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Two different modes of action of pentobarbital at glycine receptor channels

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Abstract

Glycine receptor channels are pentameric ligand-gated ion channels which respond to the binding of inhibitory transmitters by opening of a chloride-selective central pore. Pentobarbital is widely used as an anticonvulsive, hypnotic and anaesthetic drug. In the present study, the interaction between pentobarbital and glycine receptor channels was studied on outside-out patches of human embryonic kidney (HEK) 293 cells expressing $\alpha_1\beta$ glycine receptor channels. Currents elicited by 0.03 mM glycine were enhanced by pentobarbital showing potentiation of $\alpha_1\beta$ glycine receptor channels. In the presence of 1 mM glycine + pentobarbital (1 and 3 mM), desensitization was faster and the peak current amplitude decreased. After the end of glycine + pentobarbital pulses, off-currents occurred suggestive for a channel block mechanism. Pentobarbital had no agonistic effects at glycine receptor channels.

Keywords: Glycine receptor channel; Pentobarbital; Channel block; Patch-clamp; Ultra-fast application

1. Introduction

The strychnine-sensitive glycine receptor channel is a member of an ion channel superfamily which includes nicotinic acetylcholine, $GABA_A$ and serotonin ($5\text{-}HT_3$) receptor channels (Unwin, 1989; Betz, 1990). The highest density of expression was found in the brain stem and spinal cord (Langosch et al., 1990; Graham et al., 1985). They form pentamers composed of the ligand-binding α-subunits and optionally an additional β-subunit (Langosch et al., 1990). The α-subunit exists in various isoforms ($\alpha_1-\alpha_4$) whereas only one form of the β-subunit is known (Betz et al., 1991). Glycine is involved in postsynaptic inhibition by activation of the glycine receptor channels permeable to chloride ions. High levels of α_1-transcripts were found mainly in the spinal cord and the brain stem (Betz et al., 1991). The adult glycine receptors are thought to be built up of α_1 and β-subunits (Watanabe and Akagi, 1995).

Barbiturates are widely used as anticonvulsive, hypnotic and anaesthetic drugs. Potentiation and agonistic effects of barbiturates were found in previous studies at $GABA_A$ receptor channels (Evans, 1979, Robertson, 1989; Krampfl et al., 2002). In contrast, glycine receptor channels were regarded to be not sensitive to pentobarbital (Rajendra et al., 1997). It does not activate the glycine receptor channels, and a potentiation of current through glycine receptor channel was observed at 20-fold higher concentrations (1–3 mM pentobarbital) than needed for potentiation of $GABA_A$ receptor channels (Pistis et al., 1997; Belelli et al., 1999).

Concentration clamp experiments using tools for ultrafast solution exchange (Franke et al., 1987) in combination with the patch clamp technique (Hamill et al., 1981) allow precise analysis of molecular reactions between ligands and receptors. Glycine receptor channels are involved in the pathogenesis of different diseases like hyperekplexia (Shiang et al., 1993). To learn more about the pharmacology of glycine receptor channels, we investigated the interaction of pentobarbital with recombinant $\alpha_1\beta$ glycine receptor channels. We could show that pentobarbital elicited a

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channel block on glycine receptor channels similar to that described for $GABA_A$ receptor channels (Rho et al., 1996; Krampfl et al., 2002). Unlike $GABA_A$ receptor channels, agonistic effects of pentobarbital were not observed.

2. Methods

2.1. Cell expression

Transformed human embryonic kidney (HEK) 293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a 5% CO_2/95% air incubator. Cells were suspended in a buffer containing: 50 mM K_2HPO_4; 20 mM K-acetate; pH 7.35. cDNA of rat $\alpha_1\beta$ glycine receptor subunits (subcloned in pCIS2 expression vectors; Gormann et al., 1990) were added to the suspension of HEK293 cells and transfected by a device for electroporation (EquiBio, Kent, UK). To visualize transfected cells, they were cotransfected with cDNA of green fluorescent protein (GFP). Transfected cells were plated on glass coverslips and incubated for 15–24 h prior to the patch-clamp experiments.

2.2. Electrophysiological measurements

Electrophysiological experiments were performed at room temperature (20 °C), and cells were continuously superfused with extracellular solution containing: 162 mM NaCl, 5.3 mM KCl, 0.6 mM Na_2HPO_4, 0.22 mM KH_2PO_4, 15 mM HEPES, and 5.6 mM glucose. pH was adjusted to 7.4 with NaOH. Patch pipettes were pulled from thin-walled borosilicate glass tubing with filament (Clark, Pangbourne, UK) using a DMZ-Universal puller (Zeitz, Augsburg, Germany). Pipette tips were filled with high K+ solution (140 mM KCl, 2 mM $MgCl_2$, 11 mM EGTA, 10 mM glucose; pH 7.4) and had a resistance between 8 and 12 MΩ. Outside-out patches were obtained using standard methods (Hamill et al., 1981). Currents were recorded with an EPC9 patch-clamp amplifier (List Instruments, HEKA, Germany). Data were stored on hard disk with a sampling rate of 10 kHz and filtered for analysis with 2 kHz. Glycine was obtained from Sigma (St. Louis, USA).

Pentobarbital was obtained from Synopharm (Barsbüttel, Germany). Solutions were freshly prepared prior to each experiment. Pentobarbital was dissolved in extracellular solution and further diluted to final concentrations.

2.3. Fast application

Fast application of agonists or agonists + drugs was done using a piezo-driven device for concentration-clamp measurements (Franke et al., 1987). A smooth liquid filament was achieved with a single outflow (glass tubing 0.15 mm inner diameter). Different pulses of glycine with different concentrations of pentobarbital were applied to outside-out patches expressing the glycine receptor channels. Between pulses, patches were bathed in a continuously flowing background solution. To minimize run-down of currents, the interval between pulses was at least 30 s. Three to five single current traces were averaged for analysis. Desensitization was fitted with the Simplex method. The number of exponential components was incremented until addition of another current component did not significantly improve the fit (Haas and Macdonald, 1999). Experimental data were given as mean ± S.D. Statistical analysis was performed using the independent *t*-test. Time for solution exchange was <50 μs measured with an open pipette and high electrolyte gradient (Bufler et al., 1996a; Krampfl et al., 2002).

3. Results

3.1. The kinetics of glycine receptor channel currents

The experiments shown in Figs. 1 and 2 were performed by a stepwise application of a saturating glycine concentration (1 mM) + different pentobarbital concentrations to outside-out patches of HEK 293 cells transiently transfected

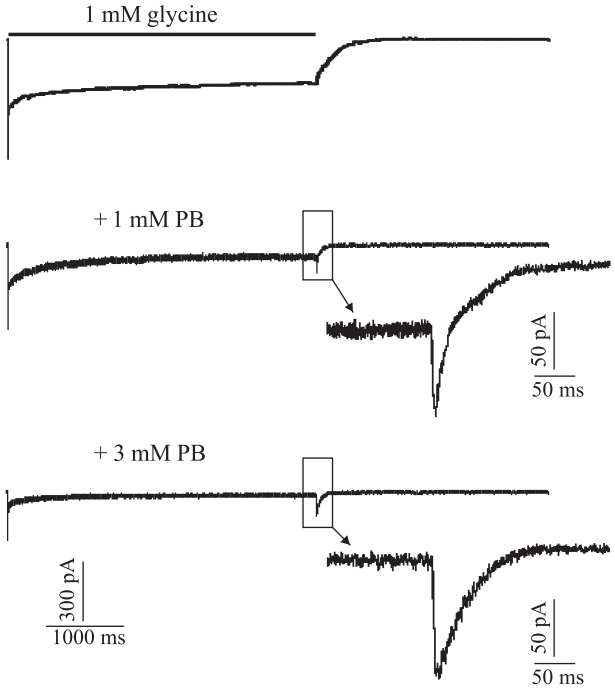

Fig. 1. Current responses of $\alpha_1\beta$ glycine receptor channels recombinantly expressed on HEK293 cells to pulses of 1 mM glycine (upper trace) or 1 mM glycine + different concentrations of pentobarbital (lower traces). Each trace is the average current of three to five single pulses. The bar above current traces indicates the time of application of the test solution (4-s pulses). The insets show the off-current induced after coapplication of different concentrations of pentobarbital. The outside-out patches from HEK293 cells were clamped to a holding potential of −60 mV during the experiments.

with cDNAs of $\alpha_1\beta$ glycine receptor channels. Application of 4-s pulses of 1 mM glycine to an outside-out patch containing $\alpha_1\beta$ glycine receptor channels resulted in a current transient with maximum amplitude of -630 pA (Fig. 1, upper trace). The current increased within <1 ms to the peak and decayed due to desensitization. The time course of desensitization was best fitted with three time constants τ_{fast}, $\tau_{intermediate}$ and τ_{slow} as previously shown (Mohammadi et al., 2003). In the experiment shown in Fig. 1, τ_{fast} had a value of 16.3 ms [mean 17.1 ± 1.8 ms ($n=7$)], $\tau_{intermediate}$ was 131 ms [mean 143 ± 17 ms ($n=7$)], and τ_{slow} was 634 ms [mean 684 ± 85 ms ($n=7$)]. The proportion of τ_{fast}, $\tau_{intermediate}$ and τ_{slow} + the steady-state current amplitude on the whole current amplitude was 0.42, 0.11 and 0.47 [mean 0.41 ± 0.06 ($n=7$), 0.13 ± 0.02 ($n=7$) and 0.49 ± 0.07 ($n=7$), respectively]. The kinetics of glycine receptor channel currents corresponded to that recently described in detail (Mohammadi et al., 2003). Addition of pentobarbital to the 1 mM glycine containing test solution had different effects (Fig. 1, lower traces): First, the peak current amplitude elicited by 1 mM glycine decreased, second, the time course of current decay was faster and third, an off-current occurred after the end of 4-s pulses. The insets show the off-currents after coapplication of 1 mM glycine + 1 or 3 mM pentobarbital at an extended time scale.

3.2. Pentobarbital effect on peak current

The dose-dependency of pentobarbital effects at glycine receptor channel currents is analyzed in Fig. 2. Pentobarbital in a concentration of 0.3 mM had no effect on the peak current amplitude [mean of the relative amplitude 1.00 ± 0.02 ($n=7$)]. At 1 and 3 mM pentobarbital, however, maximum peak current amplitude decreased dependent on the concentration. The mean values for the relative peak current amplitudes were 0.72 ± 0.05 ($n=7$) at 1 mM pentobarbital and 0.41 ± 0.04 ($n=7$) at 3 mM pentobarbital (Fig. 2A). Additionally, at concentrations ≥ 1 mM pentobarbital, an off-current was observed. The amplitude of the off-currents of $\alpha_1\beta$ glycine receptor channels after the end of 4-s pulses of 1 mM glycine + different concentrations of pentobarbital was normalized to the peak current amplitude in Fig. 2B. It increased from 0.27 ± 0.03 ($n=7$) at 1 mM pentobarbital to 0.49 ± 0.02 ($n=7$) at 3 mM pentobarbital.

3.3. Pentobarbital effect on desensitization

As was recently published (Mohammadi et al., 2003), desensitization of $\alpha_1\beta$ glycine receptor channel currents followed a complex time course which was best fitted with three exponentials at 1 mM glycine. Pentobarbital had different effects on the respective time constants of desensitization. Whereas pentobarbital had no effect on τ_{slow} [$\tau_{slow} = 684 \pm 85$ ms ($n=7$) at control and 699 ± 105 ms ($n=7$) at 1 mM glycine + 3 mM pentobarbital], τ_{fast} decreased significantly ($P<0.001$) in presence of pentobarbital. The respective values were 3.9 ± 1.1 ms ($n=7$) or 1.2 ± 0.8 ms ($n=7$) at 1 or 3 mM pentobarbital compared to control [17.1 ± 1.8 ms ($n=7$)]. Similarly, $\tau_{intermediate}$ decreased significantly ($P<0.001$) in presence of pentobarbital. The respective values were 75.3 ± 12.4 ms ($n=7$) or 36.9 ± 7.1 ms ($n=7$) at 1 or 3 mM pentobarbital compared to control [143 ± 17 ms ($n=7$)].

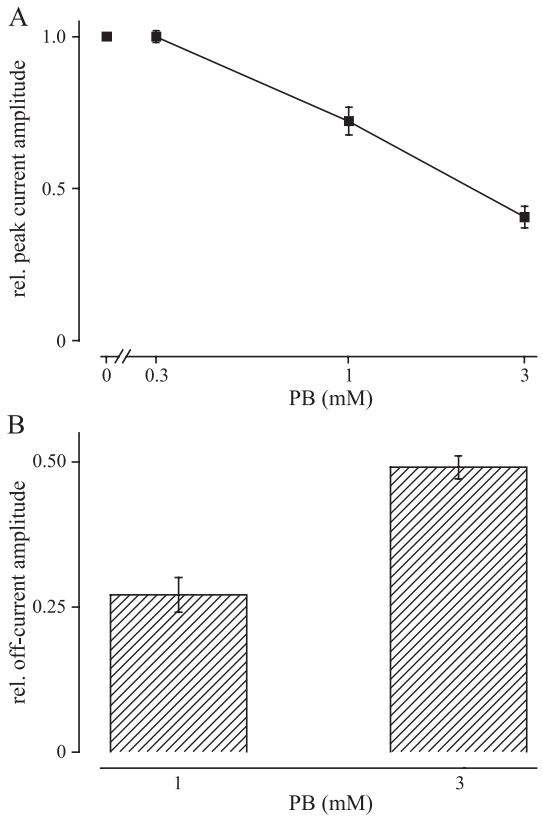

Fig. 2. (A) Dose–response curve for the relative peak current amplitude of the $\alpha_1\beta$ glycine receptor channel currents upon activation by glycine + pentobarbital as indicated. Each point is the average ± S.D. of seven independent experiments. The mean values for the relative peak current amplitudes were 0.72 ± 0.05 ($n=7$) at 1 mM pentobarbital and 0.41 ± 0.04 ($n=7$) at 3 mM pentobarbital. (B) Dose–response curve for the relative off-current amplitude of the $\alpha_1\beta$ glycine receptor channel currents upon activation by glycine + pentobarbital as indicated. The relative off-current amplitude was calculated from the maximal amplitude of the off-current to the peak current amplitude. Each bar is the average ± S.D. of seven independent experiments.

3.4. Potentiation

In the next step, we investigated the effects of pentobarbital on currents elicited by a non-saturating concentration of 0.03 mM glycine. Fig. 3A shows representative current traces generated from application of 1 mM glycine (upper trace), 0.03 mM glycine (second trace) and 0.03 mM glycine + different concentrations of pentobarbital (lower traces) as indicated. It can be easily perceived that the peak current amplitude increased when 0.01 mM pentobarbital was added but decreased at higher pentobarbital concen-

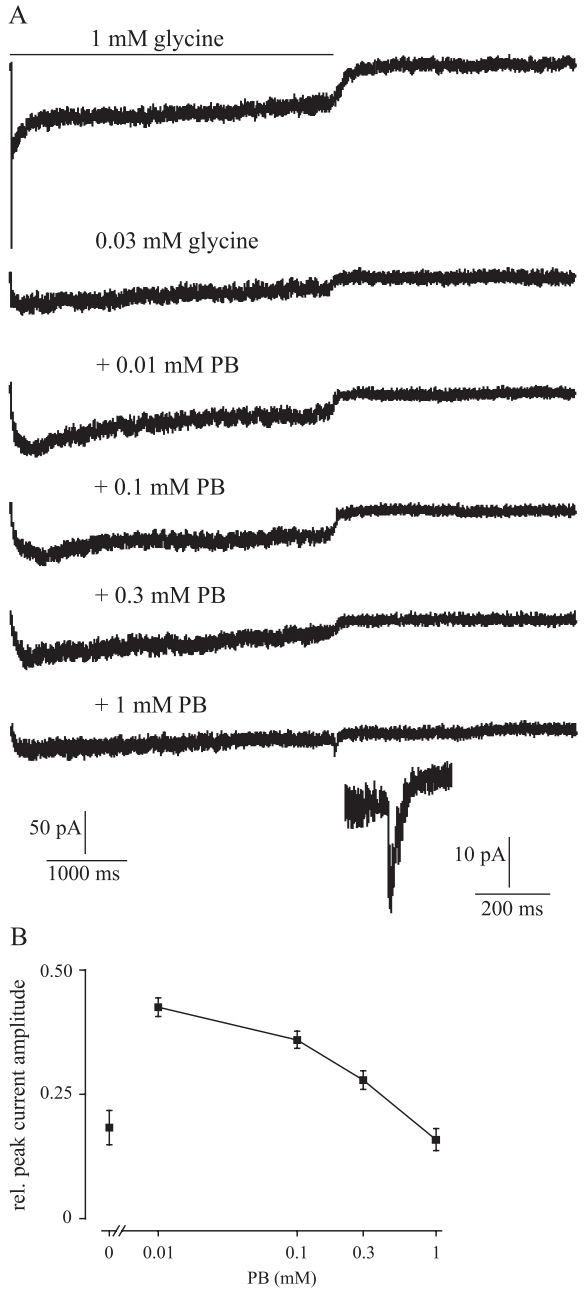

Fig. 3. (A) Current responses of $\alpha_1\beta$ glycine receptor channels recombinantly expressed on HEK293 cells to pulses of 1 mM glycine (first trace) or 0.03 mM glycine (second trace) or 0.03 mM glycine + different concentrations of pentobarbital (lower traces). Each trace is the average current of three to five single pulses. The bar above current traces indicates the time of application of the test solution (4-s pulses). The inset shows the off-current induced after coapplication of 1 mM pentobarbital. The outside-out patches from HEK293 cells were clamped to a holding potential of − 60 mV during the experiments. (B) Dose–response curve for the relative peak current amplitude of the $\alpha_1\beta$ glycine receptor channel currents upon activation by 0.03 mM glycine + pentobarbital as indicated. Each point is the average ± S.D. of three independent experiments. The value of the current amplitude elicited by 1 mM glycine is equal to 1. The amplitudes were normalized to the peak current amplitude induced by 1 mM glycine. The relative peak current amplitude by 0.03 mM glycine alone was 0.18 ± 0.03 (n = 3). In presence of 0.01 mM pentobarbital, the relative peak current amplitude was 0.42 ± 0.02 (n = 3). At 1 mM pentobarbital, it was 0.16 ± 0.02 (n = 3).

trations. Similar to the experiment of Fig. 1, an off-current after the end of a pulse of 0.03 mM glycine + 1 mM pentobarbital was observed, suggestive for a channel block mechanism (see inset of Fig. 3A). Fig. 3B shows the results of three independent experiments. The amplitudes were normalized to the peak current amplitude at 1 mM glycine. Glycine at a concentration of 0.03 mM elicited a relative amplitude of 0.18 ± 0.03 (n = 3). In presence of 0.01 mM pentobarbital, the relative peak current amplitude increased up to 0.42 ± 0.02 (n = 3), but with increasing concentrations of pentobarbital the relative amplitude decreased (Fig. 3B). At 1 mM pentobarbital, it was 0.16 ± 0.02 (n = 3) close to the value when 0.03 mM glycine was applied without pentobarbital.

3.5. Off-current

After the end of 4-s pulses of 1 mM glycine + pentobarbital ($\geq$ 1 mM), an off-current occurred, due to the transition from blocked to open states of the receptor after removal of pentobarbital (Fig. 1). Under physiological conditions of synaptic transmission, glycine is present at the postsynaptic membrane in high concentrations for a very short time period of some milliseconds. The kinetics of short pulses of 1 mM glycine parallel that of inhibitory postsynaptic currents (Legendre, 1998). To simulate synaptic transmission, short 2-ms pulses of 1 mM glycine were applied to

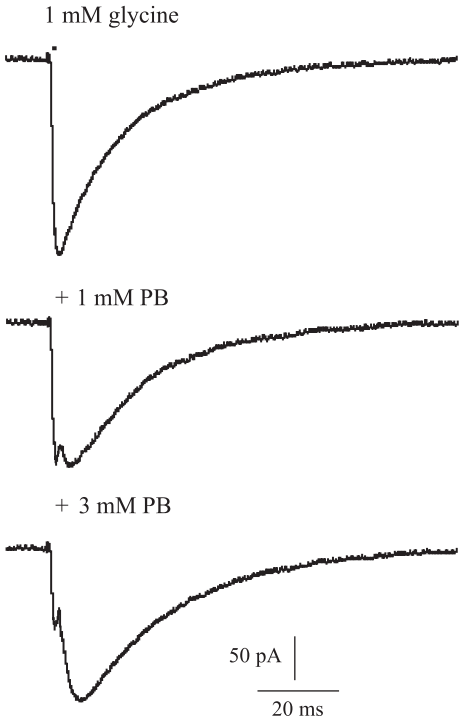

Fig. 4. Current responses of $\alpha_1\beta$ glycine receptor channels to short application (2 ms) of 1 mM glycine (upper trace) or 1 mM glycine + 1 mM or 3 mM pentobarbital (lower traces). The coapplication of pentobarbital elicited an off-current coupled an initial current (lower traces). The bar above current traces indicates the time of application of the test solution.

$\alpha_1\beta$ glycine receptor channels. The current activated by 2-ms pulses of 1 mM glycine reached the maximum current amplitude within 0.5 ms and decreased due to unbinding of glycine from the receptor (Fig. 4, upper current trace). The lower traces show currents elicited by 1 mM glycine + 1 or 3 mM pentobarbital as indicated. Two different effects were observed under this condition. (1) The peak current amplitude decreased depending on the pentobarbital concentration, and (2) an off-current occurred after cessation of the 2-ms pentobarbital pulses. The amplitude of the off-current reached values near the peak current amplitude elicited by 1 mM glycine alone. The time-dependent flow of the hyperpolarizing chloride current was calculated as the area under the current traces (pA ms). It was 44.2 pA ms at 1 mM glycine and it increased by 32% [33 ± 4% (n = 4)] to 58.4 pA ms and by 73% [69 ± 7% (n = 4)] to 76.8 pA ms at 1 and 3 mM pentobarbital, respectively.

4. Discussion

The pharmacological effects of pentobarbital at $GABA_A$ receptor channels are well known. It has different effects, first coactivation, when GABA is applied in low concentrations (Akaike et al., 1990), second a direct agonistic effect (Akaike et al., 1985, 1990; Rho et al., 1996; Akk and Steinbach, 2000; Serafini et al., 2000; Krampfl et al., 2002) and third it elicits a channel block at high concentrations (Akaike et al., 1990; Rho et al., 1996; Akk and Steinbach, 2000; Serafini et al., 2000; Krampfl et al., 2002; Eghbali et al., 2003). At glycine receptor channels, pentobarbital was shown to potentiate currents elicited by low glycine concentrations but has no agonistic effects (Pistis et al., 1997; Belelli et al., 1999). In the present study, we observed an additional channel block effect of pentobarbital at recombinant $\alpha_1\beta$ glycine receptor channels.

When pentobarbital was applied to outside-out patches containing recombinant glycine receptor channels, no current response was observed (data not shown), as recently shown (Pistis et al., 1997; Belelli et al., 1999). Coapplication of 0.01 mM pentobarbital with 0.03 mM glycine resulted in an increase of the current amplitude, corresponding to the previous studies (Pistis et al., 1997; Belelli et al., 1999). The current amplitude decreased at pentobarbital concentrations $\geq$ 0.1 mM, possibly due to channel block. When pentobarbital was applied in combination with 1 mM glycine, the peak current amplitude decreased with increasing concentrations (Figs. 1 and 2A) and a faster component of current decay occurred (Fig. 1). This decrease of the peak current amplitude combined with a faster current decay in the prolonged presence of the agonist and pentobarbital resembles observations at nicotinic acetylcholine channels when physostigmin (Bufler et al., 1996a), curare (Bufler et al., 1996b), ketamine (Scheller et al., 1996), midazolam (Hertle et al., 1997) or pentobarbital (Krampfl et al., 2000) was applied or pentobarbital to $GABA_A$ receptor channels (Krampfl et al., 2002). Because we observed no reduction of the current amplitude when glycine receptor channels were preincubated with 1 mM pentobarbital before application of the 1 mM glycine pulse (data not shown), a competitive blockade of the channels by pentobarbital could be excluded in our experiments.

Native as well as recombinant glycine receptor channels show complex kinetics of desensitization in prolonged presence of glycine with two or three time constants of desensitization with values between 10 and 3000 ms (Mohammadi et al., 2003; Legendre, 1998). The current decay after the end of short (2 ms) glycine pulse reflects unbinding of glycine from the receptor, deactivation (Mohammadi et al., 2003; Legendre, 1998). After short pulses of 1 mM glycine + pentobarbital we observed off-currents and the inhibitory chloride current flow through glycine receptor channels increased with time in presence of pentobarbital.

The occurrence of an off-current at the end of the coapplication of glycine and pentobarbital (as described above) might mean that pentobarbital washes out faster than glycine. This is surprising considering the lipophilic characteristics of pentobarbital. On the other hand, there might be different binding sites of pentobarbital at glycine receptor channels mediating potentiation and channel block. The occurrence of block only at mM concentrations as well as the fast rising phase of the off-currents could point to a low-affinity blocking site that might be localized within the hydrophilic region of the channel pore like it is known for channel block at $GABA_A$ receptor channels (Belelli et al., 1999; Akk and Steinbach, 2000). $GABA_A$ and glycine receptor channels are closely related members of the ligand-activated ion channel superfamily (Betz, 1990). In previous studies it was shown that the effects of pentobarbital at $GABA_A$ receptor channels, i.e. direct activation, coactivation and channel block, are mediated by the interaction of pentobarbital with specific binding sites (Dalziel et al., 1999; Serafini et al., 2000; Amin and Weiss, 1993; Pistis et al., 1997; Rajendra et al., 1997; Eghbali et al., 2003). Regarding the high degree of sequence homology especially within the M2 domain, i.e. the channel pore lining domain, which preserves the putative site of channel block, it is very likely that the block effect shown here corresponds to similar block effects known for pentobarbital at $GABA_A$ and nicotinic acetylcholine receptor channels (Dalziel et al., 1999; Serafini et al., 2000; Amin and Weiss, 1993; Pistis et al., 1997; Rajendra et al., 1997; Krampfl et al., 2000; Eghbali et al., 2003). Even coactivation of glycine receptor by pentobarbital resembles data on pentobarbital action at $GABA_A$ receptor channels (Serafini et al., 2000; Rajendra et al., 1997; Rho et al., 1996). Further studies on chimeras of $GABA_A$ and glycine receptor subunits might elucidate the structural differences between $GABA_A$ and glycine receptor channels accounting for the difference regarding the agonistic effect of pentobarbital on these receptors.

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