta_1\gamma_{2S}$ (B) receptors. (a) Off-currents evoked after application of 10 mM penicillin alone were blocked, in the same cell, when (b) 10 mM penicillin was applied together with 100 μM bicuculline. The drugs were applied for the period indicated by the bars.

receptors, respectively, and the effect on the off-current amplitude of 100 μM bicuculline together with 10 mM penicillin in the same cell. When 10 mM penicillin alone was applied, off-current amplitudes of 518 pA (Fig. 5A $\alpha_1\beta_1$) and 483 pA (Fig. 5B $\alpha_1\beta_1\gamma_{2S}$) were recorded whereas the application of 10 mM penicillin plus 100 μM bicuculline essentially abolished the off-current response. To test for differences in current amplitude due to cellular run-downs, off-current's responses were bracketed with GABA-activated currents. The GABA-activated currents were identical in amplitude before and after measurements of the off-currents. In cells expressing $\alpha_1\beta_1\gamma_{2S}$ receptors, the penicillin plus bicuculline off-current was in addition bracketed with off-currents generated by 10 mM penicillin alone. The average peak off-current amplitude after penicillin plus bicuculline application was terminated was 0.06 ± 0.04 ($n = 7$, $\alpha_1\beta_1$) and 0.03 ± 0.03 ($n = 6$, $\alpha_1\beta_1\gamma_{2S}$) of the control response to penicillin alone. The increased inhibition of the background current, in comparison to the effect of penicillin alone, is in accordance with the allosteric nature of the inhibition by bicuculline, but it is also possible that bicuculline inhibits some native potassium channels in the *Sf*9 cells (see Khawaled et al., 1999). We did not explore the effect further in this study.

3.3.2. Modulators

Pentobarbital is known to enhance GABA-activated currents in both $\alpha_1\beta_1$ and $\alpha_1\beta_1\gamma_{2S}$ receptors whereas incorporation of $\gamma_{1or\ 2}$ into the receptors is required for GABA-activated current enhancement at low micromolar diazepam concentrations (Barnard et al., 1998).

Fig. 6 shows the effect of 50 μM pentobarbital on the peak off-current amplitude and the block by bicuculline in the same cell. When 10 mM penicillin alone was applied, off-current amplitudes of 74 pA (Fig. 6Ai $\alpha_1\beta_1$) and 347 pA (Fig. 6Bi $\alpha_1\beta_1\gamma_{2S}$) were recorded. The amplitude of the off-current increased to 523 pA (Fig. 6Aii $\alpha_1\beta_1$) and 735 pA (Fig. 6Bii $\alpha_1\beta_1\gamma_{2S}$) when 10 mM penicillin plus 50 μM pentobarbital were applied together but no off-current was recorded when the application of 100 μM bicuculline together with the 10 mM penicillin plus 50 μM pentobarbital was terminated (Fig. 6Aiii $\alpha_1\beta_1$: Biii $\alpha_1\beta_1\gamma_{2S}$). To test for cellular run-downs, current responses to drugs to be examined were bracketed by GABA-activated currents ($\alpha_1\beta_1\gamma_{2S}$) or off-currents generated by 10 mM penicillin alone ($\alpha_1\beta_1$, $\alpha_1\beta_1\gamma_{2S}$). In all cases was the control current response recovered after the test-drug application. Pentobarbital (50 μM) when applied alone did not evoke any current in cells expressing either $\alpha_1\beta_1$ ($n = 3$) or $\alpha_1\beta_1\gamma_{2S}$ ($n = 4$) receptors. Coapplication of penicillin and 50 μM pentobarbital enhanced the average off-current peak amplitude by 2.8 ± 0.5 ($n = 12$) and 3.4 ± 0.4 ($n = 9$) of the control response with penicillin alone in cells expressing the $\alpha_1\beta_1$ or $\alpha_1\beta_1\gamma_{2S}$ receptors, respectively. The average off-current response on the removal of all three drugs (penicillin, pentobarbital and bicuculline) was 0.04 ± 0.03 ($n = 12$, $\alpha_1\beta_1$) and 0.03 ± 0.03 ($n = 7$, $\alpha_1\beta_1\gamma_{2S}$) of the response of penicillin plus 50 μM pentobarbital. As pentobarbital did not evoke any current when applied alone, the increased current amplitude recorded in the presence of pentobarbital plus penicillin must be associated with the potentiating rather than the activating property of pentobarbital at $GABA_A$ receptors.

Fig. 7 shows the effect of 1 μM diazepam on the off-current. When 10 mM penicillin alone was applied, off-current amplitudes of 210 pA (Fig. 7Ai $\alpha_1\beta_1$) and 148 pA (Fig. 7Bi $\alpha_1\beta_1\gamma_{2S}$) were recorded. When 10 mM penicillin plus 1 μM diazepam were applied to these same cells, the peak off-current amplitude was only enhanced in cells expressing the $\alpha_1\beta_1\gamma_{2S}$ receptors ($\alpha_1\beta_1$ 183 pA; $\alpha_1\beta_1\gamma_{2S}$ 354 pA) on the removal of the drugs. To test for cellular run-downs, responses to drugs examined were bracketed by off-currents generated by 10 mM penicillin alone ($\alpha_1\beta_1$, $\alpha_1\beta_1\gamma_{2S}$). In all cases, the control current response was

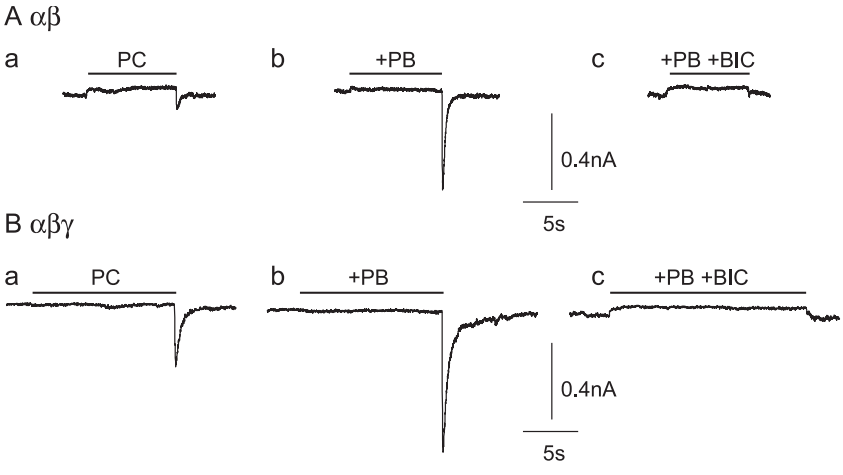

Fig. 6. Pentobarbital enhances the peak amplitude of the off-current response in $\alpha_1\beta_1$ (A) and $\alpha_1\beta_1\gamma_{2S}$ (B) receptors. (a) Off-currents evoked after application of 10 mM penicillin alone were enhanced, in the same cell, when (b) 10 mM penicillin was applied together with 50 μM pentobarbital but blocked when (c) 10 mM penicillin, 50 μM pentobarbital plus 100 μM bicuculline were applied together. The drugs were applied for the period indicated by the bars.

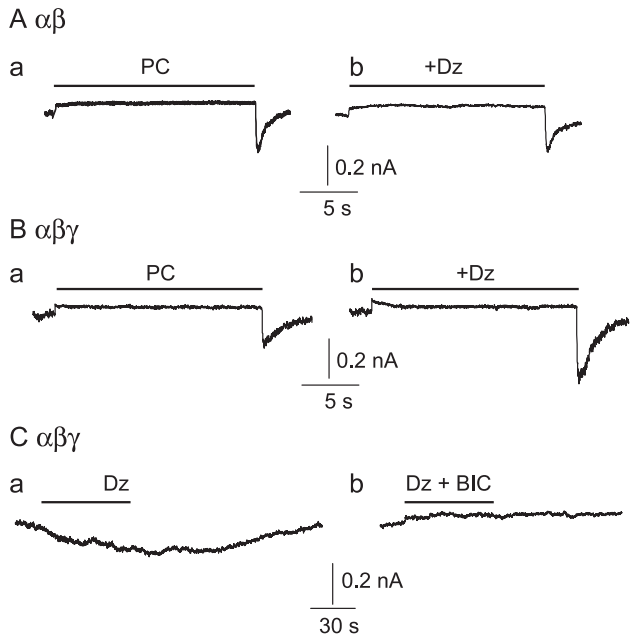

Fig. 7. Diazepam only modulates $\alpha_1\beta_1\gamma_{2S}$ channels. Penicillin (10 mM) was applied alone or together with 1 μM diazepam to cells expressing $\alpha_1\beta_1$ (A) or $\alpha_1\beta_1\gamma_{2S}$ (B) receptors. (a) Off-currents evoked after application of 10 mM penicillin alone and, in the same cell, when (b) 10 mM penicillin was applied together with 1 μM diazepam. (C) Inward current developed when 1 μM diazepam alone (b) was applied to a cell but not when 1 μM diazepam plus 100 μM bicuculline (b) were applied together to the same cell. The drugs were applied for the period of the bars.

recovered when examined after the test diazepam current response.

No enhancement of the off-current's peak amplitude was observed after exposure of the cells expressing $\alpha_1\beta_1$ receptors to 1 μM diazepam (80% confidence level). In five cells, it was 1.2 ± 0.1 of the control response whereas in cells expressing $\alpha_1\beta_1\gamma_{2S}$ receptors ($n = 9$), the average off-current enhancement on removal of penicillin plus 1 μM diazepam was 1.6 ± 0.1 of the control response with penicillin alone. Neither diazepam nor pentobarbital appeared to have large effects on the rate of decay of the off-current and the time constants (τ_c) were similar to those obtained in the absence of the modulators. The rate of decay of the off-current after removal of 10 mM penicillin plus 50 μM pentobarbital was 0.52 ± 0.10 s ($n = 7$) and 0.55 ± 0.09 s ($n = 7$) for $\alpha_1\beta_1$ and $\alpha_1\beta_1\gamma_{2S}$, respectively, and after removal of 10 mM penicillin plus 1 μM diazepam, it was 0.70 ± 0.09 s ($n = 5$) for $\alpha_1\beta_1\gamma_{2S}$.

We have shown that both diazepam and penotobarbital can enhance the off-current response. We then examined if diazepam and pentobarbital when applied alone could induce an inward current in the absence of penicillin. Fig. 7C shows an experiment where 1 μM diazepam alone and then in the presence of 100 μM bicuculline was applied for 1 min to a cell expressing $\alpha_1\beta_1\gamma_{2S}$ receptors. The peak amplitude of the 10 mM GABA-activated current in the cell was 823 pA. In the presence of 1 μM diazepam alone, an inward current of 135 pA developed (Fig. 7Ci). The current returned to the baseline level when diazepam was washed out of the bath (Fig. 7Ci). Similar results were obtained in 10 other cells. The presence of 100 μM bicuculline together with the 1 μM diazepam blocked ($n = 5$) the development of the inward current (Fig. 7Cii). The maximal value of the diazepam induced inward current as a fraction of the 10 mM GABA-activated peak current amplitude in the same cell was on the average 0.08 ± 0.02 ($n = 11$). Diazepam (1 μM) did not induce an inward current in cells expressing $\alpha_1\beta_1$ receptors ($n = 3$) or noninfected cells ($n = 4$). We then examined what effect pentobarbital had on the baseline current properties of cells expressing the receptors. Higher concentrations than 50 μM of pentobarbital were not examined as already at concentrations as low as 100 μM, pentobarbital gates the channels (Dalziel et al., 1999). When we applied 50 μM pentobarbital alone for 1 min to cells expressing $\alpha_1\beta_1\gamma_{2S}$ ($n = 4$) or $\alpha_1\beta_1$ ($n = 10$) receptors, there was no development of an inward current recorded (results not shown).

4. Discussion

Our aim was to examine if $GABA_A$ receptors made from common classes of subunit families (α, β and γ) could open in the absence of GABA and be modulated by drugs. We recorded the channel activity in the whole-cell configuration as it has been suggested that spontaneous channels may arise when patches are ripped off and away from intact cells. We chose to make the receptors from the α_1, $\beta_1$or β_2 and γ_{2S} subunits as these are expressed in many parts of the brain (Laurie et al., 1992; Wisden et al., 1992).

To examine if $\alpha_1\beta_1$ and $\alpha_1\beta_1\gamma_{2S}$ receptors open in the absence of GABA, we used penicillin (Twyman et al., 1992), an open channel blocker of $GABA_A$ receptors. The hypothesis was as follows: if the channels open spontaneously, penicillin will block the channels, collecting them in an open but blocked conformation that will be revealed as transiently open channels (the off-current) when penicillin is washed off the cell. A possible complication is if penicillin by binding to the receptor biases the equilibrium state of the closed channel towards the open state. In this study, it is not possible to separate the blocking effect of penicillin from the plausible effect penicillin may have on the latency of openings of the receptors. However, recently, Yeung et al. (2003) recorded currents generated by $GABA_A$ receptors in hippocampal neurons that were not blocked by 20 mM penicillin. Despite the absence of channel blockage, the authors reported no current enhancement by penicillin, suggesting that the plausible enhancing mechanism by penicillin was not present. In order to record the fast off-current response (ms), removal of penicillin had to be rapid and required the solution exchange around the cell to be fast. The *Sf*9 cells are small, spherical in shape and can be lifted off the bottom

of the bath. Drugs can therefore be applied and removed fast, allowing synchronous activation, blocking and unblocking of the receptors in the surface membrane of the cell. Slow removal (bath application and removal) of the penicillin masked the transient current, presumably due to nonsynchronized unblocking of the transiently opened channels. The results are consistent with the occurrence of spontaneous openings of $\alpha_1\beta_1$, $\alpha_1\beta_2\gamma_{2S}$ and $\alpha_1\beta_1\gamma_{2S}$ $GABA_A$ receptors in the absence of GABA.

The pharmacological properties of the off-current response were similar to the pharmacological properties of GABA-activated currents. The off-currents were blocked by bicuculline. As a pure competitive inhibitor of GABA binding, the bicuculline might not be expected to completely inhibit the off-current response but rather decrease the population of receptors that opened in the presence of penicillin. The receptors presumably remained closed as long as bicuculline was bound. In this context, it has been shown that bicuculline inhibits pentobarbital-gated receptors although the pentobarbital binding site is not thought to overlap with the GABA binding site (Nicoll and Wojtowicz, 1980; Lim and Birnir, 2001). Recently, bicuculline was shown to decrease the open probability of spontaneously opening $GABA_A$ single channels in hippocampal neurons (Birnir et al., 2000a,b). Our results are in agreement with the allosteric as well as the competitive nature of the inhibition of the receptors by bicuculline.

Pentobarbital in a concentration-dependent manner is a modulator, an agonist or an inhibitor at $GABA_A$ receptors whereas diazepam only has positive modulatory effects on the receptors (Barnard et al., 1998; Rabow et al., 1996). It is not known whether the different effects of pentobarbital on the receptors arise from occupation of one or more binding sites. Both pentobarbital and diazepam enhanced the off-current amplitude in $\alpha_1\beta_1\gamma_{2S}$ receptors whereas diazepam did not enhance the off-current in $\alpha_1\beta_1$ receptors in accordance with the γ_2 subunit requirement for current enhancement by low micromolar concentrations of GABA. Surprisingly, with time diazepam, and not pentobarbital, induced an inward current when the drugs were applied alone. The inward current was only recorded in cells expressing the $\alpha_1\beta_1\gamma_{2S}$ receptors. The difference in results between the two drugs when applied alone is probably related to their mechanism of modulation and possibly to the desensitization of the receptors. In GABA-activated channels, pentobarbital has been shown to increase the mean-open time and the single-channel conductance (MacDonald and Olsen, 1994; Eghbali et al., 2000) whereas diazepam is known to increase the frequency of openings, the single-channel conductance and decrease the latency of openings of $GABA_A$ receptors (MacDonald and Olsen, 1994; Eghbali et al., 1997; Guyon et al., 1999; Birnir et al., 2001; Lindquist et al., 2003) and does not act as an agonist at the receptors (Barnard et al., 1998). That only diazepam affects the opening rate of the channels (frequency and latency of openings) may possibly explain the difference observed on the baseline-current properties between diazepam and pentobarbital in this study plus the difference recorded in the decay rate of the currents when the drugs were washed off. The average decay of the off-current was only prolonged after exposure to diazepam, suggesting a longer lasting and therefore greater cumulative effect by 1 μM diazepam as compared to 50 μM pentobarbital.

In *Sf*9 cells expressing $\alpha_1\beta_1$ and $\alpha_1\beta_1\gamma_{2S}$ receptors, the instantaneous effect of penicillin is small. The estimated instantaneous effects were about 6% of the saturating GABA response in the cells, and despite the cells expressing homogenous receptor population, only about 20% to 25% of the receptors responded to penicillin. In diazepam alone, the maximal inward current was about 10% of the saturating current response to GABA in cells expressing $\alpha_1\beta_1\gamma_{2S}$ receptors. Although the frequency of the $GABA_A$ spontaneous-channel openings in this study is low, it is nevertheless significant and raises the possibility that spontaneous openings of other $GABA_A$ receptors have been overlooked.

We showed recently in hippocampal neurons that in the absence of spontaneous openings, the latency of channel opening in response to GABA or the agonist THIP can be in minutes but is decreased in the presence of diazepam (Birnir et al., 2000b, 2001; Lindquist et al., 2003). On the other hand, in the presence of spontaneous openings, the current response to GABA is fast and reaches a peak current within milliseconds (Birnir et al., 2000a), suggesting that spontaneous openings may be essential features of the molecular mechanism underlying the fast response to GABA observed e.g. during whole-cell recordings and synaptic transmission at synapses. The off-current response provides a tool to study the properties and role of spontaneously opening $GABA_A$ channels active in a cell.

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