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Desensitization of α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic Acid (AMPA) Receptors Facilitates Use-Dependent Inhibition by Pentobarbital

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ABSTRACT

Although the mechanisms underlying the use-dependent inhibition of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) by barbiturates are not well understood, it has generally been assumed to involve open channel block. We examined the properties of the inhibition of AMPARs by the barbiturate pentobarbital (PB) in acutely isolated and cultured hippocampal neurons. PB caused a use- and concentration-dependent inhibition (IC_{50} = 20.7 μM) of AMPAR-mediated currents evoked by kainate. Contrary to the properties of an open channel blocker, the inhibition by PB developed with double exponential kinetics was reduced under conditions that favor the open channel state of AMPARs and was independent of membrane voltage. In addition, the inhibition was reduced at basic pH, indicating that the uncharged form of PB is active at AMPARs. Preventing AMPAR desensitization with cyclothiazide reduced the potency of inhibition by PB and prevented its trapping after the removal of agonist. PB preferentially reduced the steady-state (IC_{50} = 92.8 μM), rather than peak (IC_{50} > 1 mM) component of responses evoked by glutamate and accelerated the onset of desensitization in a concentration-dependent manner. Miniature excitatory postsynaptic currents recorded from cultured hippocampal neurons, the time course of which is minimally influenced by desensitization, are not inhibited by PB. The sensitivity of AMPAR-mediated synaptic responses to inhibition by PB therefore depends on the contribution of desensitization to these events. Our results suggest that PB does not act as an open channel blocker of AMPARs. Rather, the sensitivity, use dependence, and trapping of inhibition by PB are determined by AMPARs desensitization.

Glutamate mediates fast excitatory neurotransmission primarily by activating the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) subtype of excitatory amino acid receptors. AMPA receptors (AMPARs) are heteromultimeric complexes assembled from four subunits (GluR1–GluR4). The biophysical properties and pharmacological sensitivity of AMPARs are determined by their subunit composition. Among the AMPAR subunits, GluR2 is unique in that its transcripts undergo RNA editing, a process that results in a glutamine (Q) to arginine (R) substitution at a residue (586) located within the channel pore (Q/R site) (Sommer et al., 1991). AMPARs comprised of the edited form of the GluR2 subunit display low Ca^{2+} permeability (Burnashev et al., 1992), outwardly rectifying current-voltage relations (Verdoorn et al., 1991), insensitivity to block by organic polycations (Bowie and Mayer, 1995), and sensitivity to inhibition by barbiturates (Taverna et al., 1994; Yamakura et al., 1995).

Barbiturates represent an important class of therapeutic agents that are used for their sedative, anesthetic, and anticonvulsant properties. The properties and molecular basis for the block of AMPARs by barbiturates has been studied in both native and recombinantly expressed receptors. In both *Xenopus laevis* oocytes and hippocampal neurons, the GluR2 subunit was shown to render AMPARs more sensitive to the depressant effects of the barbiturate pentobarbital (PB) (Taverna et al., 1994; Joo et al., 1999). A study in cultured cortical neurons revealed that the inhibition of AMPARs by PB is use-dependent, an effect interpreted as being consistent with an open-channel blocking mechanism (Marszalec and Nara-

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ABBREVIATIONS: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; AMPAR, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; GluR, glutamate receptor; PB, pentobarbital; EPSC, excitatory postsynaptic current; NMDA, *N*-methyl-D-aspartate; mEPSC, miniature excitatory postsynaptic current; TM2, transmembrane domain 2; CTZ, cyclothiazide; Ctrl, control; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid).

hashi, 1993). However, use dependence of drug action does not necessarily imply occlusion of the open pore or preferential binding to the open state (Newland and Cull-Candy, 1992; Orser et al., 1997). For example, a drug that preferentially associate with or stabilizes an agonist-bound closed state (e.g., desensitized) would produce similar use-dependent actions. Nevertheless, a channel blocking mechanism is consistent with evidence suggesting that the editing status of the Q/R site, located within the pore-forming region of the GluR2 subunit, determines the sensitivity of AMPA receptors to block by PB (Yamakura et al., 1995).

Beyond gaining a greater understanding of this important class of therapeutic agents, the importance of determining the mechanism underlying the block of AMPARs by PB is highlighted by the recent use of this compound to determine the subunit composition of AMPARs underlying excitatory postsynaptic currents (EPSCs) (Liu and Cull-Candy, 2000). Indeed, if an agonist-bound desensitized rather than open state underlies use-dependent inhibition by PB, then the sensitivity of EPSCs may depend not only on receptor subunit composition (i.e., presence or absence of GluR2 subunits) but also on the relative contribution of desensitization to the time course of synaptic events.

In the present study, we examined the mechanisms underlying the block of AMPARs by PB in CA1 pyramidal neurons acutely isolated or cultured from rat hippocampus as well as from GluR2-null mutant (−/−) and wild-type (+/+) mice. Contrary to previous suggestion of an open channel blocking mechanism, our results indicate that the potency and use dependence of PB inhibition is due to stabilization and trapping in an agonist-bound desensitized state.

Materials and Methods

Preparation of Isolated CA1 Pyramidal Neurons. CA1 pyramidal neurons were isolated from hippocampal slices prepared from either Wistar rats (14–21 days old) or GluR2-null mutant (−/−) and wild-type (+/+) (greater than 6 weeks of age) mice as described previously (Wang and MacDonald, 1995). Briefly, rats were decapitated under halothane anesthesia and their brains quickly removed and placed in cold (4°C), oxygenated extracellular solution of the following composition: 140 mM NaCl, 5.0 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 20 mM PIPES, 25 mM glucose, and 0.0003 mM tetrodotoxin, pH 7.4, osmolarity 320–335 mOsM. Hippocampi were isolated from the whole brain and cut by hand into 500- to 1000-μm-thick slices using a razor blade. Slices were then incubated at room temperature in extracellular solution containing 1.5 mg/ml protease (type XIV; Sigma Chemical, Oakville, ON, Canada). After 30 min of enzymatic digestion, slices were rinsed free of enzyme and allowed to recover for at least 1 h in a recording solution composed of 140 mM NaCl, 5.4 mM KCl, 1.3 mM $CaCl_2$, 2 mM $MgCl_2$, 25 mM HEPES, and 33 mM glucose, pH 7.4, osmolarity 320–335 mOsM. For isolation of hippocampal neurons, a single slice was transferred to a 35-mm culture dish where the CA1 region was isolated and triturated using a fire-polished Pasteur pipette. Only neurons that retained their pyramidal shape, including major primary and several secondary dendritic processes were used for recordings.

Cell Culture. Cultures of hippocampal neurons were prepared according to previously described methods from 1- to 2-day-old pups born of heterozygous GluR2 (±) mice (Joo et al., 2001). Briefly, hippocampi obtained from each group of animals were dissociated by mechanical trituration and plated on poly-D-lysine-covered glass coverslip [neonates born of GluR2 (±)]. The resulting cultures were incubated in minimal essential media (Invitrogen, Carlsbad, CA), supplemented with fetal bovine serum (100 μg/ml; Invitrogen), inactivated horse serum (100 μg/ml; Invitrogen), and insulin (8 μg/ml crystalline bovine zinc insulin; Invitrogen), at 37°C in 7% carbon dioxide. Fluorodeoxyuridine (4 mg/ml; Sigma Chemical) and uridine (10 mg/ml; Sigma Chemical) were added on day 5 to inhibit glial cell proliferation. After the first week, the cultures were maintained in only minimal essential media and 100 μg/ml horse serum until the time of recording. The genotype of all mice used in these studies was confirmed by Southern blotting or polymerase chain reaction of tail genomic DNA.

Whole-Cell Recordings. The restricted size of the isolated CA1 pyramidal cell body and proximal dendrites (~30 μm), compared with the cultured neurons, allows for greater control of the membrane voltage (space-clamp), making them ideally suited for recording responses evoked by exogenously applied agonist. Whole-cell patch-clamp recordings, performed at room temperature, were obtained using patch pipettes pulled from borosilicate glass (1.5 mm diameter; WPI, Sarasota, FL) using a two-stage vertical puller (PP-83; Narashige, Greenvale, NY). Patch electrodes had a resistance of 2 to 5 MΩ when filled with an internal solution of the following composition: 140 mM CsF, 10 mM HEPES, 11 mM EGTA, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 2 mM tetraethylammonium, and 4 mM K_2ATP, pH 7.2–7.3; osmolarity 295–305 mOsM. Patched neurons were voltage-clamped at −60 mV, lifted from the bottom of the culture dish, and carefully positioned into the stream of solution flowing from a computer-controlled, step-motor driven, multibarreled perfusion system (SF-77B; Warner Instrument, Hamden, CT). The small size of the cell body and proximal dendrites, relative to the width of the square glass tubing used (500 μm), ensured the uniform application of agonist and drug solutions (concentration-clamp). In several such recordings the solution exchange time constant, estimated from a single exponential fit of the current induced by a change in $[Na^+]_o$ (Δ = 130 mM) during a prolonged application of kainate (100 μM), was 3.1 ± 0.3 ms (n = 8). The series resistance of our recordings was monitored using hyperpolarizing voltage steps (−5 to −20 mV) applied at regular intervals (~2 min). AMPAR-mediated currents were recorded after the rapid application of the agonists kainate or glutamate. When glutamate was applied, $MgCl_2$ (2 mM) or *dl*-2-amino-5-phosphonovaleric acid (50 μM; Sigma Chemical) was added to the recording solutions to block NMDA receptors. In addition, bicuculline methiodide (10 μM; Sigma Chemical) was used in all experiments to prevent the direct activation of $GABA_A$ receptors by PB. Currents were recorded using an Axopatch 1-B and data were filtered at 2 kHz, digitized and acquired using pClamp6 program (Axon Instruments, Inc., Union City, CA). All population data are expressed as mean ± S.E.M. Statistical significance was determined using the Student's paired t test.

Recording of mEPSCs from GluR2 (+/+) and (−/−). Whole-cell recordings were obtained from cultured hippocampal neurons derived from both GluR2 (+/+) and (−/−) mice. Patch electrodes were filled with an intracellular solution of the following composition: 70 mM CsF, 60 mM CsCl, 10 mM HEPES, 2 mM $MgCl_2$, 11 mM EGTA, 2 mM TEA, 1 mM $CaCl_2$, 4 mM K_2ATP, pH 7.2–7.3; osmolarity 295–305 mOsM. AMPAR-mediated miniature excitatory postsynaptic currents (mEPSCs), recorded in the presence of 0.5 μM tetrodotoxin, were pharmacologically isolated by including 10 μM bicuculline and 40 μM *dl*-2-amino-5-phosphonovaleric acid in the external solution. Miniature EPSCs were acquired (20 kHz) using the peak-based event detection routine of the SCAN program (Strathclyde Electrophysiology Software, Glasgow, UK). The threshold for event detection was set at ~10 pA (~3 times baseline noise), and spurious events were manually rejected after visual inspection of each acquired trace. The amplitude of mEPSCs was determined for 100 to 200 events recorded before and after drug application and averaged for each cell.

Results

Kinetics of the Inhibition of AMPAR-Mediated Currents by PB. The association of a channel blocker with a

single receptor-associated binding site should proceed as a bimolecular reaction leading to the formation of a drug-receptor channel complex. Accordingly, the time course of both the onset and recovery from block are predicted to proceed with single exponential kinetics. In addition, although the forward rate constant of blockade (k_{on}) should be linearly dependent on the concentration of drug, the reverse rate constant (k_{off}) should not. Thus, the first series of experiments was designed to test whether the kinetics of barbiturate inhibition of AMPAR-mediated responses evoked by kainate are consistent with those predicted for an open-channel blocker.

In isolated CA1 pyramidal neurons voltage-clamped at −60 mV, sustained inward currents elicited by the rapid application of 100 μM kainate were rapidly and reversibly depressed by the coapplication of PB (10–1000 μM; Fig. 1A). The concentration-inhibition relationship for PB was constructed by measuring the steady-state fractional inhibition of kainate-evoked response produced at each concentration of PB (3–1000 μM; Fig. 1D). Under these conditions, the IC_{50} value for PB was 20.7 ± 1.31 μM (n = 5) and the Hill coefficient was 0.90 ± 0.01 (n = 5), suggesting a single site of action on AMPARs.

The time course of inhibition by PB was best described by a double exponential function, suggesting the involvement of multiple distinct steps. The influence of drug concentration on both the fast and slow components of block was examined to determine whether either of these components represents open channel block (Bahring and Mayer, 1998). Although the fast and slow rates of onset increased with concentration, neither showed a linear dependence on drug concentration (Fig. 1, B and C). It should be noted that even the fastest rate of onset (i.e., ~30 s^{-1} at 1 mM corresponding to a time constant of ~33 ms) was 10-fold slower than the estimated speed of solution exchange (~3 ms), indicating that the deviation from linearity was not generated by the limitations imposed by our perfusion system. The relative contribution of the fast and slow component to the onset of inhibition varied with PB concentration (Fig. 1C), the slow component decreasing with increasing concentrations of PB. In contrast, both the fast and slow rates of recovery were independent of PB concentration. The values for $k_{off\text{-}fast}$ (1.66 ± 0.17 s^{-1}) and $k_{off\text{-}slow}$ (0.34 ± 0.05 s^{-1}) were estimated using the y-axis intercepts of the plots shown in Fig. 1, B and C.

We also tested whether the inhibition by PB could be observed in the absence of receptor activation. This was done

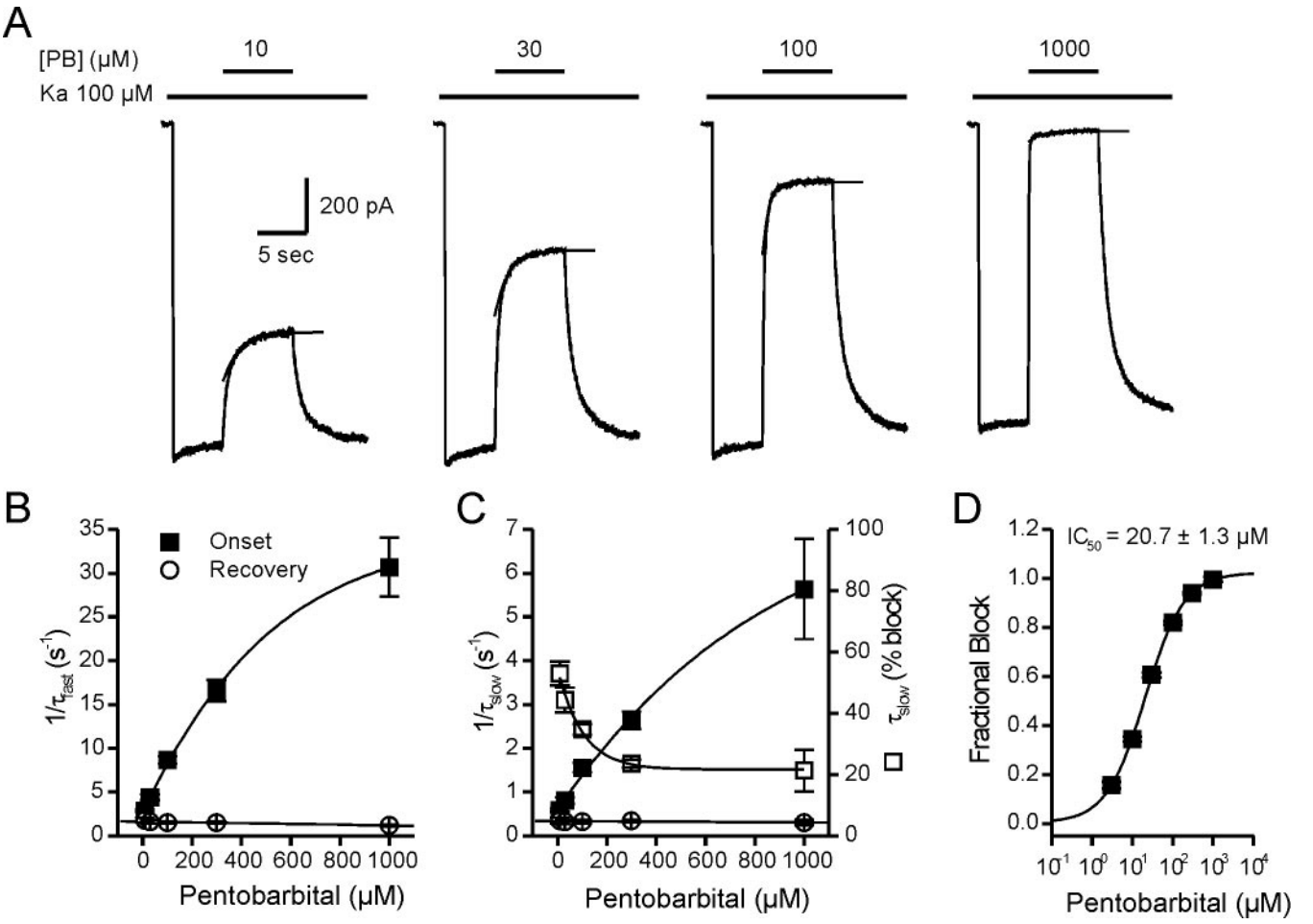

Fig. 1. Double exponential kinetics of block by PB. A, representative traces from a single cell demonstrating the blockade of kainate-evoked responses during a 7-s application of varying concentrations of PB. Onset and recovery from block were well fit using a double-exponential function. Lines drawn through the data points show the contribution of the slow component to the onset of block. B, rates for the fast component of onset (■) and recovery (○) from block ($1/\tau_{fast}$) plotted against the concentration of PB. C, rates for the slow component of onset (■) and recovery (○) from block ($1/\tau_{slow}$) are plotted against the concentration of PB as is the percentage of contribution of the slow component to the onset of block (□). D, concentration-inhibition relationship for PB block of kainate-evoked responses.

by preapplying PB for a prolonged period before the application of the agonist alone. In contrast to the rapid development of inhibition observed in the presence of agonist, applications of PB (50 μM) for 5 min in the absence of kainate produced minimal inhibition of the subsequent response to kainate (10.9 $\pm$ 2.2%, n = 3; data not shown). Thus, as reported previously, the prior association of the agonist and ensuing change in the conformation of AMPARs is required for the inhibition by PB to develop fully.

Dependence of the Degree of Blockade on the Concentration of Agonist. The degree of block produced by a fixed concentration of an open channel blocker is expected to increase with agonist concentration because this allows the blocker increased access to its binding site (Ascher et al., 1979; Chen et al., 1992). The influence of agonist concentration on the extent of blockade was determined by measuring the inhibition by 50 μM PB of currents evoked by increasing concentrations of kainate (Fig. 2, A and B). The fractional block by PB was increased by 13.5 $\pm$ 2.4% when agonist concentration was raised from 30 to 50 μM. However, when the agonist concentration was increased beyond 100 μM the inhibition was reduced. Thus, the fractional block displayed a biphasic dependence on the concentration of kainate.

The protocol illustrated in Fig. 2A allowed us to examine the effects of PB on the ability of kainate to evoke AMPAR responses. Consistent with the involvement of a mixed form of antagonism, 50 μM PB reduced the maximal response evoked by kainate and increased the EC_{50} value for kainate from 248.2 $\pm$ 18.7 to 367.5 $\pm$ 28.3 μM (Fig. 2C; n = 7, p = 0.0003).

pH Dependence of PB Effects. The potency of inhibition by PB is greatly increased by the presence of an arginine (R586, Q/R site) residue within the pore-forming TM2 region of the GluR2 subunit. Given that PB can exist in anionic form

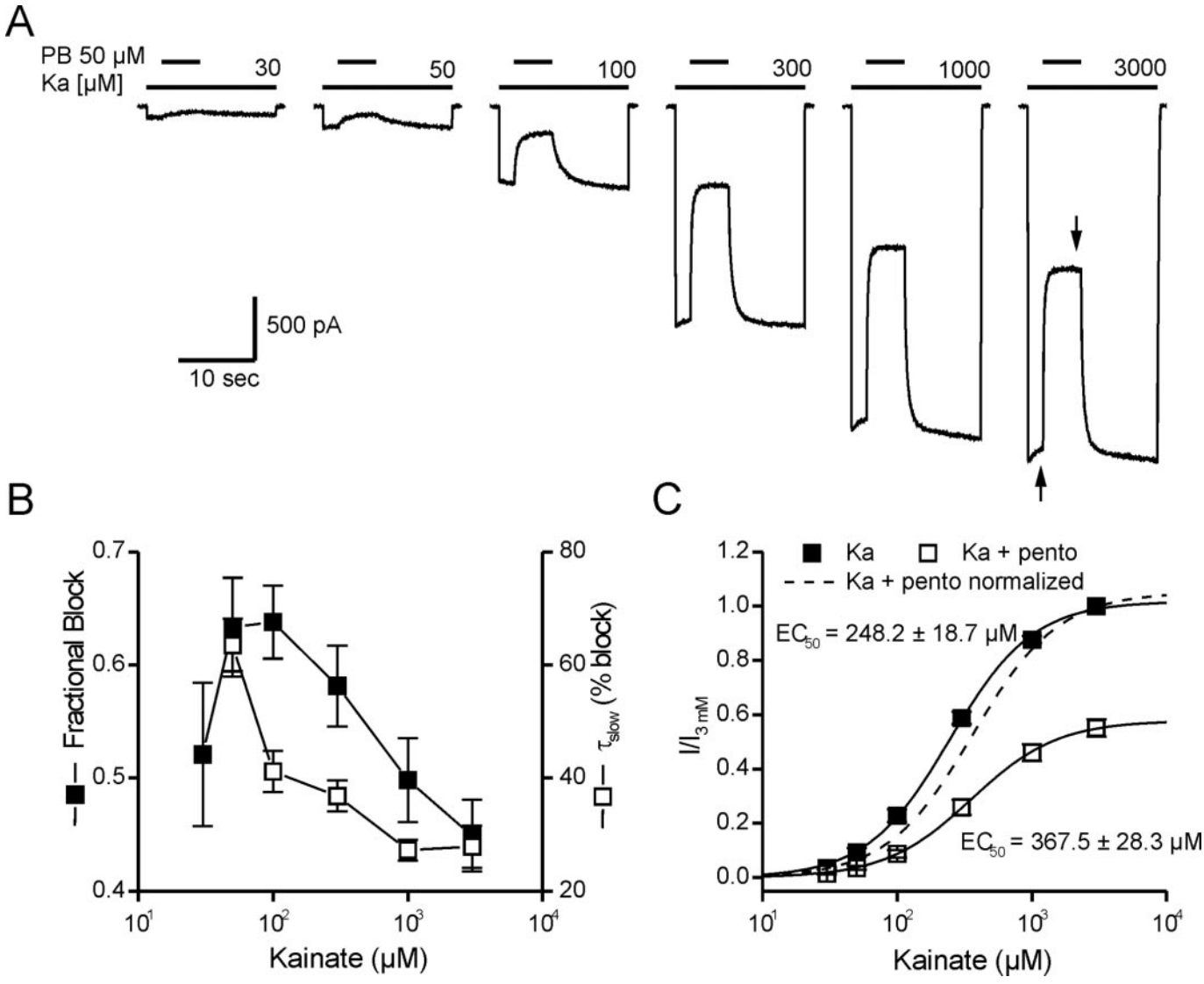

Fig. 2. Dependence of the degree of blockade on the concentration of agonist. A, illustrates the block by PB of responses elicited with increasing concentrations of kainate. Kainate response amplitudes were measured immediately before the start and end of PB applications as indicated by the arrows and normalized to the response elicited by 3 mM kainate in the absence of PB. B, fractional block [1 $-$ ($I_{PB}/I_{control}$)] by PB (■) displayed a biphasic dependence on the concentration of PB. The relative contribution of the slow component to the onset of block (□) decreased with increasing concentration of agonist. C, concentration-response relationship for kainate in the absence (■) or presence (□) of PB. Normalized response amplitudes were plotted against the concentration of kainate and fitted using the Hill equation. Dashed line represents the concentration-response curve for kainate in the presence of PB normalized to the amplitude of responses elicited by 3 mM kainate in the presence of PB.

when dissolved in an aqueous solution, it was suggested that PB binds to the positively charged R586 residue located within the pore of AMPARs (Yamakura et al., 1995). However, having a pK_a value of 8.0 (Narahashi et al., 1971), approximately 80% of PB exists in an uncharged form at physiological pH (7.4). Thus, one would expect inhibition by PB to increase under conditions that favor the charged form of the compound (i.e., basic pH). We tested the effects of increasing the extracellular pH on inhibition of AMPARs by 50 μM PB (Fig. 3). Over the pH range of 7.0 to 9.5, the charged form of PB increases from less than 10% to more than 95% (see PB titration curve in Fig. 3B). However, as the extracellular pH was increased the inhibition was reduced from 0.70 $\pm$ 0.02 (n = 7) to 0.16 $\pm$ 0.01 (n = 5, $p < 0.001$), suggesting that the uncharged form of PB inhibits AMPARs. The titration curve shown in Fig. 3B fits the experimental data well, although the derived pK_a (8.8) did not correspond to the reported pK_a of PB (8.0; Narahashi et al., 1971). This discrepancy may be due to the additional effects of pH on agonist binding and channel gating (Ihle and Patneau, 2000; Lei et al., 2000b).

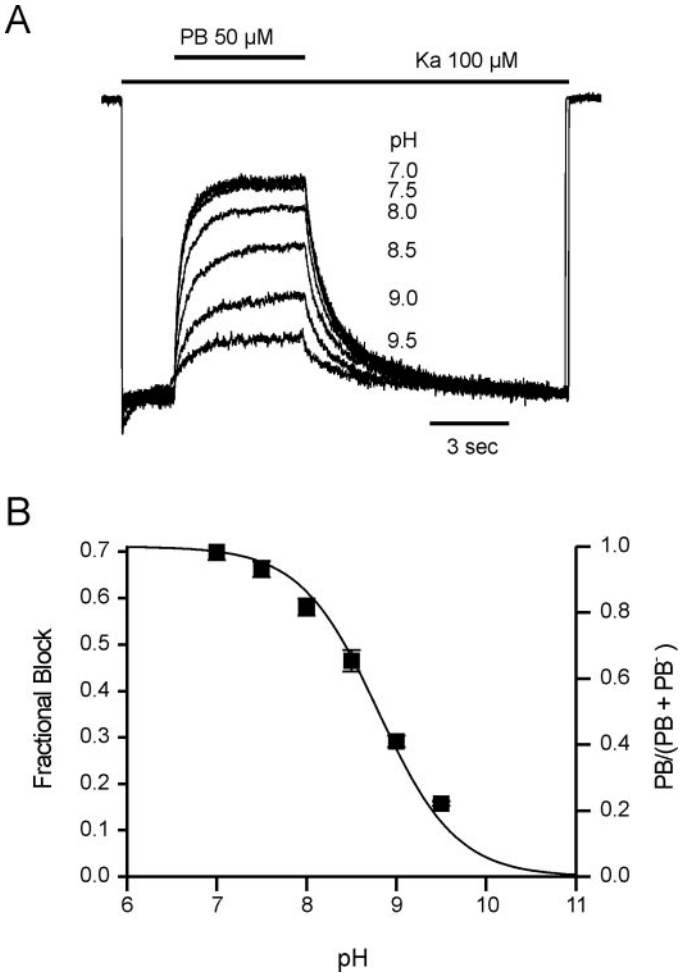

Fig. 3. Increasing extracellular pH reduces block by PB. A, superimposed traces illustrating the effect of varying the extracellular pH on the block of kainate-evoked responses by PB. The pH of all solutions applied to the cells was adjusted to the values indicated. B, plot of the fractional block by PB [$1 - (I_{PB}/I_{control})$] versus extracellular pH. The continuous line represents the titration curve for PB calculated using the function $y = 1/(1 + 10^{pH-pKa})$, where the value of pKa (8.8) was estimated by a nonlinear least-squares fit of the experimental data.

Cyclothiazide Prevents Trapping of PB after the Rapid Removal of Agonist. A common property of open channel blockers is their ability to remain bound or "trapped" to a closed state of the receptor after the unbinding of agonist (Lingle, 1983; Chen and Lipton, 1997). A trapping protocol (Fig. 4A; n = 5–8) was used to test whether PB remained trapped within AMPARs after the rapid removal of agonist. After allowing kainate-evoked currents to reach a steady state, PB was applied at a concentration (200 μM) that produces near maximal inhibition (88.7 $\pm$ 0.5%, n = 9). The block was allowed to proceed until a steady-state level of inhibition was reached, after which both kainate and PB were rapidly washed away. After variable periods of wash (30–300 s), the reapplication of agonist elicited an inward current that consisted of an initial fast component followed by a slow, biexponential return to the control current level. The initial fast component of the response (Fig. 4A, inset), presumed to represent the rapid reopening of previously unblocked receptors, was of similar amplitude as the current observed after inhibition by 200 μM PB. This indicates that a majority of receptors remained blocked despite continued wash. The slow component of the response represented the recovery from inhibition by PB. Figure 4B demonstrates that the majority of the receptors blocked after the application of PB remained so regardless of the duration of wash.

With the exception of its ability to trap, the results presented so far are not consistent with a mechanism of open channel block. We therefore speculated that the state-dependent actions of PB at AMPARs depend on the desensitized rather than opened state of the receptors. This possibility was first examined by studying the effects of cyclothiazide (CTZ), an inhibitor of AMPAR desensitization (Patneau et al., 1993; Partin et al., 1996), on the inhibition by PB.

Although kainate seems to evoke nondesensitizing AMPAR-mediated responses, desensitization nevertheless occurs but on a time scale that is too rapid to be resolved when whole-cell currents are elicited using a step-motor-driven agonist application system (Patneau et al., 1993). Consistent with this suggestion, application of 100 μM CTZ caused a greater than 10-fold enhancement (10.5 $\pm$ 1.9, n = 4) in the amplitude of kainate-evoked currents (not shown). Under these conditions, CTZ was found to abolish the trapping of PB such that near complete recovery from block was observed after brief (500-ms) washout periods (Fig. 4C). In addition, CTZ reduced the extent of inhibition by PB. Application of 500 μM PB, which previously caused a near complete block, now produced only a 71.2 $\pm$ 3.1% inhibition of the kainate-evoked currents in the presence of CTZ.

Given that glutamate induces more extensive desensitization of AMPARs compared with kainate, we therefore next examined the effects of CTZ on the PB inhibition of currents evoked by this agonist (Fig. 5). Application of 500 μM glutamate evoked large transient peak currents that decayed to a steady-state level (Fig. 5A). Coapplication of 100 to 300 μM PB during the steady-state component of the response caused 71.3 $\pm$ 5.3% inhibition. Treatment with 100 μM CTZ caused 7.4 $\pm$ 1.0-fold enhancement of the steady-state glutamate currents and reduced the block by PB to only 6.4 $\pm$ 0.7% (Fig. 5B; n = 4). Similarly, GT-21-005, a novel organic nitrate that reduces AMPAR desensitization (Toong et al., 2001), albeit less completely than CTZ, also reduced the inhibition of AMPARs by 100 μM PB (percentage of inhibition: Ctrl =

74.4 ± 1.1%; GT-21–005 = 56.0 ± 3.6%, n = 5; results not shown).

PB Accelerates the Onset of AMPAR Desensitization. We next examined the effects of PB on desensitization of AMPARs induced by glutamate. Desensitization by this agonist proceeds more slowly than with kainate, allowing us to examine the effects of PB on the transition of receptors to and from their desensitized state(s). Rapid applications of a near saturating concentration (3 mM) of glutamate evoked transient AMPAR currents (5425 ± 774 pA, n = 6) that rapidly desensitized to a steady-state level (773 ± 165 pA, n = 6). PB (3–1000 μM) was next added to both control and agonist containing solutions and several responses were recorded at each concentration to ensure that a steady-state level of inhibition had been achieved. PB was found to differentially affect the peak (I_{peak}) and steady-state (I_{SS}) components of glutamate-evoked responses (Fig. 6A). Although PB readily abolished I_{SS} (IC_{50} = 92.8 ± 15.3 μM), the maximal inhibition of I_{peak} was only 34.7 ± 3.2% (Fig. 6B; n = 6). Consequently, the I_{SS}/I_{peak} for the glutamate-evoked current (Ctrl: 0.137 ± 0.024, n = 6) was reduced by PB (1 mM; 0.012 ± 0.002, n = 6) in a concentration-dependent manner (Fig. 6C). Similarly, a concentration-dependent reduction in the time constant for the onset of desensitization (τ_{desen}) was observed in the presence of PB [Fig. 6C, τ_D (Ctrl) = 58.3 ± 8.3 ms; τ_D (PB 1 mM) = 16.6 ± 2.1 ms, n = 6]. In addition to facilitating the onset and extent of desensitization, PB produced a concentration-dependent increase in the 10 to 90% rise time of the AMPAR-mediated currents (Fig. 6D). This finding is consistent with the slight reduction in kainate potency by PB (Fig. 2B). In contrast, at concentrations of up to 300 μM, PB produced minimal changes in deactivation kinetics (Fig. 6, A and D, inset). Additionally, 50 μM PB did not affect the time course for recovery from desensitization (n = 5; not shown).

PB Inhibition of AMPARs Is Independent of Voltage. Steady-state AMPAR currents display outward rectification

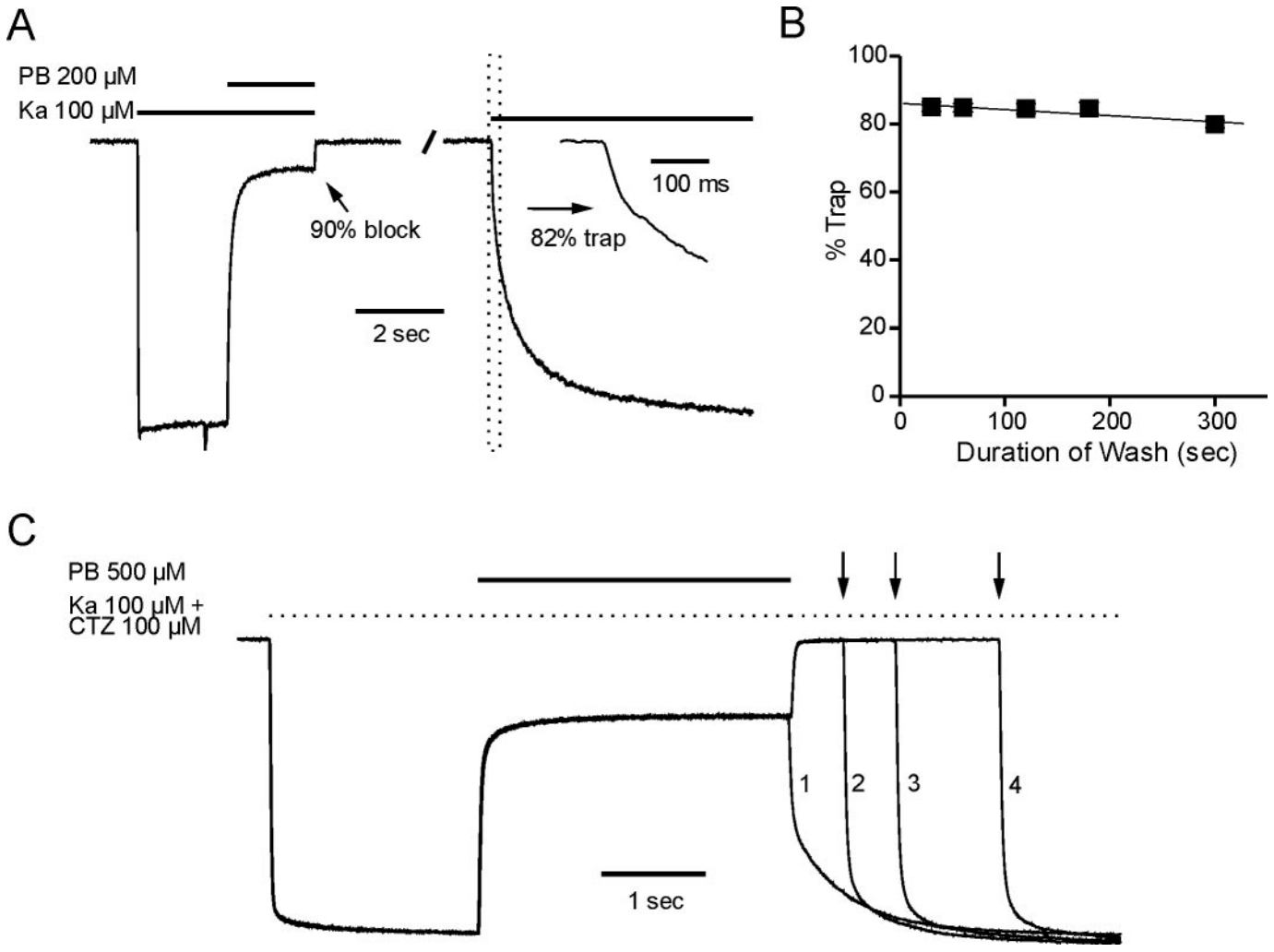

Fig. 4. In the absence of AMPAR desensitization, trapping of PB is abolished. A, representative traces demonstrating the protocol used to examine trapping of PB at AMPARs. After a 2-s application of PB (200 μM) in the continued presence of kainate, the cell was rapidly rinsed free of both PB and kainate. After variable periods of wash, reapplication of kainate elicited an initial fast response, followed by a slow recovery to control levels. Distinction between fast and slow components of recovery can be seen within the inset showing an expanded portion of the trace included within the dashed box. The amplitude of each component of the recovery from trap response (A_{fast}, A_{slow1}, and A_{slow2}) was determined from triple exponential fits of the currents evoked after reapplication of agonist. Percentage of trapping was calculated as $[(A_{slow1} + A_{slow2}/A_{fast} + A_{slow1} + A_{slow2})$/fractional block by 200 μM PB] × 100. B, summary graph illustrates the percentage of block remaining after variable periods of wash. C, superimposed traces of responses recorded in the presence of CTZ using trapping protocol as in A. In the presence of CTZ, trapping was not observed even with relatively short durations of wash consisting 0.5 (trace 2), 1 (trace 3), and 2 s (trace 4). For each trace, the timing of agonist application after wash is indicated by the arrows. The response recorded after removal of PB in the continued presence of kainate + CTZ (trace 1) is shown for comparison.

due to the voltage dependence of AMPAR desensitization (Patneau et al., 1993; Raman and Trussell, 1995). In contrast, the actions of PB on AMPAR responses evoked by kainate are reportedly independent of membrane voltage (Marszalec and Narahashi, 1993). We tested the effects of PB on AMPAR-mediated responses evoked by glutamate at membrane potentials ranging from −80 to +60 mV (Fig. 7). As previously demonstrated for GluR2-containing AMPARs, the current-voltage relationship for I_{peak} of glutamate-evoked currents was linear, whereas I_{SS} displayed prominent outward rectification (Fig. 7, B and C). Thus, I_{SS}/I_{peak} was strongly voltage-dependent, its value increasing with membrane depolarization (Fig. 7D). Consistent with the results presented in Fig. 6, 50 μM PB produced a relatively small reduction of I_{peak} (Fig. 7B, 16.7 ± 4.3% at −70 mV, $n = 4$) compared with I_{SS} (Fig. 7C; 42.5 ± 4.0% at −70 mV, $n = 4$). The reduction of I_{peak} and I_{SS} produced by PB was independent of membrane voltage. As a result, the I_{SS}/I_{peak} was reduced by ~30% at all potentials tested (Fig. 7D).

Reduced Potency of Block by PB in GluR2 (−/−) Neurons Is Associated with Accelerated Kinetics and a Reduced Contribution of the Slow Component of Block. We next examined the kinetics of PB inhibition of AMPARs in hippocampal CA1 neurons acutely isolated from GluR2 (+/+) and (−/−) mice. In neurons from GluR2 (+/+) mice (Fig. 8A) current relaxations induced by PB were best fit using a double exponential function, consistent with the results obtained in rat neurons. The rate of onset of both fast and slow components of block displayed a nonlinear dependence on the concentration of PB, whereas the rates of offset were largely independent of drug concentration (Fig. 8, B and C). As illustrated by the superimposed traces in Fig. 8A, the rate of onset and recovery from PB block was increased in neurons from GluR2 (−/−) mice. The rates of onset of the fast (Fig. 8B) and slow (Fig. 8C) components of block were independent of drug concentration, indicating that the binding of PB was no longer a rate-limiting step of the blocking reaction in GluR2-deficient AMPARs. Intriguingly, the fast and slow offset rates now demonstrated a slight inverse dependence on the concentration of PB (Fig. 8, B and C). Consistent with previous studies indicating that differences in the potency of a series of receptor antagonists, which can be estimated from $k_i = k_{off}/k_{on}$, can be attributed to differences in the rate of drug dissociation constants (MacDonald et al., 1991), comparison of the results obtained from GluR2 (+/+) and (−/−) mice revealed a much greater change in the rates of offset than onset (Table 1). In addition, the contribution of the slow component of block in GluR2 (−/−) AMPARs was reduced, especially at low concentrations of PB, suggesting that the slow component of block determines the high potency of PB for AMPARs.

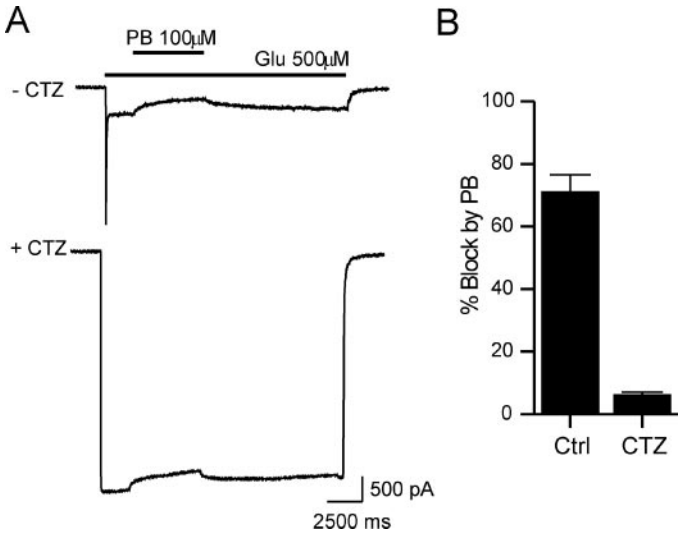

Fig. 5. Inhibition of glutamate-evoked currents by PB is reduced in the presence of CTZ. A, application of PB (100 μM) for 5 s inhibits steady-state currents evoked by glutamate (500 μM). Subsequent application of CTZ to the same cell blocked AMPAR desensitization and reduced the inhibition of glutamate-evoked currents by PB. B, bar graph of the percentage of block of glutamate-evoked steady state currents produced by PB (100 and 300 μM) in the presence and absence of CTZ.

Effects of PB on mEPSCs in Cultured Hippocampal Neurons from Both GluR2 (+/+) and (−/−) Mice. Finally, to examine the potential physiological actions of PB, we examined its effects on pharmacologically isolated AMPAR-mediated mEPSCs. Previous studies suggest that desensitization contributes little, if at all, to the time course of EPSCs at hippocampal synapses (Diamond and Jahr, 1995; Hjelmstad et al., 1999; Lei et al., 2000a). Therefore, if the sensitivity to inhibition by PB is increased by the entry of receptors into a desensitized state then PB should have minimal effects on mEPSCs. We tested this hypothesis in cultured hippocampal neurons from GluR2 (+/+) and (−/−) mice. Miniature EPSC amplitude was unaffected by the application of 100 μM PB to hippocampal cultured neurons from both GluR2 (−/−) and wild-type mice (Fig. 9A). The inability of PB to affect mEPSC amplitude might have arisen from the conditions underlying the activation of synaptic AMPARs (e.g., low transmitter release probability at a given synapse). Use of the GluR2 (−/−) neurons allowed us to compare the actions of PB with those of spermine, an open channel blocker of AMPARs devoid of the GluR2 subunit (Washburn and Dingledine, 1996). In contrast to the results obtained with PB, spermine readily reduced the amplitude of mEPSCs in GluR2 (−/−), but not GluR2 (+/+), cultured neurons (Fig. 9B). Thus, the inability of PB to affect mEPSCs provides further evidence that this compound does not act as an open channel blocker of AMPARs.

Discussion

In the present study, the mechanisms underlying the block of AMPARs by the barbiturate PB were investigated. Depression of kainate-evoked AMPAR-mediated currents by PB involved two kinetically distinct components. Neither the fast nor the slow kinetic components of inhibition displayed linear concentration dependence, implicating rate-limiting steps in the blocking reaction. PB increased the EC_{50} value for kainate, suggesting that at least one component of the block might involve a reduction in the potency of agonist. In addition, the findings that PB 1) accelerated the onset of desensitization (t_{desen}), 2) preferentially reduced the steady-state (I_{SS}) rather than peak (I_{peak}) component of glutamate-evoked currents, and 3) displayed a reduced potency in the presence of cyclothiazide, suggest that sensitivity to inhibition by PB is increased by AMPAR desensitization. Consistent with this suggestion, mEPSCs recorded in hippocampal neurons cultured from GluR2-null mutant and wild-type mice were insensitive to block by PB, presumably due the

minimal contribution of desensitization to the time course of these transient synaptic events.

Open Channel Block Does Not Underlie the Inhibition of AMPARs by PB. Previous studies have proposed that PB is an open channel blocker of AMPARs. This was based on evidence demonstrating that PB inhibition is use-dependent and sensitivity is reduced when the arginine (R586Q) present within the pore-forming TM2 region of the GluR2 subunit is replaced by glutamine. Such a mechanism implies a simple bimolecular association of the blocker with its channel associated binding site and therefore single exponential blocking kinetics is predicted. Because channel function is inhibited by occlusion of the channel pore and does not require conformational changes, the rate of onset of block should be linearly dependent on the concentration of blocker as demonstrated for a number of open channel blockers of AMPA, kainate, and NMDA receptors (Chen and Lipton, 1997; Bowie et al., 1998). In addition, given that channel opening allows greater access of the blocker to its binding site, the degree of blockade should increase with increased opened probability (Ascher et al., 1979; Chen et al., 1992). None of these predictions was borne out experimentally for the inhibition of AMPARs by PB.

The block of kainate-evoked AMPAR-mediated currents by PB was best described using a double exponential function. Multiple heteromeric complexes of AMPARs are expressed within hippocampal pyramidal neurons. The possibility that the double exponential kinetics reflect the actions of PB at distinct subpopulations of AMPARs is, however, unlikely. AMPARs from CA1 pyramidal neurons are composed predominantly of GluR1/R2 and GluR2/R3 heteromers (Wenthold et al., 1996). Previous studies have shown that AMPARs composed of these subunit combinations display nearly identical sensitivity to PB (Taverna et al., 1994) and should therefore have very similar blocking kinetics. In addition, multiple components of block are apparent even in receptors that lack the GluR2 subunit, further suggesting that subunit heterogeneity does not account for the kinetically complex behavior of the inhibition by PB.

The nonlinear concentration dependence of the rate of on-

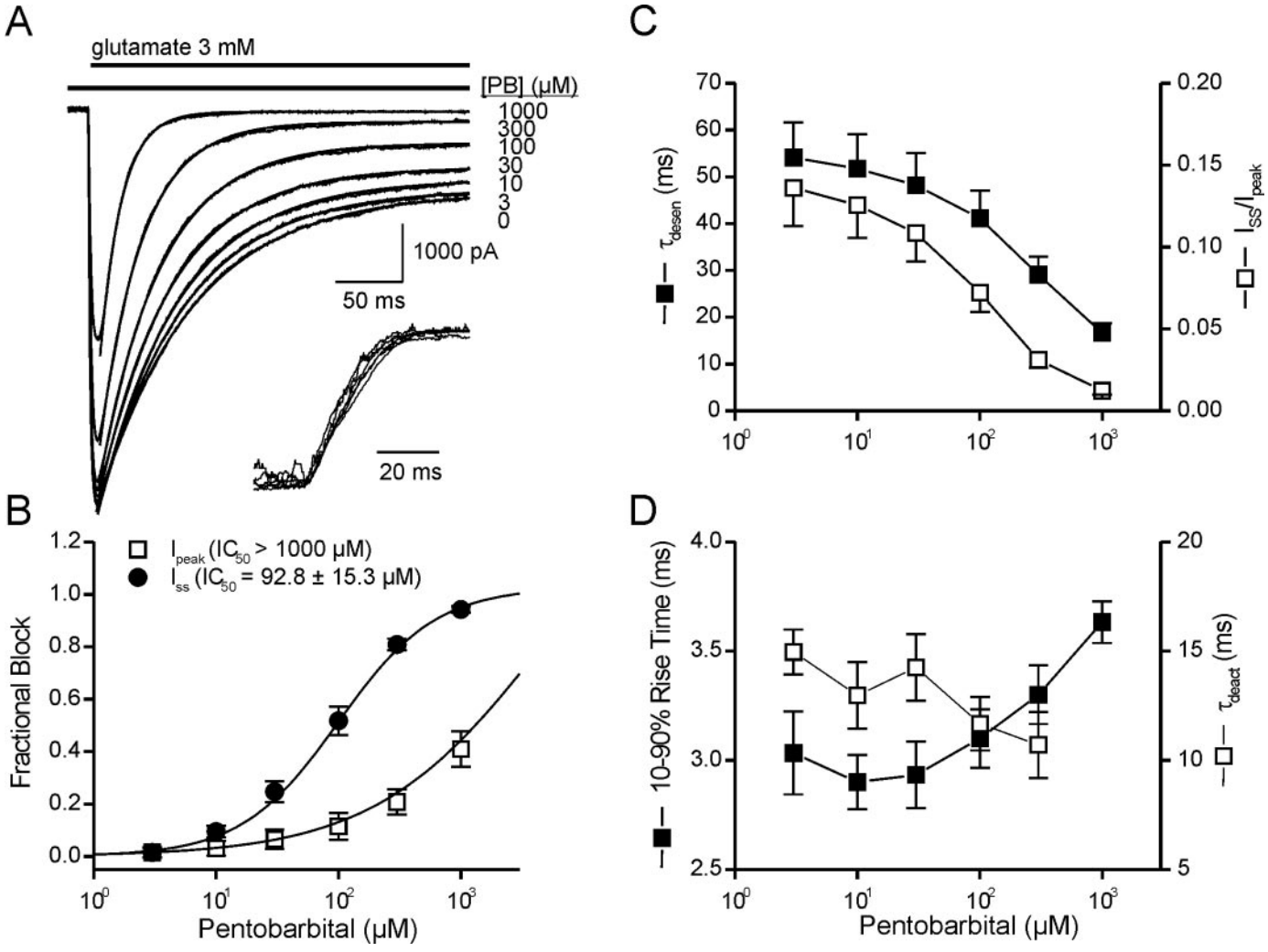

Fig. 6. PB facilitates AMPAR desensitization. A, sample traces showing the effects of increasing concentrations of PB (10–1000 μM) on AMPAR responses elicited by glutamate. Lines drawn through the traces are single exponential fits to the onset of desensitization. Inset shows deactivation responses upon termination of the glutamate application. In these experiments, PB was applied in both the extracellular and agonist-containing solution as indicated. B, concentration-inhibition analysis of the effects of PB on the I_{peak} (□) and I_{SS} (●) components of glutamate-evoked responses. The data were fit using the Hill equation. C, plots of the time constant of desensitization (τ_{desen}, ■) and I_{ss}/I_p, (□) ratio versus PB concentration. PB caused an increase in AMPAR desensitization as evidenced by the concentration-dependent reduction of both τ_D and I_{ss}/I_p. D, PB was also found to slow the 10 to 90% rise time (■) while causing a slight reduction in the time constant for deactivation (τ_{deact}, □).

set for both the fast and slow components of block implicates rate-limiting steps, possibly involving changes in receptor conformation, subsequent to the binding of PB. More complex multistate open channel blocking schemes could be considered to reconcile the complex kinetic behavior. However, the observed reduction in the degree of blockade with increasing concentration of agonist or by inhibiting AMPAR desensitization, both expected to increase channel openings, is contrary to the actions of an open channel blocker. Furthermore, it is difficult to reconcile the inability of PB to affect mEPSCs into any model of open channel block. This led us to consider the possibility that the use-dependent and trapping properties of PB may depend on an interaction of this compound with an agonist-bound, desensitized state of the receptor.

State-Dependent Block of Desensitized Receptor. Kinetic modeling studies have suggested that CTZ reduces desensitization of AMPARs by stabilizing an agonist-bound nondesensitized closed state and slowing the entry into a desensitized state (Partin et al., 1996). More recently, the molecular mechanism by which CTZ influences AMPAR gating was clarified by crystallographic experiments (Sun et al., 2002). CTZ was shown to bind within a pocket located at the interface between AMPAR subunit dimers and promote subunit dimerization, thereby preventing the conformational

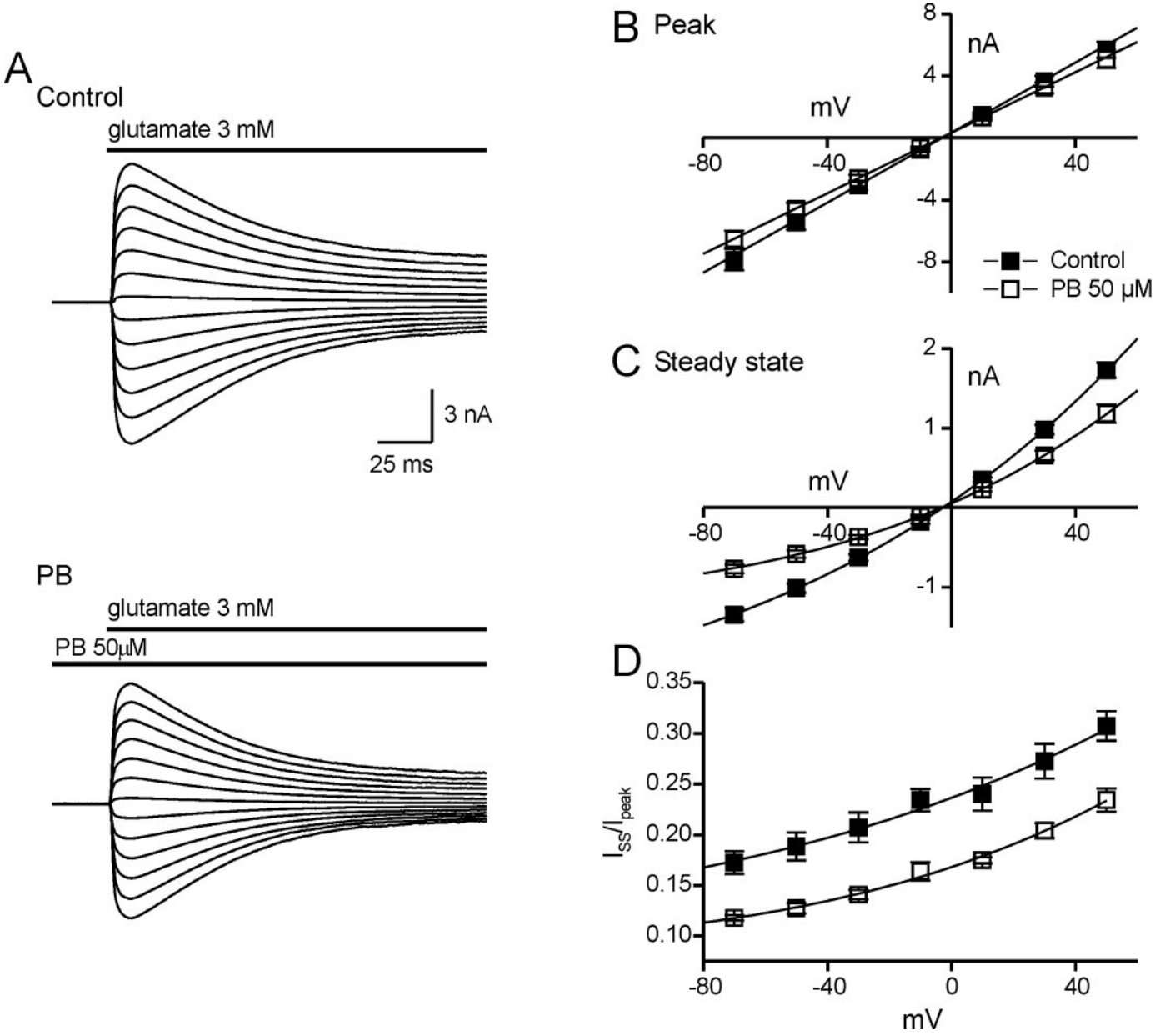

Fig. 7. The inhibition by PB is voltage-independent. A, representative traces demonstrating glutamate-evoked responses recorded at holding potentials of −70 to +50 mV before and after the application of PB to the same neuron. To ensure that equilibrium block was achieved, several responses were evoked in the presence of PB before repeating the voltage steps. B, linear current-voltage relation for I_{peak} of glutamate-evoked responses in the absence (■) and presence of PB (□). PB produced a slight reduction of I_{peak} at all holding potentials but did not alter the current-voltage relation. C, steady state current-voltage relation before (■) and after (□) the application of PB displays outward rectification due to more prominent desensitization at negative holdings. Although PB did cause a large reduction in the amplitude of I_{SS}, the current-voltage relation was not changed. D, The I_{ss}/I_{peak} ratio was increased as the holding potential was made more positive. Despite having caused a reduction in the I_{ss}/I_{peak} ratio, PB did not influence its dependence on voltage.

changes underlying desensitization. Consequently, the reduced potency and loss of trapping inhibition after treatment with CTZ suggests that each of these properties of PB requires the transition of AMPARs into a desensitized state. Moreover, abolished trapping indicates that the conformational change resulting from the interaction of CTZ with AMPARs must now allow PB to freely dissociate from a binding site of otherwise restricted access in the absence of agonist.

Although greatly reduced, partial inhibition of AMPARs by PB nevertheless occurred despite the presence of CTZ, suggesting that the association of PB does not require desensitization. Consequently, the use dependence of block results from some other agonist-induced conformational changes that allows PB to bind. Once associated with AMPARs, the acceleration of the rate of decay (τ_D) and preferential inhibition of I_{SS} suggests that PB stabilizes the desensitized state of the receptor where it then remains trapped after the removal of agonist. Computational modeling may help to resolve the potential receptor states underlying the multiple kinetic components of PB inhibition.

Role GluR2 Subunits in the Block of AMPARs by PB. Previous studies demonstrated that the sensitivity of AMPARs to PB (Taverna et al., 1994) is increased by the presence of an arginine (R) at position 586 (Q/R site) of the pore-forming TM2 region of the GluR2 subunit (Yamakura et al., 1995). At physiological pH, PB exists in either neutral or negatively charged form, whereas R586 is positively charged. Given the use dependence of PB inhibition, it is tempting to consider R586 as a critical residue underlying the "guarded" receptor binding site that only becomes accessible to PB after agonist binding. However, we find no evidence to support such an interaction. Indeed, the interaction of a charged compound to a binding site located within the channel pore would be expected to display voltage dependence, yet none was observed. Furthermore, the pH dependence of block indicated that the uncharged, rather than charged, form of PB is responsible for the inhibition of AMPARs. Thus, our re-

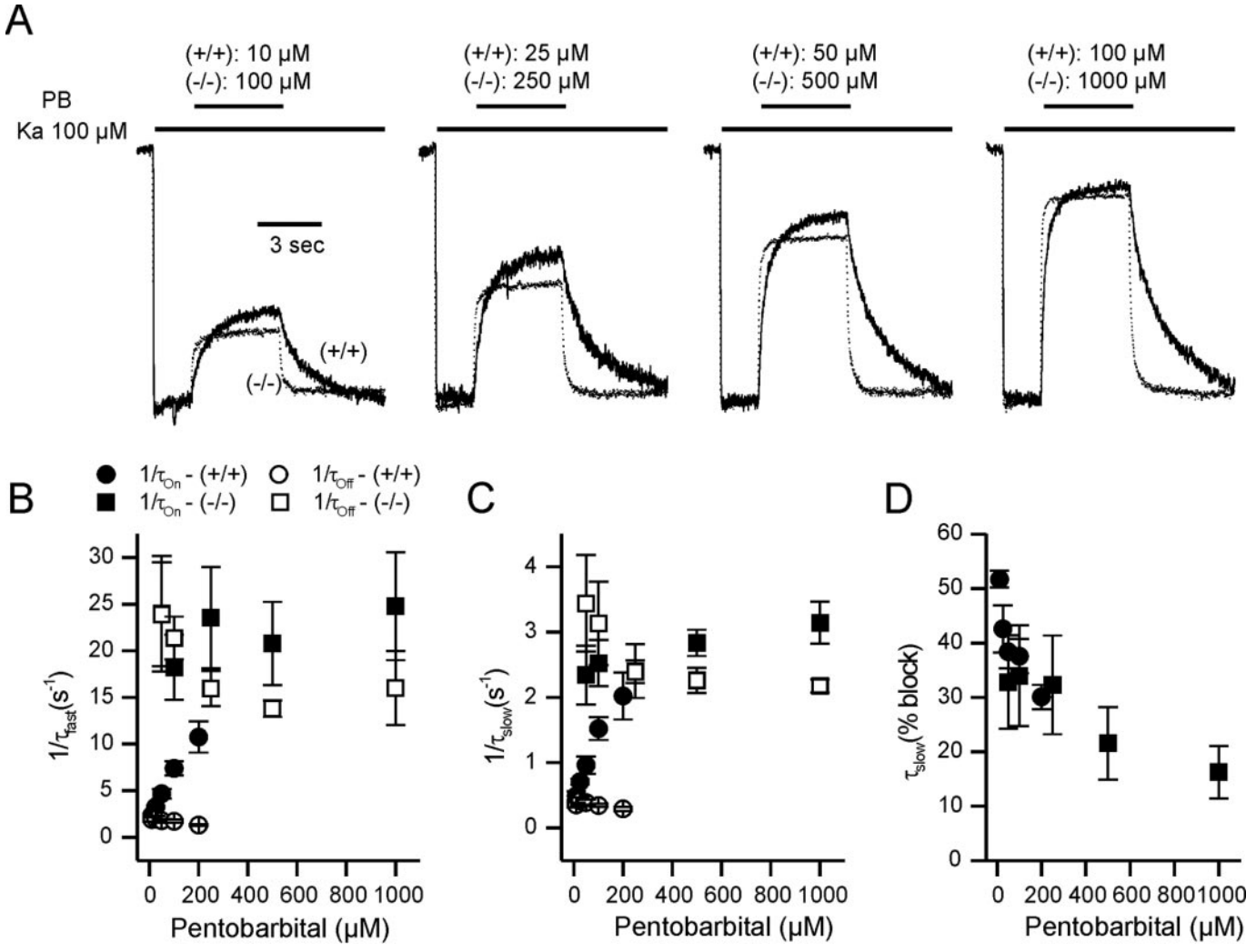

Fig. 8. Reduced block by pentobarbital in GluR2 (−/−) mice is associated with more rapid kinetics and a reduced contribution from the slow component of block. A, illustrative current traces demonstrating the reduced sensitivity to block by pentobarbital with corresponding accelerated kinetics in AMPARs lacking the GluR2 subunit. Solid and dashed traces represent responses recorded in isolated neurons obtained from (+/+) and (−/−) GluR2 mice, respectively. Note the 10-fold higher concentration of pentobarbital required in AMPARs from (−/−) mice to produce similar degree of block as in receptors from (+/+) mice. Onset and offset rates for the fast (B) and slow (C) components of block were obtained from double exponential fits of current traces and plotted against the concentration of PB. D, the relationship between the concentration of PB and the percentage of contribution of the slow component to the onset of block is plotted for responses from both (+/+) and (−/−) mice.

TABLE 1
Comparison of the onset and offset rates of the block of AMPARs by 100 μM PB in CA1 hippocampal neurons from GluR2 (+/+) and (−/−) mice

	GluR2 (+/+)	GluR2 (−/−)	Change
			%
$1/\tau_{on\text{-}fast}$ (s^{-1})	7.39 ± 0.76	18.23 ± 3.47	247
$1/\tau_{on\text{-}slow}$ (s^{-1})	1.52 ± 0.17	2.52 ± 0.35	166
$1/\tau_{off\text{-}fast}$ (s^{-1})	1.72 ± 0.17	21.37 ± 2.28	1242
$1/\tau_{off\text{-}slow}$ (s^{-1})	0.35 ± 0.03	3.13 ± 0.64	894

sults suggest that the dependence of the block by PB on the GluR2 subunit is not due to a direct interaction with a positively charged pore-lining residue.

Influence of Pore-Lining Residues on Ionotropic Receptor Desensitization. How then may pore-lining residues of the GluR2 subunit influence the state-dependent block of AMPARs by PB? Desensitization of ligand-gated ion channels is believed to involve an agonist-induced conformational change of the receptor to a nonconducting state. Recent studies have suggested, at least for the nicotinic acetylcholine (Auerbach and Akk, 1998) and NMDA receptors (Sobolevsky et al., 1999), the existence of distinct structures located within the ion permeation pathways that act as gates separately controlling receptor activation and desensitization. Regardless of whether such desensitization gates actually exists, studies of the nicotinic acetylcholine (Revah et al., 1991), 5-hydroxytryptamine$_3$ (Yakel et al., 1993) and $GABA_A$ (Gurley et al., 1995; Dibas and Dillon, 2000) receptors have shown that desensitization can be altered by point mutations within their respective pore-forming regions. In this respect, it is interesting to note that similar to PB, the picrotoxin block of $GABA_A$ receptors is use- but not voltage-dependent (Newland and Cull-Candy, 1992) and also can be reduced through mutations within the pore-forming M2 region of $GABA_A$ receptors (Gurley et al., 1995; Dibas and Dillon, 2000). Based on single channel recordings from dissociated sympathetic neurons, Newland and Cull-Candy (1992) similarly suggested that the properties of the block of $GABA_A$ receptors by picrotoxin were inconsistent with an open channel blocking mechanism. Rather, they proposed that the use dependence of picrotoxin is due to the stabilization of an agonist-bound nonconducting, possibly desensitized, state.

Given the numerous examples stated above, it seems likely that residues within the pore can similarly influence AMPAR desensitization. Thus, the reduced potency of PB observed in AMPARs lacking GluR2 (Taverna et al., 1994), as well as in those composed of GluR2 subunits with an arginine to glutamine mutations (Yamakura et al., 1995), may be attributed to the inability of these receptors to enter a desensitized state capable of being stabilized by PB binding. Such an interpretation is supported by the especially large increase in the rate of offset (~10-fold change) relative to that of the onset (~2-fold change) of block observed in the GluR2-null mutant mice.

Implications. Although PB reduces the amplitude of electrically evoked excitatory postsynaptic potentials, especially those elicited by repetitive stimulation (Winegar et al., 1988), it failed to influence the amplitude of spontaneous miniature events. This suggests that use dependence through a mechanism involving desensitization may allow greater selectivity of drug action, compared with open channel blockers, thereby allowing these agents to target rapidly firing populations of neurons during episodes of cerebral ischemia and seizure activity. Importantly, our finding of the insensitivity of mEPSCs to block by PB suggests that caution is necessary when interpreting the inability of PB to influence excitatory synaptic responses as evidence for a lack of GluR2 subunit involvement. Indeed, our results suggest that the sensitivity of synaptic responses to PB primarily depends on the contribution of receptor desensitization to the time course of synaptic events.

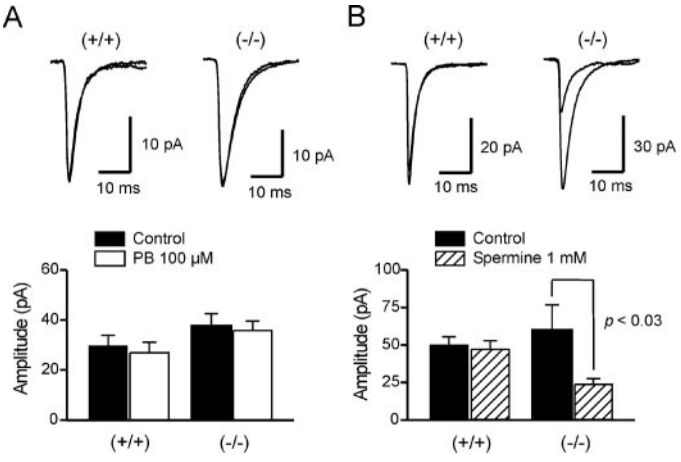

Fig. 9. Miniature EPSCs in cultured hippocampal neurons from both GluR2 (+/+) and (−/−) mice are unaffected by pentobarbital but can be readily blocked by spermine in GluR2 (−/−) cultures. A, representative traces and summary bar graph demonstrating the inability of bath applied 100 μM PB to influence the amplitude of mEPSCs recorded in cultures obtained from both GluR2 (+/+) and (−/−) mice. B, in contrast, spermine reduced the amplitude of mEPSCs in cultures from GluR2 (−/−) while having no effect in those from (+/+) mice.

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