Effects on Membrane Capacitance of Steroids with Antagonist Properties at $GABA_A$ Receptors

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ABSTRACT We investigated the electrophysiological signature of neuroactive steroid interactions with the plasma membrane. We found that charged, sulfated neuroactive steroids, those that exhibit noncompetitive antagonism of $GABA_A$ receptors, altered capacitive charge movement in response to voltage pulses in cells lacking GABA receptors. Uncharged steroids, some of which are potent enhancers of $GABA_A$ receptor activity, produced no alteration in membrane capacitance. We hypothesized that the charge movements might result from physical translocation of the charged steroid through the transmembrane voltage, as has been observed previously with several hydrophobic anions. However, the charge movements and relaxation time constants of capacitive currents did not exhibit the Boltzmann-type voltage dependence predicted by a single barrier model. Further, a fluorescently tagged analog of a sulfated neurosteroid altered membrane capacitance similar to the parent compound but produced no voltage-dependent fluorescence change, a result inconsistent with a strong change in the polar environment of the fluorophore during depolarization. These findings suggest that negatively charged sulfated steroids alter the plasma membrane capacitance without physical movement of the molecule through the electric field.

INTRODUCTION

Neuroactive steroids act potently and apparently directly to either potentiate or antagonize the actions of GABA acting at $GABA_A$ receptors. Potentiating steroids are uncharged and highly lipophilic, with log *P* values suggesting that steroids could accumulate in the plasma membrane at 10,000–100,000 times the concentration found in aqueous solution, although this has never been measured directly. This accumulation is likely relevant to potentiating steroid actions because binding sites for anesthetic neuroactive steroids were identified recently within transmembrane domains of the $GABA_A$ receptor (1), and our work has recently stressed the importance of membrane and intracellular accumulation to the longevity and the potency of receptor modulation (2–4).

By contrast, the most potent GABA-antagonist steroids are sulfated at carbon 3, of the steroid A-ring (5,6). The anionic charge of sulfated steroids keeps this class of neurosteroid from passing directly through the plasma membrane, at least at sufficiently low concentrations (7), but some of the sulfated steroids exhibit voltage dependent block of $GABA_A$ receptors (8), suggesting the possibility of physical voltage-dependent translocation of the molecule across the bilayer, as occurs with other charged lipophilic molecules (9–16).

These considerations suggest two ways in which GABA-active steroids might interact with the plasma membrane and that might be detected electrophysiologically. First, steroids might intercalate into the membrane in a way that alters the membrane capacitance, without voltage-dependent physical translocation of the steroid. This might occur, for instance, if steroids alter packing density of membrane lipids. Second, if the negatively charged sulfate group of antagonist steroids directly senses the transmembrane potential, capacitive charge movement might be generated as a result of the physical translocation of the charge across a fraction of the bilayer and through the transmembrane electrical field.

We tested uncharged and charged steroids with actions at $GABA_A$ receptors and found that only negatively charged sulfated pregnane steroids altered membrane capacitance of *Xenopus* oocytes and human embryonic kidney 293 (HEK) cells. Sulfated steroids did not affect membrane resistance. Effects on membrane capacitance did not require presence of GABA receptors. The unique effects of the charged steroids suggested the hypothesis that sulfated steroids may be physically translocated through the transmembrane electric field. In contrast to Boltzmann predictions that arise from this hypothesis, we observed little or no voltage dependence to the charge or time constants of current responses to voltage pulses over a wide range of membrane potentials. Further, a fluorescently tagged sulfated steroid, predicted to alter its fluorescence as the hydrophobicity of the fluorophore environment changes, exhibited no voltage-dependent fluorescence change. These data suggest that the main electrophysiologically detectable effect of neuroactive steroids is that of an altered membrane capacitance, imparted by antagonist sulfated pregnane steroids but not by hydroxypregnane potentiating steroids. We conclude that sulfated steroids, probably by adsorption to the membrane, alter the physical properties

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of the membrane to increase membrane capacitance, but without physical translocation of the anion. We speculate on the relevance to steroid modulation of ion channels.

MATERIALS AND METHODS

Compounds

Except for the tagged analog in Figs. 5 and 6 and the enantiomer of pregnenolone sulfate (17), steroids used in the studies were commercially available and obtained from Sigma-Aldrich (St. Louis, MO) or from Steraloids (Newport, RI). Commercially available steroids were pregnenolone sulfate; (3α,5β)-3-hydroxypregnan-20-one, also called pregnanolone and herein abbreviated 3α5βP; (3α,5β)-3-hydroxypregnan-20-one sulfate (3α5βPS); (3β,5α)-3-hydroxypregnan-20-one (3β5αP); and (3β,5α)-3-hydroxypregnan-20-one (3β5αPS). A (7-nitro-2,1,3-benzoxadiazol-4-yl)amino group was attached to carbon 2 of 3β5αPS to create a fluorescent sulfated steroid as described (C2-NBD 3β5αPS) (18). Full synthetic details will be given elsewhere. Other compounds were from Sigma.

Oocyte recordings

Stage V to VI oocytes were used for all physiology. Oocytes were harvested from sexually mature female *Xenopus laevis* frogs (*Xenopus* One, Northland, MI) under anesthesia, according to institutionally approved protocols. Oocytes were defolliculated by shaking for 20 min at 37°C in calcium-free saline solution containing collagenase (2 mg ml^{-1}). Capped mRNA, encoding rat $GABA_A$ receptor subunits α1, β2, and γ2L was transcribed in vitro using the mMessage mMachine kit (Ambion, Austin, TX) as described previously (19). cRNA (30 ng total, 1:1:1 subunit ratio) was injected 24 h after defolliculation, and recordings were carried out 24–96 h after RNA injection. γ2 subunit expression was verified by benzodiazepine (1 μM lorazepam) potentiation of responses to 2 μM GABA. Oocytes were cultured at 18°C in solution containing (in mM): NaCl 96, KCl 1, $MgCl_2$ 1, $CaCl_2$ 2, and HEPES 10 at pH 7.4, supplemented with pyruvate (5 mM), penicillin (100 U ml^{-1}), streptomycin (100 g ml^{-1}), and gentamycin (50 g ml^{-1}).

Oocytes were clamped using a virtual-ground two electrode voltage clamp (Warner OC-725C amplifier, Warner Instruments, Hamden, CT). Pipettes were filled with 3 M KCl. Bath solution included (in mM) NaCl 96, KCl 1, $MgCl_2$ 1, $CaCl_2$ 2, and HEPES 10 at pH 7.4. Data were collected using a Digidata 1322 analog/digital converter and PClamp 9 software (Molecular Devices, Sunnyvale, CA). Responses to voltage pulses were collected at open bandwidth and digitized at 50 kHz.

For most studies, we used oocytes uninjected with RNA to isolate membrane effects of the neurosteroids examined. We found no effect of the presence of $GABA_A$ receptors on the capacitive currents studied herein. Methods for estimating oocyte capacitance in the presence and absence of sulfated steroids are described in Results. For estimating charge displacement resulting from tetraphenylborate application to oocytes, we incubated oocytes overnight in 100 μM tetraphenylborate to allow drug to equilibrate with the membrane. Shorter tetraphenylborate applications of up to 2 h were not sufficient to produce capacitance changes. Unlike control oocytes, whose capacitance current relaxations were well described by a single exponential, current transients in tetraphenylborate-treated cells required a bi-exponential fit (see Fig. 4 *C*, *inset*). The fast component was similar to the single component of control cells and was assumed to be associated with basal, endogenous membrane capacitance. The slow component was assumed to represent voltage-dependent displacement of tetraphenylborate, and this component was quantified in Fig. 4, *C* and *D*. For HEK cell experiments with tetraphenylborate (Supplementary Material, Fig. S1), we detected tetraphenylborate-induced capacitance changes with lower concentrations and within 5 min of incubation (Fig. S1). These changes did not differ from those produced by overnight HEK-cell incubation in tetraphenylborate.

Cell cultures

Primary neuronal/astrocyte cultures were prepared from postnatal day 0–3 rat hippocampus as described previously (20). Cells were dissociated by papain treatment followed by mechanical dispersion onto collagen-coated 35 mm culture dishes. Culture medium was modified Eagle's medium (Invitrogen, Gaithersburg, MD) supplemented with heat-inactivated horse serum (5%), fetal bovine serum (5%), 17 mM glucose, 400 μM glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin. Cultures grew at 37°C in a humidified incubator equilibrated with 5% CO_2/95% air. Cytosine arabinoside (6.7 μM) was added 3–4 days after plating to inhibit glial division. At 4–5 days after plating, medium was supplemented by a 1/2-volume replacement with Neurobasal medium (Invitrogen) plus B27 supplement. Cultures were imaged 7–12 days after plating using conventional epi-fluorescence during application of C2-NBD 3β5αPS (18). A total of 1–6 μM C2-NBD 3β5αPS was applied by local bath perfusion through a multi-barrel pipette placed within 0.5 mm of the recorded cell. These high concentrations sometimes resulted in significantly more intracellular fluorescence (see Fig. 5) than evident at lower concentrations, where fluorescence is primarily localized to the plasma membrane (18). Emission at 535 nM was collected and analyzed using a 40× 0.8 N.A. objective and a CoolSnap ES2 camera (Photometrics, Tucson, AZ). Alternatively, 0.5 μM Bis-(1,3- dibutylbarbituric acid) trimethine oxonol (DiBAC4(3)) was applied. Images were acquired at the end of 20–30 s dwell time at the indicated voltages. Metamorph software (Molecular Devices) was used to quantify fluorescence intensity in cellular regions of interest.

HEK293 cells were grown on 35 mm culture dishes in DMEM medium with 10% fetal bovine serum and 1 mM glutamine. Small to medium-sized, isolated cells were selected for study. We avoided cells making physical contact with other cells to negate influences of gap junction coupling on the responses to voltage pulses.

Whole-cell recordings

Whole-cell recordings were made from neurons and HEK cells in a recording bath solution containing (in mM) 140 NaCl, 4 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 glucose, and 10 HEPES pH 7.3. The whole-cell pipette contained 130 cesium methanesulfonate, 4 NaCl, 0.5 $CaCl_2$, 5 EGTA, and 10 HEPES pH 7.25. Pipettes were pulled from borosilicate glass and had open tip resistances when filled with the recording solution of 1.5–4 M Ω (HEK cells) and 3–6 M Ω (neurons). Voltage was controlled using an Axopatch 200B amplifier (Molecular Devices) interfaced to a personal computer running PClamp 9 software (Molecular Devices) via a Digidata 1322 analog/digital converter (Molecular Devices). For capacitance measurements, pipette capacitance was zeroed completely using the fast capacitance compensation circuitry of the amplifier before rupturing the membrane. Data were filtered at 10 kHz using the 4-pole Bessel filter of the amplifier and were digitized at 20–200 kHz. Ten kHz and 100 kHz low-pass filter bandwidths were compared in a subset of recordings. Estimates of time constants were within 7.6 $\pm$ 3% (a difference of 11.4 μs on average); therefore, data were analyzed using a 10 kHz filter bandwidth.

All imaging and electrophysiology experiments were done at room temperature (23°C). Student's *t*-test (paired or unpaired as appropriate) was used to evaluate statistical significance. All descriptive statistics in text and graphs are presented as mean $\pm$ SE.

RESULTS

We used voltage-clamped *Xenopus* oocytes to evaluate the electrophysiological signature of steroid interactions with $GABA_A$ receptors and with the plasma membrane. Fig. 1, *A* and *B*, shows canonical effects of an exemplar 3α-hydroxypregnane steroid, pregnanolone (3α5βP; Fig. 1 *A*), and a

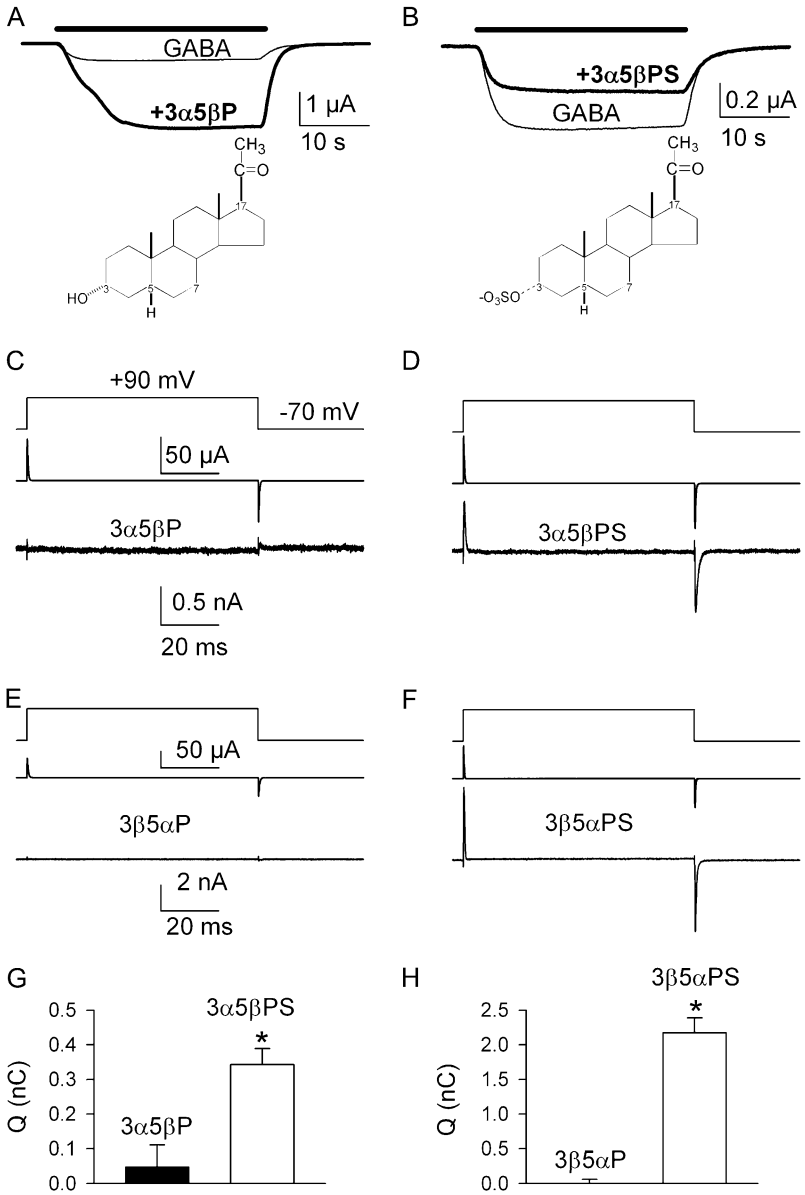

FIGURE 1 Effects on membrane properties of steroid potentiators and antagonists of $GABA_A$ receptor function. (*A*) In voltage-clamped oocytes (−70 mV) expressing the $\alpha1\beta2\gamma2$L $GABA_A$ receptor subunit combination, GABA (4 μM) application for the period denoted by the horizontal bar generated an inward current (*thin trace*) that was potentiated by the co-application of 0.5 μM 3α5βP (*thick trace*). Mean potentiation in 3 cells was 5.7 ± 0.7-fold above the baseline GABA response. (*B*) In the same oocyte as *A*, the sulfated neurosteroid 3α5βPS (10 μM) antagonized the GABA response by ~40%. The structures of the relevant steroids are shown below the panels. In four cells the average inhibition was 38 ± 2%. (*C–F*) Steroid-induced capacitive current changes in an oocyte not expressing GABA receptors. Top traces in each panel represent the voltage protocol. Middle traces represent raw current responses to the pulse protocol; responses obtained in plain saline and in 50 μM of the indicated drug are superimposed. Bottom traces represent digital subtraction of the baseline trace from the drug trace. (*C*) In the bottom trace, the negligible current responses represent current attributable to 50 μM 3α5βP, because baseline currents to the same voltage pulse have been subtracted away. (*D*) The same experiment carried out using the sulfated neurosteroid (50 μM) showed that the drug induced significant changes in the transient current responses to voltage pulses. (*E* and *F*) The same protocol as that used in *C* and *D*, except with a pair of neurosteroids with different stereochemistry at carbons 3 and 5. (*G* and *H*) Summary of the charge integral obtained by integrating the transient current onsets in traces like those in C–F (n = 3–9 oocytes for each bar). Note that uncharged steroids (*left bar* of each pair) did not generate a significant alteration ($p > 0.05$ compared with 0 change) in capacitive charge movement but that sulfated steroids (*right bar*) did ($p < 0.01$ compared with 0 change). The 3β5α sulfated steroid (*F* and *H*) generated significantly larger alterations in capacitive currents than its diastereomer (*D* and *G*; *$p < 0.05$ sulfated versus unsulfated).

noncompetitive $GABA_A$ receptor steroid antagonist, pregnanolone sulfate (3α5βPS; Fig. 1 *B*). The compounds were evaluated in oocytes injected with cRNA encoding rat $\alpha1\beta2\gamma2$L $GABA_A$ receptor subunits. Both steroid effects on GABA receptors are well documented by previous literature (21). Fig. 1 *A* shows potentiation to a moderately low concentration of GABA (4 μM). At this GABA concentration, 10 μM 3α5βPS produced ~40% inhibition, characteristic of effects of many sulfated steroids at this concentration (7,22) (Fig. 1 *B*).

These same steroids were investigated for nonspecific plasma membrane interactions using voltage-pulse protocols in naïve oocytes (not injected with exogenous RNA). Voltage pulses from −70 mV to +90 mV yielded fast capacitive currents that were unaltered by the presence of the hydroxysteroids (Fig. 1 *C*) but were detectably altered in the presence of the sulfated steroid (Fig. 1 *D*). Specifically, subtractions of current responses in the absence of drug from current responses in the presence of drug showed drug-induced outward transient currents on depolarization, and inward transient currents on repolarization (Fig. 1 *D*).

We examined the effect of altering the stereochemistry of the substituent at carbon 3 and the hydrogen atom at carbon 5. The 3β configuration of the hydroxysteroid renders it ineffective as a potentiator at $GABA_A$ receptors (21). Similar to 3α5βP, 3β5αP failed to detectably alter membrane capacitance (Fig. 1 *E*). Again, the sulfated compound with 3β and 5α stereochemistry yielded large transients (Fig. 1 *F*). Neither 3α5βPS nor 3β5αPS reliably altered any ionic currents (that are minimal in oocytes) in response to the depolarization (Fig. 1, *D* and *F*). Therefore, it is unlikely that the sulfated steroids produced their effects on capacitive currents by

altering the gating of endogenous voltage-gated currents in the oocytes.

Fig. 1, *G* and *H*, summarize the total drug-induced charge transfer obtained from drug subtractions like those shown in Fig. 1, *C–F*, for the four compounds investigated, integrated over 1 ms from the onset of the voltage step. Neither hydroxysteroid generated alterations in capacitive transients that deviated significantly from zero. By contrast, both of the corresponding charged, sulfated steroids generated significant alterations of oocyte capacitive transients. Because $3\beta5\alpha$PS yielded transients approximately fivefold larger than $3\alpha5\beta$PS, we focused our attention on the 3β sulfate for subsequent studies.

Fig. 2 *A* shows details of the $3\beta5\alpha$PS effect and clarifies the characteristics of the raw capacitive currents from which the subtracted traces in Fig. 1, *C–F*, were generated. The currents in Fig. 2 *A* represent transients in response to the initial step from −70 mV to +90 mV in the absence (*dotted line*) and presence (*solid line*) of $3\beta5\alpha$PS (same cell as Fig. 1 *F*). The change in current transients caused by drug implies a change in membrane capacitance. To estimate the change in membrane capacitance imparted by the sulfated steroid, we took membrane capacitance to equal

$$C = Q/\Delta V. \quad (1)$$

C represents membrane capacitance, *Q* the charge displacement, and ΔV the imposed voltage change. To estimate *Q* while minimizing the contributions of errors in the peak amplitude of capacitance transients induced by filtering via circuitry and pipette resistances, we fit the decay of onset capacitive transients with a single exponential from 10% of the peak and extrapolated the fit to the instant of the voltage-pulse onset (Fig. 2 *B*, *solid line*). The area under the fit (shaded area of Fig. 2 *B*) was then calculated to yield an estimate of charge displacement, which was used to calculate capacitance by Eq. 1. By this method, oocyte membrane capacitance was calculated to be 253 ± 12 nF from a sample of 11 oocytes, consistent with estimates of oocyte capacitance obtained by varied methods (23,24). $3\beta5\alpha$PS (50 μM) increased the membrane capacitance by 5 ± 2% ($p < 0.05$, paired *t*-test; Fig. 2 *B inset*).

Interestingly, with 50 ms pulses the capacitance change produced by the onset of pulses to +90 mV were not symmetrical with the return (offset) transient produced by the step back to −70 mV. With 50 ms pulses to +90 mV, there was consistently more charge recovered with the pulse offset compared with pulse onset; subtracted transients generated by the return pulse to −70 mV generated 45 ± 9% more charge transfer than the onset pulse generated ($n = 3$; Fig. 2 *C*, $p < 0.05$ compared with 0 change). In these same oocytes, when the pulse duration was decreased to 10 ms, the recovered charge at pulse offset was within 3 ± 2% of the onset transient (Fig. 2 *D*; not significantly different than 0 change). There was absolutely no asymmetry to baseline capacitive

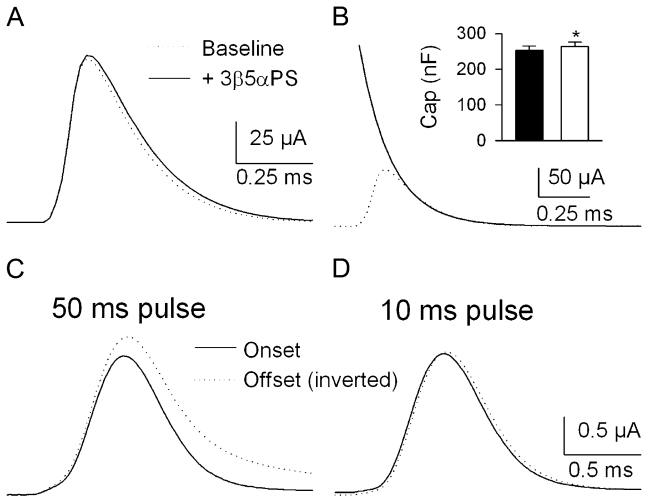

FIGURE 2 Estimates of capacitive change and conservation of charge in oocytes. (*A*) Raw, unsubtracted traces of the onset current in response to a 160 mV voltage pulse from −70 mV. The dotted trace shows the transient current response under baseline conditions (absence of drug). The solid trace shows the current response in the presence of 50 μM $3\beta5\alpha$PS. (*B*) Replot of the dotted trace (absence of steroid) from A showing a single exponential fit (*solid line*) superimposed on the relaxation of the transient, extrapolated to the onset of the voltage pulse to +90 mV. The shading under the fit represents the integral used to calculate total charge displaced. The inset shows the estimate of cell capacitance (total charge displaced, normalized to the amplitude of the voltage pulse) in the absence (*black bar*) and presence (*white bar*) of $3\beta5\alpha$PS. *$p < 0.05$, paired *t*-test, $n = 11$ oocytes. (*C* and *D*) The traces are superimposed subtractions (50 μM $3\beta5\alpha$PS minus baseline saline) showing the transient response to pulse onset (*solid line*) to +90 mV and offset (*dotted line*) on return to −70 mV. The offset responses have been inverted and superimposed for comparison with the onset responses. For long voltage pulses the charge observed on step back to −70 mV was larger than the charge at onset, but for short pulses charge was nearly completely conserved.

charge movements in these cells in the absence of drug (0 ± 0% difference in offset transients versus onset transients, $n = 3$). We interpret the asymmetry in subtracted transients with long pulses to reflect a slow drug-induced charge movement accumulated at the positive membrane potential that was recovered quickly by the return pulse. It is possible that the slow charge accumulation may represent slow asymmetric partitioning of the steroid. However, we did not observe the same asymmetry in another cell type. Patch clamped HEK cells showed no asymmetry in charge recovery with the same voltage protocol (data not shown). We conclude that the phenomenon is oocyte specific and therefore not likely relevant to receptor modulation or other cellular effects of the steroid. The asymmetry was not investigated further.

The sulfated pregnane steroid investigated in Figs. 1 and 2 shows voltage-dependent inhibition of GABA currents, whereas pregnenolone sulfate, a commonly used $GABA_A$ receptor antagonist steroid, exhibits no voltage dependence (7,25). Pregnenolone sulfate also altered capacitive currents in a fashion that was similar, though slightly smaller in magnitude, compared with those generated by $3\beta5\alpha$PS (charge

with a 160 mV step in potential was 8.2 ± 1.1 nC for pregnenolone sulfate and 11.5 ± 1.3 nC for 3β5αPS in the same oocytes; 50 ± 16% difference, $p = 0.01$, $n = 8$). Because both steroids generated relatively large capacitance changes, we conclude that steroid-induced charge movement alterations do not track qualitatively with voltage-dependent effects on $GABA_A$ receptors.

Fig. 3 shows the concentration dependence of steroid-induced charge movements. There was no clear dependence of the time constant of current relaxation on 3β5αPS concentration (Fig. 3 *A*). However, both the amplitude (Fig. 3 *B*), extrapolated from fits to subtracted traces, and total charge transfer (Fig. 3 *C*) exhibited dependence on drug concentration. Interestingly, the concentration dependence of these effects was saturable and was reasonably well described by the Hill equation (Fig. 3, *B* and *C*) with EC_{50} concentrations of 39 μM and 27 μM for charge and amplitude respectively and slope values of 1.2 and 1.4 (Fig. 3, *B* and *C*, *solid lines*). Higher concentrations could not be examined because of poor drug solubility.

The saturability with high concentrations might suggest that the capacitive changes are associated with binding to a specific site, e.g., a receptor. If bilayer interactions, rather than a proteinaceous binding site are important in the steroid-induced charge movements, we might expect little enantioselectivity of charge movements. Because we have synthesized an enantiomer of pregnenolone sulfate previously (17), we directly compared the ability of the natural and unnatural enantiomer to alter membrane capacitive currents in oocytes. Fig. 3 *D* shows currents generated by 50 μM pregnenolone sulfate and its unnatural enantiomer. Charge displacements from the unnatural enantiomer were not statistically distinguishable from those associated with pregnenolone sulfate itself in the same oocytes (2 ± 4% smaller; $p = 0.8$, $n = 6$). Accordingly, neither the amplitude nor the time constants of the drug associated changes differed between pregnenolone sulfate and its enantiomer (data not shown).

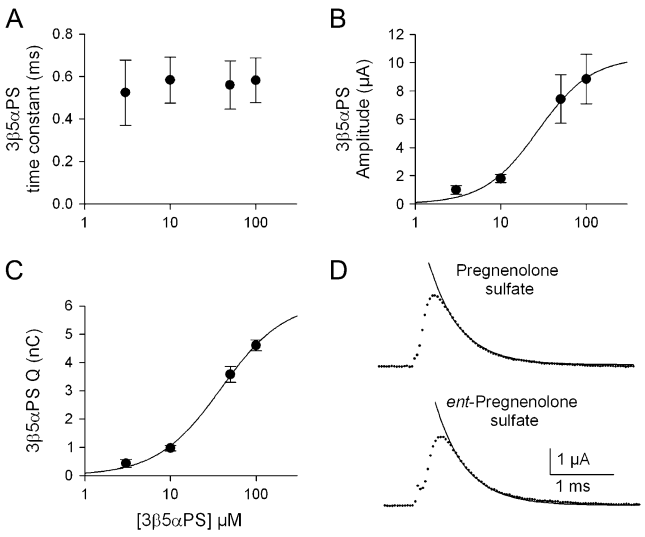

FIGURE 3 Concentration dependence of time constant (*A*), amplitude (*B*), and charge displacement (*C*) of transient onset currents. Estimates were obtained from subtraction currents. Subtracted relaxations were fit to a single exponential and extrapolated to the onset of the voltage pulse for estimates of amplitude and charge. Solid lines in *B* and *C* represent best fits of the concentration-response data to the Hill equation. $n = 4$ oocytes challenged with all four concentrations of 3β5αPS. (*D*) Lack of enantioselectivity of charge movements. Pregnenolone sulfate and its enantiomer were evaluated at 50 μM on the same oocyte. Drug minus baseline subtraction traces are shown (*dotted lines*) along with a portion of the exponential fit used to quantify drug-associated capacitive current amplitude (fit extrapolated to time zero), time constant, and total charge displacement. In this example, pregnenolone sulfate charge displacement was 1.84 nC and *ent*-pregnenolone sulfate charge displacement was 1.80 nC. Time constants were 234 μs and 232 μs, respectively.

We also tested whether surface charge influences the interaction of sulfated steroid with the plasma membrane. We manipulated surface charge screening by decreasing the concentration of divalent cations in the extracellular bath solution to 10% of normal (0.2 mM Ca^{2+} and 0.1 mM Mg^{2+}). We found that this manipulation had little effect on drug-induced capacitive change (−12 ± 5%, $n = 4$).

In summary, sulfated pregnane steroids and pregnenolone sulfate, but not hydroxypregnane steroids, alter current transients in response to voltage pulses in *Xenopus* oocytes. Because only charged neurosteroids affected membrane capacitance, we explored the effect of sulfated steroids in more detail. We hypothesized that voltage-dependent translocation of the negatively charged steroid across a fraction of the bilayer might account for the drug-induced capacitive currents observed.

If drug-induced capacitive currents result from translocation of the charged steroid between two membrane energy minima within the lipid bilayer, we expect that charge movements should obey behavior documented previously pertaining to charged hydrophobic ions (9–16). Among the predictions is a sigmoidal dependence of equilibrium charge transfer (ΔQ) on membrane potential (V):

$$\Delta Q/\Delta Q_{max} = \frac{1}{1 + \exp\dfrac{-e\beta(V - V_h)}{kT}}. \quad (2)$$

V_h represents the potential at which there are equal numbers of ions in each potential energy well, β is the fraction of the potential difference experienced by the translocating molecule, and k, T, and e have their usual meanings of Boltzmann constant, temperature Kelvin, and elementary charge per ion. In the case of 3β5αPS, the charge movement was nearly linear over the range of voltages accessible with the voltage-clamp amplifier, with only a trend toward deviation at the most deeply negative voltages (Fig. 4 *A*). The best sigmoidal fit from Eq. 2 is superimposed on the data in Fig. 4 *A*, but in fact none of the raw data points deviated significantly from a linear regression fit to the data between −40 and + 40 mV ($p > 0.05$, paired t-test, $n = 5$; raw data versus linear regression prediction).

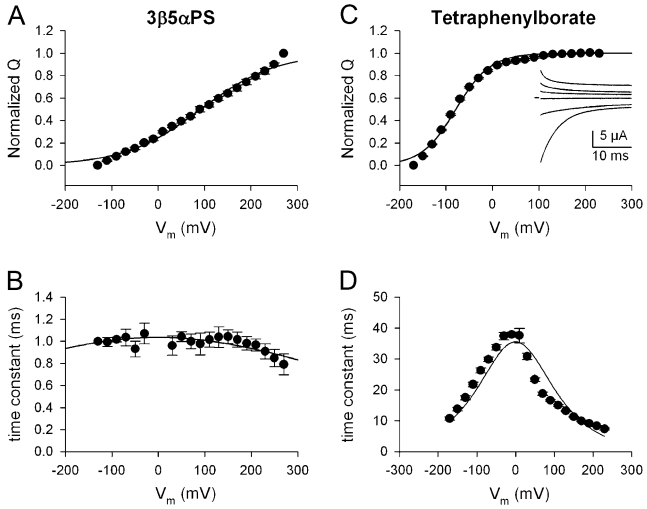

FIGURE 4 Charge displacements and time constants lack voltage dependence. Measurements were made from subtracted onset currents in response to steps from −70 mV. (*A*) Drug-induced onset charge estimates showed nearly linear (*solid circles*, $n = 5$ oocytes), rather than sigmoidal (*solid fit line*), dependence on membrane potential. (*B*) The time constants of drug-induced current relaxations did not show the expected bell-shaped dependence on membrane potential. Estimates of charge and time constants were obtained from subtraction currents, which were fit with single exponential functions extrapolated to the instant of the voltage-pulse onset. The solid lines in *A* and *B* are fits to Eqs. 2 and 3, respectively. Fit parameters were $\beta = 0.3$ and $V_h = 92$ mV (*A*) and $t_{max} = 1.03$ ms, $\beta = 0.12$, and $V_h = 0.0$ mV (*B*). (*C* and *D*) Positive control experiment using oocytes exposed to tetraphenylborate (100 μM). The inset in C shows representative capacitive currents, with the first 600 μs of capacitive current blanked, showing tetraphenylborate-induced changes in the absence of fast, endogenous capacitive currents. Voltage pulses were from a holding potential of −30 mV and were made to voltages between −200 mV and + 200 mV in 20 mV increments. The solid circles in C represent slow charge displacements, isolated with bi-exponential fits to the total capacitive transients of four oocytes. The solid line is a fit to Eq. 3 with fit parameters of $\beta = 0.7$ and $V_h = -78.7$ mV. See Results for an interpretation of the negative V_h value. Time constants of the slow component of capacitive transients are plotted in *D*. The solid line is a fit to Eq. 3 with $t_{max} = 35.6$ ms, $\beta = 0.6$, and $V_h = 0$ mV.

Similarly the time constant of capacitive current relaxations should exhibit a bell-shaped dependence on membrane potential:

$$\Delta\tau(V) = \tau_{max}\tanh\frac{[e\beta(V - V_{h})]}{2kT}. \quad (3)$$

Fig. 4 *B* shows the relationship between the steroid-induced capacitive transient time constant versus applied membrane potential. There was no detectable dependence of time constant on voltage, compared with previously reported charged lipophilic molecules (9–16). In particular, although there is doubt about the applicability of energy minima models to our data, at face value, fits in Fig. 4, *A* and *B*, suggest a small value for β, indicating that the steroid senses only a very small fraction of the transmembrane potential.

As a comparison, we examined the profile of tetraphenylborate, a well characterized hydrophobic anion (10,11,14). We found the expected sigmoidal behavior of charge displacement on voltage and the expected bell-shaped dependence of time constant on voltage (Fig. 4, *C* and *D*). Interestingly, oocytes exhibited a negatively shifted V_h value in charge versus voltage plots (Fig. 4 *C*) compared with previous studies of tetraphenylborate in artificial membranes and other cells (10,11,14). We attribute this to strong intracellular accumulation of the anion with the overnight incubation necessary to show capacitance changes (see Materials and Methods). We hypothesize that intracellular accumulation created a concentration gradient that favored an inner membrane leaflet distribution of anion. In support of this hypothesis, oocytes incubated overnight in the colored hydrophobic anion oxonol showed visible local depletion of the drug in the unstirred layer surrounding individual oocytes (data not shown). Furthermore, smaller HEK cells exhibited rapid tetraphenylborate-induced capacitance changes (within 5 min), and had V_h values closer to 0 mV (Fig. S1). Overnight incubation of oocytes in 100 μM 3β5αPS produced no sign of altered or increased capacitance changes over those produced by the brief incubations used in our studies ($n = 5$ control and 5 3β5αPS-treated oocytes, data not shown). Despite the difference in quantitative behavior of tetraphenylborate in oocytes compared with other systems, these results highlight a fundamental qualitative difference between a classically described hydrophobic anion and the sulfated steroids.

The lack of apparent voltage dependence of steroids suggests that the sulfated steroids are unlikely to translocate, either because the negative charge orients in a way that does not sense the transmembrane voltage, or because steroid is relatively immobilized in the membrane. To test this hypothesis, we turned to optical methods. Specifically, voltage-dependent translocation within the bilayer of charged fluorescent lipophilic molecules alters the fluorescence of the fluorophore reporter, an effect that has been used to provide an optical measure of membrane potential (26–28). Although not specifically used as a voltage sensor, NBD-tagged conjugates, including steroid-like molecules, increase fluorescence with increasing hydrophobicity of the solvent environment (29, 30). To provide an independent test of our conclusion that sulfated neurosteroids do not translocate across the bilayer, we tagged 3β5αPS with a fluorescent NBD group (C2-NBD 3β5αPS) (18) and tested for voltage-dependent changes in cellular fluorescence. We first verified that the tagged steroid analog obeyed the same hydrophobicity-dependent fluorescence changes as free NBD. Consistent with results with other NBD-conjugates, we found that C2-NBD 3β5αPS increased its fluorescence emission (measured at 535 nM) in ethanol compared with water by 4.6- and 5.4-fold respectively in two separate experiments.

We tested for fluorescence changes on primary cultures of hippocampal neurons, which allowed for more feasible imaging than the much larger oocytes. When tested on live, voltage-clamped hippocampal neurons, we found no change in fluorescence with the NBD-conjugated steroid when

membrane potential was pulsed from −70 mV to +40 mV for 20–30 s. The ratio of fluorescence at +40 mV relative to −70 mV from 12 regions of proximal dendrite from four cells treated with 3–6 μM of the fluorescent analog was 0.98 ± 0.1. By contrast, the well-characterized voltage-dependent fluorescent anion bis(1,3-dibutylbarbituric acid)trimethine oxonol [DiBAC4(3)] (13,28), with spectral properties similar to those of NBD, exhibited strong voltage-dependent changes in fluorescence under the same conditions (Fig. 5 *B*; +40/−70 mV ratio of 2.6 ± 0.2 in 11 membrane regions from six cells, $p < 0.05$ compared with C2-NBD 3β5αPS). The intracellular fluorescence observed in DiBAC4 (3) cells did not change with voltage (ratio 0.92 in two cells).

We also verified the ability of the NBD-tagged sulfated steroid to alter membrane capacitance. Because of limited quantities of available tagged steroid, we used human embryonic kidney cells for evaluation rather than *Xenopus* oocytes. The smaller HEK cells allowed use of local perfusion for drug delivery rather than oocyte bath exchange, dramatically reducing the amount of compound required. The use of another cell type and alternative voltage-clamp technique (whole-cell patch clamp) also afforded us the opportunity to verify several salient results from oocytes.

Like oocytes (but unlike neurons), HEK cells can be approximated electrically by a simple circuit: parallel membrane resistance and capacitance linked to the voltage clamp through an access resistance much lower than the membrane resistance (31). The simple equivalent circuit results from uncomplicated membrane geometry, especially a lack of dendrites and axons. Fig. 6 *A* shows baseline capacitive transients in HEK cells; whole-cell voltage clamp was used to step membrane potential from −70 to +20 mV. A cesium-based pipette solution was used to increase membrane resistance and to block contributions of voltage-gated potassium channels. Baseline transients were well fit by a single exponential (Fig. 6 *A*, *inset*, *solid gray line*), confirming their

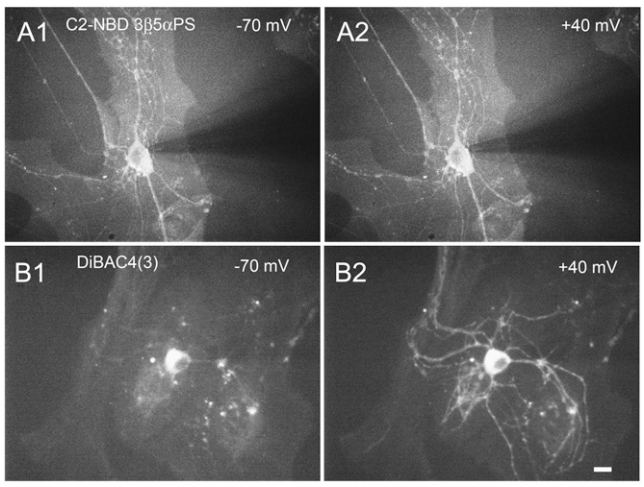

FIGURE 5 Lack of voltage-dependent fluorescence change with a sulfated NBD-tagged analog. (*A1* and *A2*) Fluorescence images obtained in the presence of 3 μM C2-NBD 3β5αPS at membrane potentials of −70 mV (*left*) and after 20 s at +40 mV (*right*). Voltage was controlled with a whole-cell patch pipette visible as a shadow on the right side of the image, through the aqueous fluorescence of the analog. Results are representative of six cells challenged with 1–6 μM C2-NBD 3β5αPS. (*Bottom panels*) Positive control using DiBAC4 (3) (0.5 μm) imaged under the same conditions as in the top panels. The fluorescence change was reversible on re-establishing a membrane potential of −70 mV (not shown). Scale bar represents 10 μm.

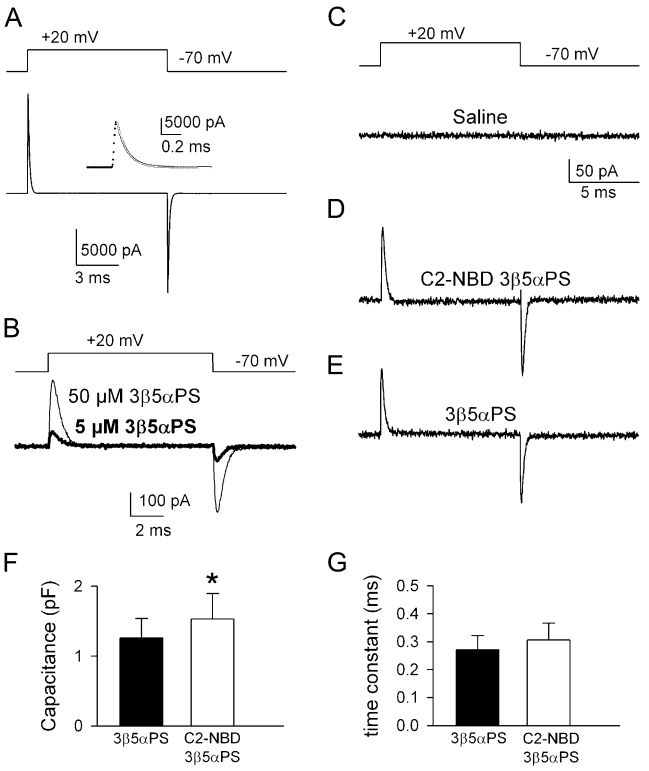

FIGURE 6 The NBD-tagged steroid C2-NBD 3β5αPS yields capacitance changes similar to the parent compound, 3β5αPS. (*A*) Raw current traces in normal saline obtained from an isolated HEK cell in response to a voltage pulse from −70 mV to +20 mV. The onset transient is shown at higher time resolution in the inset as dotted points. The gray line is a single-exponential fit, superimposed and extrapolated to the instant of the voltage change. The time constant of the transient relaxation was 102 μs. The good correspondence between fit and data is consistent with a representation of the cell as a single electrical compartment (31). (*B*) Subtracted traces obtained from a different cell exposed to 5 μM and 50 μM 3β5αPS. Transients in the presence of saline wash have been subtracted. (*C* and *D*) Subtraction currents from the cell in A. (*C*) Represents saline minus saline subtractions obtained under the same experimental conditions as with drug applications in D and E. (*D*) Represents subtraction of saline transients from transients obtained in the presence of 15 μM C2-NBD 3β5αPS. (*E*) Represents saline transients subtracted from transients obtained in the presence of 15 μM 3β5αPS. In the subtracted traces of C–E the first 50 μs of data at the onset and offset of the voltage pulse have been blanked to eliminate small, fast, capacitive changes resulting from bath level changes. The voltage protocol and calibration bars in C apply to C–E. (*F* and *G*) Summary ($n = 8$ HEK cells) of steroid-induced capacitance changes (*F*) and time constants of the capacitance changes (*G*) in HEK cells using the voltage protocol shown in A. Compounds were used at 15 μM. C2-NBD 3β5αP yielded a slightly but significantly higher change in capacitance than the parent steroid ($p = 0.03$). There was no difference in drug-induced relaxation time constant ($p > 0.09$).

compact, single-compartment structure (31). HEK cell baseline capacitance was 34.5 pF, and the time constant was 154 ± 30 μs ($n = 8$ cells). 3β5αPS altered membrane capacitance in a manner similar to that observed in oocytes (Fig. 6 *B*). Capacitive transients were concentration dependent, with 5 μM 3β5αPS increasing membrane capacitance by 2% ± 1% of the baseline and 50 μM increasing the capacitance by 12 ± 2% ($n = 7$).

To compare capacitance change induced by 3β5αPS and by the NBD-tagged analog, we compared the two drugs on the same cell at equimolar concentrations (15–50 μM). C2-NBD 3β5αPS produced similar or slightly higher alterations in capacitive current to those produced by 3β5αPS (Fig. 6, *C–G*). These results therefore verify that the tagged sulfated steroid behaves similarly to the parent compound with regard to effects on membrane capacitance. The results also extend our primary oocyte findings to mammalian cells.

DISCUSSION

Our results suggest that charged steroids interact with cells in a way that alters membrane capacitive properties. This interaction is distinct from interactions between uncharged neurosteroids and the membrane, and it apparently does not result from voltage-dependent charge movement of the anionic sulfate group itself. It is possible that these membrane interactions are important for the modulation of membrane channels, most notably the $GABA_A$ receptor, which is antagonized over the concentration range of sulfated steroids explored here.

Our evidence suggests that sulfated neurosteroids do not obey predictions of two-site, single-barrier models for hydrophobic anion translocation. Previously investigated anions include dipicrylamine (12,14,16,27), oxonols (13), and tetraphenylborate (10,11,14). In all of these cases, behavior of ions in synthetic membranes and living cells is well described by single barrier models with two energy minima representing externally facing and internally facing binding to the membrane-solution interfaces. The dependence of charge displacement on applied voltage is sigmoidal, and the relationship between current relaxation time constants and applied voltage is bell shaped. Both of these predictions were clearly violated in the case of the sulfated neurosteroid 3β5αPS.

Fluorescence imaging also was inconsistent with sulfated neurosteroid translocation. We might have expected fluorescence changes as the translocated steroid and attached NBD group were drawn into a more hydrophobic environment with depolarization. Although this negative result is not, by itself, definitive, our positive control experiments help give context to the imaging result, and the imaging is broadly consistent with the conclusions reached with biophysical measurements. Considering both the electrical changes and the fluorescence imaging, one is left with a picture of sulfated steroids adsorbing to the external leaflet of the membrane and thereby altering membrane thickness or packing order to influence the dielectric properties of the membrane, without physical translocation through the bilayer. A puzzling aspect of the phenomenon is the role of negative charge; similar steroids without a sulfate group do not detectably influence the capacitive properties of the membrane. However, cholesterol and cholesterol sulfate apparently interact distinctly with membranes (32,33), offering the core of a possible physical explanation for differences between charged and uncharged steroids.

Several other features of the capacitive changes are of note. Although we did not observe saturation of charge displacement with voltage, capacitance changes saturated at high concentrations of steroid. One possible explanation for this behavior is that a chiral steroid binding pocket, likely proteinaceous, is important for steroid interaction with the membrane and the resulting capacitive change. This possibility is rendered less likely by the similar behavior of natural and unnatural enantiomers of pregnenolone sulfate (Fig. 3 *D*). Furthermore, saturation of capacitive currents with concentration has been observed with other hydrophobic anions in artificial bilayers (14) and has been attributed to limitations on compound packing within the lattice of the plasma membrane, perhaps caused by strain on membrane polar headgroups resulting from adsorption of the anion.

Although all sulfated steroids tested generated capacitive changes, there were clear differences among diastereomeric steroid pairs. Because diastereomers, unlike enantiomers, have different physicochemical differences, this result is not necessarily surprising. For instance, it was shown recently that the hydroxysteroid diastereomer pair, pregnanolone and allopregnanolone, differ from each other in their membrane interactions, although enantiomeric pairs do not differ significantly (34).

What are the physiological implications of our observations? Steroid receptors are typically invoked to explain biological effects of steroids, including effects on neurons. However, nongenomic effects of pharmacologically administered steroids include membrane effects that can influence cell function (35,36). Likewise, the local membrane environment has been shown to affect ion channel gating (37,38). Because the log *P* value of pregnenolone sulfate is 4.81 (Advanced Chemistry Development Software V8.14, Advanced Chemistry Development, Toronto, Canada), high millimolar concentrations of membrane steroid will likely be achieved with pharmacologically relevant aqueous concentrations of sulfated steroid. High local membrane concentrations are consistent with the possibility of changes in membrane properties that may affect channel function. Furthermore, strong membrane solubility suggests that most sulfated steroids measured endogenously (39–41), will derive from membrane fractions.

Sulfated neurosteroids modulate several transmembrane receptors besides the $GABA_A$ receptor. Other targets include the NMDA receptor (42–44) and σ-receptor (45). Do the

capacitive changes reported herein have relevance to modulation of these receptor classes? We first consider GABA receptor modulation. There are tantalizing parallels between capacitance changes and GABA receptor modulation. Although virtually all sulfated steroids antagonize GABA receptors, pregnenolone sulfate and 3β5αPS seem to be stronger antagonists than 3α5βPS (6,22), correlating with the degree of capacitance change produced by different sulfated steroids in these studies. GABA receptor inhibition by pregnenolone sulfate exhibits little enantioselectivity (17); this could suggest that a membrane effect is important for inhibition. In addition the concentration dependence of capacitance change is not dissimilar from the concentration response for GABA receptor antagonism or for modulation of NMDA receptors, although this comparison is complicated by the agonist concentration dependence of antagonist potency, whereby steroid-induced inhibition increases at higher GABA concentrations (7).

Several observations, at face value, fail to support the idea that membrane effects are related to changes in receptor function, but these observations have caveats. Sulfated pregnane steroids inhibit GABA receptor function with detectable voltage dependence (8). Although this is dissimilar to the lack of voltage dependence observed in capacitance changes, pregnenolone sulfate inhibition is similar to the capacitance changes in exhibiting no detectable voltage dependence (7,25,47). Mutations have been found that affect sulfated steroid effects on GABA receptors (25,48,49). Although some of these mutations are hypothesized to directly influence steroid binding, it is difficult to exclude the possibility that the mutations instead affect transduction of distant steroid interaction (with membrane or receptor) to conformational changes that effect antagonism.

It is difficult to conceive that the membrane capacitance changes here are directly relevant to NMDA receptor modulation, because pregnenolone sulfate and 3β5αPS potentiate NMDA receptor function, but 3α5βPS antagonizes NMDA receptor function (44). Relevance of capacitance changes to other receptors/channels is also possible. For instance, sulfated steroids have presynaptic effects, some of which may be mediated by sigma receptors (50). However, presynaptic pregnenolone sulfate effects exhibit enantioselectivity (45), unlike the capacitance changes evaluated here.

In summary, our work suggests an electrophysiological signature of sulfated neurosteroids that differs from other negatively charged hydrophobic membrane probes. Rather than exhibiting behavior consistent with ion translocation in response to the membrane voltage, sulfated steroids alter the membrane capacitance similarly at all membrane potentials. Because of strong precedent that most channel modulators act through specific sites on the protein, a site-specific mode of action remains likely for sulfated steroids. However, our data raise the possibility that changes in membrane properties may participate in antagonism of GABA receptor function.

SUPPLEMENTARY MATERIAL

To view all of the supplemental files associated with this article, visit www.biophysj.org.

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