0026-895X/92/010089-15$03.00/0

MOLECULAR PHARMACOLOGY, 41:89-103

Effects of Steroids on γ-Aminobutyric Acid Receptors Expressed in *Xenopus* Oocytes by Poly(A)$^+$ RNA from Mammalian Brain and Retina

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Received June 6, 1991; Accepted September 16, 1991

SUMMARY

Electrical recordings were made in *Xenopus* oocytes to study the modulatory effects of steroids on γ-aminobutyric acid (GABA) receptors expressed by RNA from mammalian brain and retina. GABA responses expressed by rat cerebral cortex poly(A)$^+$ RNA were bicuculline-sensitive Cl^- currents mediated by $GABA_A$ receptors. GABA responses expressed by bovine retina poly(A)$^+$ RNA also were Cl^- currents but were composed of two pharmacologically distinct components, one mediated by $GABA_A$ receptors and the other by GABA receptors with novel properties, which were resistant to bicuculline but were not activated by R(+)-baclofen, a selective agonist of $GABA_B$ receptors. As reported in neurons and in other expression systems, $GABA_A$ responses expressed in oocytes by cerebral cortex RNA were strongly and stereospecifically potentiated by 5α-pregnan-3α-ol-20-one (3α-OH-DHP) and 5α-pregnan-3α,21-diol-20-one (THDOC). Threshold levels of potentiation were detectable using 1–2 nM steroid, and at concentrations of 50 and 500 nM 3α-OH-DHP shifted the EC_{50} of cortex $GABA_A$ responses from a control value of 92 ± 20 μM GABA to 40 ± 4.3 μM and 13 ± 1.8 μM, respectively. However, even at concentrations as high as 50 μM, 3α-OH-DHP did not itself elicit appreciable membrane current responses through direct activation of the cortex $GABA_A$ receptors. In addition to potentiation, 3α-OH-DHP and THDOC caused pronounced increases in the rate of desensitization of $GABA_A$ responses expressed by cortex RNA. Decay time courses of currents elicited by 1 mM GABA (90–95% of the maximum response) were fitted by the sum of two exponentials. Under control conditions, the time constant of the fast component was 4.4 ± 0.6 sec and the slow component, 22.5 ± 4.8 sec. 3α-OH-DHP at 500 nM and 5 μM reduced the time constant of the fast component by 52 ± 7% and 84 ± 5%, respectively, but showed little effect on the slow component. Unlike the potentiation effect, actions of pregnanolones on desensitization did not show stringent stereoselectivity, and 5 μM 5β-pregnan-3β-ol-20-one (3β-OH-DHP) reduced the time constant of the fast component by 59 ± 11%. Modulatory effects of 5-pregnen-3β-ol-20-one sulfate (pregnenolone-SO_4) and progesterone on $GABA_A$ receptors expressed by rat cortex RNA were predominantly inhibitory, even though at concentrations between 2 and 20 nM pregnenolone-SO_4 did cause some modest potentiation of currents elicited by 1–10 μM GABA. Pregnenolone-SO_4 at 500 nM and 5 μM blocked maximum GABA responses by 38 ± 2% and 76 ± 8%, respectively, and the same concentrations of progesterone blocked maximum currents by 14 ± 2% and 58 ± 5%. For both steroids, suppression of responses was associated with only small changes in EC_{50}, indicating that inhibition was substantially noncompetitive. Time courses of $GABA_A$ responses with pregenolone-SO_4 and progesterone did, however, appear to be different, which in turn suggested that the mechanisms underlying inhibition were not identical. Modulatory effects of steroids on $GABA_A$ receptors expressed by retina RNA were qualitatively indistinguishable from effects on cortex $GABA_A$ receptors. $GABA_A$ responses expressed by retina RNA were stereoselectively potentiated by 3α-OH-DHP and noncompetitively inhibited by pregnenolone-SO_4 and progesterone. Furthermore, 3α-OH-DHP, THDOC, and 3β-OH-DHP also caused clear increases in the rate of response desensitization. In striking contrast, bicuculline-resistant GABA responses expressed by retina RNA were essentially unaffected by any of the steroids tested. These experiments indicate that $GABA_A$ receptors expressed in oocytes by cortex or retina RNA are modulated by steroids in ways similar to those of $GABA_A$ receptors studied *in situ*. Conversely, the novel bicuculline-resistant GABA receptors expressed by retina RNA do not appear to be functionally modulated by steroids.

The major inhibitory neurotransmitter GABA is known to interact with two classes of receptors in the mammalian nervous system, denoted $GABA_A$ and $GABA_B$. Studies at the molecular level indicate that $GABA_A$ receptor complexes are composed of up to four different subunits, which exist in a variety of closely related subtypes (e.g., Refs. 1–4). Expression studies, in *Xenopus* oocytes and transfected mammalian cells, show

This work was supported by Grants MH48358-01 and NS23284 from the United States Public Health Service. L.P. received support from FIDIA S.p.a. (Abano Terme, Italy).

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ABBREVIATIONS: GABA, γ-aminobutyric acid; DMSO, dimethylsulfoxide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; $I_{G\text{-}Actr}$, membrane current elicited through activation of $GABA_A$ receptors expressed by cerebral cortex RNA; $I_{G\text{-}Aret}$, membrane current elicited through activation of $GABA_A$ receptors expressed by retina RNA; $I_{G\text{-}BR}$, membrane current elicited through activation of bicuculline-resistant GABA receptors expressed by retina RNA; 3α-OH-DHP, 5α-pregnan-3α-ol-20-one; 3β-OH-DHP, 5β-pregnan-3β-ol-20-one; pregnenolone-SO_4, 5-pregnen-3β-ol-20-one sulfate; TBPS, *t*-butylbicyclophosphorothionate; THDOC, 5α-pregnan-3α,21-diol-20-one; G protein, GTP-binding protein.

that different subunit combinations assemble to form functional receptors with different affinities for GABA, rates of desensitization, voltage dependence, and sensitivities to benzodiazepines (e.g., Refs. 5–8). $GABA_B$ receptors have not yet been cloned but are probably members of a G protein-coupled family of receptors (9).

Xenopus oocytes injected with poly(A)$^+$ RNA extracted from mammalian cerebral cortex or chick optic lobe express a mixture of $GABA_A$ subunits that assemble to form functional receptors with properties similar to those of $GABA_A$ receptors studied *in situ* (e.g., Refs. 10–14). $GABA_A$ receptors expressed in oocytes are ligand-gated Cl^- channels that show desensitization and are blocked by picrotoxin, competitively antagonized by bicuculline, and potentiated by both benzodiazepines and barbiturates (10–14). Expression of functional $GABA_B$ receptors in oocytes has recently been reported using rat cerebellum RNA (15), although electrical responses were detected in only a small fraction (<10%) of oocytes tested. $GABA_B$ responses expressed by cerebellum RNA are carried substantially by K^+, are activated by R(+)-baclofen, are antagonized by phaclofen, and are insensitive to bicuculline (15). Whether it is possible to detect expression of $GABA_B$ receptors using rat cerebral cortex RNA remains uncertain (16).[2]

Poly(A)$^+$ RNA extracted from mammalian retina, like cerebral cortex RNA, expresses $GABA_A$ receptors. But in addition, retina RNA expresses GABA receptors that are resistant to bicuculline, show little desensitization, and are not significantly modulated by either benzodiazepines, β-carbolines, or barbiturates (17).[2] Unlike $GABA_B$ receptors expressed by cerebellum RNA, the bicuculline-resistant GABA receptors expressed by retina RNA are neither activated by R(+)-baclofen nor antagonized by phaclofen or 2-hydroxysaclofen, indicating that they do not belong to the $GABA_B$ class of receptor (17, 18). Moreover, like $GABA_A$ receptors these retina GABA receptors gate membrane currents that are carried substantially by Cl^- and show sensitivity to picrotoxin (17).

It is now clear that a variety of steroids have strong modulatory actions on $GABA_A$ receptors, effects that have potential physiological relevance in mammalian nervous systems (e.g., Refs. 19–21). For example, pregnanolone isomers such as 3α-OH-DHP and THDOC potently and stereoselectively potentiate responses mediated by $GABA_A$ receptors (e.g., Refs. 19–24). Evidence from binding studies and functional assays suggests that these steroids act through multiple sites on the receptor complex that are distinct from modulatory sites for benzodiazepines or barbiturates (e.g., Refs. 22–25), but even this fundamental aspect of mechanism remains unresolved (e.g., Ref. 26). 3α-OH-DHP has been shown to increase burst duration of GABA-gated single-channel currents in bovine chromaffin cells (22), whereas studies on recombinant human $GABA_A$ receptors have indicated that the principle effect is to increase the frequency of channel opening (24).

In addition to positive modulation by 3α-hydroxy-pregnanolones, the neurosteroid pregnenolone-SO_4 and various glucocorticoids appear to have complex interactions with $GABA_A$ receptors, which include antagonist-type effects (e.g., Refs. 27 and 28). For example, pregnenolone-SO_4 at micromolar concentrations has been shown to inhibit $GABA_A$ responses in rat cortical neurons (27), primarily by reducing the frequency of channel opening (29).

[2] R. M. Woodward and R. Miledi, unpublished results.

In terms of electrical properties, the bicuculline-resistant GABA receptors expressed in oocytes by retina RNA appear to resemble $GABA_A$ receptors that are insensitive to both benzodiazepines or barbiturates (17). It was, therefore, of interest to determine whether these novel bicuculline-resistant GABA receptors were either positively or negatively modulated by steroids.

Materials and Methods

RNA extraction and size fractionation. Poly(A)$^+$ RNA was extracted from bovine retina, rat cerebral cortex, and rat spinal cord, using the phenol/chloroform procedure. Methods were largely as described previously (see Ref. 30), but with some important modifications. Briefly, fresh bovine eyeballs were obtained from a local slaughterhouse, placed in ice in the dark, and transported (1–1.5 hr) to the laboratory. All subsequent procedures were performed under room light. The eyeballs were hemidissected along the ora serrata, vitreous was removed, and retina was carefully peeled away with forceps and spatula. Retina was recovered essentially free of pigment epithelium, and pieces were immediately frozen in liquid nitrogen, weighed, and stored at −80°. Cerebral cortex was dissected from rats sacrificed by rapid decapitation. Cortex was frozen in liquid nitrogen, and likewise weighed and stored at −80°.

Frozen tissue was homogenized (30–60 sec), using a Polytron homogenizer at medium speed, in phenol equilibrated by the addition of 55 ml of homogenization buffer for every 500 g of phenol used (loose crystals). Homogenization buffer was (in mM) Tris·HCl, 200, pH 9.0; NaCl, 50; EDTA, 10; sodium dodecyl sulfate, 0.5%; with heparin added at 1 mg/ml just before use. Individual preparations normally used between 6 and 12 g of bovine retina and between 6 and 10 g of rat cerebral cortex (wet weight); optimum yields were from 7–8 g of tissue. The homogenized mixture was shaken for 5 min and centrifuged to separate phases. Aqueous layer and interphase were extracted twice with an equal volume of chloroform/isoamyl alcohol (49:1), for 5 min each time. The aqueous layer was then extracted with an equal volume of equilibrated phenol/chloroform/isoamyl alcohol (50:49:1), for 5 min, and centrifuged. This was repeated till no interphase was visible (two or three times). A final extraction with chloroform/isoamyl alcohol was carried out, and total nucleic acids were precipitated by 3–4 hr in 200 mM NaCl with 2.5 volumes of 200 proof ethanol, at −20°. The nucleic acid pellet was dissolved in 20 mM HEPES, pH 7.5 (approximately 2 g/ml tissue), solid NaCl was slowly added to 3 M, and total RNA was allowed to precipitate at −15° for 12–18 hr. The RNA pellet was washed three times in 10 ml of sodium acetate (pH 6.0), with the pellet each time being well dispersed with a glass rod. The pellet was finally washed with 75% ethanol and either stored at −20° or used directly for chromatography on oligo(dT) columns (Collaborative Research Type 3).

In this study, four separate poly(A)$^+$ RNA extractions were made from bovine retina, two from rat cortex, and one from rat spinal cord. Total poly(A)$^+$ RNA from retina typically expressed GABA-activated membrane currents that were <40 nA at a holding potential of −60 mV and were often marginal for detailed pharmacological studies. To increase the size of GABA responses expressed by retina RNA, total poly(A)$^+$ RNA was size-fractionated on 10–30% (w/v) sucrose density gradients (31). Gradients were divided into 30 fractions and enrichment of RNAs encoding functional retina GABA receptors was assayed by electrical recordings in oocytes (17, 32). For fractions of retina RNA showing the highest activity, size-fractionation resulted in a 5–10-fold increase in expressional potency for GABA responses, but did not qualitatively distort any of the fundamental properties of responses expressed by total poly(A)$^+$ RNA. In the main, size-fractionation was simply a means of amplifying the retina responses to facilitate pharmacological characterization (17). It was also clear, however, that the relative proportions of bicuculline-sensitive and bicuculline-resistant currents did vary somewhat between different fractions. This suggested that RNA molecules encoding retina $GABA_A$ subunits and bicuculline-

resistant GABA receptors, although approximately the same size, were at least partially separated on 10–30% gradients.

Expression of GABA receptors in oocytes. Follicle-enclosed *Xenopus* oocytes, at stages V and VI of development (33), were dissected from the ovary and stored in Barth's medium [in mM, NaCl, 88; KCl, 1; $Ca(NO_3)_2$, 0.33; $CaCl_2$, 0.41; $MgSO_4$, 0.82; $NaHCO_3$, 2.4; HEPES, 5; pH 7.4; usually with 0.1 mg/ml gentamycin]. Oocytes were microinjected with 75–100 ng of total poly(A)$^+$ RNA from rat cerebral cortex, rat spinal cord, or bovine retina and approximately 25 ng of size-fractionated bovine retina RNA, all dissolved in 50 nl of H_2O. Two days after injection, oocytes were defolliculated by 0.75–1-hr treatment with 0.5–1.0 mg/ml collagenase (Sigma type I) (34).

Electrophysiology. Electrical recordings were normally made between 3 and 10 days after injection of oocytes, using a conventional two-electrode voltage clamp at a holding potential of −60 mV (35). Oocytes were placed in a 0.1-ml chamber and continuously superfused at 5–15 ml/min with frog Ringer solution (in mM, NaCl, 115; KCl, 2; $CaCl_2$, 1.8; HEPES, 5; pH 7.0). All drugs were applied to oocytes by bath perfusion in Ringer solutions. Membrane current responses were recorded on a chart recorder and, when necessary for subsequent analysis, on a Racal Thermionic Store-4 FM tape recorder or a Toshiba Digital-4 videotape recorder/Unitrade data acquisition and storage system.

Notation and distinction of membrane current responses. To minimize the length of subscripts used to identify membrane currents in the text, currents elicited by GABA have been abbreviated from I_{GABA} to I_G. As mentioned in the introduction, poly(A)$^+$ RNA extracted from rat cerebral cortex expresses GABA responses, in oocytes, mediated by $GABA_A$ receptors. These membrane current responses have been denoted $I_{G\text{-}Actx}$. Poly(A)$^+$ RNA from bovine retina expresses GABA-activated currents with two superimposed components, distinguished by their sensitivity to bicuculline. In terms of electrical properties and pharmacology, the component blocked by bicuculline is mediated by retina $GABA_A$ receptors and has been denoted $I_{G\text{-}Aret}$. The component that shows bicuculline resistance is mediated by retina GABA receptors with novel pharmacology and has been denoted $I_{G\text{-}BR}$ (17).

Under control conditions, $I_{G\text{-}BR}$ could be strongly activated by concentrations of GABA that either were subthreshold or caused only weak activation of $I_{G\text{-}Aret}$ (17). For example, 10 μM GABA elicited >90% of maximum $I_{G\text{-}BR}$ but caused only threshold activation of $I_{G\text{-}Aret}$. Effects of steroids specifically on $I_{G\text{-}BR}$ were, therefore, readily measured by using low concentrations of GABA to selectively activate this current, and 0.1–1 mM bicuculline methobromide to abolish $I_{G\text{-}Aret}$ activated by higher concentrations of GABA. As mentioned above, the relative sizes of the two components of retina GABA responses varied somewhat between different RNA preparations and fractionations and also showed dependence on the RNA fraction injected. Thus, in different oocytes, $I_{G\text{-}Aret}$ could constitute 5–60% of maximum GABA responses expressed by retina RNA. In this study, comparison was made between the modulatory effects of steroids on $I_{G\text{-}Actx}$, $I_{G\text{-}BR}$, and $I_{G\text{-}Aret}$, but quantitative analyses were generally restricted to comparisons between $I_{G\text{-}Actx}$ and $I_{G\text{-}BR}$. Qualitative illustration of the modulatory effects of steroids on $I_{G\text{-}Aret}$ was made using oocytes that showed strong expression of this component.

Measurement of concentration-response curves. Recording conditions in oocytes are sufficiently stable to allow construction of multiple concentration-response curves in the same cell. Curves were determined by measuring the peak currents elicited by exposures to increasing concentrations of GABA, with the exposures being separated by appropriate intervals of wash. For responses mediated by $GABA_A$ receptors, currents elicited by 1–10 μM GABA were separated by intervals of 1–3 min, whereas currents elicited by 0.1–3.0 mM GABA required intervals of 15–30 min to ensure full resensitization of the response. Bicuculline-resistant GABA responses expressed by retina RNA showed little desensitization (17) and were, therefore, separated by intervals of only 2–5 min. Modulatory effects of steroids were assayed by repeating concentration-response curves in the presence of steroid, with each GABA exposure being preceded by 2-min steroid preincubations. The long lasting effects of many of the steroids tested made it necessary to first measure a full concentration-response curve under control conditions and then sequentially repeat the full curve with steroid.

Data analysis. Membrane current responses were normalized and expressed as a fraction of the maximum response. EC_{50} values and slope factors (pseudo-Hill coefficients) were calculated from GABA concentration-response curves, using a nonlinear least squares curve-fitting program based on a four-parameter logistic equation (36).

Calculation of time constants. Current traces were stored on a Nicolet 310 digital oscilloscope, and response decays were subsequently analyzed on an IBM PC-AT, as the sum of two exponentials. Fitting of exponentials was done using the simplex algorithm for minimization of nonlinear equations, with a program developed in the laboratory. For reasons that remain unclear, the rate of $I_{G\text{-}Actx}$ desensitization can show appreciable variation among oocytes taken from different frogs[2]; all comparisons of time constants were, therefore, made between oocytes injected at the same time and taken from the same animal. In addition, a previous study on nicotinic acetylcholine receptors expressed in oocytes had shown that antibiotics such as gentamycin strongly increased the rate of response desensitization (37). Preliminary experiments suggested that gentamycin had little effect on the desensitization of GABA responses[2] but, nevertheless, we specifically used oocytes that had not been exposed to antibiotics for measurement of decay time constants. Finally, to obviate effects on desensitization that were simply due to potentiation of GABA responses by steroids, decay time constants were determined for currents elicited by 1 mM GABA, which constituted >90% of maximum responses.

Steroids. The steroids used in this study were 3α-OH-DHP (elsewhere termed allopregnanolone), 5β-pregnan-3α-ol-20-one (elsewhere termed pregnanolone), 5α-pregnan-3β-ol-20-one, 3β-OH-DHP, THDOC (elsewhere termed allotetrahydrodeoxycorticosterone), pregnenolone-SO_4, and progesterone. All steroids were initially made up as 0.05, 0.5, 5.0, and 50 mM stocks in ethanol or DMSO. For Ringer solutions of steroids, ethanol or DMSO was diluted to ≤0.1% (v/v) and when applied alone showed no significant effects on membrane current responses elicited by GABA. In some oocytes, 0.1% ethanol did cause small (1–2 nA) outward currents on the unstimulated oocyte membrane, and when necessary these currents were subtracted from apparent effects on GABA responses. All drugs were obtained from Sigma.

Results

Effects of 3α-OH-DHP on GABA responses expressed by cortex RNA. GABA responses expressed in oocytes by rat cortex poly(A)$^+$ RNA ($I_{G\text{-}Actx}$) were strongly potentiated by 3α-OH-DHP (Fig. 1, A and B). Potentiation of currents elicited by 10 μM GABA was detectable with as little as 1–2 nM 3α-OH-DHP. At concentrations between 10 and 1000 nM, 3α-OH-DHP caused clear shifts to the left in $I_{G\text{-}Actx}$ concentration-response curves, without significantly changing the slope of the curve or the maximum current (Fig. 2A). In 50 and 500 nM 3α-OH-DHP, the EC_{50} for $I_{G\text{-}Actx}$ was shifted from 92 ± 19 μM (n = 8) to 40 ± 4.3 μM (n = 4) and 13 ± 1.8 μM (n = 5), respectively (all values given as mean ± standard deviation). Slope factors (pseudo-Hill coefficients) for $I_{G\text{-}Actx}$ concentration-response curves were 1.0 ± 0.2 under control conditions, 1.0 ± 0.1 in 50 nM 3α-OH-DHP, and again 1.0 ± 0.1 in 500 nM 3α-OH-DHP. Unless otherwise stated, EC_{50} values and slope factors in all following control $I_{G\text{-}Actx}$ concentration-responses curves were not significantly different from values quoted here.

THDOC and 5β-pregnan-3α-ol-20-one also caused strong positive modulation of $I_{G\text{-}Actx}$. When assayed on currents elicited by 10 μM GABA, concentrations of steroid necessary to induce threshold levels of potentiation were between 2 and 5 nM THDOC, and levels of potentiation using 50 and 500 nM

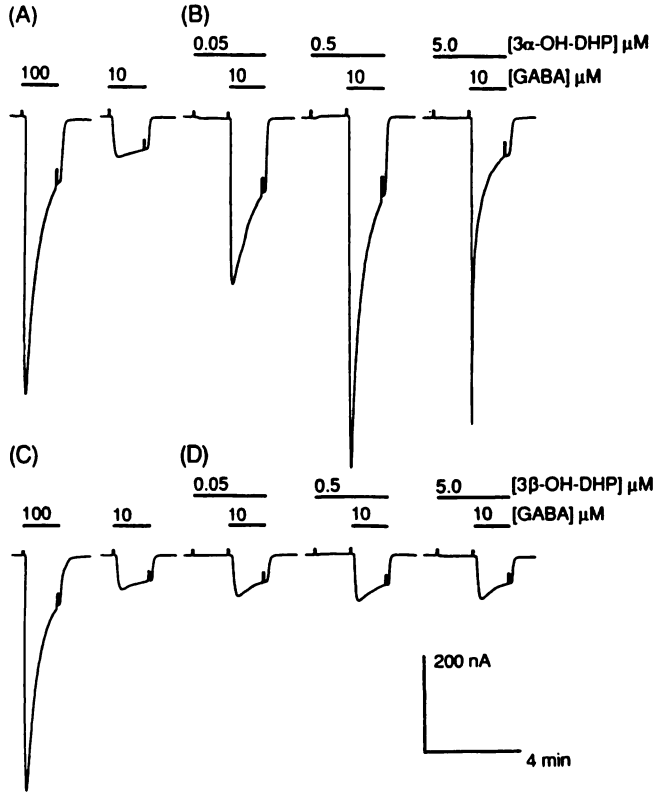

Fig. 1. Actions of 3α-OH-DHP and 3β-OH-DHP on $I_{G\text{-}Actx}$. A and B, Effects of 3α-OH-DHP; records taken from the same cell. A, Control responses, defining GABA sensitivity before application of 3α-OH-DHP. B, Potentiation of 10 μM GABA response by 3α-OH-DHP. Note increased rate of response desensitization in 5 μM 3α-OH-DHP. C and D, Effects of 3β-OH-DHP; records taken from the same cell. C, Control responses. D, Effect of 3β-OH-DHP on currents elicited by 10 μM GABA. The holding potential was −60 mV, with periodic 10-mV depolarizing steps (duration, 5 sec) used to monitor membrane conductance. The dead time of the perfusion system for drug applications was 5–10 sec. To minimize desensitization, exposures were separated by 5–20-min intervals of wash. Inward current is denoted by downward deflection, and capacitative transients (partially picked up by the chart recorder on steps in potential) have been deleted during preparation of figures. Unless otherwise stated, these recording conditions were used in all following figures.

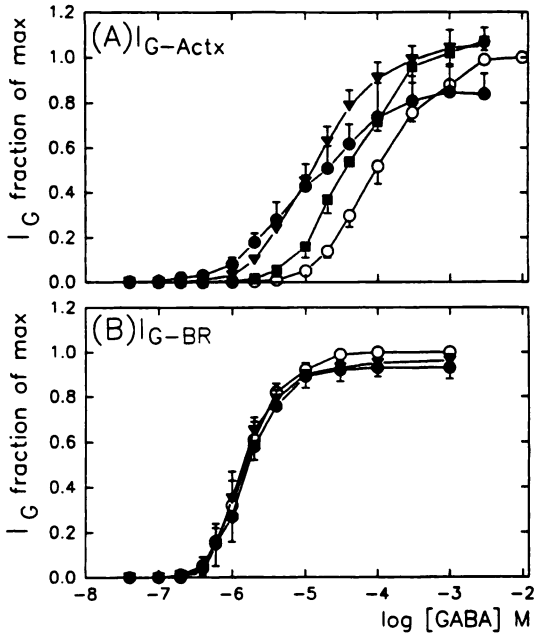

Fig. 2. Concentration-response curves comparing effects of 3α-OH-DHP on $I_{G\text{-}Actx}$ and $I_{G\text{-}BR}$. A, $I_{G\text{-}Actx}$. ○, GABA control (n = 8); ■, GABA plus 50 nM 3α-OH-DHP (n = 4); ▼, GABA plus 500 nM 3α-OH-DHP (n = 5); ●, GABA plus 5 μM 3α-OH-DHP (n = 5). B, $I_{G\text{-}BR}$. ○, GABA control (n = 7); ▼, GABA plus 500 nM 3α-OH-DHP (n = 3); ●, GABA plus 5 μM 3α-OH-DHP (n = 5). Control curves were constructed in single oocytes, and GABA exposures were then repeated with different concentrations of steroid. In this and all following graphs, data points are the mean ± standard deviation of membrane current elicited by GABA (I_G), expressed as a fraction of the maximum control response, with *error bars* omitted when less than the size of the *symbols*. For GABA responses expressed by retina RNA, 0.1–1 mM bicuculline methobromide was used to abolish $I_{G\text{-}Actx}$, allowing measurements of the effects of steroids selectively on $I_{G\text{-}BR}$.

THDOC were similar to those described for 3α-OH-DHP. 5β-Pregnan-3α-ol-20-one was only slightly less active.

When 3α-OH-DHP was increased to 5 μM, concentrations of GABA required to elicit threshold responses were shifted from control values of 1–2 μM to as low as 40 nM, indicating a further increase in the degree of potentiation of $I_{G\text{-}Actx}$. Nevertheless, at concentrations of >10 μM GABA, levels of potentiation in 5 μM 3α-OH-DHP appeared to be lower than those seen in 500 nM 3α-OH-DHP, resulting in a slight decrease in slope factor to 0.8 ± 0.1 and a 16 ± 9% reduction in maximum response (n = 5) (Fig. 2A). The major reason for the reduction in levels of potentiation apparent when using 5 μM 3α-OH-DHP is also illustrated in Fig. 1. As concentrations of 3α-OH-DHP were increased it became clear that the steroid not only potentiated $I_{G\text{-}Actx}$ but also markedly increased the rate of response desensitization. For example, the current elicited by 100 μM GABA in the absence of steroid (Fig. 1A) was approximately the same amplitude as that elicited by 10 μM GABA in 5 μM 3α-OH-DHP, but the rate of response decay was clearly increased in the presence of steroid (Fig. 1B). Thus, the apparent reductions in levels of potentiation seen with 5 μM 3α-OH-DHP were at least partly due to limitations in the speed of GABA delivery from our perfusion system, and even when high flow rates (e.g., 15 ml/min) were used, desensitization was sufficient to cut short the rising phase and thereby decrease the peak current. THDOC and 5β-pregnan-3α-ol-20-one at 0.5–5 μM caused similar pronounced increases in the rate of desensitization of $I_{G\text{-}Actx}$.

Interestingly, 3α-OH-DHP did not itself elicit appreciable membrane current responses, even when tested on oocytes that showed strong expression of $I_{G\text{-}Actx}$. For example, 5–50 μM 3α-OH-DHP elicited inward currents of only 1–2 nA in oocytes that showed response thresholds at 0.4 μM GABA and gave 100–120-nA responses to 10 μM GABA (n = 4). The currents elicited by 3α-OH-DHP were too small for determination of changes in membrane conductance but were still apparently inward at a holding potential of 0 mV. This suggested that 3α-OH-DHP responses were not carried exclusively by Cl^- which has a reversal potential between −20 to −30 mV in oocytes, and, indeed, might arise independently of any interaction with $GABA_A$ receptors. Further electrical and pharmacological characterization of currents elicited by 3α-OH-DHP was rendered unfeasible by the small size of these responses, which are close to the limits of resolution for recording whole-cell currents in voltage-clamped oocytes.

The potentiating effects of 3α-OH-DHP, THDOC, and 5β-pregnan-3α-ol-20-one on $I_{G\text{-}Actx}$ were all long lasting and occurred in the absence of any simultaneous application of GABA. For example, after a 2-min exposure to 5 μM 3α-OH-DHP,

applied alone, strong potentiation was still apparent even after a 1.5–2.0-hr wash. These persistent actions of steroids clearly differed from the potentiating effects of pentobarbital, which were largely washed out in 5–10 min (see also Ref. 12). The effects of 3α-OH-DHP, THDOC, and 5β-pregnan-3α-ol-20-one on desensitization of $I_{G\text{-}Actx}$ were also slow to wash out, and after 2-min exposures to 5 μM 3α-OH-DHP increases in the rate of response desensitization were usually still apparent after 1.5 hr.

Effects of 3α-OH-DHP on GABA responses expressed by retina RNA. In contrast to the multiple effects seen on $I_{G\text{-}Actx}$, 3α-OH-DHP showed no significant modulation of the bicuculline-resistant GABA responses expressed by retina RNA ($I_{G\text{-}BR}$) (Figs. 2B and 3, A and B). Threshold currents, elicited by 0.2–0.4 μM GABA, showed no potentiation by 0.05–5 μM 3α-OH-DHP, and there were no significant shifts in $I_{G\text{-}BR}$ concentration-response curves. The EC_{50} and slope factors in the absence of steroid were 1.5 ± 0.2 μM GABA and 1.8 ± 0.3 (n = 7), respectively, and these values were effectively unaltered in 0.05–5 μM 3α-OH-DHP (Fig. 2B). EC_{50} and slope factors of all following $I_{G\text{-}BR}$ concentration-response curves, measured under control conditions, were not significantly different from values quoted here.

In our initial characterization of $I_{G\text{-}BR}$ (17), the EC_{50} value (1.38 ± 0.25 μM; n = 21) was calculated directly from double-logarithmic plots and slope (2.75 ± 0.26; n = 21) specifically from the rising phase of concentration-response curves (i.e., currents elicited by 0.2–2 μM GABA). Reanalysis of the same data using the curve-fitting procedure gave an EC_{50} of 1.5 ± 0.4 μM and slope factor of 1.9 ± 0.5, all within the standard deviations of values measured in the present study. The only potentially significant difference is between the slope values quoted in the original study (17) and the slope factors determined by curve-fitting, which are consistently lower. This discrepancy arises because the curve-fitting program considers all values in the concentration-response curve, whereas the original calculations were based solely on the rising phase of the curve. This reestimation of the slope factor for $I_{G\text{-}BR}$ might be important, because the original value of 2.75 ± 0.26 clearly suggested that the gating of the bicuculline-resistant GABA receptors involved binding of more than two molecules of GABA. In contrast, the value of 1.9 ± 0.5 calculated by curve fitting implies that gating does not necessarily involve binding of more than two GABA molecules, although it does not rule out this possibility. Slopes of $I_{G\text{-}Actx}$ concentration-response curves were similarly lower when calculated by curve-fitting, i.e., 1.63 ± 0.09 (n = 21) calculated from the rising phase of the curve (17), compared with a value of 1.1 ± 0.2 calculated from the same data by curve-fitting. In the case of $I_{G\text{-}Actx}$, slope factors calculated by curve-fitting to the full concentration-response curve probably underestimate the real values, because the analysis fails to account for the influence of desensitization, which compromises measurement of peak responses elicited by higher concentrations of GABA.

The only potential action of 0.5–5 μM 3α-OH-DHP on $I_{G\text{-}BR}$ was 5–10% inhibition of maximum responses (Fig. 2B), but

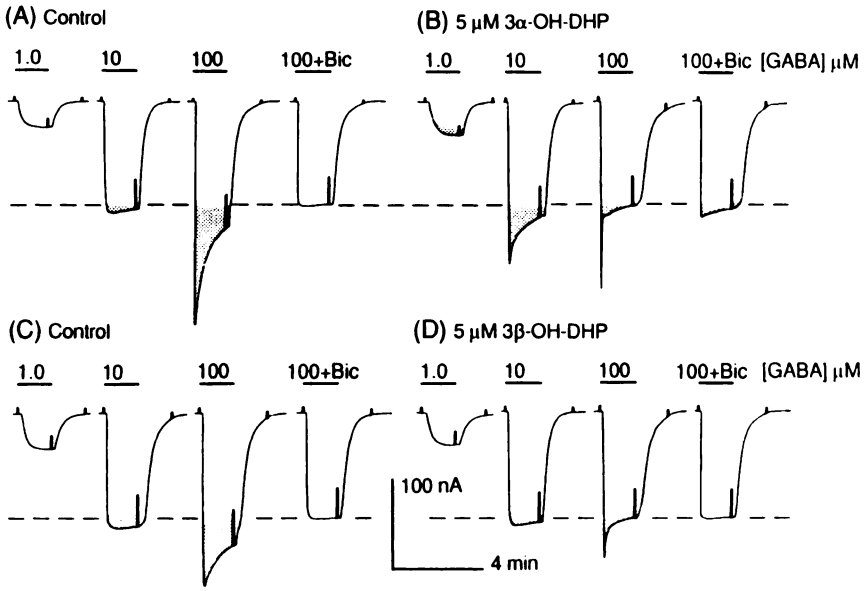

Fig. 3. Actions of 3α-OH-DHP and 3β-OH-DHP on $I_{G\text{-}BR}$ and $I_{G\text{-}Arel}$. A and B, Effects of 3α-OH-DHP; records taken from the same cell. A, Examples of control GABA responses before application of steroid. Response elicited by 1 μM GABA was wholly $I_{G\text{-}BR}$, with no detectable bicuculline-sensitive current. Response elicited by 10 μM GABA was maximal for $I_{G\text{-}BR}$, together with a threshold response of $I_{G\text{-}Arel}$. Response elicited by 100 μM GABA was again maximal for $I_{G\text{-}BR}$, together with a substantial $I_{G\text{-}Arel}$, which showed desensitization. Response elicited by 100 μM GABA plus 100 μM bicuculline methobromide (*Bic*) showed abolition of $I_{G\text{-}Arel}$ and preservation of maximum $I_{G\text{-}BR}$. In this and all following records of GABA responses expressed by retina RNA, $I_{G\text{-}Arel}$ has been indicated by *shading*, and the amplitude of maximum $I_{G\text{-}BR}$ shown by a *broken line*. B, GABA responses during incubation with 5 μM 3α-OH-DHP. Response elicited by 1 μM GABA was control $I_{G\text{-}BR}$ and in addition a small $I_{G\text{-}Arel}$. (Current elicited by 1 μM GABA plus 100 μM bicuculline is indicated below *shading*.) Response elicited by 10 μM GABA approached maximum for $I_{G\text{-}BR}$, together with a strongly potentiated $I_{G\text{-}Arel}$. Response elicited by 100 μM GABA again approached maximum for $I_{G\text{-}BR}$, with $I_{G\text{-}Arel}$ showing a sharply increased rate of desensitization. Response in 100 μM bicuculline showed that, with 5 μM 3α-OH-DHP, 100 μM bicuculline substantially blocks $I_{G\text{-}Arel}$ but is an insufficient concentration to abolish this component. C and D, Effects of 3β-OH-DHP; records taken from the same cell. C, Control GABA responses before application of steroid; currents as described in A. D, GABA responses during incubation in 5 μM 3β-OH-DHP. Response elicited by 1 μM GABA was control $I_{G\text{-}BR}$ with no additional $I_{G\text{-}Arel}$. Response elicited by 10 μM GABA approached maximum for $I_{G\text{-}BR}$ and showed no potentiation of $I_{G\text{-}Arel}$. Response elicited by 100 μM GABA was maximal for $I_{G\text{-}BR}$, with $I_{G\text{-}Arel}$ showing an increased rate of desensitization. Response in 100 μM bicuculline showed abolition of $I_{G\text{-}Arel}$.

even this small effect was inconsistent. Furthermore, in two experiments carried out to investigate this effect specifically, 20 μM 3αOH-DHP caused <5% reductions in the current elicited by 10 μM GABA. As previously described (17), $I_{G\text{-}BR}$ showed only low levels of desensitization. For example, 4-min exposures to 1 mM GABA caused <10% desensitization of the current, compared with >90% desensitization of $I_{G\text{-}Actx}$. Consistent with the absence of potentiation effects, 0.5–5 μM 3α-OH-DHP caused no appreciable increase in the rate at which $I_{G\text{-}BR}$ desensitized.

Whereas $I_{G\text{-}BR}$ was essentially unaffected by 3α-OH-DHP, the bicuculline-sensitive component of GABA responses expressed by retina RNA ($I_{G\text{-}Aret}$) was strongly potentiated (Fig. 3, A and B). In 5 μM 3α-OH-DHP, threshold concentrations for eliciting $I_{G\text{-}Aret}$ were reduced from 5–10 μM to approximately 0.1 μM GABA, with responses elicited by 10 μM GABA showing 10–20-fold amplification. In addition, 0.5–5 μM 3α-OH-DHP also caused clear increases in the rate at which $I_{G\text{-}Aret}$ desensitized (Fig. 3B). THDOC, at 0.5–5 μM, had similar potentiating effects on $I_{G\text{-}Aret}$ and also increased the rate of response desensitization. Measurement of $I_{G\text{-}BR}$ in 5 μM 3α-OH-DHP was complicated slightly because $I_{G\text{-}Aret}$ elicited by 100 μM GABA was only partially blocked by 100 μM bicuculline (e.g., Fig. 3B). Increasing the concentration of bicuculline to 1 mM effectively abolished the current without blocking $I_{G\text{-}BR}$ (data not shown).

Effects of 3β-OH-DHP on GABA responses expressed by cortex RNA. Studies on $GABA_A$ receptors *in situ* have shown that 3β-hydroxy isomers of pregnanolone cause comparatively little potentiation of membrane current responses (e.g., Refs. 19 and 22–24). Following preliminary experiments, we elected to use 3β-OH-DHP, in preference to 5α-pregnan-3β-ol-20-one, because the former appeared to show lower levels of potentiation.

As illustrated in Fig. 1, C and D, 0.05–5 μM 3β-OH-DHP caused only marginal potentiation of $I_{G\text{-}Actx}$. When assayed on currents elicited by 10 μM GABA, levels of potentiation ranged between 10 and 20%. Given the high potency of 3α-hydroxypregnanolones, it remains unclear whether these levels of potentiation were actually caused by 3β-OH-DHP or were due to low levels of contamination by active isomers. 3β-OH-DHP at 0.5 and 5 μM caused no significant shift to the left in $I_{G\text{-}Actx}$ concentration-response curves but did appear to reduce the maximum current (Fig. 4A). For example, currents elicited by 100 μM GABA were reduced 19 ± 11% by 5 μM 3β-OH-DHP and the maximum response was reduced by 28 ± 1% (n = 3). As described for 3α-OH-DHP, the apparent reduction in maximum $I_{G\text{-}Actx}$ was due, at least in part, to 3β-OH-DHP causing significant increases in the rate of response desensitization. In the case of 3β-OH-DHP, this apparent inhibition was more pronounced than that seen with 3α-OH-pregnanolones, probably because there was no significant potentiation to obscure the effect.

Effects of 3β-OH-DHP on GABA responses expressed by retina RNA. As described for 3α-OH-DHP, $I_{G\text{-}BR}$ was essentially unaffected by 0.5–5 μM 3β-OH-DHP (Figs. 3, C and D, and 4B). In some cases, there appeared to be 5–10% reductions in maximum $I_{G\text{-}BR}$, but these inhibitory effects were inconsistent, and in two experiments 20 μM 3β-OH-DHP caused only 5 and 7% reductions in the current elicited by 10 μM GABA. At 0.5–5 μM, 3β-OH-DHP also had no clear effect on the desensitization of $I_{G\text{-}BR}$. Using the same concentrations, 3β-OH-DHP showed no appreciable potentiation of $I_{G\text{-}Aret}$ but, as

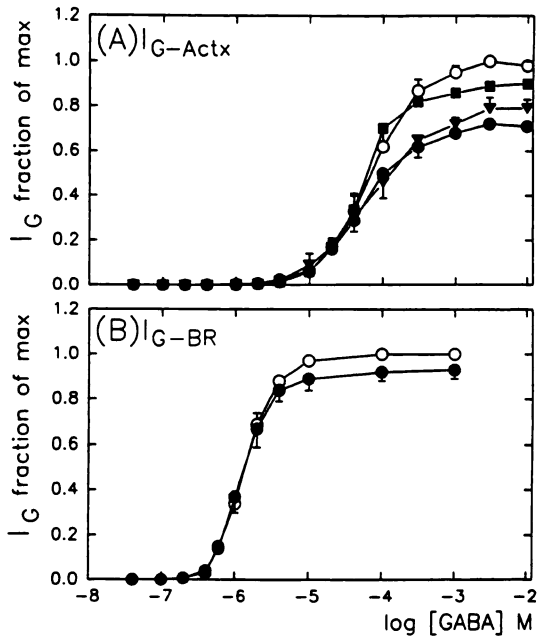

Fig. 4. Concentration-response curves comparing effects of 3β-OH-DHP on $I_{G\text{-}Actx}$ and $I_{G\text{-}BR}$. A, $I_{G\text{-}Actx}$. ○, GABA control (n = 5); ■, GABA plus 50 nM 3β-OH-DHP (n = 3); ▼, GABA plus 500 nM 3β-OH-DHP (n = 3); ●, GABA plus 5 μM 3β-OH-DHP (n = 3). B, $I_{G\text{-}BR}$. ○, GABA control (n = 6); ●, GABA plus 5 μM 3β-OH-DHP (n = 6).

described for $I_{G\text{-}Actx}$, did cause clear increases in the rate of response desensitization (Fig. 3D).

Effects of 3α-OH-DHP and 3β-OH-DHP on desensitization of $I_{G\text{-}Actx}$. Potentiation of $GABA_A$ responses by progesterone metabolites has been characterized in a variety of assay systems (e.g., Refs. 19, 20, and 22–24), whereas effects on the rate of $GABA_A$ receptor desensitization remain largely undocumented. We, therefore, made further studies to characterize this aspect of $GABA_A$ receptor modulation by steroids.

Sample records illustrating the effect of 5α-OH-DHP on the rate of $I_{G\text{-}Actx}$ desensitization are given in Fig. 5. Although the decay of $I_{G\text{-}Actx}$ under control conditions could in some cases be adequately fitted by a single exponential, accurate fitting normally required the sum of two exponentials (Fig. 5A). This level of complexity in the process of receptor desensitization became increasingly apparent when responses were repeated in 3α-OH-DHP, where it was clear that the time course of the decay had at least two components (Fig. 5, B and C). To investigate the effects of steroids on each component separately, all time courses of $I_{G\text{-}Actx}$ desensitization were, therefore, similarly analyzed in terms of two components. Examples showing the effects of 3α-OH-DHP and 3β-OH-DHP on time constants of $I_{G\text{-}Actx}$ desensitization are given in Table 1. In these experiments, control levels were first established by one to three exposures to 1 mM GABA, separated by 20–30-min intervals of wash (Table 1A). Rates of response desensitization were relatively stable upon repeated exposures, although there tended to be modest decreases over prolonged periods of recording (i.e., 2–3 hr). Application of 10 mM GABA did not appear to cause further significant increases in rates of response desensitization (Table 1B), indicating that control rates were already close to maximal using 1 mM GABA. Likewise, after 2-min preincubations the steroid vehicles ethanol (Table 1C) and DMSO, at 0.1% (v/v), caused no appreciable changes in the rate of response decay. As suggested in Fig. 5, the main effect

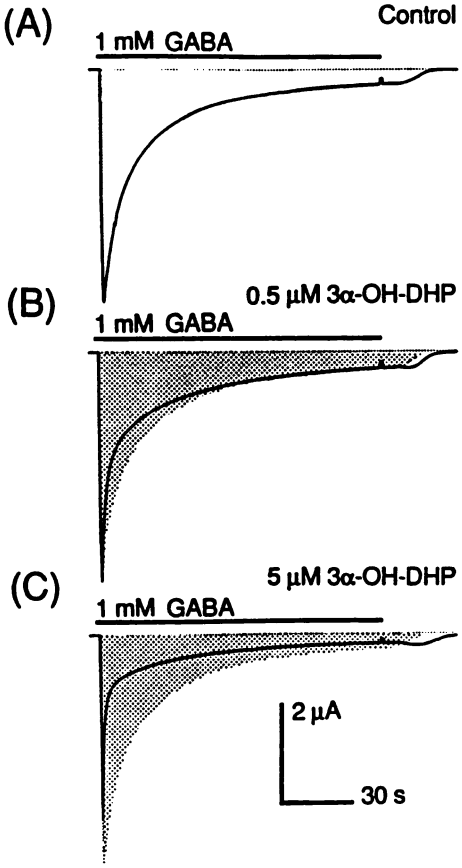

Fig. 5. Effect of 3α-OH-DHP on desensitization of $I_{G\text{-}Actx}$; records taken from the same cell. A, Control response before application of steroid. B, Response in 500 nM 3α-OH-DHP. C, Response in 5 μM 3α-OH-DHP. For comparison, *shaded areas* indicate time course of the control response. In each case, GABA responses were activated after 2-min preincubation in steroid and were separated by 30-min intervals of wash.

TABLE 1

Effects of 3α-OH-DHP and 3β-OH-DHP on the desensitization of GABA responses expressed by rat cerebral cortex RNA

All values are given as the mean ± standard deviation of *n*, independent determinations. Data were obtained using oocytes from the same frog, and the time course of desensitization was fitted by the sum of two exponentials. τ_{fast} and τ_{slow} denote the time constants of fast and slow components, respectively.

Drug concentration	τ_{fast}	τ_{slow}	*n*
	sec	sec	
A. GABA, 1 mM (control)	4.4 ± 0.6	22.5 ± 4.8	18
B. GABA, 10 mM	4.0 ± 1.9	25.1 ± 2.4	4
C. GABA, 1 mM, + 0.1% ethanol	4.4 ± 1.2	25.8 ± 2.5	4
D. GABA, 1 mM, + 50 nM 3α-OH-DHP	3.9 ± 0.5	26.6 ± 2.9	5
E. GABA, 1 mM, + 500 nM 3α-OH-DHP	2.1 ± 0.3	26.6 ± 2.8	6
F. GABA, 1 mM, + 5.0 μM 3α-OH-DHP	0.7 ± 0.2	26.5 ± 4.4	9
G. GABA, 1 mM, + 500 nM 3β-OH-DHP	2.8 ± 0.4	26.6 ± 3.5	3
H. GABA, 1 mM, + 5.0 μM 3β-OH-DHP	1.8 ± 0.5	27.2 ± 4.3	3

of 3α-OH-DHP was on the fast component of $I_{G\text{-}Actx}$ desensitization. Actions of 3α-OH-DHP showed thresholds between 50 and 100 nM, with 500 nM and 5 μM 3α-OH-DHP reducing the time constant of the fast component by 52 ± 7 and 84 ± 5%, respectively (Table 1, E and F). In other terms, 5 μM 3α-OH-DHP caused a >5-fold increase in the rate of desensitization of the fast component. 3β-OH-DHP also caused clear increases in the rate of desensitization of the fast component, with 500 nM and 5 μM 3β-OH-DHP reducing the time constant by 36 ± 9 and 59 ± 11%, respectively (Table 1, G and H) (e.g., Fig. 6D). For both steroids, increases in the rate of desensitization of the fast component were accompanied by modest decreases in the rate of the slow component. In all cases these decreases were ≤21%, which was the deviation seen in control responses.

The effect of pregnanolones on $I_{G\text{-}Actx}$ desensitization was then compared with the actions of a barbiturate and a benzodiazepine, both of which had been shown to potentiate $GABA_A$ responses expressed in oocytes (11–13). In four experiments using currents elicited by 1 mM GABA, pentobarbital at 10–100 μM caused no increase in the rate of $I_{G\text{-}Actx}$ desensitization but, on the contrary, appeared to decrease the rate of decay by 10–20% (e.g., Fig. 6C). In three experiments, diazepam at 1–10 μM also caused no significant increase in the rate of $I_{G\text{-}Actx}$ desensitization but did cause modest reductions in maximum currents (e.g., Fig. 6B).

To investigate whether the effect on desensitization was specific to $GABA_A$ receptors, actions of steroids were then assayed on strychnine-sensitive glycine receptors expressed in oocytes by poly(A)$^+$ RNA extracted from adult rat spinal cord. As previously reported (38), oocytes injected with spinal cord RNA showed strong expression of glycine responses but only weak expression of GABA responses. 3α-OH-DHP, THDOC, and 5β-pregnan-3α-ol-20-one at concentrations of 0.5 or 5 μM all caused no appreciable potentiation of glycine responses (e.g., Fig. 7, A and D) and, furthermore, no significant increases in the rate at which glycine responses desensitized (e.g., Fig. 7, B and E). 3β-OH-DHP likewise had no clear effect on the desensitization of glycine responses. In contrast, small $GABA_A$ responses expressed in the same oocytes by spinal cord RNA were strongly potentiated by 5 μM 3α-OH-DHP or THDOC and, furthermore, showed pronounced increases in the rate of desensitization (e.g., Fig. 7, C and F).

Effects of pregnenolone-SO_4 on GABA responses expressed by cortex RNA. Pregnenolone-SO_4 was first assayed on $I_{G\text{-}Actx}$ elicited by 10 μM GABA and at concentrations between 2 and 20 nM caused relatively modest (10–50%) potentiation of the response. However, at concentrations of >20 nM pregnen-

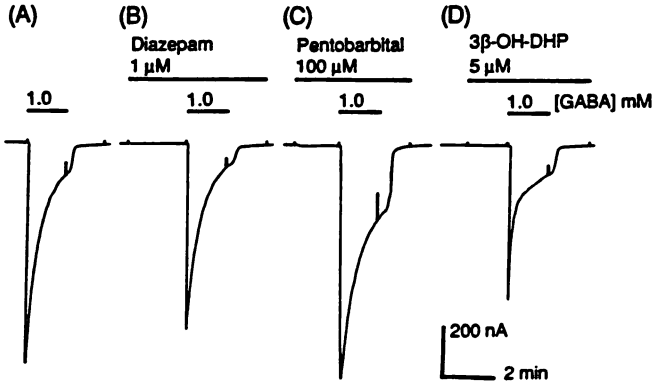

Fig. 6. Comparing effects of diazepam, pentobarbital, and 3β-OH-DHP on desensitization of $I_{G\text{-}Actx}$. The same oocyte was repeatedly exposed to 1 mM GABA at 30-min intervals. A, Control response. B, Response with 1 μM diazepam; desensitization was largely unaffected. C, Response with 100 μM pentobarbital; rate of desensitization was decreased. D, Response with 5 μM 3β-OH-DHP; rate of desensitization was increased.

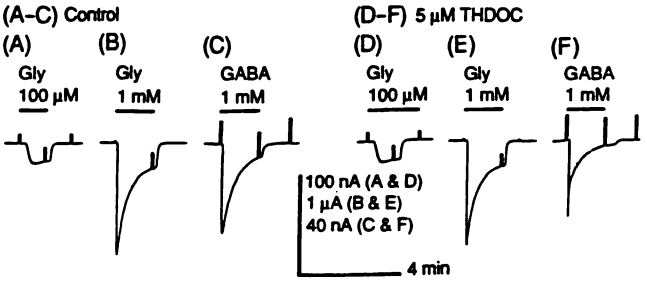

Fig. 7. Effect of THDOC on strychnine-sensitive glycine responses expressed by rat spinal cord RNA. A–C, Control responses elicited by glycine and GABA before steroid exposure. D–F, Responses elicited in the same oocyte by repeated exposures to glycine and GABA during incubation with 5 μM THDOC.

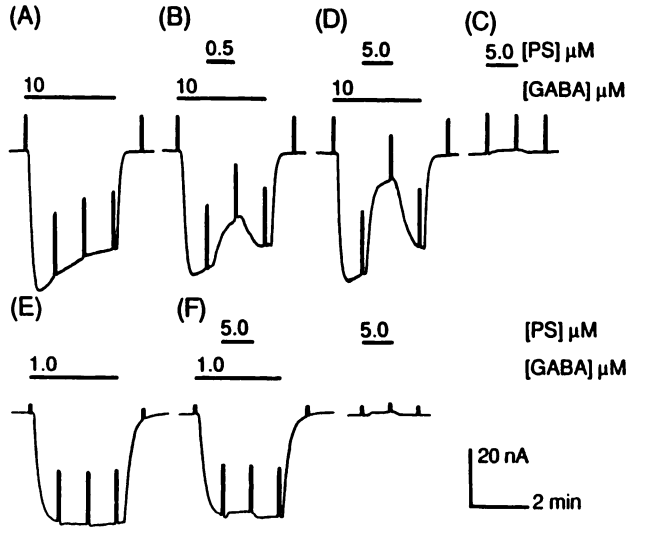

Fig. 8. Comparing blocking actions of pregnenolone-SO_4 (*PS*) on $I_{G\text{-}Actx}$ and $I_{G\text{-}BR}$. A–D, Effects on $I_{G\text{-}Actx}$; records taken from the same cell. A, Control response elicited by an extended exposure to 10 μM GABA. B, Blocking effect of 0.5 μM pregnenolone-SO_4. C, Blocking effect of 5 μM pregnenolone-SO_4; note rapid washout. D, Small outward current elicited by pregnenolone-SO_4 when applied alone. E–G, Effects on $I_{G\text{-}BR}$; records taken from the same cell. E, Control response elicited by an extended exposure to 1 μM GABA. F, Effect of 5 μM pregnenolone-SO_4 on GABA response. Final record, small outward current elicited by pregnenolone-SO_4 when applied alone. Note that the apparent slight reduction in $I_{G\text{-}BR}$ (F) was largely artifactual, resulting from a small outward current elicited on the unstimulated membrane.

olone-SO_4 caused no further increases in GABA responses, and above >100 nM effects became predominantly inhibitory. When tested on responses elicited by extended exposures to 10 μM GABA, 500 nM and 5 μM pregnenolone-SO_4 reduced control currents by approximately 35% and 75%, respectively (e.g., Fig. 8, A–D). Unlike the persistent modulatory effects caused by 3α-OH-DHP and THDOC, blocking actions of pregnenolone-SO_4 appeared to be rapidly removed upon wash, with effects of 5 μM pregnenolone-SO_4 cleared by >90% within 1 min (Fig. 8D).

Assaying of the actions of pregnenolone-SO_4 on $I_{G\text{-}Actx}$ elicited by 0.1–1 mM GABA revealed further complexities in the effects of this steroid. With 5 μM pregnenolone-SO_4, responses elicited by 1 mM GABA were reduced to brief (1–2 sec) spikes of current, followed by small maintained currents that were <10% of the maximum control response (Fig. 9, A–C). Furthermore, although blocking effects were largely removed during 20-min intervals of wash, there still appeared to be residual increases

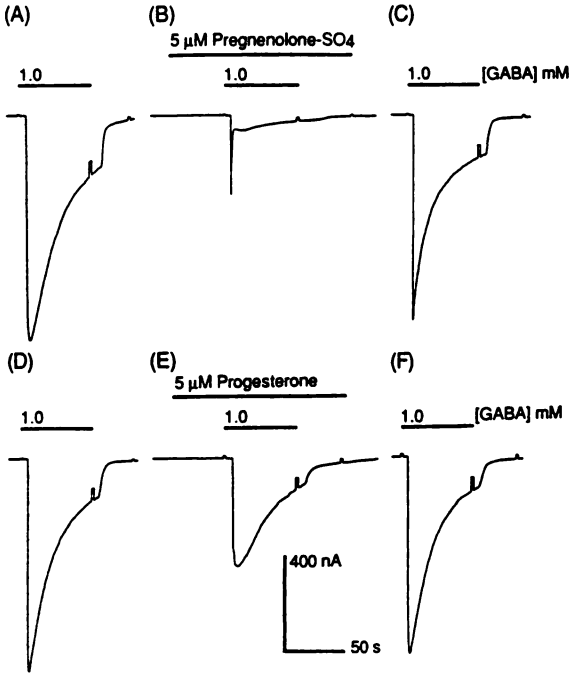

Fig. 9. Comparing blocking actions of pregnenolone-SO_4 and progesterone on $I_{G\text{-}Actx}$. A–C, Effects of pregnenolone-SO_4; records taken from the same cell. A, Control GABA response before application of steroid. B, Response in 5 μM pregnenolone-SO_4; current showed initial activation but was rapidly blocked. C, Response after washout of pregnenolone-SO_4; major blocking effect was removed but there was still an apparent residual increase in the rate of desensitization. D–F, Effects of progesterone; records taken from the same cell. D, Control response before application of steroid. E, Response in 5 μM progesterone; current was partially blocked but showed slow time course. F, Response after washout of progesterone; blocking effect was washed out, with no residual increase in rate of desensitization. GABA responses were separated by 30-min intervals of wash.

in the rate of response desensitization (e.g., Fig. 9C). These experiments suggested that, in addition to facilitatory and inhibitory actions, pregnenolone-SO_4 also affected the desensitization of $I_{G\text{-}Actx}$. As described above, the blocking actions of pregnenolone-SO_4 were washed out relatively rapidly and could, therefore, be at least partially distinguished from apparent effects on desensitization, which were more persistent. For example, pre-exposure to 5 μM pregnenolone-SO_4 applied alone, followed by a 3-min wash, caused no clear inhibition of $I_{G\text{-}Actx}$ but did appear to increase the rate of desensitization. Nevertheless, because of the predominance of inhibitory activity, detailed analysis of the effect of pregnenolone-SO_4 on response desensitization was not possible. Rough comparisons with 3α-OH-DHP, THDOC, and 3β-OH-DHP suggested that pregnenolone-SO_4 was significantly less active than any of the pregnanolone isomers.

The inhibitory actions of pregnenolone-SO_4 on $I_{G\text{-}Actx}$ resembled, at least superficially, those of drugs showing a "use-dependent" component to their blocking effects. For $GABA_A$ responses, these include picrotoxin (39)[2] and TBPS, which has been characterized in detail on $GABA_A$ receptors expressed in oocytes by chick brain RNA (40). The blocking effects of pregnenolone-SO_4 and picrotoxin were, therefore, compared, to investigate whether inhibition occurred through similar or demonstrably different mechanisms. For these experiments, we looked at the time courses and levels of inhibition of GABA responses repeated during extended applications of picrotoxin

or pregnenolone-SO_4; a representative experiments is illustrated in Fig. 10. Extended incubations in picrotoxin or pregnenolone-SO_4 were started by coapplication with GABA (first records in Fig. 10, B and E) and were then maintained continuously for 1 hr. During these prolonged applications the oocytes were repeatedly exposed to 100 μM GABA for 1 min at 15-min intervals for a total of five exposures, three examples of which are shown in Fig. 10. Apparent use-dependent blocking effects were detectable during the response elicited by coapplication of GABA and picrotoxin, which consisted of an spike of current, suggesting initial activation and then rapid blocking of the channel, followed by a maintained current, reflecting the full level of inhibition (Fig. 10B). Repeated applications of GABA in the continuous presence of picrotoxin then showed that the full blocking level was maintained, with no recurrence of the use-dependent component (Fig. 10B). In contrast, although blocking effects in pregnenolone-SO_4 also appeared to show use dependence, characterized by an initial brief spike of current (as described for picrotoxin), the full level of inhibition was not thereafter maintained, and the apparent use-dependent component could be successively reactivated even after 1 hr of continuous incubation with steroid and repeated applications of GABA (Fig. 10E).

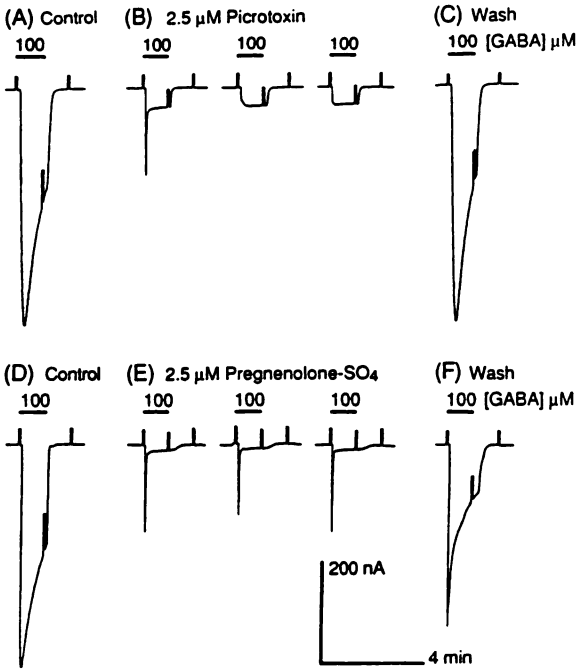

Fig. 10. Comparison of blocking actions of picrotoxin and pregnenolone-SO_4 on $I_{G\text{-}Actx}$ in the same oocyte. A, Initial control response. B, Currents elicited by 1-min GABA applications repeated at 15-min intervals during a continuous 1-hr incubation in picrotoxin; three examples of a total of five. *First record*, current elicited by initial coapplication of GABA and picrotoxin. GABA was then washed out but application of picrotoxin was maintained throughout. *Second record*, current elicited by second application of GABA, 15 min into the extended incubation with picrotoxin. *Third record*, current elicited by the fifth application of GABA, 1 hr into the incubation with picrotoxin. Washout of picrotoxin was started after this response. C, Current elicited by GABA after 15-min washout of picrotoxin. D, Response after 30-min picrotoxin washout; control for subsequent experiments using pregnenolone-SO_4. E, Responses elicited by 1-min GABA applications repeated at 15-min intervals during a continuous 1-hr incubation in pregnenolone-SO_4. Procedures identical to those described for picrotoxin. *First record*, initial coapplication of GABA and pregnenolone-SO_4. *Second record*, second application of GABA, 15 min into the incubation with pregnenolone-SO_4. *Third record*, fifth application of GABA, 1 hr into the incubation with pregnenolone-SO_4. F, Response after 30-min washout of pregnenolone-SO_4; note apparent residual effect on desensitization.

Effects of pregnenolone-SO_4 on $I_{G\text{-}Actx}$ concentration-response curves were determined and showed that inhibition of responses was associated with only modest increases in EC_{50} (Fig. 11A). For example, under control conditions the EC_{50} was 70 ± 2.7 μM GABA (n = 10), and with 0.05, 0.5, and 5 μM pregnenolone-SO_4 the EC_{50} was 80 ± 1.9 μM (n = 3), 96 ± 3.8 μM (n = 5), and 126 ± 11 μM (n = 3), respectively. Thus, although inhibition was predominantly noncompetitive, the progressive increase in EC_{50} seen as concentrations of pregnenolone-SO_4 were raised suggested that there could also be a weak competitive component to the blocking action. Measuring the peak current spikes elicited with steroid, 50 nM pregnenolone-SO_4 reduced maximum GABA responses by 16 ± 8% (n = 3), 500 nM by 38 ± 2% (n = 4), and 5 μM by 76 ± 8% (n = 3) (Fig. 11A). Comparing currents measured after the initial spike, 0.5 μM pregnenolone-SO_4 reduced corresponding control currents by >75% and 5 μM pregnenolone-SO_4, by >90%.

Pregnenolone-SO_4 also had blocking actions on glycine responses expressed in oocytes by adult rat spinal cord RNA. For example, 5 μM pregnenolone-SO_4 reduced currents elicited by 1 mM glycine between 50 and 60%. Interestingly, although blocking effects were pronounced, the time course of glycine responses in 5 μM pregnenolone-SO_4 was slow and did not show the brief spikes of current that characterized inhibition of $I_{G\text{-}Actx}$.

Effects of pregnenolone-SO_4 on GABA responses expressed by retina RNA. In contrast to the complex actions on $I_{G\text{-}Actx}$, pregnenolone-SO_4 showed little or no modulation of $I_{G\text{-}BR}$ (Fig. 12, A and B), and 0.5–5 μM pregnenolone-SO_4 showed no clear blocking actions on the current elicited by 1 μM GABA

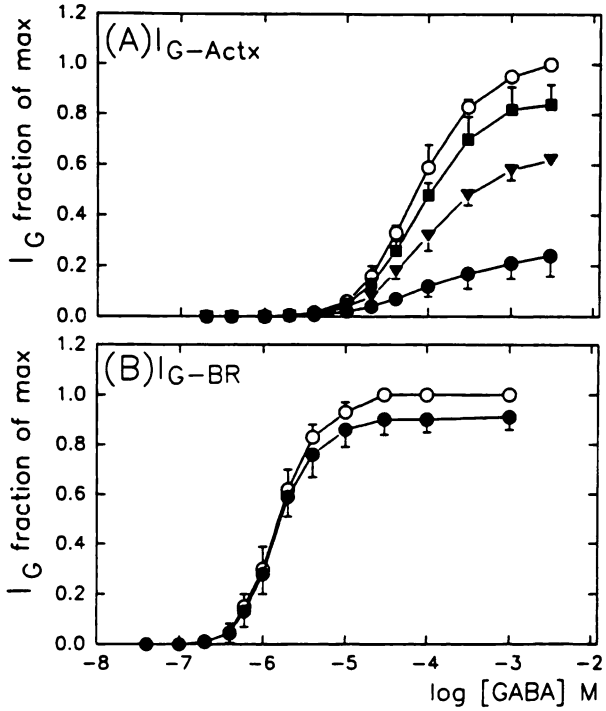

Fig. 11. Concentration-response curves comparing effects of pregnenolone-SO_4 on $I_{G\text{-}Actx}$ and $I_{G\text{-}BR}$. A, $I_{G\text{-}Actx}$. ○, GABA control (n = 10); ■, GABA plus 50 nM pregnenolone-SO_4 (n = 3); ▼, GABA plus 500 nM pregnenolone-SO_4 (n = 4); ●, GABA plus 5 μM pregnenolone-SO_4 (n = 3). B, $I_{G\text{-}BR}$. ○, GABA control (n = 4); ●, GABA plus 5 μM pregnenolone-SO_4 (n = 4).

(Fig. 8, E–G). For example, in these experiments the EC_{50} and slope factor for $I_{G\text{-}BR}$ under control conditions were 1.3 ± 0.5 μM and 2.1 ± 0.1 (n = 4), respectively. In 5 μM pregnenolone-SO_4 the values were 1.2 ± 0.1 μM and 2.0 ± 0.1 (n = 4), respectively, insignificantly different from controls. The only possible effect of pregnenolone-SO_4 on $I_{G\text{-}BR}$ concentration-response curves was to cause a slight reduction in maximum current (Fig. 11B). However, these apparent inhibitory effects were always weak, generally <10%, and in three separate experiments concentrations as high as 20 μM pregnenolone-SO_4 caused only 10–12% reductions in currents elicited by 10 μM GABA. Pregnenolone-SO_4 at 5 μM also had no clear effect on the rate of $I_{G\text{-}BR}$ desensitization.

$I_{G\text{-}Aret}$, like $GABA_A$ responses expressed by cortex RNA, was strongly inhibited by 0.5–5 μM pregnenolone-SO_4 (Fig. 12, A and B). Threshold concentrations for detecting blocking actions were approximately 100 nM, and in 5 μM pregnenolone-SO_4 responses elicited by 100 μM GABA were again reduced to brief spikes of current, ranging between 10 and 20% of control responses (Fig. 12B, *arrow*).

Effects of progesterone on GABA responses expressed by cortex RNA. Currents elicited by 10 μM GABA were used to assay for any potentiation of $I_{G\text{-}Actx}$ by progesterone. At 5 μM, progesterone caused little or no increase in response, and 50 μM progesterone reduced responses by 40–60%. When the effect of progesterone was tested on currents elicited by 1 mM GABA, it was clear that the major actions of 0.5–5 μM progesterone on $I_{G\text{-}Actx}$ were inhibitory (Fig. 9, D–F). However, unlike GABA responses in pregnenolone-SO_4, currents elicited by 1 mM GABA in 5 μM progesterone appeared to show slow time courses (Fig. 9E). Furthermore, blocking effects of progesterone were largely removed during 20-min intervals of wash, and there was no clear residual effect on the rate of $I_{G\text{-}Actx}$ desensitization (Fig. 9F). Blocking actions of 0.5–5 μM progesterone on $I_{G\text{-}Actx}$ concentration-response curves were associated with little change in EC_{50}, indicating that inhibition was largely noncompetitive (Fig. 13A). For example, the EC_{50} under control conditions was 77 ± 3.6 μM GABA (n = 6), and in 0.5 and 5 μM progesterone was 88 ± 0.1 μM and 61 ± 3.8 μM (n = 3), respectively. Progesterone was significantly less active than pregnenolone-SO_4 as an inhibitor of $I_{G\text{-}Actx}$, with 500 nM progesterone reducing maximum responses by 14 ± 2%, and 5 μM progesterone by 58 ± 5% (n = 3) (Fig. 13A).

Effects of progesterone on GABA responses expressed by retina RNA. As described for pregnenolone-SO_4, 0.05–5 μM progesterone was essentially inactive on $I_{G\text{-}BR}$ (Fig. 12, C and D). Again, the only potential effect of 5 μM progesterone was a marginal inhibition of maximum response (Fig. 13B), but in two separate experiments concentrations as high as 20 μM progesterone caused only 5 and 6% reductions in the current elicited by 10 μM GABA. At concentrations up to 5 μM, progesterone also had no clear effect on the rate of desensitization of $I_{G\text{-}BR}$.

Progesterone caused no obvious potentiation of $I_{G\text{-}Aret}$ and, as described for $GABA_A$ responses expressed by cortex RNA, micromolar concentrations were clearly inhibitory (Fig. 12, C and D). For example, in three experiments $I_{G\text{-}Aret}$ elicited by 100 μM GABA was reduced 50–65% by 5 μM progesterone. As seen with $I_{G\text{-}Actx}$, time courses of $I_{G\text{-}Aret}$ elicited in progesterone were slow, compared with the spikes of current seen in pregnenolone-SO_4, and progesterone appeared to have no residual effect on the rate at which $I_{G\text{-}Aret}$ desensitized (Fig. 12D).

Discussion

The main purpose of this study was to compare the modulatory effects of steroids on GABA receptors expressed in

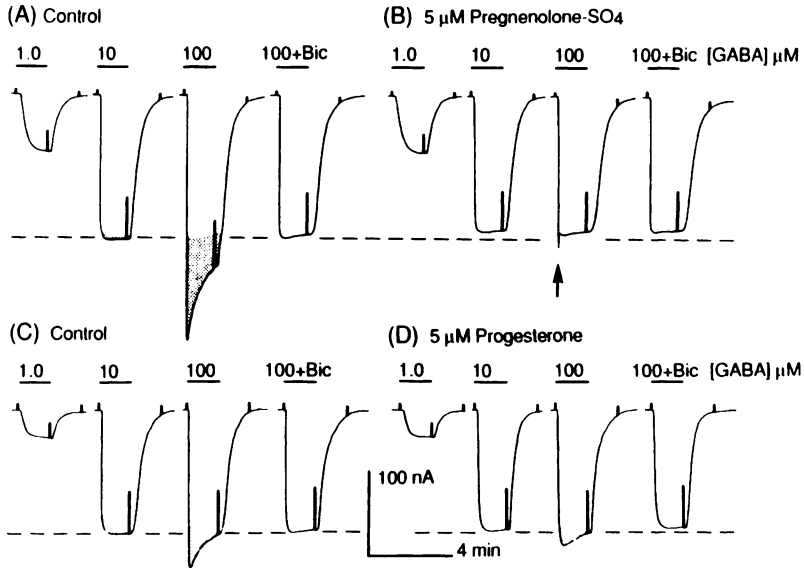

Fig. 12. Actions of pregnenolone-SO_4 and progesterone on $I_{G\text{-}BR}$ and $I_{G\text{-}Aret}$. A and B, Effects of pregnenolone-SO_4; records taken from the same cell. A, Control GABA responses before application of steroid; currents are as described in Fig. 3A. B, GABA responses during incubation in 5 μM pregnenolone-SO_4. Response elicited by 1 μM GABA was control $I_{G\text{-}BR}$. Response elicited by 10 μM GABA approached maximum for $I_{G\text{-}BR}$, with no detectable $I_{G\text{-}Aret}$. Response elicited by 100 μM GABA was again maximum for $I_{G\text{-}BR}$, with a small transient activation of $I_{G\text{-}Aret}$ (*arrow*). Response in 100 μM bicuculline showed that $I_{G\text{-}BR}$ was only marginally reduced by 5 μM pregnenolone-SO_4. C and D, Effects of progesterone; records taken from the same cell. C, Control GABA responses before application of steroid, as described in Fig. 3A. D, GABA responses during incubation in 5 μM progesterone. Response elicited by 1 μM GABA was control $I_{G\text{-}BR}$. Response elicited by 10 μM GABA approached maximum for $I_{G\text{-}BR}$ and showed control levels of $I_{G\text{-}Aret}$. Response elicited by 100 μM GABA was, again, maximal for $I_{G\text{-}BR}$, whereas $I_{G\text{-}Aret}$ was blocked approximately 50%; note that the reduced current had a slow time course. Response in 100 μM bicuculline showed that $I_{G\text{-}BR}$ was only marginally reduced by 5 μM progesterone.

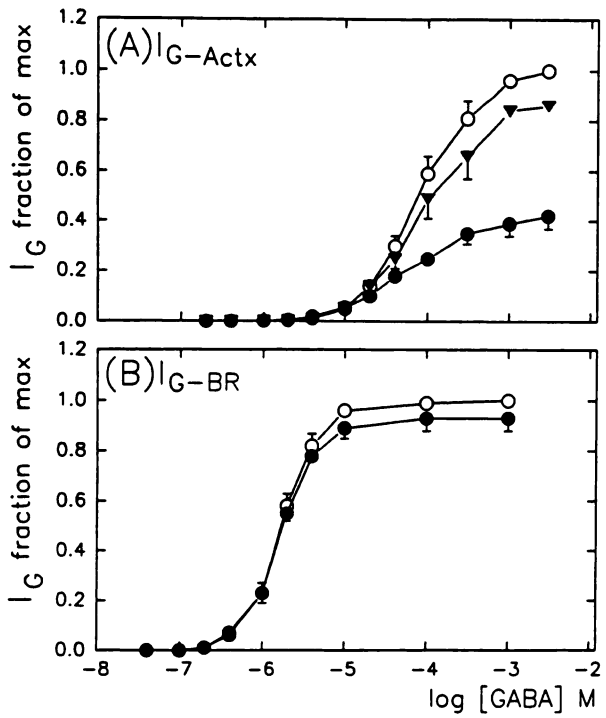

Fig. 13. Concentration-response curves comparing effects of progesterone on $I_{G\text{-}Actx}$ and $I_{G\text{-}BR}$. A, $I_{G\text{-}Actx}$. ○, GABA control (n = 6); ▼, GABA plus 500 nM progesterone (n = 3); ●, GABA plus 5 μM progesterone (n = 3). B, $I_{G\text{-}BR}$. ○, GABA control (n = 3); ●, GABA plus 5 μM progesterone (n = 3).

oocytes by RNA from mammalian brain with effects on GABA receptors expressed by RNA from mammalian retina. These will be discussed separately below.

Similarities between $GABA_A$ receptors expressed in oocytes and those studied *in situ*. As reported for $GABA_A$ receptors in cultured rat hippocampal neurons (19), bovine adrenomedullary chromaffin cells (22), and recombinant receptors expressed in a cell line (24), responses mediated by rat cerebral cortex $GABA_A$ receptors expressed in oocytes were strongly potentiated by 3α-OH-DHP, THDOC, and 5β-pregnan-3α-ol-20-one. Thresholds for detecting potentiation by 3α-OH-DHP and THDOC were between 1 and 5 nM, similar to those reported in chromaffin cells (22) or neurons (e.g., Refs. 19 and 25), and the effect also showed pronounced stereoselectivity, with 3β-hydroxy isomers having little or no activity. Positive modulation of $GABA_A$ receptors expressed in oocytes resulted in clear leftward shifts in concentration-response curves and lowering of GABA concentrations required to elicit threshold responses from micromolar to nanomolar ranges.

Currents elicited through direct activation of $GABA_A$ receptors by pregnanolones. The only clear difference between the actions of 3α-OH-pregnanolones on rat cortex $GABA_A$ receptors expressed in oocytes and those on $GABA_A$ receptors studied in other systems was that, when applied alone, the steroids did not activate appreciable membrane current responses. For example, in bovine adrenomedullary chromaffin cells THDOC and 5β-pregnan-3α-ol-20-one, at concentrations of >1 μM, were shown to elicit Cl^- currents through direct activation of $GABA_A$ receptors (22). These currents were antagonized by bicuculline and potentiated by benzodiazepines and barbiturates (22). Similarly, micromolar concentrations of 3α-OH-DHP and THDOC were shown to cause direct activation of bicuculline-sensitive Cl^- currents in cells expressing recombinant $GABA_A$ receptors (24). In clear contrast, even at concentrations as high as 50 μM, 3α-OH-DHP did not appear to elicit significant membrane currents through activation of rat cerebral cortex $GABA_A$ receptors expressed in oocytes. At present, it remains unclear whether the absence of this aspect of steroidal modulation is actually intrinsic to the structure of rat cortex $GABA_A$ receptors or is simply a consequence of expressing the receptors in oocytes; due to, for example, misprocessing of RNAs, incorrect assembly of receptors, or expression in a foreign lipid environment. Interestingly, recent studies on recombinant rat $GABA_A$ receptors expressed in oocytes have shown that subunit composition appears to regulate the degree to which pentobarbital directly activates receptors (8). This raises the possibility that the ability of steroids to cause direct activation of $GABA_A$ receptors is dependent on subunit composition.

Steroid-induced increase in the rate of desensitization of $GABA_A$ receptors expressed in oocytes. In addition to confirming the well documented potentiation of $GABA_A$ responses by 3α-OH-pregnanolones, our experiments also demonstrated that pregnanolones cause pronounced increases in the rate of response desensitization. Before discussing these results, it is important to stress that the time resolution of our experiments was somewhat limited by the perfusion system and recording conditions. Even using fast flow (15 ml/min) and a small chamber (0.1 ml), the large size of oocytes (1.0–1.3 mm diameters) tended to make simultaneous application of GABA to the whole surface difficult. This was probably compounded by the dense covering of microvilli beneath the vitelline envelope (e.g., Refs. 33 and 34), a microenvironment into which drugs have to diffuse before interacting with receptors. For these reasons, time constants calculated for the fast component of $I_{G\text{-}Actx}$ decay are probably significant underestimates of the real rate of desensitization (see Ref. 14).

Studies using the "concentration-clamp" technique on frog sensory neurons report time constants of approximately 3 sec for the fast component of $GABA_A$ receptor desensitization (41). GABA-induced $^{36}Cl^-$ influx assays on mouse spinal cord neurons report times for half-maximal desensitization of 4 sec (42). U-tube drug application on recombinant $GABA_A$ receptors expressed in a cell line shows time constants for desensitization ranging between 1 and 10 sec, depending on subunit composition (7). In our experiments, the time constant for the fast component of $I_{G\text{-}Actx}$ desensitization under control conditions was 4.4 ± 0.6 sec, certainly comparable to that determined in other systems. But with 5 μM 3α-OH-DHP and 3β-OH-DHP, peak measurable currents were reduced by 16 ± 9% and 28 ± 1%, respectively, in turn suggesting that decay time constants measured in these experiments were underestimates.

It was clear that the effect of steroids on desensitization of $I_{G\text{-}Actx}$ did not show the stringent stereoselectivity that characterized potentiation. At 5 μM, 3α-OH-DHP did appear to have higher activity than 3β-hydroxy isomers, reducing τ_{fast} by 84 ± 5%, compared with 3β-OH-DHP, which reduced τ_{fast} by 59 ± 11%. However, due to the absence of any potentiation effect, peak currents measured in 3β-OH-DHP were more strongly reduced than those in 3α-OH-DHP, and this would tend to cause some additional underestimate in τ_{fast}. Lack of stringent stereoselectivity and effects on desensitization by pregnenolone-SO_4 both suggest that this aspect of $GABA_A$ receptor modulation by steroids is not simply a facet of potentiation,

implying that the two effects are distinct. In addition, pentobarbital also caused strong potentiation of $GABA_A$ responses but actually decreased the rate of desensitization (see Ref. 12), and diazepam modestly potentiated responses without causing any increases in the rate of decay (see Refs. 13 and 14). These experiments all indicate that potentiation of $GABA_A$ responses in oocytes is not necessarily accompanied by increases in the rate of desensitization and, conversely, that increases in desensitization are not necessarily associated with potentiation of responses.

Recent studies on rat dorsal root ganglion cells have shown that a number of general anaesthetics potentiate $GABA_A$ responses and also cause increases in the rate of response desensitization (43). This raises the question of whether the effect of steroids on desensitization is due to specific actions on $GABA_A$ receptors or nonspecific effects, for example, perturbations of the fluidity of lipid bilayers (26, 44). Two observations begin to address this question. Firstly, although pregnanolones had pronounced effects, it was also clear that progesterone caused little or no increase in the rate at which $GABA_A$ responses desensitized. This indicates that the effect is specific to particular types of steroid and is not a general effect common to this class of molecule. Secondly, experiments using oocytes injected with rat spinal cord RNA showed that 3α-OH-DHP, 3β-OH-DHP, and THDOC did not appreciably increase desensitization of glycine responses but in the same cells had strong effects on GABA responses. These results indicate either that $GABA_A$ receptors are particularly sensitive to nonspecific effects or that increases in the rate of response desensitization are due to direct interaction of steroids with receptors. If the effect on desensitization is indeed due to direct actions with $GABA_A$ receptor complexes, then this steroid modulatory site does not appear to show the same stereoselectivity as the site, or sites, mediating potentiation of GABA responses.

Mechanisms underlying $GABA_A$ receptor desensitization are poorly understood. Analogies drawn with G protein-coupled receptors (45) and nicotinic acetylcholine receptors (46) have suggested that phosphorylation of the receptor is an important initial step in the process. The β-subunits of $GABA_A$ receptors contain consensus sequences for phosphorylation (1) and have been reported to be substrates for both protein kinases C and A (47). Nevertheless, more recent evidence suggests that phosphorylation by these kinases plays no direct role in receptor desensitization (42), although it might be involved in the longer term process of receptor "run-down" (48). Without further knowledge of the mechanisms involved in $GABA_A$ receptor desensitization, speculation as to how steroids interact with this process is necessarily restricted. In general terms, it seems most likely that after exposure to pregnanolones the $GABA_A$ receptor complex is itself allosterically modulated, either through direct interactions or nonspecific effects on the membrane, such that it desensitizes rapidly upon activation by GABA. Alternatively, it remains possible that this effect of steroids is not directly on receptors but is due to modulation of a separate component within the desensitization mechanism, for example, through regulation of the activity of a protein kinase. In either case, it will first be important to investigate whether steroids actually regulate $GABA_A$ receptor desensitization in neurons and other cell types, to determine whether this facet of their actions is for some reason particular to receptors expressed in oocytes.

A further consideration is that, under control conditions, decays can in some cases be fitted by a single exponential. This raises the possibility that steroids are not simply facilitating the normal mechanism by which the $GABA_A$ receptors desensitize but are regulating the receptor in a manner such that desensitization occurs via a novel mechanism that is either inactive or only weakly active under control conditions. When comparing responses in different oocytes, the variations in rate and complexity of $GABA_A$ receptor desensitization might then, at least in part, be a reflection of variations in levels of endogenous steroidogenesis in oocytes. This would also be consistent with the observation that time constants tend to increase over extended periods of recording, where modulatory steroids that had accumulated during oocyte storage would be slowly cleared from the oocyte membrane. We should stress, however, that at present there is no direct evidence in oocytes for modulation of $GABA_A$ receptors by endogenous steroids.

Finally, expression studies using recombinant $GABA_A$ receptor subunits have demonstrated that a multiplicity of subunit combinations can assemble to form functional $GABA_A$ receptors (e.g., Refs. 1–8). Furthermore, the properties of $GABA_A$ receptors clearly depend on subunit composition and, in particular, some subunit combinations form receptors that desensitize with a single component, whereas others show at least two components (7). THDOC and 3α-OH-DHP have been shown to potentiate responses mediated by $GABA_A$ receptors formed from a number of subunit combinations (24), but actions on response desensitization were not reported. In our experiments, oocytes were injected with total cortex poly(A)$^+$ RNA and, hence, expressed a variety of different $GABA_A$ receptor subunits and probably a heterogeneous population of functional receptors. It therefore remains possible that the apparently selective effect of steroids on the fast component of desensitization is due to interactions with specific subpopulations of $GABA_A$ receptors.

Mechanisms underlying inhibition of $GABA_A$ receptors by pregnenolone-SO_4 and progesterone. The various modulatory effects of pregnenolone-SO_4 on $GABA_A$ responses and the mechanisms by which pregnenolone-SO_4 interacts with $GABA_A$ receptors remain unresolved. Pioneering studies suggested that pregnenolone-SO_4 interacted competitively with TBPS at the convulsant binding site (49). This appeared to be supported by electrical recordings from rat cortical neurons, which showed that pregnenolone-SO_4 and picrotoxin blocked currents primarily by decreasing the frequency of single-channel opening (29). Conversely, nanomolar concentrations of pregnenolone-SO_4 were also shown to facilitate muscimol binding (50), suggesting that the steroid might actually potentiate $GABA_A$ responses. In addition, recent binding studies have suggested that pregnenolone-SO_4 acts at distinct and possibly multiple sites that might regulate the convulsant binding site allosterically (e.g., Refs. 51 and 52).

For rat cortex $GABA_A$ receptors expressed in oocytes, 5–20 nM pregnenolone-SO_4 did indeed cause modest potentiation of currents elicited by 10 μM GABA, but at concentrations of >100 nM the dominant effect of pregnenolone-SO_4 was inhibitory. Inhibition of $GABA_A$ responses by pregnenolone-SO_4 was complex and, like use-dependent channel blockers acting at the convulsant site (e.g., picrotoxin and TBPS), blocking actions of pregnenolone-SO_4 appeared either to require activation of the receptor by GABA or at least to be facilitated by channel opening. However, unlike the blocking effects of picrotoxin and TBPS, blocking actions of pregnenolone-SO_4 were not main-

tained, even in the continuous presence of steroid, and hence were not cumulative. This suggests that, during incubations with pregnenolone-SO_4, $GABA_A$ receptors either remain unaffected or are only partially blocked by the steroid. Upon activation of receptors by GABA, the channels open briefly but are then rapidly and potently blocked. It remains unclear whether this block is allosteric, possibly due to stabilization of a closed form of the receptor, or whether it is due to direct "plugging" at a site within the lumen of the Cl^- channel. Whatever, once GABA is washed from the receptor the channels do not appear to remain blocked, even though incubation in pregnenolone-SO_4 has been maintained throughout, and the receptors can be reactivated, albeit briefly, by subsequent applications of GABA. In the case of maintained incubations in picrotoxin or TBPS, the channel does remain blocked after the initial exposure to GABA, and the receptor can be reactivated only after the inhibitor itself has been washed out. These experiments do not directly address questions relating to sites of action but do suggest that the mechanism underlying the blocking actions of pregnenolone-SO_4 differs from that of picrotoxin or TBPS and seems to be consistent with pregnenolone-SO_4 acting at a site, or sites, that are allosteric to the convulsant binding site.

Progesterone has been shown to potentiate $GABA_A$ responses in chick spinal cord neurons and to antagonize currents mediated by glycine receptors (53). In our experiments, 0.05–50 μM progesterone caused little or no potentiation of currents mediated by rat cortex $GABA_A$ receptors, and at concentrations of >200 nM the dominant effect on GABA responses was inhibitory. These differences could be species or tissue dependent or, again, a result of expressing receptors in the foreign environment of the oocyte. Time courses of GABA responses elicited during incubations in progesterone were slow, and there was no obvious indication of increases in the rate of response desensitization. These results suggest that the mechanisms by which pregnenolone-SO_4 and progesterone inhibit $GABA_A$ responses could be different. However, at present it still remains unclear whether this actually reflects a difference in mechanisms or whether the time resolution in our experiments was simply insufficient to detect any brief current spikes in progesterone. Experiments using rapid drug application techniques will be needed to resolve the issue. In this context, it is interesting to note that, although micromolar concentrations of pregnenolone-SO_4 clearly inhibited glycine responses, the response time courses were also slow, likewise suggesting that the mechanisms by which pregnenolone-SO_4 inhibits glycine and $GABA_A$ responses could be different. Finally, as with the various effects of pregnanolone isomers, it remains possible that, depending on subunit composition, different subpopulations of $GABA_A$ receptors showed variations in sensitivity to the blocking actions of pregnenolone-SO_4 or progesterone.

Effects of steroids on GABA responses expressed by retina RNA. $GABA_A$ responses expressed by retina RNA were strongly modulated by steroids. In all respects, actions of steroids on this component of retina GABA responses appeared to be qualitatively similar to modulatory effects seen on responses expressed by cortex RNA. The bicuculline-sensitive currents expressed by retina RNA were strongly potentiated by 3α-OH-DHP and THDOC but not by 3β-OH-DHP, confirming that the effect was stereoselective. Pregnenolone-SO_4 and progesterone inhibited retina and cortex $GABA_A$ responses with similar potency, and the time courses of retina $GABA_A$ responses in the presence of the two steroids were fast and slow, respectively. In addition, 3α-OH-DHP, THDOC, and 3β-HO-DHP all caused clear increases in the rate at which retina $GABA_A$ responses desensitized. Taken together, these experiments serve to confirm that oocytes injected with retina RNA express functional $GABA_A$ receptors with properties similar to those described *in situ*.

In direct contrast, bicuculline-resistant GABA responses expressed by retina RNA were almost wholly unaffected by any of the steroids used in this study.

As previously described (17), the GABA receptors mediating bicuculline-resistant responses showed higher affinity and levels of cooperativity than $GABA_A$ receptors expressed by cerebral cortex RNA. Thus, even though 5 μM 3α-OH-DHP reduced EC_{50} values for cortex $GABA_A$ receptors from 70–110 μM to approximately 10 μM, these were still significantly higher than the EC_{50} values of 1–2 μM for the bicuculline-resistant response. This raises questions as to whether detection of any potentiation of $I_{G\text{-}BR}$ by 3α-OH-pregnanolones could have been expected.

Although EC_{50} values for $I_{G\text{-}BR}$ were invariably lower than those for $I_{G\text{-}Actx}$ in steroids, evidence for potentiation of bicuculline-resistant responses, if any were present, should still have been measurable when effects of 3α-OH-pregnanolones on currents elicited by low concentrations of GABA were examined. For example, under control conditions GABA concentrations necessary to elicit threshold $I_{G\text{-}Actx}$ were 0.4–1 μM, whereas in 5 μM 3α-OH-DHP responses could be activated by concentrations of GABA as low as 40 nM. In contrast, GABA concentrations required to elicit threshold $I_{G\text{-}BR}$ were relatively low, between 200 and 400 nM, but were not appreciably reduced by 5.0 μM 3α-OH-DHP. Because response thresholds for eliciting $I_{G\text{-}Actx}$ were approximately 10-fold lower in 3α-OH-DHP, this suggests that reductions in response threshold should have been clearly detectable if the bicuculline-resistant GABA receptor was under any appreciable positive modulation by 3α-OH-DHP.

The marginal blocking effects of most steroids on maximum $I_{G\text{-}BR}$ were inconsistent and were not appreciably enhanced using higher concentrations of steroid. In some cases these apparent blocking effects were probably artifactual, arising through slight run-down of the response during extended periods of recording, effects for which adequate controls were not always included. Our results, therefore, suggest that the bicuculline-resistant GABA receptors expressed by retina RNA do not show functional modulation by any of the steroids tested in this study. Bicuculline-resistant GABA responses are, however, sensitive to picrotoxin (17),[2] and insensitivity to pregnenolone-SO_4 provides at least indirect evidence to suggest that these two drugs do not act at the same site.

In conclusion, studies at the molecular level will be required to determine why the novel bicuculline-resistant GABA receptors expressed by retinal RNAs are insensitive to modulators. Interestingly, the cDNA encoding a GABA receptor that is highly enriched in mammalian retina, denoted $GABA_{\rho 1}$, has recently been cloned (54). This receptor has sequence homology with $GABA_A$ receptor subunits and forms ligand-gated Cl^- channels that show high sensitivity to picrotoxin when expressed in oocytes (54). Further pharmacological and molecular characterization should determine what role this receptor plays in mediating the bicuculline-resistant GABA responses expressed by retina poly(A)$^+$ RNA. Comparative structure-function studies might then be helpful for investigating the proc-

esses underlying modulation of $GABA_A$ receptors by steroids, benzodiazepines, and barbiturates and might also provide insights into the mechanisms by which $GABA_A$ receptors desensitize.

Acknowledgments

We thank Dr. M. M. Panicker for microinjection of oocytes, Mr. Q. T. Nguyen for providing spinal cord RNA, Ms. L. Jaimes for technical assistance, and Mr. Rico Miledi for developing analysis software. L.P. also thanks Prof. A. Giotti for helpful discussions.

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