Cellular/Molecular

Slow Actions of Neuroactive Steroids at $GABA_A$ Receptors

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Neuroactive steroids are potent and efficacious modulators of $GABA_A$ receptor activity and are potent sedatives and anesthetics. These positive modulators of $GABA_A$ receptors both potentiate the actions of GABA at the receptor and, at higher concentrations, directly gate the channel. The contribution of direct gating to the cellular and behavioral effects of neuroactive steroids is considered of little significance because it has been generally found that concentrations well above those needed for anesthesia are required to gate channels. By studying solitary glutamatergic neurons devoid of synaptic GABA input, we show that direct gating occurs and significantly alters membrane excitability at concentrations $\leq$100 nM. We propose that the relevance of direct gating has been overlooked partly because of the extremely slow kinetics of receptor activation and deactivation. We show that slow deactivation of directly gated currents does not result from an inherently tight ligand–receptor interaction because the slow deactivation is markedly accelerated by γ-cyclodextrin application. We hypothesize that steroids access the relevant $GABA_A$ receptor site from a non-aqueous reservoir, likely the plasma membrane, and that it is slow reservoir accumulation and departure that accounts for the slow kinetics of receptor gating by neuroactive steroids.

Key words: **allopregnanolone; GABA; neurosteroid; anesthesia; inhibition; hippocampus**

Introduction

Neuroactive steroids are potent modulators of $GABA_A$ receptor activity and are thus of interest for their sedative, anxiolytic, anticonvulsant, and anesthetic properties. It has been known for two decades that GABA-potentiating steroids, such as the endogenous steroids (3α,5α)-3,21-dihydroxypregnan-20-one and (3α,5α)-3-hydroxypregnan-20-one (3α5αP), have at least two actions at $GABA_A$ receptors, analogous to barbiturates and many other anesthetics. Neuroactive steroids potentiate the actions of GABA and, at higher concentrations, directly gate the $GABA_A$ receptor chloride channel (Callachan et al., 1987) at a site distinct from the GABA binding site (Ueno et al., 1997). Generally, concentrations in excess of 10 μM steroid are reported to directly gate the receptor channel (Callachan et al., 1987; Cottrell et al., 1987; Puia et al., 1990), although some studies have found that lower concentrations (high nanomolar to low micromolar) are effective (Liu et al., 2002; Wohlfarth et al., 2002). An additional complication in many studies is that it is difficult to separate direct gating from potentiation of endogenous, tonic GABA activity. Therefore, the contributions of direct gating to GABAergic effects at low steroid concentrations remain unclear. If lower concentrations indeed effectively directly gate the $GABA_A$ channel, it is possible that direct gating may play an important role in the cellular and behavioral effects of neuroactive steroids.

We examined the direct effects of the neurosteroid 3α5αP on solitary hippocampal neurons grown in synaptic isolation. We studied only glutamatergic neurons to limit the possibility of inadvertently studying potentiation of endogenous GABA acting tonically at receptors. We found evidence that 3α5αP directly gates $GABA_A$ receptors at concentrations of ~100 nM. However, the kinetics of current development and offset are extremely slow. Slow current development likely accounts for the failure of previous studies to appreciate direct gating at these low concentrations. We hypothesized that the slow kinetics of these currents arises from accumulation of steroid into a reservoir, likely the plasma membrane, from which the ligand accesses a site on the GABA receptor. Support for this idea came from studies using γ-cyclodextrin as a soluble, hydrophobic "sponge" (Adam et al., 2002). The presence of γ-cyclodextrin significantly speeded the offset kinetics of directly gated neurosteroid currents. The actions were clearly distinct from bicuculline, a noncompetitive antagonist with respect to steroid direct gating. We conclude that the receptor site for direct gating may be near the protein–membrane interface and that direct gating is likely to participate significantly in the actions of neurosteroids at behaviorally relevant concentrations.

Materials and Methods

Hippocampal microcultures. Primary microcultures of hippocampal cells were prepared from 1–3 d postnatal Sprague Dawley rats, as described previously (Mennerick et al., 1995). Halothane-anesthetized rats were decapitated, and the hippocampi were removed. Hippocampi were cut into 500-μm-thick transverse slices. Single-cell suspensions were pre-

Received April 14, 2004; revised June 8, 2004; accepted June 9, 2004.

This work was supported by a gift from the Bantly Foundation (C.F.Z.) and National Institutes of Health Grants GM 47969 (D.F.C., C.F.Z.), NS40488 (S.M.), and AA12952 (S.M.). We thank Alex Evers, Joe Henry Steinbach, Gustav Akk, Keith Isenberg, and laboratory members for discussion.

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DOI:10.1523/JNEUROSCI.1399-04.2004

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pared with 1 mg/ml papain digestion in oxygenated Leibovitz L-15 medium, followed by mechanical trituration in modified Eagle's medium containing 5% horse serum, 5% FCS, 17 mM D-glucose, 400 μM glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin. Cells were plated in the modified Eagle's medium at a density of 75 cells/mm^2 onto 35 mm plastic culture dishes precoated with collagen microdroplets sprayed on a layer of 0.15% agarose. The antimitotic cytosine arabinoside (5–10 μM) was added on the third day after plating to halt glial proliferation. Electrophysiology was performed 10–18 d after plating.

Culture electrophysiology. Whole-cell recordings were performed on hippocampal microculture neurons, using an Axopatch 1D amplifier (Axon Instruments, Foster City, CA) interfaced to a Pentium III-based computer via a Digidata 1322 acquisition board (Axon Instruments). At the time of experiments, culture medium was replaced with an extracellular recording solution consisting of (in mM): 138 NaCl, 4 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 glucose, 10 HEPES, and 0.025 D-APV, pH 7.25. Recordings were at room temperature. Access resistance (5–10 MΩ) was compensated electronically 90–100% for synaptic currents. For steroid-gated currents, which were much smaller in amplitude, no access resistance compensation was performed. The standard pipette solution contained (in mM): 140 cesium methanesulfonate, 4 NaCl, 0.5 $CaCl_2$, 5 EGTA, and 10 HEPES, pH 7.25. Exogenous drugs were applied with a multi-barrel pipette coupled with miniature solenoid valves that allowed rapid switching (~100 msec on whole cells). Autaptic release of neurotransmitter was evoked in voltage-clamped solitary neurons with a 2 msec voltage pulse to 0 mV from a holding potential of −70 mV (Bekkers and Stevens, 1991; Mennerick et al., 1995). Release was evoked every 25 sec for synaptic responses, and drug exposure was initiated ~20 sec before stimulation.

Xenopus *oocyte expression studies.* Stage V–VI oocytes were harvested from sexually mature female *Xenopus laevis* (*Xenopus* One, Northland, MI) under 0.1% tricaine (3-aminobenzoic acid ethyl ester) anesthesia, according to institutionally approved protocols. Oocytes were defolliculated by shaking for 20 min at 37°C in collagenase (2 mg/ml) dissolved in calcium-free solution containing (in mM): 96 NaCl, 2 KCl, 1 $MgCl_2$, and 5 HEPES at pH 7.4. Capped mRNA, encoding rat $GABA_A$ receptor α1, β2, and γ2L subunits, was transcribed *in vitro* using the mMESSAGE mMachine kit (Ambion, Austin, TX) from linearized pBluescript vectors containing receptor coding regions. Subunit transcripts were injected in equal parts (20–40 ng of total RNA) 8–24 hr after defolliculation. Oocytes were incubated up to 5 d at 18°C in ND96 medium containing (in mM): 96 NaCl, 1 KCl, 1 $MgCl_2$, 2 $CaCl_2$, and 10 HEPES at pH 7.4, supplemented with pyruvate (5 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), and gentamycin (50 μg/ml).

Oocyte electrophysiology. Two-electrode voltage-clamp experiments were performed with a Warner OC725 amplifier 2–5 d 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 ~1 MΩ. Drugs were applied from a common tip via a gravity-driven multi-barrel drug delivery system. Unless indicated otherwise, drugs were co-applied with no pre-application period. Cells were clamped at −70 mV for all experiments, and the current at the end of 20–30 sec drug applications was measured for quantification of current amplitudes.

Data analysis. Data acquisition and analysis were performed with pCLAMP 9.0 software (Axon Instruments). Data plotting and curve fitting were done with Sigma Plot 8.0 software (SPSS, Chicago, IL). Data are presented in the text and figures as mean ± SE. Statistical differences were determined using a two-tailed Student's *t* test. Fitting of the dose–response relationships were performed using the Hill equation as described in the legend to Figure 4. Potentiation in text and figures (see Figs. 4*D*, 7*B–D*) is calculated as $R_s/R_G - 1$, where R_s is the potentiated response in the combined presence of steroid and GABA, and R_G is the response to GABA alone. Thus, responses to GABA alone are represented by potentiation values of zero.

Drugs. All drugs were from Sigma (St. Louis, MO), except (3α,5α)-17-phenylandrost-16-en-3-ol (17PA), which was synthesized as described (Mennerick et al., 2004). Steroids and 17PA were prepared as stock solutions in DMSO. Final DMSO concentration was always below 0.13%, and solutions were matched for DMSO concentration.

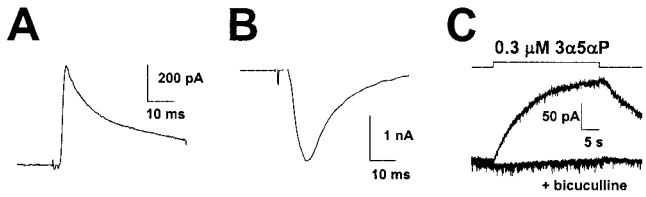

Figure 1. 3α5αP currents from solitary glutamatergic hippocampal islands. *A*, Example IPSC elicited from −50 mV. The current is a result of subtracting one sweep in the presence of 25 μM bicuculline from a sweep in the absence of bicuculline. *B*, Example EPSC elicited from another solitary neuron from −50 mV. The subtraction was performed with 1 μM NBQX (2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonoamide). The pipette solution for both recordings contained cesium methanesulfonate. *C*, Current gated from a holding potential of −20 mV by 300 nM 3α5αP in the presence and absence of bicuculline (25 μM). Bicuculline was present throughout the trace. Note that in this and subsequent figures, there is a slight lag between the switch in solutions, denoted by the top trace, and onset or offset of the measured current. Partly, this lag results from a combination of exchange time in the dead space of the perfuser tip and distance of the perfuser from the recorded cell. After accounting for this lag, liquid junction potential exchanges suggested an exchange time much faster (~100 msec) than the rise and decay of the 3α5αP-gated currents.

Results

Direct gating at low steroid concentrations

Figure 1, *A* and *B*, shows representative autaptic currents used to define the transmitter phenotype of the solitary cell under study (Bekkers and Stevens, 1991; Mennerick et al., 1995). Cells were clamped at −50 mV, and the voltage was briefly stepped to 0 mV to elicit autaptic transmission. Solitary GABA cells (Fig. 1*A*) were distinguished easily from solitary glutamate cells (Fig. 1*B*) by outward versus inward autaptic currents, when filled with the cesium methanesulfonate solution used in the whole-cell patch pipette. Thus, all neurons in the subsequent experiments were confirmed solitary glutamatergic cells. Glutamatergic cells grown under these conditions are known to express GABA receptors (Rao et al., 2000), so we were able to study the response of these cells to neuroactive steroids acting at these nonsynaptic receptors. Qualitatively similar results were observed in GABAergic neurons, although spontaneous synaptic responses clearly contaminated responses to exogenous agonists recorded from GABAergic neurons (data not shown).

After cell identification, we clamped glutamatergic neurons to −20 mV to provide ~45 mV of driving force on Cl^- currents, based on our solution compositions. When we applied 300 nM 3α5αP, we found a slowly developing, bicuculline-sensitive outward current in all cells tested for the present experiments (Fig. 1*C*). On removal of 3α5αP from the perfusing saline, the current slowly returned to baseline. The slow kinetics of the current did not result from technical limitations on solution exchange times with the multi-barrel pipette used for drug delivery. Liquid junction potential exchanges were <100 msec at an open patch pipette tip (data not shown), and the rise and decay of GABA-gated currents in whole-cell recordings was consistent with rapid drug access to cells (see Figs. 3–5).

We tested whether the 3α5αP response truly represented direct gating rather than potentiation of GABA coming from nonsynaptic sources (Brickley et al., 1996; Nusser and Mody, 2002; Caraiscos et al., 2004). First, to test for the presence of ambient GABA, we challenged solitary excitatory neurons with a high concentration (30 μM) of the antagonist SR-95531 (gabazine).

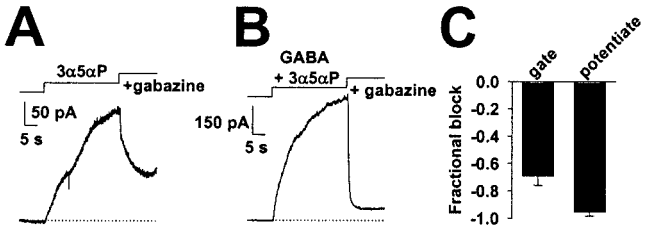

Figure 2. Incomplete block of 3α5αP-gated currents by gabazine. *A*, Example trace from an experiment using 3α5αP (300 nM) alone and plus gabazine (10 μM). *B*, Example trace from the same cell showing the effect of gabazine on GABA (0.5 μM) currents potentiated by 300 nM 3α5αP. Note the more complete block compared with the administration of 3α5αP alone. *C*, Summary of experiments comparing the degree of gabazine block of directly gated currents with potentiated currents as in *A* and *B*. $n = 4$ cells in which both potentiation and gating were measured. The difference between the degree of block was significant ($p < 0.01$) by paired *t* test.

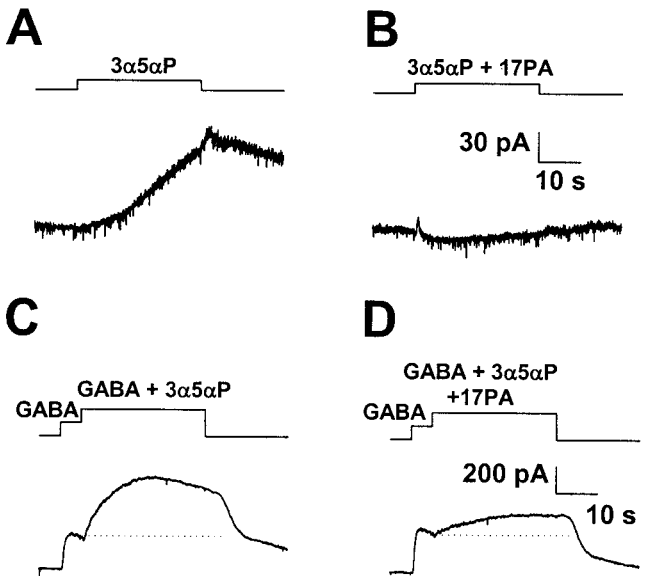

Figure 3. A steroid antagonist completely blocks responses to 3α5αP alone. All responses are from the same cell. *A*, Response to 300 nM 3α5αP in a solitary neuron. *B*, Response to the coapplication of 300 nM 3α5αP with 10 μM 17PA. Note the complete loss of current. *C*, A 5 sec application of 0.5 μM GABA was followed with a 30 sec coapplication of GABA plus 300 nM 3α5αP. The dotted line denotes the amplitude of the GABA current at the end of the 5 sec pre-application of GABA. *D*, Same protocol as *C*, except that 10 μM 17PA was present during the coapplication phase of the experiment. Note that 17PA incompletely blocked the potentiation.

Consistent with negligible GABA presence, we found that gabazine produced a 1.2 ± 0.4 pA outward current ($n = 19$ cells).

To further test whether steroid responses of solitary glutamate cells represent direct gating, we exploited known pharmacological properties of steroid direct gating. In the absence of GABA binding sites, steroids still effectively gate receptors (Ueno et al., 1997), indicating that steroids bind to a site other than the GABA site. Nevertheless, in wild-type receptors, bicuculline and gabazine, both thought to be GABA-site ligands, inhibit steroid-gated currents, suggesting these agents allosterically promote nonconducting states of the receptor. Interestingly, bicuculline produces nearly a complete block of steroid-gated currents, whereas gabazine produces partial block at saturating concentrations (Ueno et al., 1997). We sought to exploit this differential efficacy of bicuculline versus gabazine. We reasoned that gabazine block of 3α5αP currents should be incomplete if responses result from direct gating. In contrast, if ambient GABA is involved in generating steroid-gated currents, bicuculline and gabazine should both block responses nearly completely at high concentrations.

Consistent with our expectations, 10 μM gabazine failed to completely block responses to 3α5αP alone (Fig. 2*A*). When 3α5αP was used to potentiate the response of a low concentration of exogenous GABA, the block was much more complete (Fig. 2*B*,*C*) ($n = 4$). In another sample of four neurons, 10 μM gabazine inhibited directly gated 3α5αP currents by 46 ± 9%. Block increased to only 55 ± 10% at a 10-fold higher gabazine concentration (100 μM), consistent with previous work showing that 10 μM gabazine is nearly saturating (Ueno et al., 1997). These results confirm the idea that 3α5αP directly activates $GABA_A$ receptors in the high nanomolar concentration range.

In a final experiment to confirm direct gating under the conditions of our experiments, we used a recently characterized steroid antagonist, 17PA. 17PA has no effect on GABA responses or potentiation of GABA responses by benzodiazepines or barbiturates, but 17PA significantly antagonizes 5α-reduced steroid effects at $GABA_A$ receptors (Mennerick et al., 2004). 17PA antagonizes direct gating more strongly than potentiation (Mennerick et al., 2004), so we tested 17PA against responses to 300 nM 3α5αP alone and 300 nM 3α5αP used as a potentiator of responses to 0.5 μM GABA. Figure 3 shows that in a solitary glutamatergic neuron, responses to 3α5αP alone were completely antagonized by 10 μM 17PA, but in the same cell, significant potentiation was still detectable in the combined presence of GABA, 3α5αP, and 17PA. On average, the direct 3α5αP current was inhibited by 103 ± 4% by 10 μM 17PA ($n = 4$). In the same cells, potentiation was 129 ± 31% in the absence of 17PA and retained a residual potentiation of 35 ± 17% in the presence of 17PA. The results are unlikely to be explained by a low ambient GABA concentration present during 3α5αP wash-on, because in other experiments we found that varying GABA concentration over the range of 0.2–2 μM had no consistent effect on the amount of potentiation antagonism produced by 17PA (data not shown). In summary, results from Figures 2 and 3 support the idea that responses to 3α5αP represent directly gated currents rather than potentiation of ambient GABA effects.

To examine more systematically the concentration range over which 3α5αP directly gates receptors, we performed concentration–response analyses. Figure 4 compares the concentration–response profiles of 3α5αP used alone (Fig. 4*A*,*B*) or as a potentiator of responses to a low GABA concentration (0.5 μM) (Fig. 4*C*,*D*). Figure 4, *E* and *F*, shows the concentration–response profile for GABA itself. The 3α5αP direct gating experiment yielded an EC_{50} value of 1.7 μM and a Hill coefficient of 0.9. The potentiation experiment yielded an EC_{50} value of 0.075 μM and a Hill coefficient of 1.5. These results, especially for direct gating, should be taken as estimates only because responses to the lowest concentration of steroid probably did not reach steady state by the end of the 30 sec application, and peak amplitudes of currents at the highest concentrations may have been underestimated by an apparent desensitization, which appeared with a time course only slightly slower than the onset of current (Fig. 4*A*). These caveats notwithstanding, it is clear that the qualitative result from previous studies was replicated in our study, that potentiation occurs at significantly lower steroid concentrations than direct gating. However, our results demonstrate that direct gating occurs at lower concentrations than previously appreciated and suggest that direct gating may be relevant to the physiological effects of neuroactive steroids.

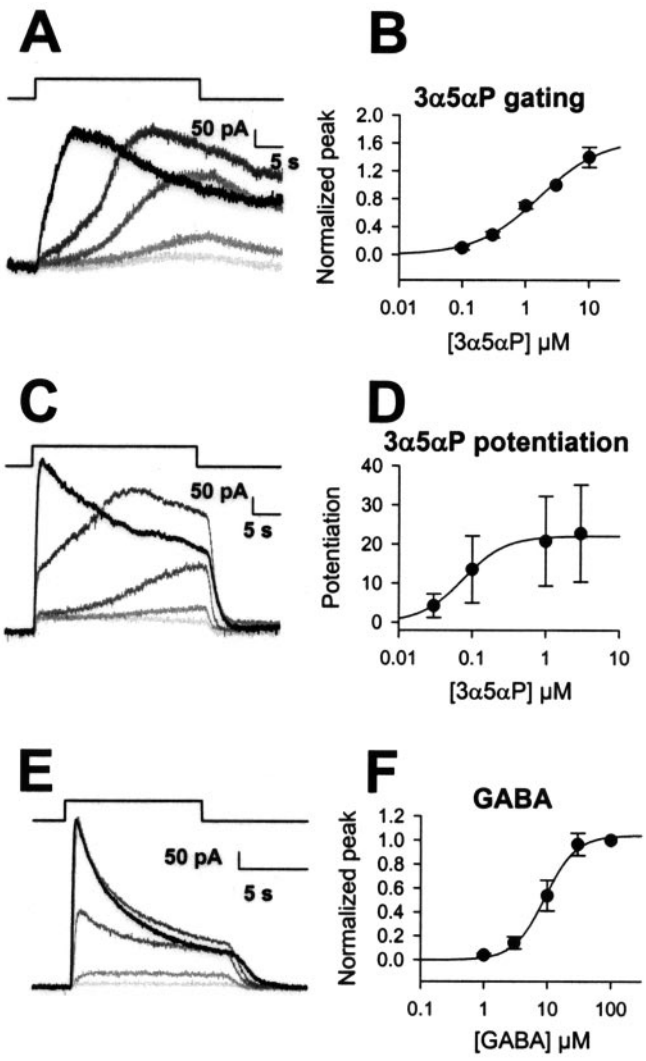

Figure 4. Concentration–response profiles for direct 3α5αP effects ($n = 6$) (*A*, *B*), potentiation of 0.5 μM GABA responses ($n = 4$) (*C*, *D*), and direct GABA responses ($n = 4$) (*E*, *F*). The shading of traces represents increasing concentrations of 3α5αP (*A*, *C*) or GABA (*E*). In *C*, the lightest trace represents the response to GABA alone, from which potentiation was calculated (GABA response is represented as zero, and potentiation was calculated as described in Materials and Methods). Peak amplitudes of responses were measured for summary plots. For all summary plots, the solid lines represent fits to the Hill equation, $ax^n/(b^n + x^n)$, where *a* is the maximum response, *b* is the half-maximum concentration (EC_{50}), *x* is the potentiator concentration, and *n* is the Hill coefficient. Fit parameters are given in the text for *B* and *D*. For GABA responses, the EC_{50} value was 9.1 μM, and the Hill coefficient was 1.8.

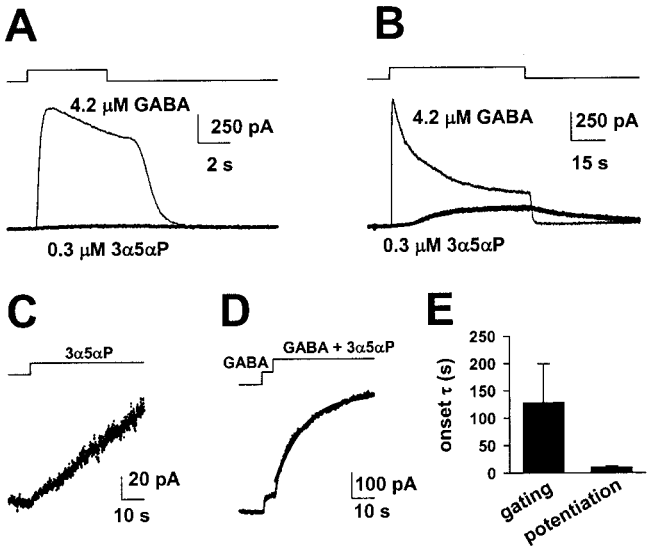

Figure 5. Comparison of GABA-gated and 3α5αP-gated currents. *A*, Comparison of responses to 5 sec applications of concentrations calculated to be 20% of maximum for GABA and 3α5αP. Note that the slower lag for drug washout compared with wash-in was a result of a slower rate of perfusion (and therefore exchange time) for the saline wash. The slower saline wash was used to maintain cell viability for longer periods of time. *B*, Comparison of responses in the same cell as in *A* to 60 sec applications of the same concentrations of agonists. *C*, *D*, Direct gating has slower onset than potentiation. Comparison of direct gating onset (*C*) with potentiation onset (*D*) in the same cell at the same concentration of 3α5αP (100 nM). In *D*, GABA was used at 0.5 μM. Solid lines represent single-exponential fits to the development of the steroid-induced current or current potentiation. In this example, the time constant of current development was 328 sec, and the time constant of potentiation development was 13 sec. *E*, Summary of onset time constants obtained from four neurons in which direct gating and potentiation were directly compared.

Slow kinetics of direct gating

We suspect that direct gating by low steroid concentrations has not been appreciated by previous studies partly because of the extremely slow kinetics of current development. With drug exposures of <5 sec, typical of many studies, directly gated 3α5αP currents are likely to be undetectable. This is highlighted by the experiments in Figure 5. In Figure 5, *A* and *B*, the kinetics of GABA responses were compared with the kinetics of directly gated 3α5αP responses. For both drugs, a concentration 20% of maximum was used, as estimated from the concentration–response data in Figure 4 (0.3 μM for 3α5αP and 4.2 μM for GABA). When a 5 sec application of drugs was compared, little steroid response was detectable (21 ± 2 pA; $n = 3$ neurons). In contrast, when a 60 sec exposure to both drugs was used, a delayed, slowly rising current developed in response to 3α5αP application (124 ± 12 pA; $n = 3$). After accounting for the desensitization of the GABA response over this same time period, the steady-state response from steroid was 31 ± 10% of the GABA response ($n = 3$ neurons), although comparison of peak currents suggests that steroid gates much less efficiently than GABA at an equivalent (percentage of maximum) concentration (peak GABA current, 1057 ± 88 pA; $n = 3$).

Figure 5, *C–E*, shows a comparison of rise times of directly gated currents (Fig. 5*C*) with potentiation of 0.5 μM GABA responses (Fig. 5*D*). In four neurons in which both direct gating and potentiation onset at 0.1 μM 3α5αP were tested, the directly gated current was always slower to rise (81 ± 7% slower; $p < 0.05$), estimated from single-exponential fits to the rising phase of the current (Fig. 5*C–E*).

It is clear from these experiments that the rise of steroid responses is orders of magnitude slower than expected from a diffusion-limited, ligand–receptor interaction. Although GABA association itself is slower than the diffusion limit (Jones et al., 1998), steroid association is clearly much slower than this. What is the reason for the slow steroid kinetics? A recent study reported slow neurosteroid effects at GABA receptors and found evidence that the slow kinetics resulted from activation of a pertussis toxin-sensitive G-protein (Liu et al., 2002). Presumably, time delays associated G-protein activation and associated intracellular signaling cascades could account for the slow 3α5αP currents. However, in our hands, overnight treatment with 300 ng/ml pertussis toxin produced no effect on 3α5αP-gated currents (Fig. 6*B*,*D*,*F*), although in the same cultures, pertussis toxin treatment effectively blocked adenosine receptor-mediated synaptic depression (Fig. 6*A*,*C*,*E*). We conclude that the slow kinetics of

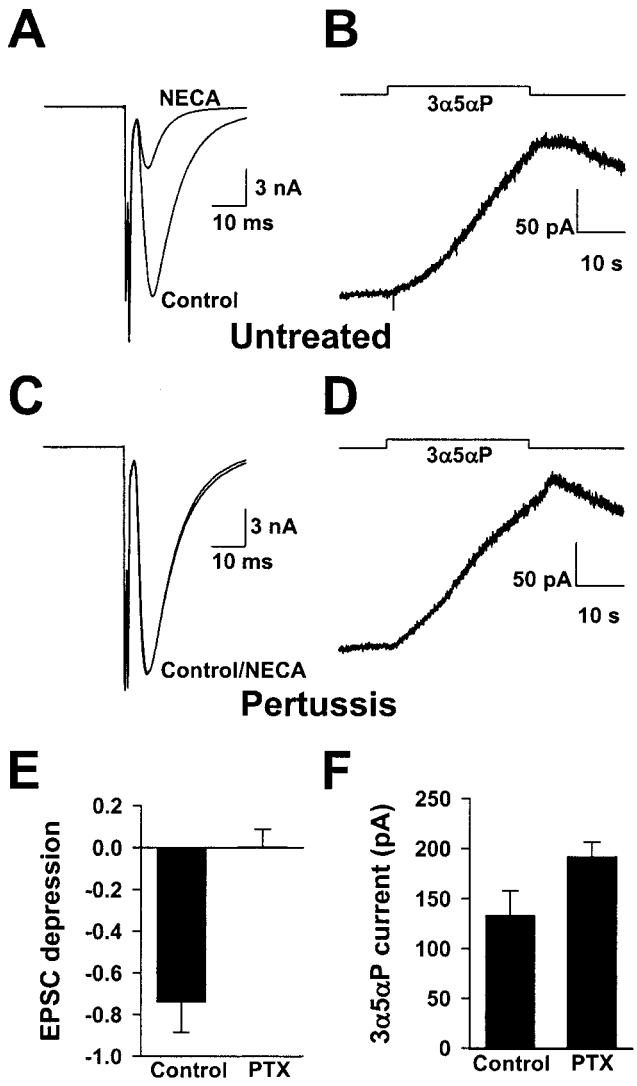

Figure 6. No effect of pertussis toxin on responses of glutamatergic hippocampal neurons to 3α5αP. *A*, *B*, Control neuron, the synaptic depression induced by 0.1 μM 5′-*N*-ethylcarboxamidoadenosine (NECA), and the response of the same neuron to a 30 sec application of 300 nM 3α5αP. *C*, *D*, Neuron treated with pertussis toxin. Note the lack of NECA effect on EPSC but robust 3α5αP current. *E*, Summary of the effect of overnight pertussis toxin treatment on the NECA effect on EPSC amplitudes ($n = 9$ control and 9 pertussis-treated cells). Five control and four pertussis toxin-treated neurons were recorded using a K^+-based pipette solution to account for the possibility that Cs^+ loading occluded NECA-induced synaptic depression. There was no consistent difference among Cs^+-loaded cells and K^+-loaded cells, so results were pooled for the summary. *F*, Summary of pertussis toxin effects on amplitude of 3α5αP currents, measured at the end of a 30 sec pulse of 300 nM 3α5αP ($n = 4$ control neurons and 5 pertussis toxin-treated neurons).

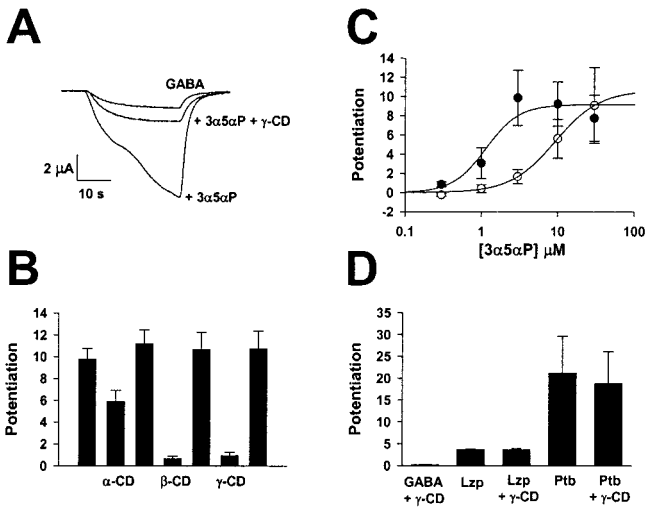

Figure 7. Cyclodextrin effects on 3α5αP potentiation at recombinant receptors. *A*, Example of the effect of γ-cyclodextrin (γ-CD) on 3α5αP potentiation of GABA responses. *B*, Summary of the effects of α-, β-, and γ-cyclodextrin on 3α5αP responses. Concentrations were fixed at 500 μM cyclodextrin and 0.5 μM 3α5αP ($n = 4$ cells). *C*, 3α5αP concentration–response relationship obtained in the presence and absence of 500 μM γ-cyclodextrin. GABA was constant at 2 μM ($n = 4–8$ at each concentration). Solid lines represent a fit to the Hill equation with the Hill coefficient constrained to ≤2. Note the lack of effect of γ-cyclodextrin at saturating 3α5αP concentrations (fits predicted a maximum potentiation of 9.2 and 10.6 for absence and presence of γ-cyclodextrin, respectively). The EC_{50} value for 3α5αP potentiation shifted from 1.1 to 9.2 μM. *D*, No effect of γ-cyclodextrin on responses to GABA alone ($n = 6$) or to potentiation by lorazepam (Lzp; 1 μM; $n = 4$) and pentobarbital (Ptb; 100 μM; $n = 4$) was observed.

3α5αP-gated currents do not involve pertussis toxin-sensitive G-proteins.

Cyclodextrins sequester neurosteroids

We next considered the possibility that the slow 3α5αP-gated currents may be governed by steroid accumulation and departure from a non-aqueous reservoir (perhaps the plasma membrane), from which steroid accesses the relevant $GABA_A$ receptor site. In this model, the reservoir concentration of steroid, rather than the aqueous concentration, may be most directly relevant for steroid gating. This hypothesis predicts that the very slow deactivation of directly gated currents (Figs. 1, 3, 4, 6) is not a result of a high-affinity ligand–receptor interaction, which typically dictates slow deactivation kinetics (Jones and Westbrook, 1995; Bianchi and Macdonald, 2001). Rather, slow deactivation is the result of slow reservoir emptying into the aqueous medium.

We probed these ideas using cyclodextrins, cyclic sugar molecules with a hydrophobic core region within which cholesterol (Szejtli, 1998) and at least some steroids (Adam et al., 2002) fit. Cyclodextrins have been used to manipulate plasma membrane cholesterol content (Yancey et al., 1996; Haynes et al., 2000; Westover et al., 2003). The association of the hydrophilic groups of the cyclodextrin with polar phosphate head groups is believed to provide a hydrophobic alternative to the lipid into which cholesterol can diffuse and thus be removed rapidly from the cell membrane (Christian et al., 1997). Presumably, through the same mechanism, cyclodextrins could sponge neuroactive steroids from the membrane or other non-aqueous reservoirs. Crystallization of the neuromuscular blocking steroid rocuronium with a γ-cyclodextrin (a cyclodextrin with seven cyclic sugars) has been achieved (Bom et al., 2002), and GABA-active steroids are often delivered *in vivo* using cyclodextrins as solubilizing agents (Wang et al., 1997). These lines of evidence suggest that cyclodextrins may be effective tools for manipulating neurosteroid actions.

To test the use of cyclodextrins as molecular neurosteroid sponges, we performed preliminary experiments in *Xenopus* oocytes. We challenged oocytes expressing the α1β2γ2L subunit combination with GABA, GABA plus 0.5 μM 3α5αP, or GABA plus 3α5α*p* plus 500 μM cyclodextrins (mixed together before coapplication) (Fig. 7*A*). We tried variants of α-cyclodextrins (six sugars), β-cyclodextrins (seven sugars), and γ-cyclodextrins (eight sugars). We found that β- and γ-cyclodextrins effectively blocked potentiation, whereas α-cyclodextrin was relatively ineffective (Fig. 7*A*,*B*),

presumably because the hydrophobic pocket of the α-cyclodextrin is too small for an optimal steroid fit (Szejtli, 1998). We also tried several substituted β- and γ-cyclodextrins, including methyl-β-cyclodextrin and 2-hydroxypropyl-γ-cyclodextrin. These had similar effects to the parent cyclodextrin (data not shown). Because γ-cyclodextrin has been reported to have less effect on membrane cholesterol than β-cyclodextrins (Ohtani et al., 1989), we chose γ-cyclodextrin for additional characterization.

The effect of γ-cyclodextrin was not through direct antagonism of GABA receptors. First, increasing the 3α5αP concentration to saturating values eliminated the γ-cyclodextrin effect (Fig. 7*C*). Also, 500 μM and 5 mM γ-cyclodextrin (Fig. 7*D* and data not shown) had no effect on responses to GABA alone. Furthermore, 500 μM γ-cyclodextrin had no significant effect on the potentiation of GABA responses by the benzodiazepine lorazepam or by the barbiturate pentobarbital (Fig. 7*D*). These results suggest that neuroactive steroids, like other steroids and cholesterol, are effectively bound and sequestered by cyclodextrins. Inspection of the shift in the 3α5αP concentration–response curve in Figure 7*C* suggests a dissociation constant of ~70 μM for the γ-cyclodextrin–3α5αP interaction.

Cyclodextrins speed deactivation kinetics in hippocampal neurons

In hippocampal neurons, we tested the ability of γ-cyclodextrin to speed the deactivation kinetics of 3α5αP-gated currents. We reasoned that if the slow dissociation of ligand from receptor explains the slow deactivation kinetics of directly gated currents, then cyclodextrin will be unable to alter the deactivation kinetics of 3α5αP-gated currents. In contrast, if steroid is repeatedly binding and unbinding receptor from a putative non-aqueous reservoir, the rate-limiting factor for deactivation may be reservoir emptying rather than the intrinsic kinetics of ligand dissociation. In this case, we might speed deactivation with cyclodextrin application. Figure 8, *A* and *B*, shows the results of an experiment in which we washed 300 nM 3α5αP away after a 30 sec application either with normal saline or with saline plus 500 μM γ-cyclodextrin. γ-Cyclodextrin speeded deactivation kinetics markedly, consistent with the idea that the intrinsic dissociation kinetics of steroid from receptor are not rate limiting for current deactivation.

An alternative explanation for the results shown in Figure 8, *A* and *B*, is that γ-cyclodextrin acts as a noncompetitive steroid antagonist in addition to, or rather than as, a steroid sponge. Although the experiments in oocytes suggest that any direct antagonist activity at GABA receptors is minimal, the experiments do not fully exclude the possibility of antagonism specific to steroid effects. We reasoned that if γ-cyclodextrin were acting as a noncompetitive steroid antagonist, then rapid washout of steroid after γ-cyclodextrin-induced deactivation should result in a rebound current, reflecting the continued presence of steroid after γ-cyclodextrin washout (Bianchi and Macdonald, 2001).

Figure 8, *C–F*, compares the effects of a brief combined 3α5αP plus γ-cyclodextrin exposure with a brief bicuculline exposure. Bicuculline was used in this case as a noncompetitive antagonist with respect to steroid agonist (Ueno et al., 1997). The experiment shows that after wash-in of γ-cyclodextrin, the current only minimally rebounds, consistent with the loss of steroid to γ-cyclodextrin. In contrast, wash with bicuculline produced the predicted rebound current, reflecting continued presence of steroid after washout of antagonist. To show that γ-cyclodextrin washout was effective in these experiments, we performed an additional control experiment, depicted in Figure 8*F*. In this experiment, we exploited potentiated GABA responses to examine

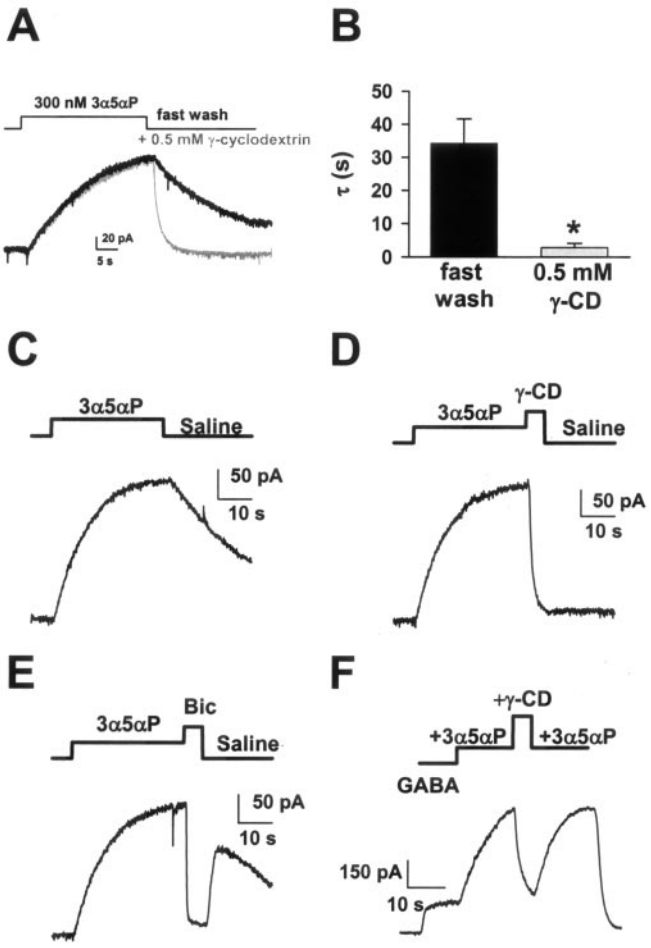

Figure 8. γ-Cyclodextrin effects on direct gating offset kinetics in hippocampal neurons. *A*, Example showing speeding of the offset of 3α5αP (300 nM) current in a hippocampal neuron by 500 μM γ-cyclodextrin. Note that although slow saline washes were used for some experiments (see Fig. 4), the solution exchange times for saline and cyclodextrin solutions in the present experiment were matched. *B*, Summary of results from four neurons treated as in *A*. Deactivation was fit with a single-exponential function, the average time constant of which is plotted (*$p = 0.01$). *C–E*, Comparison of brief γ-cyclodextrin and bicuculline effects on deactivation of directly gated currents. All data in *C–E* are from a single neuron. Protocols are as indicated. Concentrations of drugs were 300 nM 3α5αP, 500 μM γ-cyclodextrin, and 25 μM bicuculline. *F*, Control experiment for the speed of γ-cyclodextrin washout. The experimental protocol is as indicated, with GABA at 0.5 μM, 3α5αP at 0.1 μM, and γ-cyclodextrin at 500 μM. Note that 3α5αP was co-applied with GABA and γ-cyclodextrin was co-applied with GABA and 3α5αP where indicated. Note that the redevelopment of potentiated current after γ-cyclodextrin exposure was similar to initial onset of potentiation suggesting that γ-cyclodextrin washout is not rate limiting.

the speed with which we could remove γ-cyclodextrin. In all cells tested ($n = 6$), the redevelopment of 3α5αP-potentiated current after brief γ-cyclodextrin exposure was somewhat faster (time constant, 47 ± 3% faster) than the time constant of initial 3α5αP potentiation, suggesting that γ-cyclodextrin washout is not rate limiting. The results clearly show that γ-cyclodextrin washout did not limit our ability to resolve a rebound current in Figure 8*D*.

Direct gating and neuronal excitability

Finally, to assess directly the effect of direct gating on neuronal excitability, we explored the effect of 3α5αP on action potential responses to depolarizing current injection. We found that 100 nM 3α5αP produced a clear decrease in excitability in all neurons tested, assessed by the number of spikes fired in response to

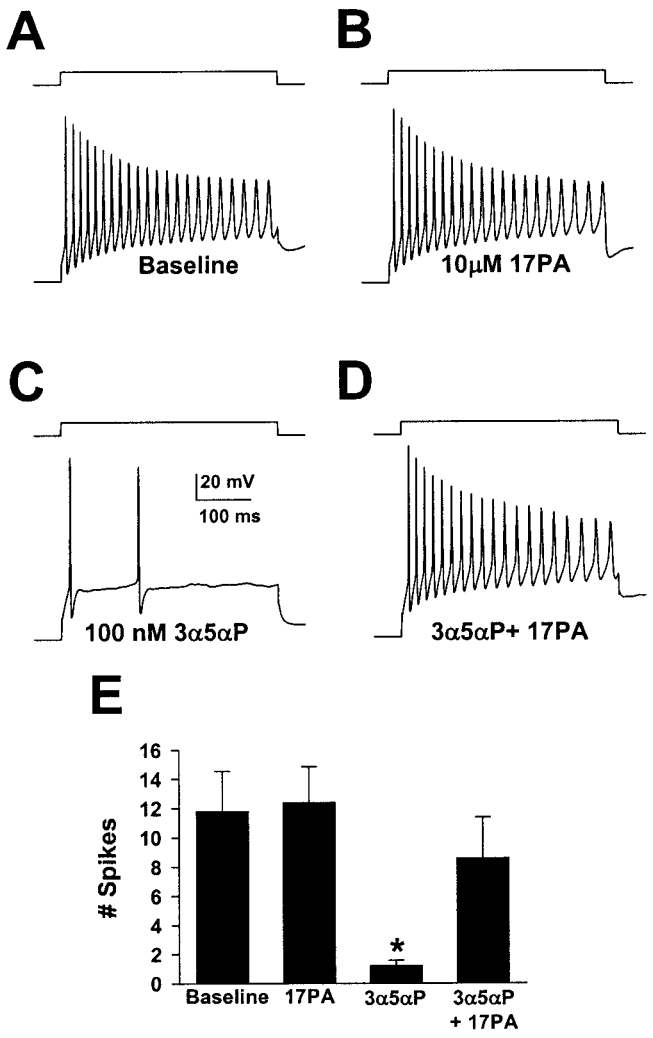

Figure 9. The effect of direct gating on neuronal excitability. *A–D*, Solitary glutamate neurons were treated as indicated. Cells were exposed to drugs for 40 sec before a 400 msec direct depolarizing current injection (300 pA for this cell; top traces) to elicit action potentials (bottom traces). The calibration bar in *C* applies to *A–D*. *E*, Summary of the number of action potentials elicited under the labeled conditions in five solitary neurons. $*p < 0.05$ significantly different from control and from steroid plus 17PA.

depolarizing current injection (Fig. 9). To determine whether the decrease in excitability was the result of neurosteroid effects at the $GABA_A$ receptor, we co-applied 17PA. We found the decrease in spiking was primarily prevented by 17PA (Fig. 9*C*,*E*), which in the absence of 3α5αP had no effect on neuronal spiking (Fig. 9). Taken together, our results suggest that direct gating may represent a significant mechanism by which neuroactive steroids can alter neuronal excitability.

Discussion

The main results of this study show that neuroactive steroids directly gate the $GABA_A$ receptor at low concentrations, likely relevant to their behavioral effects. This direct action of steroids may have previously been unappreciated partly because of the brief exposures used in many previous studies relative to the slow development of directly gated currents. We show that slow deactivation kinetics do not result from an inherently high-affinity interaction between steroid and receptor. Instead, slow deactivation likely results from slow removal of steroid from a cyclodextrin-accessible reservoir, which we hypothesize is the plasma membrane.

Consistent with previous work, we find that potentiation occurs at lower concentrations than direct gating and generates much larger current amplitudes. Thus, *in situ*, where average ambient GABA concentrations are estimated at ≤ 1 μM (Lerma et al., 1986), potentiation may play a much larger role than direct gating in setting membrane excitability. Nevertheless, in local areas of minimal GABA concentrations, perhaps created by paucity of GABA terminals or by particularly avid uptake, direct gating may play a more prominent role. Even with ambient GABA present, a small percentage of receptors will be bound by GABA (~8.4% at 0.5 μM GABA and 100 nM 3α5αP; calculated from values in Fig. 4), leaving many receptors available for gating by steroid.

Several factors may have hindered previous observations of direct gating by low neuroactive steroid concentrations. First, we propose that the extremely slow development of currents has precluded observation of directly gated currents in many previous studies, in which application times of <1 sec are routine. Although slow responses may have been a limitation on experimental observations, it is unlikely the slow kinetics of current development are an important limitation on the *in vivo* relevance of direct gating. Endogenous neuroactive steroids are probably present in nearly steady-state concentrations; they are not synaptically released and equilibrate rapidly across the plasma membrane. Therefore, any *in situ* contribution of direct gating is likely to be tonic.

Second, we observed that oocytes expressing recombinant receptors are less sensitive than hippocampal neurons to potentiation, for which we observe a difference of >10-fold in EC_{50} values (Figs. 4*D*, 7*C*) and to direct gating (data not shown for oocytes). Therefore, receptor subunit composition, expression environment, or differences in post-translational state may also contribute to the failure of some previous studies to detect low-concentration direct gating.

Slow kinetics of steroid-gated currents have also been previously observed in neuronal cultures (Liu et al., 2002). In this previous study, biphasic GABA receptor-mediated currents were observed in response to steroid applied alone. In our studies, we never observed biphasic responses to 3α5αP applied alone. The difference might be explained by differences in experimental conditions. We exclusively used solitary glutamatergic cells to avoid contribution of responses from synaptic or ambient GABA. It is possible that the biphasic currents arose in the previous study as a result of a combination of potentiation of ambient or synaptic GABA responses with a slower directly gated response. The previous study also suggested that the slow 3α5αP responses involved a pertussis toxin-sensitive G-protein. Using adenosine modulation of EPSCs as a positive control for pertussis toxin sensitivity, we failed to find a role for a pertussis toxin-sensitive G-protein in the slow, directly gated currents.

Instead, to account for our evidence of a cyclodextrin-accessible reservoir that is rate limiting in the deactivation of 3α5αP-gated currents, we suggest the hypothesis that membrane concentration of steroid is an important determinant of the rate of current development and offset. Our hypothesis is based on ability of γ-cyclodextrin to speed deactivation of steroid-gated responses and based on the known hydrophobicity of neuroactive steroids (log *p* value of 4.89 for 3α5αP, calculated using Advanced Chemistry Development, version 4.67; Toronto, Ontario, Canada). Important corollaries of this hypothesis are that the steroid binding site may be near the membrane–protein in-

terface and that the membrane partition coefficient could be an important determinant of steroid potency. Although the plasma membrane is the most likely candidate for the cyclodextrin-accessible reservoir, we cannot exclude the possibility of proteinaceous reservoirs. Regardless of the exact nature of the reservoir, our results clearly show that the slow deactivation kinetics of 3α5αP-gated currents do not result from an inherently tight association between 3α5αP and the receptor site.

Cyclodextrins have been widely used as molecular sponges to remove membrane cholesterol (Yancey et al., 1996; Haynes et al., 2000; Westover et al., 2003). Interestingly, the kinetics of cholesterol removal appear to be significantly slower (15 sec to 21 min) than our ability to deactivate GABA receptor currents. At least part of the slow kinetics of cholesterol removal may be governed by slow movement of cholesterol from the inner membrane leaflet to the outer leaflet (Haynes et al., 2000), where it is accessible to extracellularly applied cyclodextrin. If the reservoir for neuroactive steroids represents the plasma membrane, then the very rapid termination of steroid-gated currents by cyclodextrin suggests that steroid associates with membrane in a manner readily accessible to extracellular saline. We cannot exclude the possibility of more deeply embedded pools of steroid within the membrane (including the inner membrane leaflet) or within intracellular compartments, but it appears that only a pool readily accessible by cyclodextrin applied in the aqueous medium is immediately relevant to direct gating.

We should also note that although the kinetics of direct gating are particularly slow, development and offset of potentiation are also quite slow (Fig. 5). Therefore, we do not exclude an important effect of similar, cyclodextrin-sensitive mechanisms to potentiation of GABA-gated currents. In fact, because the sites for potentiation and direct gating on the GABA receptor are unknown, it is possible that similar or the same site mediates both effects of steroids.

We considered several alternative interpretations to the reservoir hypothesis that might account for cyclodextrin effects. It is possible that cyclodextrins have direct antagonistic effects on the GABA receptor that account for the accelerated deactivation observed in Figure 8. We view direct antagonism as unlikely for several reasons. First, we found no effect of 500 μM γ-cyclodextrin on currents gated by GABA in either oocytes (Fig. 7) or hippocampal neurons (data not shown). Second, we found no effect of γ-cyclodextrin on potentiation of GABA responses by pentobarbital or lorazepam (Fig. 7). Third, in hippocampal neurons, we observed no rebound current after cyclodextrin washout, in contrast to noncompetitive blockers of steroid-gated currents (Fig. 8). A competitive interaction between cyclodextrin and steroid at the receptor is possible, but if cyclodextrin accelerates deactivation by competing for the steroid site, this mechanism bolsters our basic conclusion that the inherent steroid–receptor interaction is not high affinity because steroid must dissociate before a competitor can bind (Bianchi and Macdonald, 2001).

As noted, our favored interpretation of the cyclodextrin effect suggests that the inherent association between steroid and receptor is rather low affinity. However, we cannot fully exclude a more complicated model in which a multivalent interaction between steroid and receptor is necessary for gating. It is possible that part of the steroid dissociates rapidly, rendering the steroid accessible to cyclodextrin, whereas another part of the steroid remains bound to the receptor. Cyclodextrin binding would be sufficient to render the steroid inactive, although steroid is still tethered to the receptor. Such models are formally possible, but based on parsimony, we favor a simpler mechanism of complete ligand dissociation.

A final alternative is that cyclodextrin does not directly affect a non-aqueous reservoir of accumulated steroid but rather has its effects by altering some intrinsic membrane component (e.g., cholesterol). An effect on cholesterol is unlikely because γ-cyclodextrin is less efficient than methyl-β-cyclodextrin at removing cholesterol (Ohtani et al., 1989). Also, the concentrations of methyl-β-cyclodextrin used to manipulate membrane cholesterol are typically ≥10-fold greater than the concentrations of γ-cyclodextrin used in our experiments (Haynes et al., 2000; Westover et al., 2003). Furthermore, the lack of effect of γ-cyclodextrin on GABA-gated responses makes it unlikely that the general membrane perturbation is responsible for the cyclodextrin effects on steroid-gated currents. Finally, when we performed protocols designed to deplete membrane cholesterol, by incubating cells for 10–20 min at 37°C in 5 mM methyl-β-cyclodextrin, we found no effect on the ability of 3α5αP to directly gate currents after incubation (data not shown). In summary, our favored interpretation that cyclodextrin removes neurosteroid from the plasma membrane or other non-aqueous reservoir to terminate GABA receptor-mediated responses is consistent with the known ability of cyclodextrins to sequester neuroactive steroids and represents the most parsimonious explanation for the experimental observations.

We conclude that direct gating deserves consideration as relevant to the cellular and behavioral effects of neuroactive steroids. Consideration of the temporal aspects of direct gating led us to the conclusion that slow kinetics are not governed by simple ligand–receptor interactions. Rather, we hypothesize that access to the steroid direct gating (and perhaps potentiation) site occurs via the plasma membrane and it is the membrane concentration of steroid that governs direct gating.

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