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ACCELERATED COMMUNICATION

Enantiomers of Neuroactive Steroids Support a Specific Interaction with the GABA-C Receptor as the Mechanism of Steroid Action

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ABSTRACT

Neuroactive steroids can either potentiate or inhibit a variety of membrane channels. Most studies have suggested that the effects are mediated by specific association of the steroid with the affected channel. However, a recent study of the ρ1 (GABA-C) receptor (*Mol Pharmacol* **66:**56–69, 2004) concluded that the actions were consistent with an action of the steroid in the lipid bilayer to alter the lateral pressure profile in the membrane. The enantiomers of an optically active compound are expected to have identical physical properties, including interactions with hydrophobic portions of the cell membrane. We have used two pairs of enantiomers (pregnanolone and *ent*-pregnanolone, allopregnanolone and *ent*-allopregnanolone) and show that the ability to potentiate (allopregnanolone) or inhibit (pregnanolone) the ρ1 receptor is enantioselective. Therefore, these results strongly suggest that the actions of these neuroactive steroids are mediated by interactions with chiral regions of the target protein, rather than by a change in membrane properties (including lateral pressure).

Many mechanisms have been proposed to underlie the actions of psychoactive compounds. In general, the mechanisms fall into two classes: mechanisms involving specific interactions with target molecules (e.g., sites on membrane proteins) and mechanisms involving more general effects on the milieu in which the target molecule is found (e.g., effects on membrane lipid packing, mobility or order). Neuroactive steroids are a particular type of compound, which can both modulate the function of membrane channels and also intercalate into biological membranes. Indeed, it seems very likely that a preferred route of access for neuroactive steroids may be through the lipid membrane (Akk et al., 2005). However, the question arises of whether effects are mediated through a direct interaction with the receptor protein. Previous studies of neuroactive steroids have shown that the enantiomer of allopregnanolone (3α5αP) is much less effective at potentiating responses of the GABA-A receptor (Wittmer et al., 1996, Covey et al., 2000), the enantiomer of 17β-estradiol is ineffective at potentiating the human nicotinic α4β2 receptor (Paradiso et al., 2001), and the enantiomer of (3α,5α,17β)-3-hydroxyandrostan-17-carbonitrile is less effective at blocking the nicotinic α4β2 receptor (Paradiso et al., 2000). These observations are consistent with the idea that steroids interact with chiral sites on the target proteins. However, there are two caveats to this interpretation. The first is that not all steroids show enantioselectivity in actions (for example, Nilsson et al., 1998; Covey et al., 2000). The second is that biological membranes contain optically active components in the lipid milieu that might interact differently with the steroid enantiomers.

A recent study (Morris and Amin, 2004) of the actions of

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ABBREVIATIONS: 3α5αP, allopregnanolone ((3α,5α)-3-hydroxypregnan-20-one); 3α5βP, pregnanolone ((3α,5β)-3-hydroxypregnan-20-one); DMSO, dimethyl sulfoxide.

pregnanolone [3α5βP; (3α,5β)-3-hydroxypregnan-20-one] and allopregnanolone [3α5αP; (3α,5α)-3-hydroxypregnan-20-one] on the ρ1 GABA-C receptor concluded that the actions of these neuroactive steroids were most consistent with the idea that the steroids altered the lateral pressure profile in the membrane (Cantor, 1997). 3α5αP potentiates the receptor response to low concentrations of GABA, whereas 3α5βP inhibits (Morris et al., 1999, Goutman and Calvo, 2004).

We examined the actions of the natural and unnatural forms of these steroids on the responses of ρ1 receptors (the unnatural form is indicated by the prefix *ent*-; for example, *ent*-3α5αP). We confirmed the findings that 3α5βP inhibits the response to low concentrations of GABA, whereas 3α5αP potentiates the receptor response. In contrast, the enantiomers of the natural steroids are much less effective at either potentiating (3α5αP) or inhibiting (3α5βP) the activation of the ρ1 receptor.

We also examined the biophysical interactions of each enantiomer pair with natural and artificial membranes (J.-M. Alakoskela, D. F. Covey, and P. K. J. Kinnunen, submitted). 3α5αP and *ent*-3α5αP show no difference in effects on a variety of measures, including packing of the interior or headgroups, mobility of hydrocarbon chains, phase transitions, or association of the steroid with headgroup or interior region of the leaflet. Likewise, 3α5βP and *ent*-3α5βP show no differences in any measured parameter. The data indicate that the enantiomers have identical interactions with membranes for all the parameters examined. As expected, 3α5αP and 3α5βP are clearly distinct from each other in their biophysical effects on membranes. Therefore, although 3α5αP and 3α5βP clearly differ from each other in terms of effects on membrane properties, there is no indication that the two enantiomers of a given steroid (that is, 3α5αP and *ent*-3α5αP or 3α5βP and *ent*-3α5βP) do.

These data do not support the idea that steroids act on the ρ1 receptor by affecting properties of the lipid bilayer. Rather, they are more consistent with the idea that the steroids interact with specific sites, probably on the receptor protein, that recognize specific structural features of the steroid.

Materials and Methods

Experiments were conducted using two-electrode voltage clamp of *Xenopus laevis* oocytes, as described previously (Steinbach et al., 2000; Paradiso et al., 2001). A full-length construct of the human ρ1 receptor was provided by D. Weiss (University of Texas Health Center, San Antonio, TX; see Amin and Weiss, 1996), and transferred to pcDNA3 (Invitrogen, Carlsbad, CA). The construct was sequenced and found to correspond to the published sequence (GenBank accession number M62400). cRNA was synthesized using mMessage mMachine T7 kit (Ambion, Austin, TX). Eight nanograms were injected into each oocyte in 23 nl. Oocytes were incubated for 40 to 60 h at 18°C before being studied electrophysiologically.

3α5αP and 3α5βP were purchased from Sigma (St. Louis, MO), and enantiomers were prepared as described previously (Hu et al., 1997; Nilsson et al., 1998). Steroids were dissolved in DMSO at a concentration of 10 mM, then diluted to the appropriate final concentration in recording saline. The maximal final concentration used was 20 μM to avoid problems with steroid solubility. Solutions were applied using glass reservoirs and fluorocarbon or metal tubing to reduce adsorption.

As reported previously (Morris et al., 1999; Goutman and Calvo, 2004), the effects of steroids reversed very slowly. Because we were concerned about time-dependent changes in control responses during the protracted washes, most experiments were performed by paired applications. At first, 2 applications of GABA alone (200 nM) were applied to measure control responses. Then GABA (200 nM) was applied with 10 μM steroid (either the native or unnatural form). A second control application was then made, followed by GABA with the other member of the enantiomeric pair. A final control application was then made. Responses were measured from the peak (average current for an approximately 5-s interval centered at the peak) to the baseline (average current for an approximately 5-s interval before the response). The effect of steroid was measured by the relative response in the presence of steroid to the control response preceding the application of steroid. All applications were separated by at least 3 min. The order of application (natural or unnatural) was switched between eggs. Subsequent analysis of the effects of the steroids on responses showed that there was no effect of order of application—that is, the relative potentiation of 3α5αP was the same irrespective of whether it was applied before or after *ent*-3α5αP. The effects of the enantiomeric forms were then compared for each oocyte. Steroids were tested on six separate sets of oocytes injected with ρ1 cRNA. The maximal concentration of DMSO used was 0.2%. When this concentration of DMSO was applied (without steroid), it had no effect on responses to 200 nM GABA (relative response 0.97 ± 0.05; three oocytes tested). All results are reported as mean ± S.D. (number of observations).

Results

The responses of the injected oocytes to GABA alone were similar to those reported previously (Amin and Weiss, 1996; Morris et al., 1999; Goutman and Calvo, 2004). The concentration-response relationship is described by the Hill equation with an EC_{50} value of 700 ± 70 nM and a Hill coefficient of 2.0 ± 0.2 (mean ± S.D. of fits to curves from five oocytes). A GABA concentration of 200 nM was chosen for studies of steroids because it corresponded to the concentration required to elicit a response of approximately 10% of the maximum response (Morris and Amin, 2004). (The predicted response to 200 nM is 6.4% of maximum.)

The essential results are shown in Fig. 1. This figure shows the responses of an oocyte expressing the ρ1 receptor to 200 nM GABA, GABA plus 10 μM 3α5βP, and GABA plus 10 μM *ent*-3α5βP (Fig. 1A). Note that 3α5βP blocks the response, whereas *ent*-3α5βP seems to potentiate. Similar data are shown for responses of a different oocyte to 10 μM 3α5αP and *ent*-3α5αP (Fig. 1D). In this case, the enantiomer has no effect, whereas the natural form potentiates. The concentration of 10 μM steroid was chosen because it has been reported to be maximally effective at inhibiting and close to maximal at potentiating responses (Morris et al., 1999; see also Fig. 2). In all, paired responses were obtained from eight oocytes for 3α5αP and *ent*-3α5αP, and nine oocytes for 3α5βP and *ent*-3α5βP. In every oocyte tested, *ent*-3α5αP potentiated less than 3α5αP whereas *ent*-3α5βP inhibited less than 3α5βP (and, actually, *ent*-3α5βP potentiated responses). If we assume that the natural and unnatural forms are equally effective and potent, then this result would be obtained by chance in less than 0.4% of sets of eight pairs and 0.2% for nine pairs. Therefore, we conclude that the natural and unnatural forms are not equivalent in actions on the ρ1 receptor. Likewise, in five pairs of oocytes tested with 1 μM steroids and five other pairs tested with 20 μM steroids, the enantiomer always produced less potentiation (3α5αP) or less inhibition (3α5βP), which would happen in fewer than

4% of pairs if the enantiomer was equivalent to the natural steroid.

The paired comparisons were used because of concerns about slow reversibility of steroid effects (see *Materials and Methods*). Therefore, the quantitative values for potentiation and inhibition are somewhat less critical for testing the hypothesis. However, the differences are clearly apparent in the parametric data. The concentration-effect relationships for the natural and unnatural forms of the steroids are shown in Fig. 2. The potentiation curve for 3α5αP is very similar to that reported earlier (Morris et al., 1999), whereas *ent*-3α5αP shows essentially no effect over the same concentration range. The inhibition curve for 3α5βP is also very similar to that reported earlier (Morris et al., 1999). However, *ent*-3α5βP shows a potentiating, rather than inhibiting, action at higher concentrations. For neither steroid pair does the unnatural form show similar effects to the natural form.

In studies of neuroactive steroids at other transmitter-gated channels, the inhibiting and potentiating effects seem to be independent (e.g., Akk et al., 2001; Paradiso et al., 2001). Therefore, we performed some experiments to indicate whether 3α5αP and 3α5βP seem to have independent (that is, multiplicative) actions on the ρ1 receptor. To do this, we applied 200 nM GABA plus 10 μM 3α5αP for approximately 100 s, then switched to 200 nM GABA plus 10 μM 3α5αP plus 10 μM 3α5βP. As shown in Fig. 3, a rapid inhibition occurs. The amount of inhibition was assessed by comparing the response at the maximal inhibition to the response immediately before the blocker was applied. The amount of inhibition for the potentiated response (response reduced to 0.38 $\pm$ 0.04, results from three oocytes) is indistinguishable from that obtained for responses to GABA alone (0.39 $\pm$ 0.08, n = 9 oocytes; Fig. 2). The response just before the application of 3α5βP was potentiated to an average of 1.47 ($\pm$0.08, n = 3) times the control response to 200 nM GABA, measured at the same time in the application, which is similar to the data shown in Fig. 2 (1.41 $\pm$ 0.17, n = 8). It is difficult to evaluate the predictions of the lateral pressure mechanism when a mixture of steroids is applied. However, the data suggest that the potentiating effects of 3α5αP and the inhibiting effects of 3α5βP have independent mechanisms, and the net effect is produced by the multiplication of the potentiation and inhibition. We also performed similar experiments using enantiomer pairs. 3α5βP (10 μM) blocks responses to the application of 200 nM GABA plus 10 μM *ent*-3α5βP (relative response in the presence of 3α5βP was reduced to 0.36 $\pm$ 0.03) whereas 10 μM *ent*-3α5αP has little effect on responses potentiated by 3α5αP (relative response 0.91 $\pm$ 0.05).

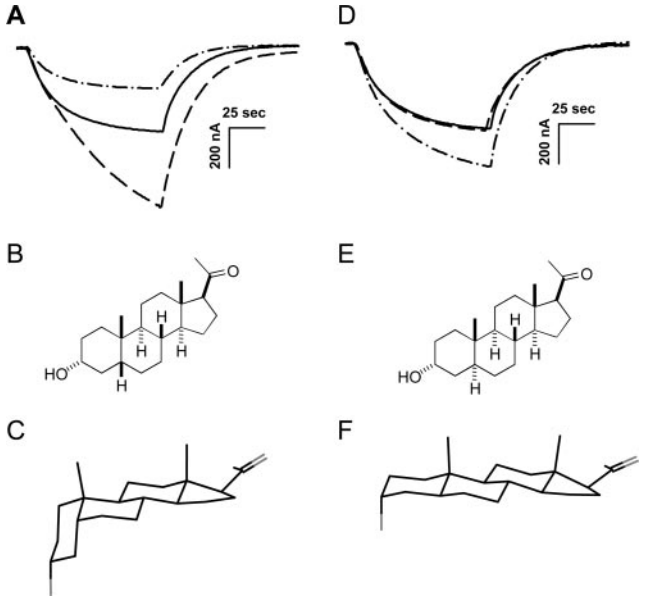

Fig. 1. Responses of oocytes to GABA and enantiomer pairs. Top left (A), response of an oocyte to 200 nM GABA (solid line), then to 200 nM GABA plus 10 μM *ent*-3α5βP (dashed line), then to 200 nM GABA plus 10 μM 3α5βP (dot-dashed line). Note that the natural enantiomer inhibits the response, whereas the unnatural form potentiates. Top right (D), shows the responses of a different oocyte to 200 nM GABA, then to 200 nM GABA plus 10 μM *ent*-3α5αP (dashed line), then to 200 nM GABA plus 10 μM 3α5αP (dot-dashed line). In this case, the natural form potentiates whereas the unnatural form has no effect. B and C, structures of 3α5βP as a flat structure and in a three-dimensional view; E and F, similar structures for 3α5αP.

Discussion

Our results demonstrate that the enantiomer of 3α5αP is inactive on the ρ1 receptor, whereas the enantiomer of 3α5βP has an effect opposite that of the natural compound.

In a full study (J.-M. Alakoskela, D. F. Covey, and P. K. J. Kinnunen, submitted) of the interactions of 3α5αP, 3α5βP, and their enantiomers with lipid membranes, the members of an enantiomer pair showed no difference in effects on a variety of measures, including packing of the interior or headgroups, mobility of hydrocarbon chains, hydration of the

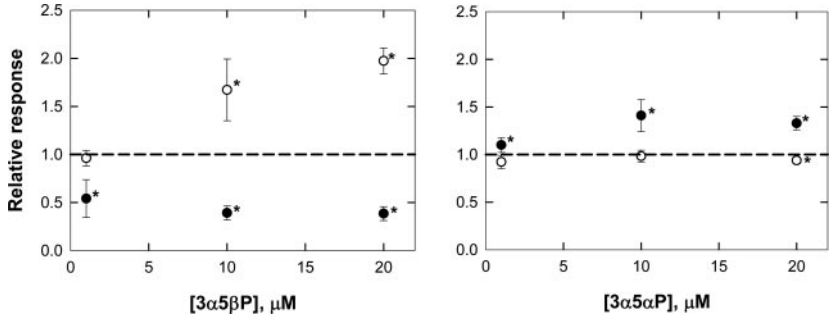

Fig. 2. Summary of effects of steroids on responses to 200 nM GABA. Left, summary of the effects of 3α5βP (filled symbols) and its enantiomer (open symbols) on the response to 200 nM GABA. An asterisk indicates that the relative response is significantly different from 1 ($P < 0.05$, t test). The relative responses to 3α5βP and *ent*-3α5βP are significantly different from each other at each concentration tested. The data were obtained from five oocytes (1 μM), nine oocytes (10 μM), and five oocytes (20 μM). Right, summary of similar data for the effects of 3α5αP (filled symbols) and its enantiomer (open symbols). The relative responses to 3α5αP and *ent*-3α5αP are significantly different from each other at all concentration tested. The data were obtained from five oocytes (1 μM), eight oocytes (10 μM) and five oocytes (20 μM).

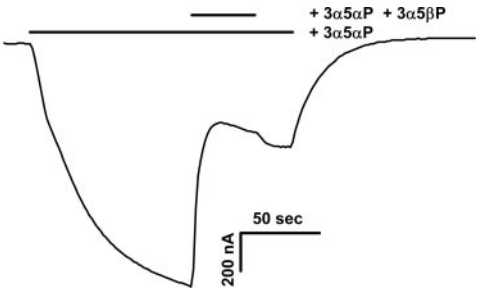

Fig. 3. 3α5βP inhibits responses potentiated by 3α5αP. GABA (200 nM) plus 10 μM 3α5αP was applied to an oocyte for approximately 100 s; then the solution was changed to 200 nM GABA plus 10 μM 3α5αP plus 10 μM 3α5βP for approximately 50 s, and then the solution was switched back to 200 nM GABA plus 10 μM 3α5αP for approximately 20 s. 3α5βP shows a rapid inhibitory effect, which is only partially reversed. On average, the response in the presence of 3α5αP is potentiated by 1.47-fold, whereas the addition of 3α5βP reduces the potentiated response to 0.38 ± 0.04.

headgroup region, phase transitions, surface potential, and association of the steroid with headgroup or interior region of the leaflet. In contrast, the 5α- and 5β-reduced steroids differ in most of the measures, as expected. These data strongly suggest that the enantiomers of a given steroid interact identically with the membrane.

No technique can directly measure the lateral pressure profile, which can only be calculated or simulated. However, its integral moments, the first of which is related to the splay curvature elastic modulus and spontaneous curvature, and the second to the Gaussian curvature elastic modulus (Cantor, 1999), can both be calculated from simulations (Gullingsrud and Schulten, 2004) and can be measured as well. We did not directly measure these parameters. In addition, simulations of the behavior of cholesterol-containing bilayers suggest that the lateral pressure profile may be very complex, having an alternating array of very strong tension (contracting) and pressure (expanding) components (Patra, 2005). It is not possible to predict how a membrane protein, of irregular shape and presently unknown volume changes during gating, would interact with such a spatially complex pressure distribution. Hence, even the measurement of the first two integral moments could not confirm the pressure profiles to this level of detail. Therefore, the possibility exists that the members of an enantiomer pair could have different effects on the lateral pressure profile even though they have identical effects on all the parameters we measured.

Subject to that caveat, the present results indicate that these neurosteroids, and probably other steroids, act on the ρ1 receptor by interacting with a chiral site, probably on the receptor protein. It is somewhat surprising that *ent*-3α5βP acts as a potentiator. If the potentiation and inhibition are mediated by interactions at specific sites, this observation suggests that *ent*-3α5βP might have some efficacy at the site for potentiation. Indeed, previous studies of the GABA-A receptor have found that the enantiomers of 5β-reduced steroids retain more ability to potentiate than do enantiomers of 5α-reduced steroids (Covey et al., 2000). Our results also suggest that 3α5βP and 3α5αP have independent actions to (respectively) inhibit and potentiate responses of ρ1 receptors. It seems less likely that a mechanism mediated by effects on lateral pressure would show such a simple interaction as more steroid is incorporated into the membrane.

In any case, the strong enantioselectivity for both 3α5βP and 3α5αP suggests that an action in the membrane to change a membrane property, such as lateral pressure, is less likely than a direct interaction with a chiral site, probably on the ρ1 receptor.

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