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Effects on γ-Aminobutyric Acid $(GABA)_A$ Receptors of a Neuroactive Steroid That Negatively Modulates Glutamate Neurotransmission and Augments GABA Neurotransmission

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ABSTRACT

Neurosteroids positively and negatively modulate γ-aminobutyric acid $(GABA)_A$ receptors and glutamate receptors, which underlie most fast inhibition and excitation in the central nervous system. We report the identification of a neuroactive steroid, (3α,5β)-20-oxo-pregnane-3-carboxylic acid (3α5βPC), with unique cellular actions. 3α5βPC positively modulates $GABA_A$ receptor function and negatively modulates *N*-methyl-D-aspartate (NMDA) receptor function, a combination that may be of particular clinical benefit. 3α5βPC promotes net $GABA_A$ potentiation at low steroid concentrations (<10 μM) and at negative membrane potentials. At higher concentrations, the steroid also blocks GABA receptors. Because this block would presumably counteract the NMDA receptor blocking actions of 3α5βPC, we characterize the GABA receptor block in some detail. Agonist concentration, depolarization, and high extracellular pH increase the block. The apparent p*K* for both potentiation and block was 6.4 to 6.9, substantially higher than expected from carboxylated steroid in an aqueous environment. Block is not dependent on the stereochemistry of the carboxylic acid at carbon 3 and is relatively insensitive to placement of the carboxylic acid at the opposite end of the steroid (carbon 24). Potentiation is critically dependent on the stereochemistry of the carboxylic acid group at carbon 3. Consistent with the pH dependence of potentiation, effects of the amide derivative (3α,5β)-20-oxo-pregnane-3-carboxamide, suggest that the unionized form of 3α5βPC is important for potentiation, whereas the ionized form is probably responsible for block. Further refinement of the neuroactive steroid to promote GABA potentiation and NMDA receptor block and diminish GABA receptor block may lead to a clinically useful neuroactive steroid.

Neurosteroids have received recent wide attention because of their endogenous presence in the central nervous system at concentrations that may modulate GABAergic and/or glutamatergic synaptic communication (Baulieu, 1998; Concas et al., 1998). Synthetic analogs of endogenous neurosteroids may be clinically useful neuroprotectants, anesthetics, and anticonvulsants (Gasior et al., 1999; Zorumski et al., 2000). Generally, augmentation of GABAergic transmission and block of NMDA receptor-mediated transmission are associated with anesthetic, anticonvulsant, and antiexcitotoxic properties (Macdonald and Greenfield, 1997). Unfortunately, whereas many neuroactive steroids have activity at both of these receptor types, no known endogenous or synthetic steroid dampens NMDA receptor signaling without also inhibiting GABAergic signaling. These are opposing cellular effects with regard to the clinically desirable properties mentioned above.

Pregnane steroid derivatives with a sulfate or other negatively charged substituent at the carbon 3 (C3) position in the α-configuration are negative modulators of NMDA receptors through noncompetitive, voltage-independent block (Park-Chung et al., 1994). The β-configuration of the ring fusion at C5 is also important for blocking action, as β sulfate substitution at C3 retains NMDA receptor blocking activity if the steroid is 5β-reduced (Park-Chung et al., 1994; Weaver et al., 2000). Hemisuccinate and other hemiester α-substituents at C3 retain NMDA receptor blocking activity (Weaver et al., 1997, 2000). Unfortunately, the neuroprotective profile of each of these NMDA receptor antagonists is compromised by the fact that each of these derivatives also blocks $GABA_A$ receptor activity (Park-Chung et al., 1999). Additionally, whereas (3α,5β)-3-hydroxypregnan-20-one hemisuccinate, the hemisuccinate analog of naturally occurring preg-

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ABBREVIATIONS: GABA, γ-aminobutyric acid; NMDA, *N*-methyl-D-aspartate; C3, carbon 3; 3α5βPC, (3α,5β)-20-oxo-pregnane-3-carboxylic acid; IPSC, inhibitory postsynaptic current; 3α5βPS, 3α-hydroxy-5β-pregnan-20-one sulfate; 3α5βPA, (3α,5β)-20-oxo-pregnane-3-carboxamide; EPSC, excitatory postsynaptic current; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid; I/V, current/voltage.

nanolone sulfate, has been shown to be neuroprotective (Weaver et al., 1997), this analog is subject to hydrolysis of the hemisuccinate group.

We report here the cellular characterization of a novel neurosteroid analog, (3α,5β)-20-oxo-pregnane-3-carboxylic acid (3α5βPC), with both NMDA antagonist and GABA-potentiating actions. We synthesized this steroid as a nonhydrolyzable analog of (3α,5β)-3-hydroxypregnan-20-one hemisuccinate (Weaver et al., 1997). Like the hemisuccinate, at physiological pH the carboxylic acid group of 3α5βPC should be largely deprotonated, making 3α5βPC a likely NMDA receptor blocker. However, like GABA-potentiating steroids, which contain a 3α-hydroxyl group as a hydrogen bond donor (Phillipps, 1975), the —COOH of un-ionized 3α5βPC is a similarly located hydrogen bond donating group, and therefore un-ionized 3α5βPC might be expected to potentiate GABA receptors.

3α5βPC exhibits complex actions on $GABA_A$ receptors, with potentiation, voltage-dependent block, and direct gating of $GABA_A$ receptors. At low concentrations of drug and at physiological pH, net potentiation of $GABA_A$ receptor function and IPSCs are observed. At higher concentrations, the postsynaptic $GABA_A$ potentiation is decreased by steroid-induced block of receptor function. Block shows little stereoselectivity and is relatively insensitive to placement of the carboxylate at either end of the steroid ring structure. The pH dependence of both block and potentiation by carboxylated steroids suggests a higher apparent pK than expected of these organic acids in water. This probably reflects the influence of membrane/protein constituents on the pK of the carboxylate group.

Materials and Methods

Hippocampal Cultures. Primary hippocampal microcultures were prepared from 1- to 3-day-old postnatal albino rats using established methods (Mennerick et al., 1995). Under halothane anesthesia, rats were decapitated, and the hippocampi were dissected and cut into 500-μm-thick transverse slices. The slices were dissociated with 1 mg/ml papain in oxygenated Leibovitz L-15 medium and mechanically triturated in modified Eagle's medium containing 5% horse serum, 5% fetal calf serum, 17 mM D-glucose, 400 μM glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin. Isolated cells were plated (75 cells/mm^2) onto plastic culture dishes coated first with 0.15% agarose then with atomized droplets of rat tail collagen. To halt glial proliferation, cultures were treated with 10 μM cytosine arabinoside after 3 days in vitro. Experiments were performed in cultures that were 4 to 14 days old.

***Xenopus laevis* Oocytes.** Stage V-VI oocytes were harvested from sexually mature female *X. laevis* (Xenopus One, Northland, MI) under 0.1% tricaine (3-aminobenzoic acid ethyl ester) anesthesia. Oocytes were defolliculated by shaking for 20 min at 37°C in collagenase (2 mg/ml) dissolved in calcium-free solution containing: 96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$ and 5 mM HEPES at pH 7.4. Capped mRNA, transcribed in vitro (mMessage mMachine; Ambion, Austin, TX) from linearized plasmids containing receptor-coding regions, were injected into oocytes 24 h after defolliculation. Oocytes were incubated for up to 2 weeks at 18°C in ND96 medium containing 96 mM NaCl, 1 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, and 10 mM HEPES at pH 7.4 supplemented with 5 mM pyruvate and the above-mentioned antibiotics. The cDNAs for the $GABA_A$ receptor subunits were provided by A. Tobin [University of California, Los Angeles (α1)], P. Malherbe [Hoffman-La Roche, Switzerland (β2)], and C. Fraser [National Institute on Alcohol Abuse and Alcoholism, Bethesda, MD (γ2L)].

Electrophysiology. For synaptic studies, the growth medium was exchanged for a solution containing 138 mM NaCl, 4 mM KCl, 10 mM HEPES, 10 mM D-glucose, 2 mM $CaCl_2$, and 1 mM $MgCl_2$, pH 7.25. Whole-cell voltage-clamp recordings of autaptic currents were performed from neurons on single-neuron islands using recording pipettes with open tip resistances of 2 to 5 MΩ. The pipette solution contained 140 mM KCl, 4 mM NaCl, 5 mM EGTA, 0.5 mM $CaCl_2$, 10 mM HEPES, pH 7.25. Neurons were recorded using an Axopatch 1-D patch-clamp amplifier (Axon Instruments, Foster City, CA), and series resistance (<10 MΩ) was compensated 90 to 100% during experiments. Synaptic transmission was activated by stimulating neurons with 1.5-ms voltage steps from −70 to +30 mV at intervals of 20 to 30 s. During experiments, microislands were continuously superfused locally using a gravity-driven multibarrel pipette with a common exit port. The tip of this local perfusion system was placed ~400 μm from the microisland being recorded, and solution flowed at a rate of 0.1 to 0.5 ml/min. All recordings were done at room temperature (~22°C) on the stage of a Nikon inverted microscope equipped with phase-contrast optics. Studies examining exogenous applications of agonists were performed using whole-cell recordings as described above, except the pipette solution contained 140 mM CsCl in place of KCl. Experiments examining low-Mg^{2+}-induced action potentials (Fig. 1F) were performed in the current-clamp mode of the patch amplifier on islands containing small networks of neurons.

For oocyte recordings, experiments were performed with a virtual ground, two-electrode voltage clamp using a Dagan CA-1B amplifier 1 to 10 days after RNA injection. The extracellular recording solution was ND96 medium (with no supplements). Intracellular recording pipettes were filled with 3 M KCl and had open tip resistances of ~1MΩ. Drugs were applied from a common tip via a gravity-driven multibarrel drug-delivery system. Acetic acid and NaOH were used to adjust the pH of extracellular solutions.

For synaptic studies, averages of two to eight traces per experimental condition were used for analysis and display. Currents were filtered at 1 to 5 kHz using a four-pole Bessel filter and were digitized using pClamp, version 6.0 (Axon Instruments). Data were analyzed off-line using the pClamp software. Unless otherwise noted, results represent mean ± S.E.M. Statistical differences were determined using two-tailed t tests.

Drugs. Unless otherwise stated, drugs were from Sigma (St. Louis, MO). Pregnenolone sulfate and (3α,5β)-pregnan-20-one sulfate (3α5βPS) were obtained from Steraloids (Newport, RI) and from Sigma. The 3β5βPS was prepared as described elsewhere (Park-Chung et al., 1997) and was the generous gift of Dr. Robert H. Purdy (Scripps Research Institute, La Jolla, CA). A preliminary account of the synthesis of 3α5βPC and 3β5βPC has been published (Zeng et al., 1999). Full synthetic details will be published elsewhere. The methyl ester of 3α5βPC was prepared by reacting the acid with diazomethane dissolved in ether. The (3α,5β)-20-oxo-pregnane-3-carboxamide (3α5β PA) was prepared from 3α5βPC by converting this carboxylic acid to the acid chloride and then reacting this intermediate with ammonia dissolved in methylene chloride.

Results

Alteration of Synaptic Activity by 3α5βPC. Figure 1A shows the structure of 3α5βPC. 3α5βPC selectively depressed the slow NMDA receptor component of EPSCs in hippocampal neurons, consistent with the effect of other neuroactive steroids with a charged moiety in the α-configuration at C3 (Park-Chung et al., 1997). 3α5βPC (20 μM) produced only 8 ± 5% depression of the NMDA component (N = 3; data not shown), whereas 50 μM 3α5βPC produced 20 ± 4% depression (N = 9; Fig. 1, B and C). Effects on the fast α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor component of EPSCs were negligible with 8 ± 5% potentiation produced at 50 μM (Fig. 1, B and C).

To explore the effects of 3α5βPC on GABA transmission,

we examined the effect of 3α5βPC on GABAergic IPSCs in solitary hippocampal neurons. Figure 1D shows the effect of 3α5βPC on GABAergic IPSCs over a range of concentrations. Clear potentiating effects were observed at moderately low concentrations, making 3α5βPC the first described neuroactive steroid to both block NMDA receptor-mediated neurotransmission and potentiate GABAergic neurotransmission. In 10 of 14 neurons examined with 50 μM 3α5βPC, the peak amplitude of the IPSC was reversibly increased by >25% (106 ± 52% increase; $N = 14$). However, because of variabil-

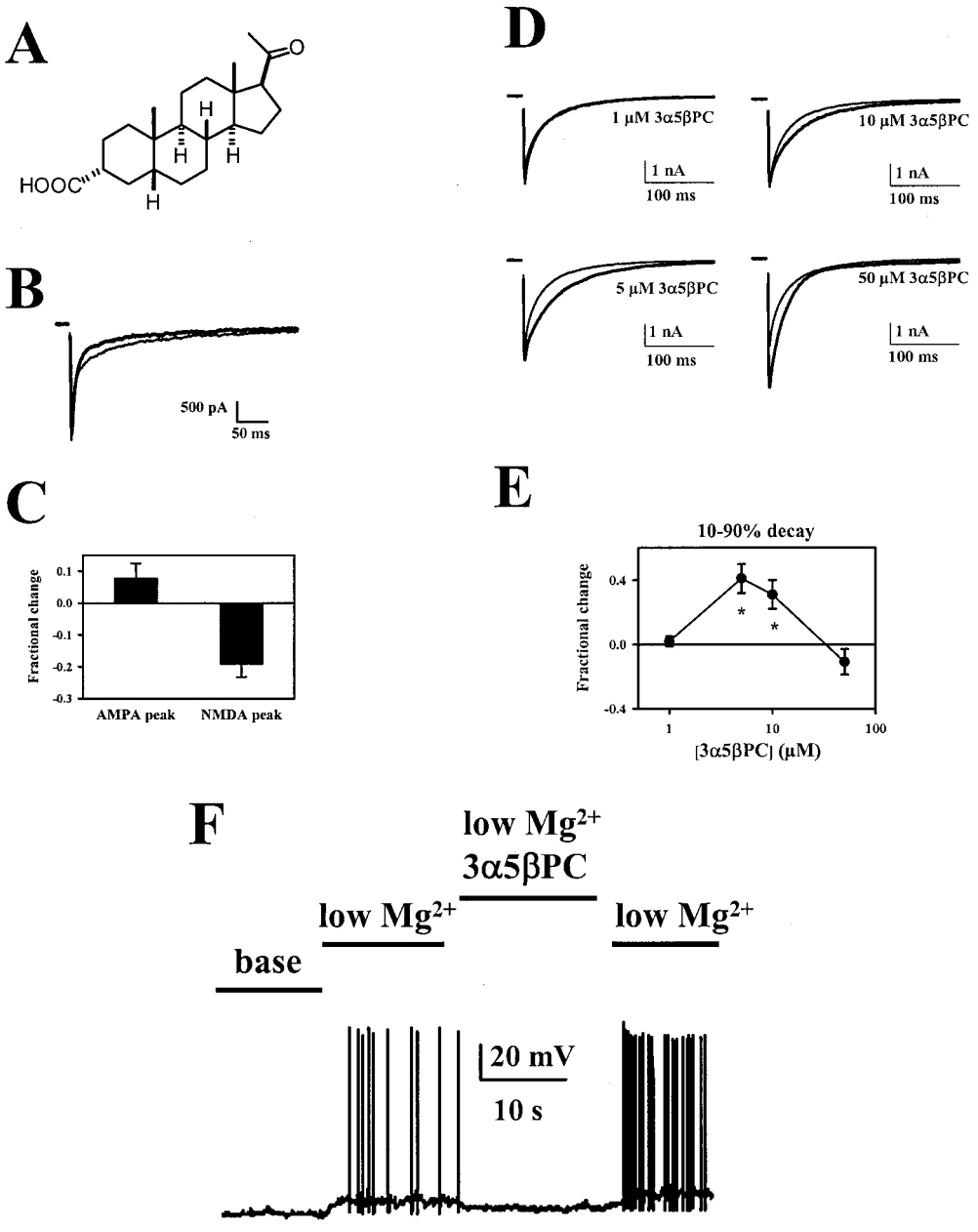

Fig. 1. Synaptic effects of 3α5βPC. A, structure of 3α5βPC showing the pregnane core and stereochemistry of the C3 and C5 substituents. B and C, 3α5βPC selectively depressed the slow NMDA receptor component of EPSCs. B, autaptic EPSCs were elicited from solitary glutamatergic neurons in microcultures with a 2-ms voltage pulse to 0 mV (from a holding potential of −70 mV). The extracellular bath solution contained no added Mg^{2+} and 10 μM glycine to unmask NMDA receptors. The traces represent average responses obtained in the absence (thin trace) and presence (thick trace) of 50 μM 3α5βPC. Note that the peak EPSC was not affected by the drug, but the slow NMDA component was selectively diminished. Transient currents associated with stimulation have been blanked in this and subsequent figures for clarity. C, summary of the effects of 50 μM 3α5βPC on peak EPSC and the NMDA component (measured 30 ms after stimulation) in nine neurons. D and E, concentration-response effects of 3α5βPC on hippocampal IPSCs. D, top to bottom, represent the effect of 3α5βPC on an IPSC from a solitary GABAergic cell (all from the same cell). In all cases, the thick trace represents the IPSC in the presence of drug, whereas the thin trace represents the IPSC in the absence of drug. As noted in the text, the effect on peak amplitude seen with 50 μM 3α5βPC was variable from cell to cell. E, summary data representing the concentration dependence of 3α5βPC on IPSC 10 to 90% decay time. Asterisks denote significantly different than zero ($p < 0.05$, two-tailed t test). The values for the 10 to 90% decay times from which the normalized data in Fig. 1E were derived were baseline, 114 ± 13 ms; 1 μM 3α5βPC, 105 ± 10 ms; 5 μM 3α5βPC, 143 ± 10 ms; 10 μM 3α5βPC, 132 ± 7 ms; and 50 μM 3α5βPC, 101 ± 16 ms. F, in a current-clamp recording from a small network of neurons grown in a microculture, action potential activity was increased by removing extracellular Mg^{2+} and adding glycine to potentiate NMDA receptor activity (low Mg^{2+}). 3α5βPC (5 μM) reversibly eliminated the firing. The apparent increase in firing upon removal of drug in this cell was not consistently observed in other neurons.

ity in the magnitude of this effect, changes in the peak amplitude reached only trend-level significance ($p = 0.07$; $N = 14$). The more prominent effect of the steroid was the prolongation of IPSC decay time course (Fig. 1D), similar to actions of other neuroactive steroids and other $GABA_A$ potentiators. However, the effects of 3α5βPC on IPSC decays decreased at higher concentrations, yielding a bell-shaped concentration-response relationship (Fig. 1E). At concentrations >10 μM, the prolongation of IPSCs by 3α5βPC became less apparent, such that effects of 50 μM 3α5βPC were not significantly different than in the absence of drug (Fig. 1E).

In all neurons tested, regardless of whether they exhibited a glutamatergic or GABAergic autaptic postsynaptic current, higher concentrations of steroid gated a steady inward current probably resulting from direct gating of $GABA_A$ receptors by 3α5βPC, a common effect of GABA-potentiating neuroactive steroids (Majewska, 1992). In five GABAergic cells, the respective concentrations of 5, 10, and 50 μM 3α5βPC gated currents of 15 ± 8, 33 ± 14, and 56 ± 26 pA. Although we did not study the directly gated current in detail, it is possible that this tonic activation of $GABA_A$ receptors could be physiologically and clinically significant (Bai et al., 2001).

To verify that the net cellular effect of 3α5βPC is indeed inhibitory, we examined the effect of 3α5βPC on action potential firing activity in small groups of hippocampal neurons grown in microcultures. Firing within networks was induced by lowering extracellular Mg^{2+} to 0.1 mM and adding saturating concentrations of extracellular glycine to augment NMDA receptor function. Similar conditions have been previously shown to enhance action potential activity in primary cultures (Segal and Furshpan, 1990). Concentrations between 5 and 50 μM 3α5βPC stopped low Mg^{2+}-induced spiking in all cells tested ($N = 7$). The effect of 5 μM 3α5βPC on one cell is depicted in Fig. 1F.

3α5βPC Potentiates and Blocks $GABA_A$ Receptors. In subsequent experiments we sought to explain the complicated actions of 3α5βPC on IPSC decays using a combination of biophysical and pharmacological approaches. Prolongation of the decay phase of IPSCs is a common effect of GABA potentiators, including neuroactive steroids, benzodiazepines, and barbiturates, and probably underlies at least part of the clinical utility of these drugs. The reversal of IPSC prolongation at high steroid concentrations thus presumably undermines a potentially important property of the drug, especially because these high concentrations of steroid overlap with the concentrations that affect NMDA receptor-mediated EPSCs. We therefore probed structural and functional mechanisms of IPSC block by 3α5βPC in more detail.

We hypothesized that 3α5βPC possesses both potentiating and blocking activity at postsynaptic $GABA_A$ receptors, with potentiating effects dominating at low concentrations of drug and block dominating at higher concentrations. This would explain the apparent reversal of IPSC prolongations at higher concentrations (Fig. 1, D and E). To verify that postsynaptic effects explain the effects observed on both EPSCs and IPSCs, we used exogenous applications of agonists at AMPA/kainic acid, NMDA, and GABA receptors to isolate postsynaptic effects of 3α5βPC (Fig. 2). Consistent with synaptic data, at −70 mV there was no effect of 3α5βPC on responses to 100 μM kainic acid, a nondesensitizing AMPA receptor agonist. Again, consistent with synaptic data, block of NMDA responses (Fig. 2B) and potentiation of GABA

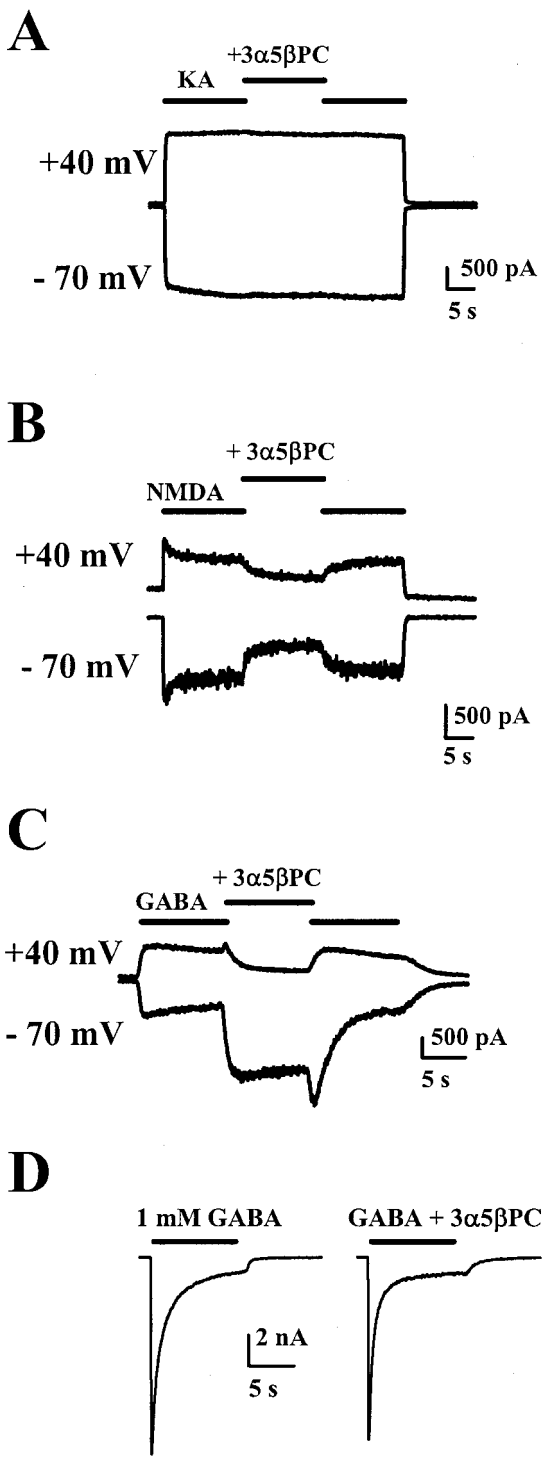

Fig. 2. Postsynaptic effects of 3α5βPC reveals block of NMDA receptors and both potentiation and block of $GABA_A$ receptors. A, responses to the nondesensitizing AMPA receptor agonist kainic acid (KA, 100 μM) at two membrane potentials are represented. There was no effect of 3α5βPC (50 μM) at either potential. B, responses to 100 μM NMDA in another neuron. Extracellular Mg^{2+} was omitted and 10 μM glycine was added to all solutions. In addition, extracellular calcium concentration was lowered to 0.2 mM to reduce Ca^{2+}-dependent desensitization of NMDA receptors (Clark et al., 1990; Legendre et al., 1993). 3α5βPC (50 μM) inhibited responses to NMDA with little voltage dependence. C, responses to GABA (2 μM) in the absence and presence of 50 μM 3α5βPC. Note the potentiation of GABA responses at −70 mV but block of responsiveness at + 40 mV. D, responses to 1 mM GABA at −70 mV in the absence (left) and presence (right) of 10 μM 3α5βPC.

receptor responses (Fig. 2C) were prominent at negative membrane potentials. Interestingly, upon washout of drug during GABA applications, a small off-response was noted in all cells tested (Fig. 2C; $N = 5$). This could reflect the hypothesized blocking action of 3α5βPC, which upon washout is relieved faster than potentiation is relieved.

In an attempt to isolate the $GABA_A$ receptor blocking effect, we reasoned that the ionized (—COO^-) form of 3α5βPC should be prominent at physiological pH and that the associated negative charge may impart a voltage dependence to any GABA receptor-blocking effects of the drug. Consistent with a voltage-dependent block of $GABA_A$ receptors by 3α5βPC, we observed that at positive membrane potentials, instead of potentiation, net inhibition of GABA responses was observed (Fig. 2C). No overshooting off-response was observed at the positive potential, possibly reflecting slower relief from block at the positive potential (Fig. 2C). There was no apparent voltage dependence to the blocking effect of 50 μM 3α5βPC at NMDA receptors (Fig. 2B; 42 ± 3% depression at −70 mV, $N = 6$ and 45 ± 3% depression at +40 mV, $N = 4$; $p > 0.5$).

If 3α5βPC-mediated block of GABA receptors explains the reversal of IPSC prolongations at concentrations >5 μM then the 3α5βPC may block GABA receptors at negative membrane potentials or with application of high concentrations of GABA, such as are thought to be achieved briefly in the synaptic cleft during GABA neurotransmission. We found that when preapplied or coapplied with 1 mM GABA, 10 μM 3α5βPC produced no obvious potentiation at −70 mV ($N = 7$). Rather, 3α5βPC depressed peak GABA responses (by 24 ± 4%) and increased the rate of apparent desensitization (Fig. 2D). The 10 to 90% decay time during GABA applications was speeded by 58 ± 4% in the presence of steroid ($N = 7$). Taken together, these results suggest that block of 3α5βPC is fostered by high steroid concentrations, high GABA concentrations, and probably positive membrane potentials. Although several explanations for dependence of 3α5βPC block on GABA concentration are possible, further evidence for apparent use dependence and voltage dependence of block are presented below (Figs. 3 and 4)

We were able to separate potentiation and block of $GABA_A$ receptors by examining current relaxations in response to voltage pulses in the presence of 2 μM GABA plus or minus 10 to 50 μM 3α5βPC. Voltage-dependent conductances in hippocampal neurons were inhibited with a combination of extracellular tetrodotoxin (500 nM) and Cd^{2+} (50 μM) to block sodium and calcium conductances, respectively, and intracellular Cs^+ to block potassium conductances. Residual voltage-gated membrane conductances and leak currents were subtracted from GABA-induced currents digitally offline. GABA current/voltage (I/V) curves examined at membrane potentials between −50 and +50 mV showed outward rectification of the steady-state GABA current, similar to that seen with many GABA receptor subunit combinations (Segal and Barker, 1984; Burgard et al., 1996; Fig. 3, B and D) and a nearly linear instantaneous I/V relationship, measured immediately after the step to the test potential (Fig. 3D). The linear instantaneous I/V relationship suggests that the outward rectification observed in steady-state I/V curves derives from a voltage dependence of GABA binding or gating steps rather than from inherent rectification of single-channel conductance (Fig. 4). In the presence of 3α5βPC, potentiation dominated at negative membrane potentials in both the steady-state and instantaneous I/V relationships (Fig. 3, E and F). In contrast, at positive potentials, block of steady-state responses was apparent at positive membrane potentials (Fig. 3F). Inspection of raw traces revealed an inward, time-dependent relaxation of GABA currents to a steady-state level smaller than that gated by GABA alone (Fig. 3C). These results are consistent with a voltage-dependent (and/or gating-dependent) and time-dependent block of GABA receptors by 3α5βPC.

Structural Requirements for Block and Potentiation. Stereoselectivity of 3α5βPC effects on GABA receptors

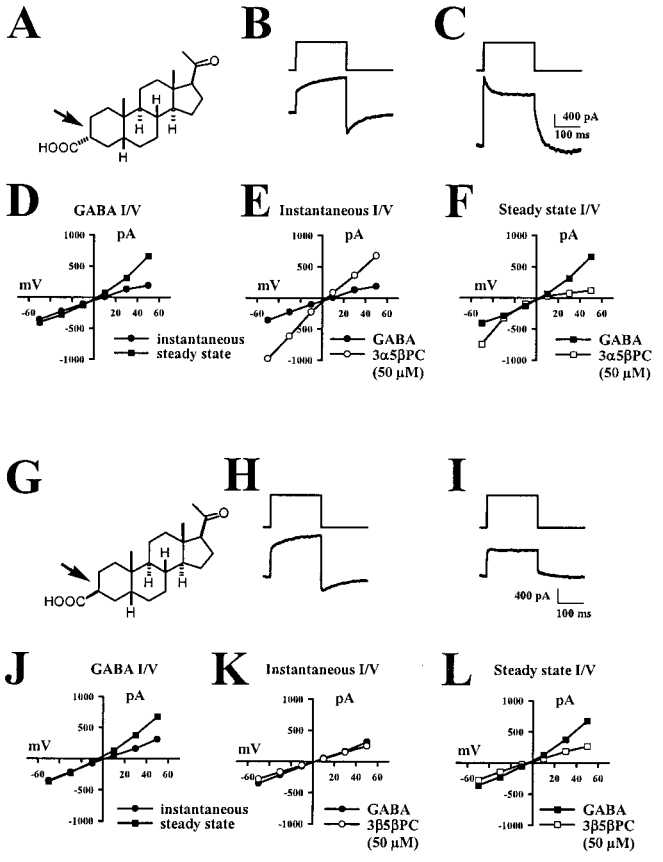

Fig. 3. Potentiation is stereoselective about C3 but block is not. A, structure of 3α5βPC with emphasis (arrow) on the stereochemistry of the C3 substituent. B, leak subtracted GABA currents from a hippocampal neuron. A voltage pulse from −70 to +50 mV (top trace) was delivered in the absence and presence of 2 μM GABA. The current in the absence of GABA was digitally subtracted to reveal the GABA-gated current (bottom trace). Note the outward voltage-dependent relaxation of the GABA current. C, same protocol was applied to the same cell, except that 3α5βPC (50 μM) was also present in the GABA solution. Note the steady-state potentiation of GABA current apparent at −70 mV (before the voltage pulse) and the potentiation of the instantaneous GABA current observed at +50 mV, which gives rise to an inward relaxation and block of current relative to B at steady state. D–F, I/V relationships obtained from pulses to various membrane potentials. D, I/V relationship for GABA in the absence of modulator. Instantaneous current was measured between 3 and 5 ms after the voltage pulse. Steady-state current was measured at the end of the 200-ms voltage pulse. Note the nearly linear instantaneous I/V and outward rectification of the steady-state I/V. E, instantaneous I/V for GABA alone is replotted from D with the instantaneous current I/V relationship obtained in the presence of GABA plus 50 μM 3α5βPC. F, steady-state current I/V relationship for GABA alone is replotted from D along with the steady-state I/V relationship measured in the presence of GABA and 50 μM 3α5βPC. G–L, same protocols as in A–F were applied to a different cell using the diastereomer 50 μM 3β5βPC. Note that potentiation is absent from both the instantaneous and steady-state currents at all potentials, but block is similar to that observed with 3α5βPC.

was examined to gain insight into the stereochemical requirements for potentiation and block. We synthesized the 3β-diastereomer (3β5βPC; Fig. 3G) and characterized 3β5βPC in the same voltage-pulse protocol used to examine 3α5βPC. Interestingly, 3β5βPC exhibited voltage-dependent block similar to that exhibited by the 3α-diastereomer, but the 3β-compound elicited no potentiation (Fig. 3, H–L). These results suggest that potentiation is highly stereoselective whereas block is not.

With the benefit of a diastereomer that exhibited only block, we were able to examine the voltage dependence and [GABA] dependence of block in more detail. For these experiments we examined block of recombinant receptors expressed in *X. laevis* oocytes, which have the advantage of small background conductances and fewer concerns about spatial voltage clamp of neuronal dendritic trees. When examined in *X. laevis* oocytes expressing the α1β2γ2L subunit combination, GABA I/V curves in response to 2 μM GABA were very similar to those obtained in hippocampal neurons. Figure 4A shows a steady-state I/V relationship to 2 μM GABA from a representative oocyte. The steady-state currents outwardly rectified, as observed in hippocampal neurons (Fig. 3). 3β5βPC (10 μM) exhibited block at positive potentials but had almost no effect at negative potentials. Although this pattern of block could represent inherent voltage dependence to 3β5βPC block, it is possible that the block at positive potentials is related directly to the increased gating of GABA receptors at positive potentials (evident in the outwardly rectifying I/V relationship). By this hypothesis, voltage dependence of block occurs indirectly because of the apparent use dependence of 3β5βPC block.

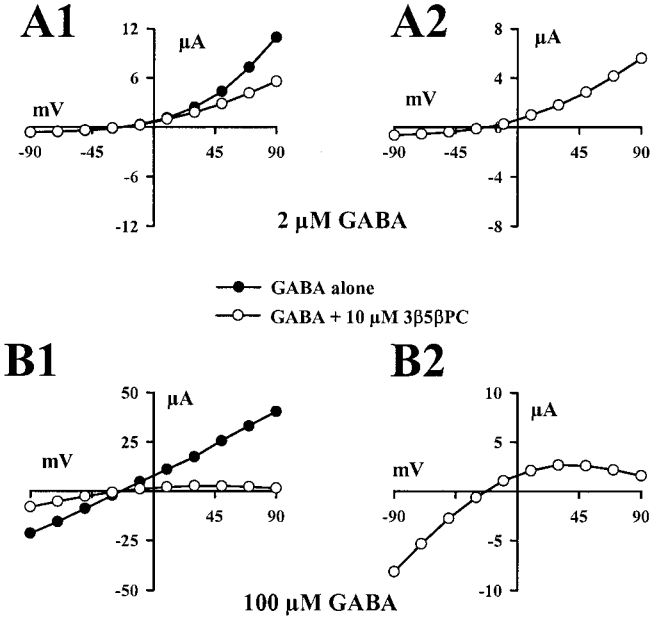

Fig. 4. Voltage and [GABA] dependence of 3β5βPC block. A1, I/V relationship obtained from steady-state responses to 2 μM GABA at various membrane potentials from an *X. laevis* oocyte expressing recombinant $GABA_A$ receptors (α1β2γ2L subunit combination). A voltage-pulse subtraction protocol similar to that used in hippocampal neurons (Fig. 3) was used. ●, GABA alone. ○, currents in response to GABA plus 10 μM 3β5βPC. A2, data in the presence of 3β5βPC were replotted on an expanded *y*-axis to highlight the shape of the I/V curve. B1 and B2, same protocols applied to the same oocyte in A, except that 100 μM GABA was used as the agonist (plus 10 μM 3β5βPC for open circles). Note the linearity of the 100 μM GABA I/V curve and the persistence of voltage-dependent block.

To determine whether there is inherent voltage dependence of 3β5βPC block, we examined the I/V relationship of responses to a high concentration of GABA (100 μM). The EC_{50} concentration for GABA in our experimental conditions was ~10 μM with a Hill coefficient near 2 (data not shown). Therefore, 100 μM GABA represents a concentration nearly maximum. Figure 4B shows I/V relationships for 100 μM GABA in the presence and absence of 10 μM 3β5βPC obtained from the same oocyte represented in A. Note that the steady-state current in the absence of steroid increased ~34-fold at −90 mV (from 0.6 to 21.5 μA), consistent with a much higher probability of channel opening at 100 μM GABA. The I/V relationship for 100 μM GABA was nearly linear, in contrast to the outwardly rectifying I/V relationship for 2 μM GABA (Fig. 4, A1 and B1, solid symbols). This result suggests that the voltage-dependent steps in GABA receptor gating are no longer rate limiting in the presence of high concentrations of GABA. However, block by 3β5βPC still exhibited notable voltage dependence. In contrast to the linear I/V curve for GABA alone, the I/V curve for 100 μM GABA in the presence of 10 μM 3β5βPC exhibited inward rectification (Fig. 4B2). This result suggests that 3β5βPC block possesses inherent voltage dependence, separate from its apparent use dependence. In addition, block by 10 μM 3β5βPC at negative potentials, although negligible in the presence of 2 μM GABA, was dramatically increased in the presence of 100 μM GABA (Fig. 4, A1 and B1). Thus, 3β5βPC block also exhibits clear dependence upon GABA concentration.

To further test the structural requirements for block by carboxylated steroids, we examined lithocholic acid, a bile steroid with a carboxylate group at C24. Lithocholic acid, like 3β5βPC, blocked GABA receptors in a [GABA]-dependent manner (Fig. 5, B and C), suggesting GABA receptor block is relatively insensitive to placement of the carboxylate. Lithocholic acid was apparently a somewhat weaker blocker of $GABA_A$ receptors than 3β5βPC, with 50 μM lithocholic acid inhibiting GABA responses by 71 ± 6% (N = 6) compared with 84 ± 3% block by 3β5βPC under the same conditions (Fig. 5C; +90 mV, 20 μM GABA). Interestingly, we observed no evidence of potentiation of GABA responses by lithocholic acid at any voltage or GABA concentration. This result, similar to the results of C3 diastereomers (Fig. 3), suggests that potentiation is more susceptible to structural modifications of carboxylated steroids than block.

The results of Figs. 2 to 5 suggest that block of GABA receptors probably explains the complicated concentration effects of 3α5βPC on IPSCs (Fig. 1, D and E). As a direct test that GABA receptor block is relevant to IPSCs, we examined the effect of 3β5βPC on IPSCs. The 3β5βPC diastereomer (50 μM) truncated the time course of IPSCs as expected (Fig. 6, A and B). In five neurons, the peak IPSC was depressed by 3β5βPC by 16 ± 3%. The 10 to 90% decay time was decreased by 49 ± 5%, from 112 ± 10 to 56 ± 3 ms. This result is consistent with the idea that the blocking action of 3α5βPC can explain the apparent reversal of IPSC prolongations at high steroid concentrations.

Dependence of Potentiation and Block on pH. Studies of the structural requirements of neuroactive steroid potentiation and block have previously suggested that a hydrogen bond donor at C3 is necessary for potentiation, whereas a negative charge at C3 is important for block (Phillipps, 1975). The predicted pK of the carboxylate group in 3α5βPC and

3β5βPC is ~5.0 (Fini et al., 1987; Loudon, 1995). However, the p*K* of organic acids is dramatically altered by changing the solvent dielectric constant or hydrogen bonding ability (Fini et al., 1987; Smejtek et al., 1987). To explore the role of the ionization state of 3α5βPC in the potentiating and blocking action at $GABA_A$ receptors, we examined the effect of altered extracellular pH on 3α5βPC potentiation and block. For these studies, we examined the effect of steroids on *X. laevis* oocytes expressing the $GABA_A$ receptor subunit combination α1β2γ2L, because these cells tolerate large and repeated shifts in pH (Fig. 7). As in hippocampal neurons, 50 μM 3α5βPC potentiated oocyte responses to 2 μM GABA at −70 mV and physiological pH (Fig. 7, A1 and A3). Consistent with previous results, low pH usually diminished responsiveness to GABA (Fig. 7A2; Zhai et al., 1998). Importantly, lowering the pH to 5.8 increased the net potentiation of 3α5βPC measured at −70 mV. Examination of steady-state I/V curves at pH 7.4 showed evidence for potentiation at negative membrane potentials and block at positive membrane potentials, again nearly identical to data from hippocampal neurons (Fig. 7A3). At pH 5.8, steady-state GABA responses were potentiated at all membrane potentials (Fig. 7A4). This result suggests that un-ionized 3α5βPC may relieve block, augment potentiation, or both.

To determine whether potentiation, independent of block, is affected by pH, we isolated the potentiating effect of 3α5βPC with low GABA concentrations (2 μM), low 3α5βPC concentration (5 μM), and a negative membrane potential (−70 mV). As shown by the titration curve in Fig. 7B, potentiation grew as pH was lowered, but the apparent p*K* was higher than predicted for an organic acid in water (p*K* of ~5). The apparent p*K* of 3α5βPC when interacting with receptor was ~6.4 from the fit to data in Fig. 7B. The saturation of responses at low pH values did not result from achieving maximum potentiation by steroid, because at pH 5.8, doubling the total steroid concentration from 5 to 10 μM increased the GABA responses a further 3.3 ± 0.2-fold ($N = 3$ oocytes; data not shown).

Potentiation by 3α5βPC does not seem dependent upon charge neutrality per se at C3, as the methyl ester derivative of 3α5βPC, which is electroneutral at C3 but is not a hydrogen bond donor, was inactive at concentrations up to 50 μM. In three oocytes treated with 2 μM GABA, responses at −50 mV were potentiated by only 8 ± 9% a 50 μM concentration of the methyl ester derivative, compared with 391 ± 10% potentiation for 50 μM 3α5βPC under the same conditions ($N = 4$; data not shown).

On the other hand, 3α5βPA, the amide derivative of 3α5βPC, which is electroneutral at C3 and a weaker hydrogen bond donor than un-ionized 3α5βPC, potentiated responses to 2 μM GABA in a concentration-dependent manner (Fig. 6C). In three *X. laevis* oocytes examined, responses were potentiated by 23 ± 7, 233 ± 47, and 326 ± 70% at 1, 10, and 50 μM, respectively. Unlike 3α5βPC, steady-state I/V plots of responses to 2 μM GABA in the presence of 50 μM 3α5βPA were potentiated at all potentials, with no evidence of block at positive potentials (data not shown). Also unlike 3α5βPC, potentiation by a subsaturating concentration of 3α5βPA showed very little pH dependence (cf. Figure 6, A and C). At pH 7.4, 5 μM 3α5βPA potentiated responses to 2 μM GABA by 75 ± 7%; at pH 5.8 responses were potentiated by 109 ± 14% ($N = 3$ oocytes). This result is consistent with the expectation that 3α5βPA is not ionized over the entire pH range examined. The result is also consistent with the hypothesis that the increased 3α5βPC potentiation at low pH results from a change in the concentration of un-ionized steroid and from titration of a residue on the receptor protein. Consequently, the high p*K* value for 3α5βPC suggests that the environment in which the bound steroid is located has a lower dielectric constant or hydrogen bonds more weakly than water (Smejtek et al., 1987).

To test the effect of low pH on GABA receptor block, independent of potentiation, we examined the effect of low pH on block by the 3β-diastereomer and lithocholic acid (Fig. 8). We used a combination of 20 μM GABA and 50 μM 3β5βPC to induce severe block across a range of membrane potentials. 3β5βPC (50 μM) at −90 mV depressed responses to 2 μM GABA by 28 ± 7% but depressed responses to 20 μM GABA by 67 ± 6% ($N = 5$ oocytes at each GABA concentration). These data are consistent with the observed dependence of block by 3α5βPC on GABA concentration (Figs. 2, C and D, and 4). Surprisingly, despite the large block with elevated GABA concentration, low pH (5.8) caused nearly complete relief from block at all membrane potentials (Fig. 8, A and B). Depression of GABA responses was 84 ± 3% at +90 mV and pH 7.4, but depression was only 3 ± 3% at +90 mV and pH 5.8 ($N = 5$; Fig. 8D). Lithocholic acid behaved similarly to 3β5βPC (Fig. 8C). As with 3α5βPC potentiation, titration curves for both 3β5βPC and lithocholic acid (Fig. 8, B and C) revealed high apparent p*K* values (6.9 and 6.4, respectively).

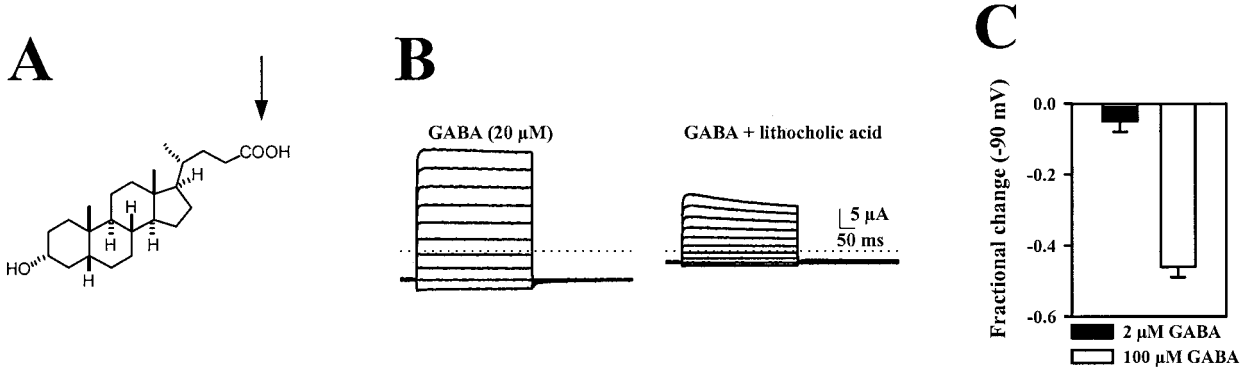

Fig. 5. Effect of lithocholic acid on GABA responses. A, structure of lithocholic acid with emphasis on placement of the carboxylate group (arrow) at C24. B, effect of lithocholic acid on responses to 20 μM GABA in an oocyte expressing the α1β2γ2 subunit combination. C, summary of the GABA-dependence of lithocholic acid. Average block (10 μM lithocholic acid) of responses to 2 and 100 μM GABA is shown.

To determine whether the ionization state of a receptor residue probably contributes to the effects of pH on 3β5βPC block, we examined the effect of pH on several sulfated steroids whose blocking actions are similar to those of 3β5βPC and probably act at a similar site. Ionization of these steroids, even if the local environment shifts the p*K* value by 2 units, should not be affected between pH 7.4 and 5.8 because of the extremely low p*K* of the sulfate group (Loudon, 1995). At pH 5.8, block of GABA (20 μM) responses by 2 μM 3α5βPS was decreased only slightly at all membrane potentials examined (Fig. 8D). Like 3α5βPC and 3β5βPC, block by 2 μM 3α5βPS was dependent upon GABA concentration, depressing responses to 2 μM GABA (−90 mV) by 13 ± 3% and responses to 20 μM GABA by 58 ± 2%. Also like 3β5βPC, 3α5βPS exhibited apparent voltage dependence, especially at low GABA concentrations. At +90 mV, block was increased from 13 ± 3% (−90 mV) to 30 ± 5% ($N = 6$). Because of the apparent use dependence of 3α5βPS block, the slight decrease in the effect of drug at pH 5.8 (Fig. 8D) may be related to the reduced amplitude of GABA responses at low pH. We found that pregnenolone sulfate (2 μM) block was also only slightly affected by pH 5.8 (91 ± 2% depression, $N = 4$ at pH 7.4, versus 76 ± 5% depression, $N = 2$ at pH 5.8). In addition, 3β5βPS and dehydroepiandrosterone sulfate block were similarly weakly affected by pH (data not shown). These data are consistent with the idea that the effect of pH on carboxylated steroid block results primarily from the ionization state of the steroid rather than to sites on the receptor. These experiments also showed that only low micromolar concentrations of sulfated steroids are needed to match the degree of block given by the carboxylated steroids, suggesting that block by sulfated steroids is substantially more potent (~25 fold) than block by carboxylated steroids. In summary, these experiments suggest that both potentiation and block by carboxylated steroids are dramatically and inversely affected by pH

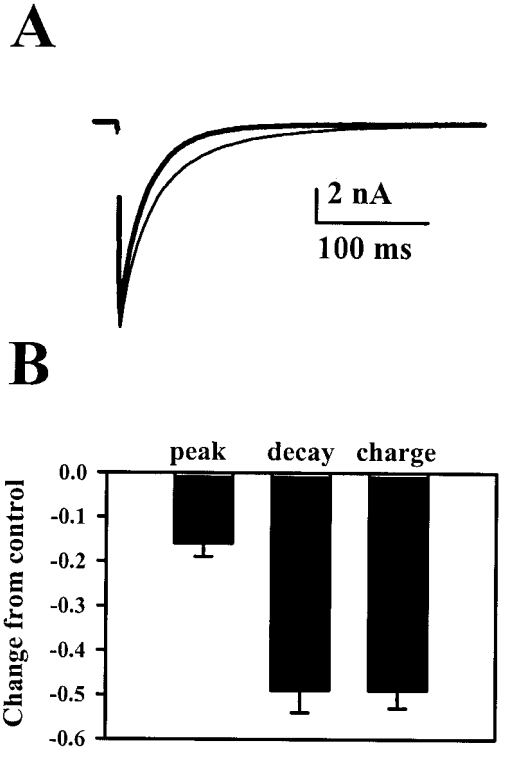

Fig. 6. 3β5βPC truncated the decay of IPSCs. A, thin trace represents control IPSCs. The thick trace represents averaged sweeps in the presence of 50 μM 3β5βPC. B, summary of effects of 50 μM 3β5βPC on the peak amplitude, 10 to 90% decay time, and total charge transfer from five neurons.

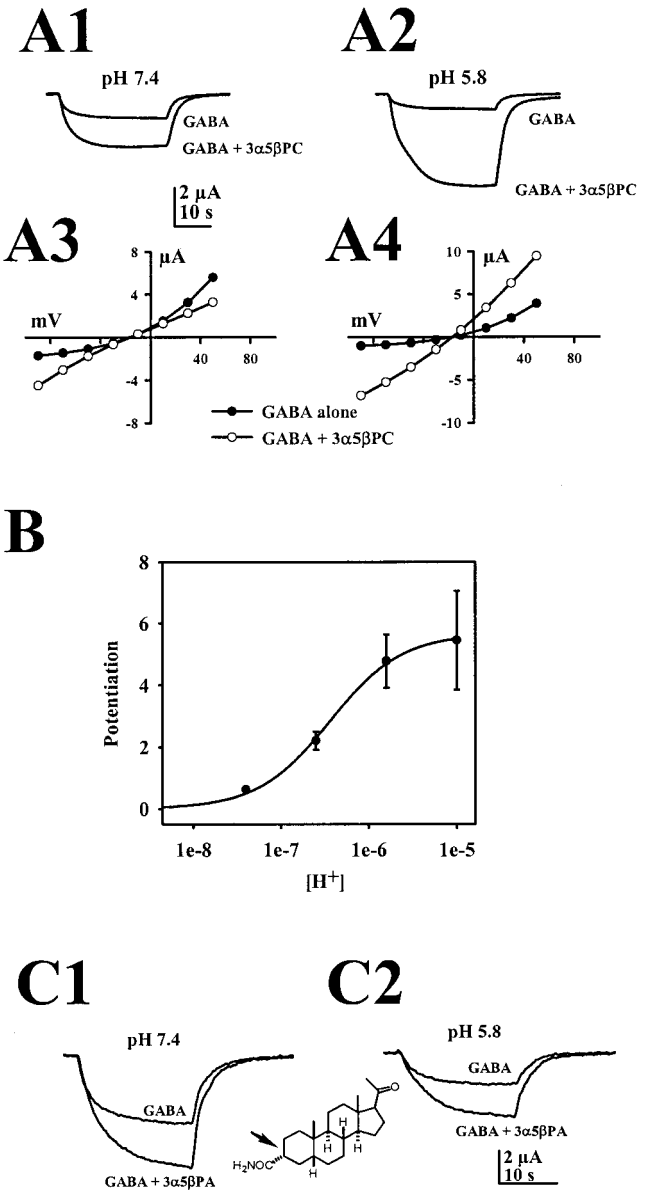

Fig. 7. Potentiating effect of 3α5βPC was pH-dependent. A1 and A2, traces from an *X. laevis* oocyte expressing an α1β2γ2L subunit combination. A1, at pH 7.4 and a membrane potential of −70 mV, 50 μM 3α5βPC potentiated the response to 2 μM GABA. A2, at the same membrane potential but pH 5.8, the response to GABA alone was slightly reduced but the potentiation is much larger than at the higher pH. A3 and A4, from the same cell, steady-state I/V curves showed loss of block and apparent enhanced potentiation at low pH. A3, steady-state I/V curve at pH 7.4. The potentiation and block at negative and positive membrane potentials at pH 7.4 are similar to results in hippocampal neurons (Fig. 3F). A4, steady-state I/V curve at pH 5.8. Note the potentiation of steady-state GABA responses at all membrane potentials. B, titration of the potentiating effect of 3α5βPC. Potentiation, independent of block, was isolated by using low concentrations of GABA (2 μM) and 3α5βPC (5 μM) and by examining negative membrane potentials −70 mV. Potentiation was calculated relative to the steady-state GABA response at each pH value. The solid line represents a titration curve with an apparent p*K* of 6.4. C1 and C2, potentiation of the amide derivative of 3α5βPC is pH-independent. Protocol was similar to that used for A1 and A2. The inset shows the structure of 3α5βPA. Holding potential was −70 mV.

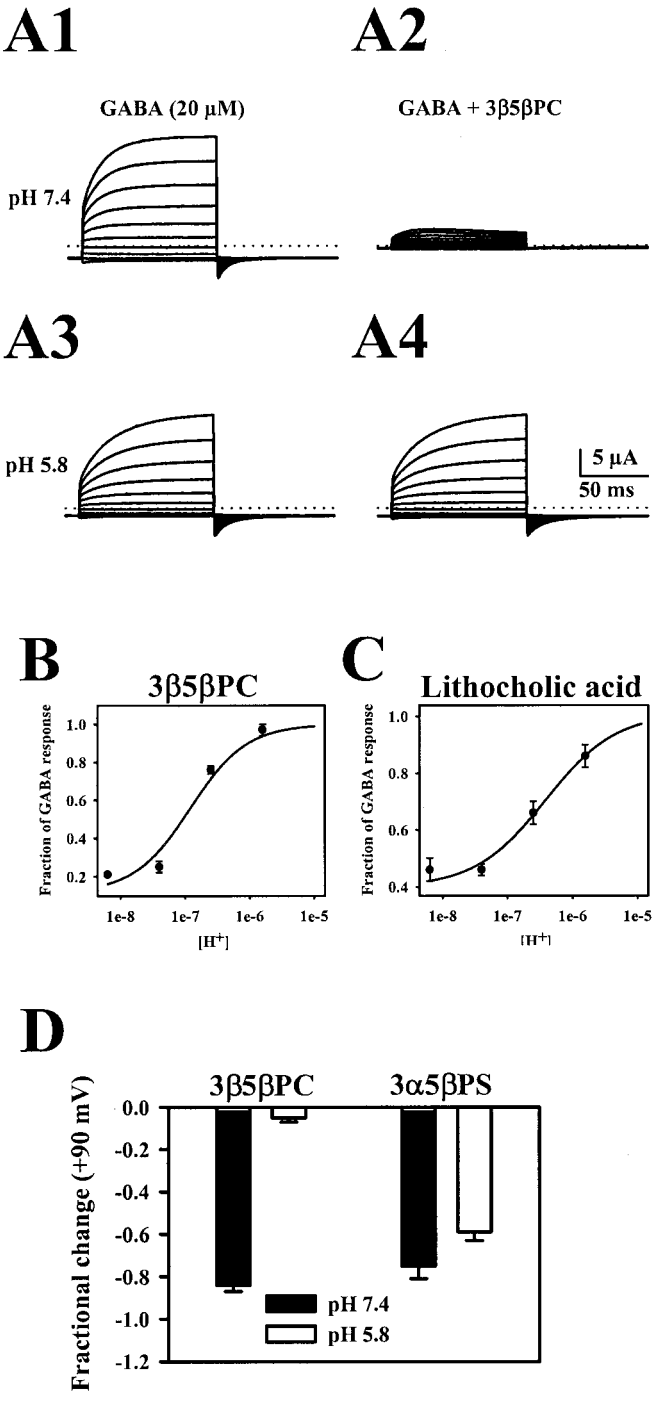

Fig. 8. A1–A4, low pH diminished block by 3β5βPC. Voltage pulses in the presence and absence of GABA were applied in 20-mV increments to potentials between −90 and +90 mV. The traces shown are subtractions of traces in the absence of GABA from those in the presence of GABA. A1, at pH 7.4 responses to 20 μM GABA showed typical outward rectification. Twenty μM GABA was used to enhance the effect of 3β5βPC. A2, 3β5βPC (50 μM) depressed GABA responsiveness at all membrane potentials. A3, GABA responsiveness was depressed slightly by pH 5.8. A4, 3β5βPC depression was nearly eliminated at low pH. B and C, pH dependence of block by 3β5βPC (B, $N = 4$) and by lithocholic acid (C, $N = 5$). Apparent pK values for the blockers were 6.9 and 6.4, respectively. D, low pH only slightly affected the block of GABA responses by 3α5βPS (2 μM). Because the block by 3α5βPS, like that of 3β5βPC, is [GABA]-dependent, the small amount of reduction in effectiveness at low pH could be due to the decreased GABA responsiveness at pH 5.8. The summary reflects fractional block of steady-state response to 20 μM GABA at +90 mV for 50 μM 3β5βPC ($N = 5$; different sample than Fig. 8B) and 2 μM 3α5βPS ($N = 4$).

and are consistent with the idea that the local environment of the steroid dramatically affects the steroid pK.

Discussion

Novel Combination of Cellular Actions for 3α5βPC. The unique attribute of the neuroactive steroid 3α5βPC is the ability of this compound to potentiate $GABA_A$ receptor function and inhibit NMDA receptor function. This combination of cellular effects may enhance the clinical profile over previously characterized anesthetic neuroactive steroids. While block of NMDA receptors is perhaps sufficient to protect against various forms of glutamate-mediated toxicity (Michaelis, 1998), maximum block of NMDA receptors by neuroactive steroids is typically less than 100%. Therefore, the GABA-blocking actions of these same steroids may promote an increase in synaptic activity that at least partially undermines the direct effects on NMDA receptors. Likewise, GABA-potentiating actions should enhance the anesthetic properties of NMDA receptor blockers, because $GABA_A$ receptor potentiation and NMDA receptor block are the cellular actions most closely correlated with anesthetic properties (Franks and Lieb, 1994).

While 3α5βPC is a lead for developing neuroactive steroids with favorable clinical properties, several features of 3α5βPC may not be desirable or optimal for anticonvulsant, anesthetic, and neuroprotective properties. The most serious problem with this compound may be the blocking effect on $GABA_A$ receptors at high micromolar concentrations of drug. Therefore, this work focused on defining structural and functional aspects of carboxylated steroids at $GABA_A$ receptors. Block of $GABA_A$ receptors requires significantly higher concentrations of steroid than endogenous sulfated steroids, but these same high micromolar concentrations are required for activity at NMDA receptors. Given that 50 μM 3α5βPC produces no net change in IPSC time course but significantly dampens NMDA EPSCs (Fig. 1), 3α5βPC could be a better neuroprotective agent than previously reported C3 sulfates and hemiesters, all of which reportedly block $GABA_A$ receptors (Park-Chung et al., 1999). Acidosis produced during acute central nervous system insults such as anoxia would probably minimize $GABA_A$ block and maximize the potentiating effects of 3α5βPC (Figs. 7 and 8).

On the other hand, minimizing block of $GABA_A$ receptors while retaining or enhancing NMDA receptor block would probably produce a neuroactive steroid with better clinical utility. Given the resistance of $GABA_A$ receptor block to major changes in the stereochemistry or placement of the carboxylic acid (Figs. 3 and 5), it may prove difficult to eliminate block. Rather, ongoing work in our laboratories is aimed at defining and optimizing structural requirements for NMDA receptor block, which may prove a more fruitful strategy for improving the clinical usefulness of carboxylated steroids.

Action at $GABA_A$ Receptors: Site(s) of Action. Given that 3α5βPC both potentiates and blocks GABA receptors, it is worth considering current evidence for possible sites on the $GABA_A$ receptor that may mediate these actions. Potentiation by 3α5βPC exhibits clear stereoselectivity, whereas block does not. Likewise, concentration-response data at synapses (Fig. 1E) suggest that potentiation is most prominent at low micromolar concentrations of steroid, whereas block becomes apparent at concentrations >10 μM. Potentiation and block by 3α5βPC can also be temporally separated during voltage jumps

to positive potentials (Fig. 3, A–F). Finally, low pH has opposite effects on block and potentiation, nearly eliminating block whereas enhancing potentiation. These results are all consistent with the hypothesis that potentiation and block occur at separate sites on the $GABA_A$ receptor. Previous work also suggests that positive modulators of $GABA_A$ receptors, such as (3α,5α)-3-hydroxypregnan-20-one, act at a different $GABA_A$ receptor-associated site than blocking steroids such as pregnenolone sulfate (Zaman et al., 1992; Park-Chung et al., 1999). Unfortunately, specific potentiating and blocking sites on the $GABA_A$ receptor have thus far eluded identification, although a point mutation in transmembrane domain 2 dramatically reduces block by pregnenolone sulfate, perhaps by altering the transduction mechanism of block (Akk et al., 2001). Interestingly, it was previously shown that 3α5βPS and pregnenolone sulfate lack enantioselectivity for block of $GABA_A$ receptors, possibly suggesting the lack of a conventional chiral protein-ligand recognition site for pregnane and pregnene series blockers (Nilsson et al., 1998).

Potentiation by 3α5βPC and block by 3β5βPC and lithocholic acid are pH-dependent. The apparent pK values for potentiation and block were 6.4 to 6.9. Because these values are approximately 1.5 to 2 units above the pK of lithocholic acid in water (Fini et al., 1987), we suggest that the pK of carboxylated steroids is significantly altered by the local environment in which the steroids act. The pK for many organic acids, including lithocholic acid, is increased from ~5 in water to ~8 in aqueous organic solvents (Fini et al., 1987). The pK of organic acids is also raised by association of the acid with membrane (Smejtek et al., 1987). Therefore, the local environment of the carboxylated steroids can dramatically affect the pK value and probably accounts for the high apparent pK values observed in the present study.

We found that potentiation effects at GABA receptors probably are mediated by the un-ionized form of 3α5βPC. Although potentiation by 3α5βPC was increased by decreased ionization, electroneutrality at C3 is not sufficient for potentiation. Another requirement seems to be that the substituent at C3 be a hydrogen bond donor (Phillipps, 1975). Consistent with this hypothesis, the methyl ester derivative of 3α5βPC was inactive at GABA receptors, and the amide derivative of 3α5βPC was an effective but pH-independent potentiator. The un-ionized form of 3α5βPC probably constitutes slightly less than 10% of the total steroid at physiological pH (assuming a pK of 6.4), and significant potentiation occurs at 5 μM total steroid. This suggests that protonated 3α5βPC is probably active at less than 500 nM, making the un-ionized form of 3α5βPC slightly lower in potency than other unsulfated neuroactive steroids that potentiate GABA responsiveness.

Given that potentiation and block by 3α5βPC probably occur through different sites (Park-Chung et al., 1999), future work will be aimed at structural modifications to 3α5βPC that maximize potentiation but minimize block. Ideally, we seek a compound for which only the un-ionized form will potently interact with GABA receptors but that retains block of NMDA receptors.

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