

# Novel type of $G_{q/11}$ protein-coupled neurosteroid receptor sensitive to endocrine disrupting chemicals in mast cell line (RBL-2H3)

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**1** Agonistic neurosteroids, including pregnenolone, dehydroepiandrosterone and its sulfate (DHEAS), caused rapid degranulation in measurements of  $\beta$ -hexosaminidase ( $\beta$ -HEX) release from a mast cell line, RBL-2H3. This degranulation was blocked by BSA-conjugated progesterone (PROG-BSA) or 17 $\beta$ -estradiol, both of which are antagonistic neurosteroids.

**2** DHEAS-induced  $\beta$ -HEX release was blocked by U-73122 or xestospongine C, but not by PTX or EGTA. DHEAS-induced  $\beta$ -HEX release was also abolished by  $G_{q/11}$ -AS, but not by  $G_{q/11}$ -MS. Pharmacological analyses revealed that the neurosteroids stimulated a putative membrane receptor through activation of the novel  $G_{q/11}$  and phospholipase C.

**3** While representative endocrine-disrupting chemicals (EDCs) did not show any degranulation or nocifensive actions by themselves, they blocked the DHEAS-induced degranulation.

**4** The binding of a PROG-BSA-fluorescein isothiocyanate conjugate (PROG-BSA-FITC) to cells was inhibited by neurosteroids and EDCs.

**5** In the algogenic-induced biting and licking responses test, DHEAS caused agonistic nocifensive actions in a dose-dependent manner between 1 and 10 fmol (i.p.). DHEAS-induced nocifensive actions were abolished by PROG-BSA or nonylphenol.

**6** Taken together, these results suggest that a  $G_{q/11}$ -coupled neurosteroid receptor may regulate the neuroimmunological activity related to sensory stimulation and that some EDCs have antagonistic actions for this receptor.

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**Keywords:** Neurosteroid; endocrine-disrupting chemical; GPCR; mast cell;  $\beta$ -hexosaminidase release

**Abbreviations:** AS-ODN, antisense oligodeoxynucleotide; DBP, di-*n*-butyl phthalate; DEP, di-2-ethylhexyl phthalate; DES, diethylstilbestrol; DHEAS, dehydroepiandrosterone sulfate; DPH, diphenhydramine; E<sub>2</sub>, 17 $\beta$ -estradiol; MS-ODN, missense oligodeoxynucleotide; NP, nonylphenol; OP, 4-octylphenol; PLC, phospholipase C; PREGS, pregnenolone sulfate; PROG, progesterone; PTX, pertussis toxin;  $\beta$ -HEX,  $\beta$ -hexosaminidase

## Introduction

Steroids synthesized *de novo* in the central and peripheral nervous systems are called neurosteroids, and have a wide variety of physiological and pharmacological functions (Compagnone & Mellon, 2000; Plassart-Schiess & Baulieu, 2001). Although steroid hormones are known to have genomic actions through intracellular steroid hormone receptors (Beat & Klug, 2000), there is also increasing evidence of rapid nongenomic steroid actions (Rupprecht & Holsboer, 1999; Falkenstein *et al.*, 2000). The latter nongenomic actions of neurosteroids include allosteric actions on ligand-gated channels such as GABA<sub>A</sub> (Majewska *et al.*, 1986) and NMDA (Wu *et al.*, 1991) receptors, and these actions influence neuronal

excitability (Majewska *et al.*, 1986). Recently, it has been found that progesterone binds to the oxytocin receptor, a member of the G protein-coupled receptor (GPCR) family, and suppresses oxytocin-induced responses (Grazzini *et al.*, 1998). On the other hand, we have reported that some neurosteroids share pharmacological actions with a putative G<sub>i</sub>-coupled sigma ( $\sigma$ ) receptor in the brain through reconstitution experiments (Ueda *et al.*, 2001a). Thus, neurosteroids may have nongenomic mechanisms through GPCRs in addition to ligand-gated channels.

Recently, we reported that pregnenolone sulfate (PREGS) and dehydroepiandrosterone sulfate (DHEAS), two representative neurosteroids, induce nociceptive flexor responses through two novel types of neurosteroid receptors, termed neuronal NS1/ $\sigma$ -type and non-neuronal NS2-type (Ueda *et al.*, 2001a). The NS2-type neurosteroid receptor probably exists on mast cells to induce histamine release, since the nociceptive responses and plasma extravasation after neurosteroid stimulation were completely blocked by diphenhydramine (DPH), a histamine antagonist (Ueda *et al.*, 2001a; Uchida *et al.*, 2003). While studying the physiological and pathophysiological roles of this peripheral neurosteroid receptor, we found that its

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neurosteroid-induced actions were abolished by 1,1-dichloro-2,2-bis(*p*-chlorophenyl) ethylene (DDE), an endocrine-disrupting chemical (EDC) that possesses estrogenic actions (Uchida *et al.*, 2003). Therefore, the peripheral neurosteroid receptor may be a target for some EDCs as well as nuclear steroid receptors. In this study, we demonstrate the involvement of the putative G<sub>q/11</sub>-coupled neurosteroid receptor in the mast cell degranulation induced by neurosteroids and EDCs.

## Methods

### Materials

The following chemicals were obtained from Sigma (St Louis, MO, U.S.A.): PREGS, dehydroepiandrosterone (DHEA), DHEAS, progesterone (PROG), progesterone 3(*O*-carboxymethyl) oxime conjugated to bovine serum albumin (PROG-BSA) (steroids : BSA = 38 : 1), PROG-BSA-fluorescein isothiocyanate conjugate (PROG-BSA-FITC), 17 $\beta$ -estradiol (E<sub>2</sub>), E<sub>2</sub>-6-*O*-carboxy-methyl oxime-BSA (E<sub>2</sub>-BSA) (steroids : BSA = 32 : 1), *N*-acetyl- $\beta$ -D-glucosaminide, oxytocin, quercetin and RU486. ICI-182,780 was purchased from WAKO (Osaka, Japan). Nonylphenol (NP), 4-octylphenol (OP), di-*n*-butyl phthalate (DBP), di-2-ethylhexyl phthalate (DEP), and diethylstilbestrol (DES) were purchased from Kanto Kagaku (Tokyo, Japan). Xestospongine C (araguspogine E) was a gift from Professor Kobayashi (Kobayashi *et al.*, 1989). The antisense oligodeoxynucleotide (AS-ODN, 5'-ATGGACTCAGAGT-3') for rat G<sub>q/11</sub> and its missense oligodeoxynucleotide (MS-ODN, 5'-AGTGACCTCAGGAT-3') were synthesized (Ueda & Inoue, 2000). The AS-ODN (5'-TCGGCAGCCATGATCTCT-3') for rat 25-DX and its MS-ODN (5'-TCCGCTGCG ATCATGTCA-3') were synthesized. All ODNs were purchased from Qiagen (Tokyo, Japan). G<sub>q/11</sub> was obtained from NEN Life Science Products, Boston, MA, U.S.A. Antiserum rabbit against 25-DX (IZA) was a gift from Professor Okamoto (Raza *et al.*, 2001). We calculated the concentration of BSA-bound steroids as reported previously (Ueda *et al.*, 2001a).

### Cell culture

RBL-2H3 cells were maintained in minimum essential medium supplemented with 10% FBS, L-glutamine and penicillin/streptomycin. In most experiments, unless otherwise stated, RBL-2H3 cells were treated with 30  $\mu$ M quercetin for 48 h, as described previously (Senyshyn *et al.*, 1998). Quercetin was prepared as a 60 mM stock solution in propylene glycol. For each experiment, the cells were incubated overnight in complete growth medium and then with pertussis toxin (PTX; 100 ng ml<sup>-1</sup>) for 12 h, or U-73122 (100 nM), U-73343 (100 nM), xestospongine C (1  $\mu$ M), EGTA (1 mM), wortmannin (1  $\mu$ M), RU-486 (1  $\mu$ M) or ICI-182,780 (1  $\mu$ M) for 10 min before addition of the test drugs. The AS-ODN or MS-ODN for G<sub>q/11</sub> (20  $\mu$ M), 25-DX (20  $\mu$ M) was pretreated every 8 for 48 h before addition of the test drug for measurement of  $\beta$ -hexosaminidase ( $\beta$ -HEX) release. Western blot analysis was performed as reported previously (Ueda & Inoue, 2000), using the antiserum against G<sub>q/11</sub> (1 : 1000 dilution) or 25-DX (1 : 1000 dilution).

### Measurement of $\beta$ -HEX release

The experiment was performed according to previous reports (Hong-Geller & Cerione, 2000; Zussman & Sagi-Eisenberg, 2000). RBL-2H3 cells were seeded in 24-well plates, grown to confluency, incubated in growth medium containing 30  $\mu$ M quercetin, and then washed twice with HEPES-Tyrod's BSA buffer (140 mM NaCl, 2.7 mM KCl, 1.8 mM CaCl<sub>2</sub>, 5.6 mM glucose, 12 mM NaHCO<sub>3</sub>, 0.37 mM NaH<sub>2</sub>PO<sub>4</sub> · 2H<sub>2</sub>O, 25 mM HEPES, 0.49 mM MgCl<sub>2</sub>, 0.1% BSA, pH 7.4). The cells were then stimulated with various drugs for 10 min or indicated periods at 37°C, and 100  $\mu$ l aliquots of the supernatants were collected and incubated with 50  $\mu$ l of 10 mM *N*-acetyl- $\beta$ -D-glucosaminide in 0.05 M citrate buffer (pH 4.5) for 1 h at 37°C. To determine the total amount of  $\beta$ -HEX, the cells were lysed with 0.1% Triton X-100. The absorbance at 405 nm was read in a microplate reader (Bio-Rad 550; Nippon Bio-Rad Laboratories, Osaka, Japan).

### PROG-BSA-FITC binding assay

The experiment was performed according to previous reports (Benten *et al.*, 1999; Nadal *et al.*, 2000). RBL-2H3 cells (1.0  $\times$  10<sup>5</sup> cells/well) cultured on polylysine-coated coverslips for 24 h were fixed with 4% (wt/vol) paraformaldehyde for 30 min, and then washed three times with phosphate-buffered saline (PBS). The cells were incubated with 1  $\mu$ M PROG-BSA-FITC at room temperature for 2 h, and then washed. FITC-labeled cells were analyzed using a confocal laser scanning microscope (FLUOVIEW; Olympus, Tokyo, Japan), after excitation of the FITC fluorescence by a 488 nm argon laser line.

### Animals and behavioral studies

The algogenic-induced biting and licking (ABL) test was performed as described previously (Uchida *et al.*, 2003). Male ddY mice weighing 20–22 g were used in all experiments. The experimental procedures were approved by the Nagasaki University Animal Care Committee and complied with the recommendations of the International Association for the Study of Pain (Zimmermann, 1983). Test drugs were intraplantarly (i.pl.) administered into the hind paw. Mice were adapted to individual transparent plastic cages, which served as observation chambers, for 1 h prior to the i.pl. injection. Immediately after the injection, each mouse was replaced in the cage over a mirror and behavioral testing was initiated. The total amount of time showing nocifensive responses such as biting and licking behaviors was measured for 10 min after the i.pl. injection. We first dissolved neurosteroids and EDCs in 100% methanol to 20 mM and diluted to a concentration of 1–30 fmol/20  $\mu$ l (or 5 pM–1.5 nM) in physiological saline immediately before use. As stock DHEAS is highly diluted for *in vivo* use, any significant effects of methanol would be avoided. Test agents were administered by i.pl. injection in a volume of 20  $\mu$ l. Mice were only used once.

### Statistical analysis

The results were expressed as the mean  $\pm$  s.e. and analyzed by a one-way ANOVA with Scheffe's test. The criterion of significance was set at \**P* < 0.05.

## Results

### Neurosteroids induce rapid degranulation of RBL-2H3 cells

Degranulation of mast cells was determined by measuring the release of granule-associated  $\beta$ -HEX. Significant and maximum  $\beta$ -HEX releases were observed as early as 3 and 10 min after the addition of DHEAS (1  $\mu$ M), respectively (Figure 1a). Similar agonistic activity was also observed with 1  $\mu$ M DHEA, DHEAS or PREGS, whereas no changes were observed with 1  $\mu$ M PROG, E<sub>2</sub>, PROG-BSA or E<sub>2</sub>-BSA (Figure 1b). DHEAS did not cause any remarkable changes in the cell morphology or cell survival activity, as evaluated by the trypan blue exclusion assay (data not shown).

### PROG-BSA and E<sub>2</sub> antagonize the degranulation

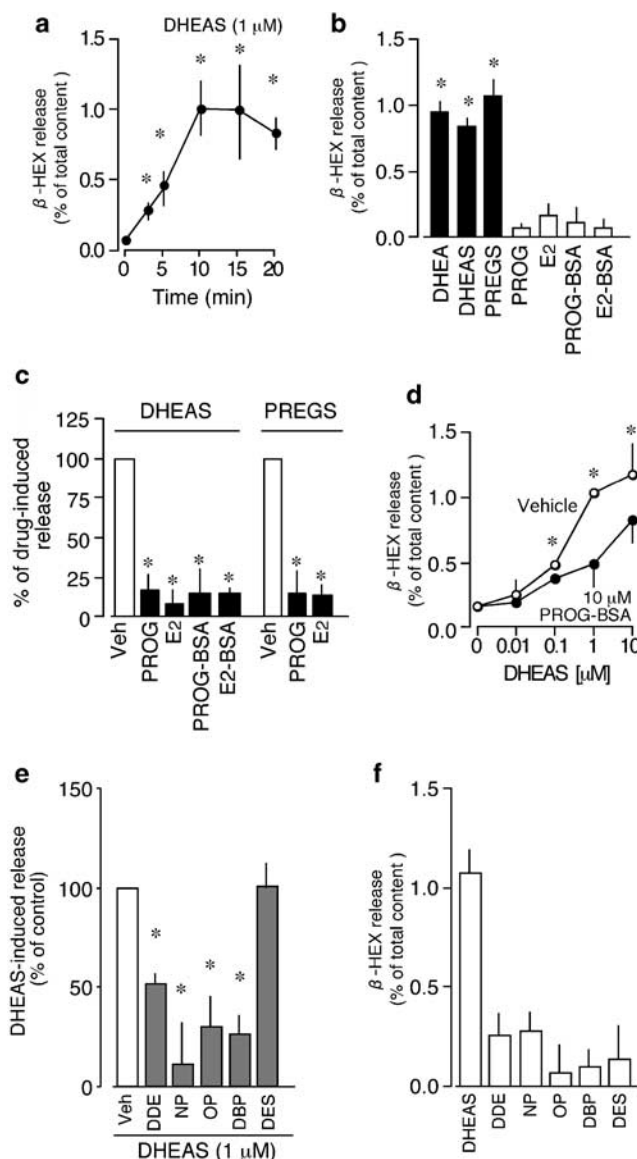
DHEAS (1  $\mu$ M)-induced degranulation was markedly blocked by PROG, E<sub>2</sub>, PROG-BSA or E<sub>2</sub>-BSA (Figure 1c). Similar antagonism was also observed for PREGS-induced release. As shown in Figure 1d, the concentration-dependent release by DHEAS (10 nM–10  $\mu$ M) was shifted to the right by 10  $\mu$ M PROG-BSA.

### EDCs antagonize DHEAS-induced $\beta$ -HEX release

As shown in Figure 1e, DHEAS (1  $\mu$ M)-induced  $\beta$ -HEX release was significantly inhibited by various EDCs, including 1  $\mu$ M NP, OP, DBP and DEP, but not by 1  $\mu$ M DES. However, no significant changes in the degranulation were observed with these EDCs (Figure 1f).

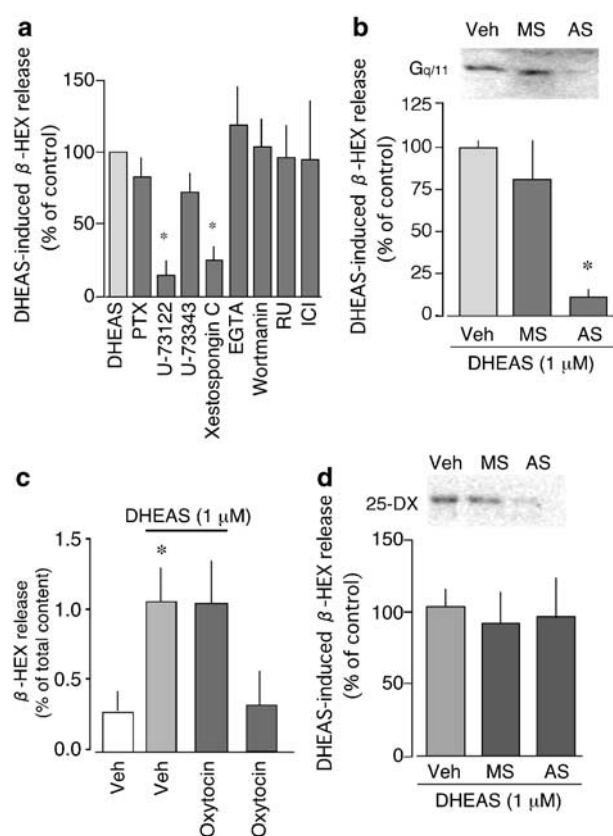
### Signal transduction of DHEAS-induced degranulation

As shown in Figure 2, DHEAS-induced  $\beta$ -HEX release was not affected by PTX (100 ng ml<sup>-1</sup>) pretreatment, which significantly inhibited Compound 48/80 (10  $\mu$ g ml<sup>-1</sup>)-induced  $\beta$ -HEX release (13  $\pm$  3% of the total content,  $n$  = 6) by approximately 70%, consistent with a previous report (Senyshyn *et al.*, 1998). On the other hand, the DHEAS-induced degranulation was significantly abolished by U-73122 (100 nM), a phospholipase C (PLC) inhibitor, but not by U-73343 (100 nM), an inactive derivative of U-73122. Xestospingon C (1  $\mu$ M), an allosteric inositol trisphosphate (InsP<sub>3</sub>) receptor antagonist (Gafni *et al.*, 1997) also blocked the degranulation, while no significant change was observed with EGTA (1 mM) or wortmannin (1  $\mu$ M), a PI3 kinase inhibitor (Figure 2a). These results suggest that Ca<sup>2+</sup> mobilization from intracellular stores plays a more important role in DHEAS-induced degranulation than Ca<sup>2+</sup> influx from the extracellular space. Furthermore, intracellular PROG or E<sub>2</sub> receptor antagonists, such as RU486 (RU; 1  $\mu$ M) or ICI-182,780 (ICI; 1  $\mu$ M) (Baulieu, 1989; Howell *et al.*, 2000), did not cause any significant reduction in the DHEAS-induced degranulation (Figure 2a). Pretreatment with the AS-ODN (20  $\mu$ M) for G $\alpha_{q/11}$ , but not the MS-ODN (20  $\mu$ M), markedly reduced the amount of G $\alpha_{q/11}$  protein in RBL-2H3 cells and inhibited DHEAS (1  $\mu$ M)-induced degranulation (Figure 2b). None of the inhibitors showed any significant effect on their



**Figure 1** Neurosteroid-induced  $\beta$ -HEX release from RBL-2H3 cells and its blockade by PROG and EDCs. RBL-2H3 cells were incubated with 30  $\mu$ M quercetin for 48 h in normal growth medium. Data represent the mean  $\pm$  s.e. from more than three separate experiments. \* $P$  < 0.05, compared with the vehicle-treated group. (a) Time course of DHEAS-induced  $\beta$ -HEX release. Confluent cultures in 24-well plates were stimulated with DHEAS (1  $\mu$ M) and  $\beta$ -HEX release was evaluated using supernatants from the cells at the indicated periods. The results represent the ratio (%) of  $\beta$ -HEX release to the total content. (b) Degranulation by several neurosteroids (1  $\mu$ M). (c) Antagonistic effects of PROG, E<sub>2</sub> and their BSA-conjugates (10  $\mu$ M) on DHEAS- and PREGS (1  $\mu$ M)-induced  $\beta$ -HEX release. The results represent the  $\beta$ -HEX release (%) by various neurosteroids (1  $\mu$ M). (d) Competitive blockade of DHEAS-induced degranulation by PROG-BSA (10  $\mu$ M). (e) Lack of  $\beta$ -HEX release by EDCs (1  $\mu$ M). (f) Antagonism of DHEAS-induced  $\beta$ -HEX release by EDCs (10  $\mu$ M).

own. On the other hand, oxytocin showed no antagonistic effects on DHEAS-induced  $\beta$ -HEX release (Figure 2c). In addition, treatment with oxytocin alone caused no significant effects on  $\beta$ -HEX release. Furthermore, AS-ODN treatment for 25-DX, a membrane-associated PROG-binding protein,

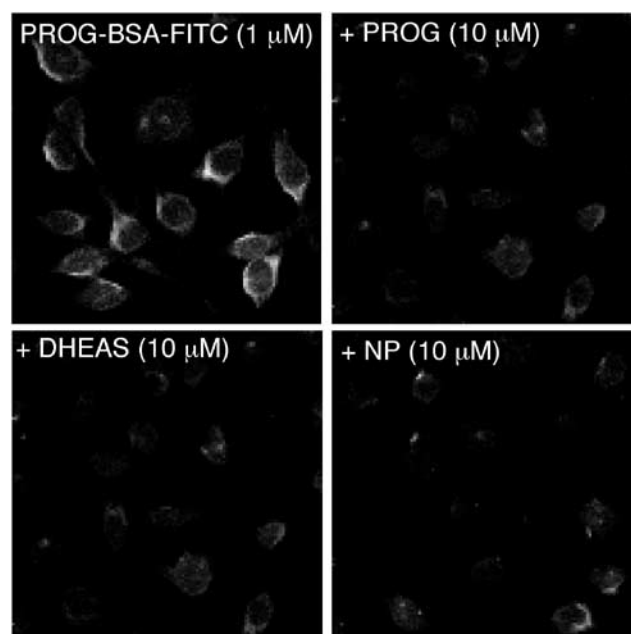


**Figure 2** G<sub>q/11</sub> and PLC activation-mediated  $\beta$ -HEX release by DHEAS. The results represent the mean  $\pm$  s.e. from more than three separate experiments. \* $P < 0.05$ , compared with the DHEAS- or PREGS-treated group. (a) Effects of several inhibitors on DHEAS (1  $\mu$ M)-induced  $\beta$ -HEX release. PTX, U-73343 and U-73122 were used at 10  $\mu$ M. Xestospongin C, wortmannin, RU-486 (RU) and ICI-182,734 (ICI) were used at 1  $\mu$ M. EGTA was used at 1 mM. (b) Effects of the G<sub>q/11</sub> AS-ODN on DHEAS (1  $\mu$ M)-induced  $\beta$ -HEX release. Inset: reduction in the G<sub>q/11</sub> level as assessed by Western blotting analysis (2  $\mu$ g protein per lane, 12% acrylamide gel). Results represent DHEAS-induced  $\beta$ -HEX release (%) using RBL-2H3 cells pretreated with AS-ODN or MS-ODN for G<sub>q/11</sub>. (c) No effect of oxytocine on DHEAS-induced  $\beta$ -HEX release. Oxytocin (10  $\mu$ M) was added with or without DHEAS (1  $\mu$ M). (d) No effect of DHEAS (1  $\mu$ M)-induced  $\beta$ -HEX release by 25-DX AS-ODN. Inset: reduction in the 25-DX level by AS-ODN or MS-ODN in Western blotting analysis (1.5  $\mu$ g protein per lane, 12% acrylamide gel). Veh: vehicle-, AS; AS-ODN-, MS; MS-ODN-treatment.

had no effect on the DHEAS-induced  $\beta$ -HEX release (Figure 2d).

#### Blockade of PROG-BSA-FITC binding to the cell membrane by PROG, DHEAS and NP

RBL-2H3 cells cultured on polylysine-coated coverslips were fixed with paraformaldehyde, washed three times with PBS and used for binding experiments with 1  $\mu$ M PROG-BSA-FITC. FITC-labeled cells were analyzed using a confocal laser scanning microscope. Fluorescence was only observed at the cell surface, and the addition of a high concentration (10  $\mu$ M) of PROG, DHEAS or NP completely abolished the binding (Figure 3). Similar inhibition was also observed with OP or DDE at 1  $\mu$ M (data not shown).



**Figure 3** PROG-BSA-FITC binding to RBL-2H3 cells. PROG-BSA-FITC (1  $\mu$ M) binding to RBL-2H3 cells was performed in the absence or presence of PROG (10  $\mu$ M), DHEAS (10  $\mu$ M) or NP (10  $\mu$ M). The details are described in the Methods section.

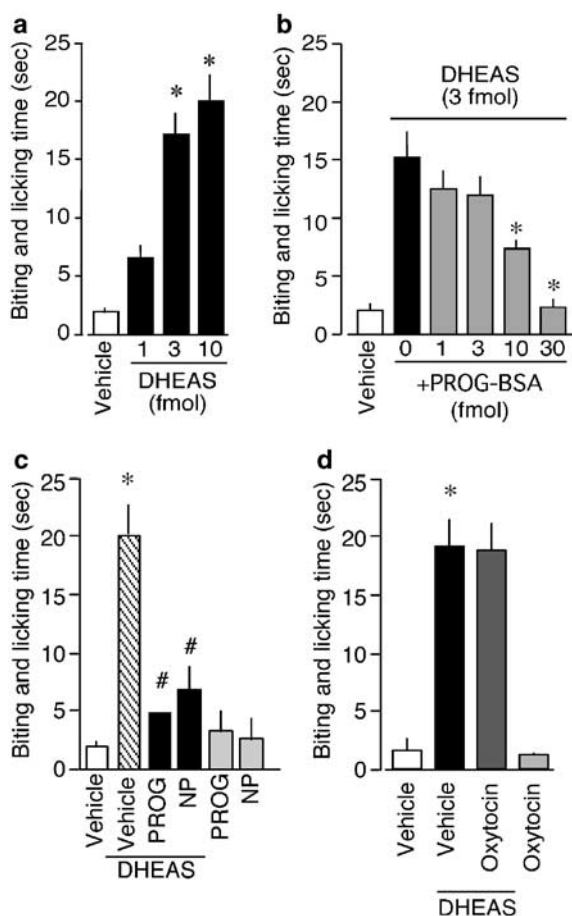
#### Blockade of DHEAS-induced nocifensive responses by PROG-BSA and EDCs

Neurosteroid-induced nocifensive responses were evaluated by the average total period (s) showing licking and biting of the paw. As shown in Figure 4a, DHEAS dose-dependently induced the nocifensive responses between 1 and 10 fmol (i.p.). The DHEAS (3 fmol, i.p.)-induced effects were concentration-dependently inhibited by PROG-BSA (1–30 fmol, i.p.), a membrane-impermeable neurosteroid receptor antagonist (Figure 4b). The DHEAS-induced nocifensive responses were also inhibited by PROG and NP, both of which had no effects when administered alone (Figure 4c). On the other hand, oxytocin showed no antagonistic effects on DHEAS-induced nocifensive responses (Figure 4d).

## Discussion

The present study is the first to demonstrate that neurosteroids stimulate degranulation of mast cells through a G<sub>q/11</sub> protein-coupled membrane receptor, and that some EDCs share this putative GPCR with neurosteroids; furthermore, it should be a nongenomic action. Some neurosteroids have agonistic actions, while others have antagonistic actions. All EDCs used in this study have antagonistic actions.

In previous studies, we demonstrated that DHEAS and PREGS stimulated [<sup>35</sup>S] GTP $\gamma$ S binding to brain membranes and induced nociception *in vivo*, while PROG abolished these actions (Ueda *et al.*, 2001a,b). In the brain membranes, G<sub>i</sub> activity was regulated by these neurosteroids as well as by sigma ( $\sigma$ ) ligands. These observations are supported by a recent report that neurosteroids increase the probability of glutamate release through a G<sub>i</sub>-coupled  $\sigma$  receptor (Meyer



**Figure 4** DHEAS-induced nocifensive responses. The results represent the mean  $\pm$  s.e. of the total period(s) showing nocifensive biting and licking behaviors from more than three separate experiments. (a) Dose-dependent DHEAS (1–10 fmol)-induced nocifensive behaviors. \* $P < 0.05$ , compared with the vehicle-treated group. (b) Blockade of DHEAS (3 fmol, i.p.)-induced nocifensive responses by coadministration of PROG-BSA (1–30 fmol, i.p.). \* $P < 0.05$ , compared with the DHEAS (10 fmol, i.p.)-treated group. (c) Blockade of DHEAS-induced nocifensive responses by coadministration of progesterone (PROG) or nonphenol (NP) at 10 fmol (i.p.). # $P < 0.05$ , compared with the group without PROG-BSA. (d) No effect of oxytocin (10 fmol) on DHEAS (3 fmol)-induced nocifensive responses. \* $P < 0.05$ , compared with the vehicle-treated group.

*et al.*, 2002). In *in vivo* experiments, both neurosteroids and  $\sigma$  agonists caused nociception through a common receptor (NS1/ $\sigma$ ) in the presence of DPH to block histamine actions. In such *in vivo* experiments, we also found that very low doses (1–100 fmol) of neurosteroids caused nociceptive flexor responses in a DPH-reversible manner, suggesting histamine release from mast cells. We proposed that such indirect actions were mediated by the NS2 subtype of the putative neurosteroid receptor (Ueda *et al.*, 2001a).

Neurosteroid-induced degranulation, as evaluated by  $\beta$ -HEX release from RBL-2H3 cells, was observed as early as 3 min (Figure 1a). The degranulation induced by DHEAS or PREGS was abolished by PROG, E<sub>2</sub> or their membrane-impermeable BSA-conjugates (Figure 1c). On the other hand, PROG-BSA-FITC binding was observed at the cell surface and this binding was abolished by PROG or DHEAS (Figure 3). These findings suggest that both DHEAS and

PREGS behave as agonists, while PROG, E<sub>2</sub> and their BSA-conjugates act as competitive antagonists at the cell surface binding sites, consistent with our previous report (Uchida *et al.*, 2003). The DHEAS-induced degranulation was PTX-insensitive, but abolished by pretreatment with the AS-ODN for G<sub>q/11</sub>. These findings suggest that the neurosteroid-induced degranulation occurs through a G<sub>q/11</sub>-coupled NS2 receptor in mast cells, in contrast to the neuronal G<sub>i</sub>-coupled NS1/ $\sigma$  receptor.

Although at least two distinct subtypes of neurosteroid receptors have been postulated in our studies, it should be noted that DHEAS and PREGS have behaved as agonists, while PROG acted as an antagonist. Recently, it has been reported that PROG could be a ligand of the oxytocin receptor, a member of the GPCR family (Grazzini *et al.*, 1998). However, oxytocin produced no responses in RBL-2H3 cells, and did not abolish DHEAS-induced  $\beta$ -HEX release (Figure 2c). Moreover, oxytocin also showed no inhibitory effect on PROG-BSA-FITC binding (Supplementary data 1). These results suggested that the putative neurosteroid receptor in mast cells is unlikely to be the oxytocin receptor. Recently, another membrane-associated PROG-binding protein, 25-DX, was cloned (Selmin *et al.*, 1996). The 25-DX gene encodes a 25-kDa single transmembrane protein (Falkenstein *et al.*, 1998) with a high affinity for PROG (Meyer *et al.*, 1998). However, the protein level of 25-DX in RBL-2H3 cells was too low for detection in Western blotting analyses using a specific antibody (Raza *et al.*, 2001), and AS-ODN treatment for 25-DX had no effect on the DHEAS-induced  $\beta$ -HEX release (Figure 2d). Thus, all these results suggest that the G<sub>q/11</sub>-coupled NS2 receptor is a novel receptor.

EDCs are thought to have serious effects on reproductive organs through disturbance of endocrine homeostasis. Although it is generally accepted that EDCs act through nuclear steroid hormone receptors, recent findings have revealed that they also have nongenomic actions through membrane receptors (Nadal *et al.*, 2000). We have previously demonstrated that an EDC, *p-p'*-DDE (1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene), blocked DHEAS-induced extravasation (Uchida *et al.*, 2003). Here, we have demonstrated that various other kinds of EDCs have similar antagonistic activities against neurosteroid-induced degranulation. However, it should be noted that the potent estrogenic compound DES had no effect on the DHEAS-induced degranulation, even though E<sub>2</sub> showed a potent antagonistic effect (Figure 1c). It is therefore evident that the neurosteroid-induced degranulation does not occur in parallel with nuclear steroid receptor activation.

The neurosteroid-induced degranulation level was only 1–2% of the total content, which is much less than that mediated by the IgE receptor (10–15%). Since we have observed that DHEAS causes significant extravasation (Uchida *et al.*, 2003) and nocifensive responses (Figure 4) partly through a histamine release, it is evident that neurosteroid-mediated degranulation has some pathophysiological roles. On the other hand, as the DHEAS concentration for an injection to cause significant nocifensive responses (3 fmol/20  $\mu$ l or 1.5 nM) appears to be higher than that required for *in vitro* degranulation (0.1  $\mu$ M), the possibility that the neurosteroid-induced degranulation and *in vivo* nocifensive responses are separate events cannot be excluded. In this respect, we have demonstrated that such DHEAS-induced behavioral changes are

partly attributed to direct stimulation on nociceptor endings (Ueda *et al.*, 2001a). However, as the sensitivity to DHEAS appeared to be much lower than the indirect mechanism through histamine release, this possibility is unlikely to be valid. Instead, we are speculating that the high sensitivity to DHEAS in nociceptive responses may be attributed to a local amplification mechanism, which was first proposed in the case of nociceptin/orphanin FQ (Ueda & Inoue, 2000). The nociceptor stimulation by histamine released from mast cells following DHEAS stimulation would be also amplified through a local substance P release (Ueda & Inoue, 2000).

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