Signal Transduction of DHEAS-induced Degranulation

As shown in Fig. 2, DHEAS-induced -HEX release was not affected by PTX (100 ng • ml^{-1}) pretreatment, which significantly inhibited Compound 48/80 (10 g • ml^{-1})-induced -HEX release (13 ± 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. Xestospongin C (1 M), an allosteric inositol trisphosphate ($InsP_3$) receptor antagonist (Gafni et al. 1997) also blocked the degranulation, while no significant change was observed with EGTA (1 mM) or wortmannin (1 M), a PI3 kinase inhibitor (Fig. 2A). These results suggest that Ca^{2+} mobilization from intracellular stores plays a more important role in DHEAS-induced degranulation than Ca^{2+} influx from the extracellular space. Furthermore, intracellular PROG or E_2 receptor antagonists, such RU486 or ICI-182,780 (Baulieu 1989; Howell et al. 2000), degranulation

Pretreatment with the AS-ODN (20 M) for $G_{q/11}$, but not the MS-ODN (20 M), markedly reduced the amount of $G_{q/11}$ protein in RBL-2H3 cells and inhibited DHEAS (1 M)-induced degranulation (Fig. 2B).

On the other hand, oxytocin showed no antagonistic effects on DHEAS-induced HEX release (Fig. 2C). In addition, treatment with oxytocin alone caused no significant effects on -HEX release. Furthermore, AS-ODN treatment for 25-DX, a membrane-associated PROG-binding protein, had no effect on the DHEAS-induced -HEX release (Fig. 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 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 M) of PROG, DHEAS or NP completely abolished the binding (Fig. 3). Similar inhibition was also observed with OP or DDE at 1 M (data not shown).

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 Fig. 4A, DHEAS dose-dependently induced the nocifensive responses between 1-10 fmol (i.pl.). The DHEAS (3 fmol, i.pl.)-induced effects were concentration-dependently inhibited by PROG-BSA (1-30 fmol, i.pl.), a membrane-impermeable neurosteroid receptor antagonist (Fig. 4B). The DHEAS-induced nocifensive responses were also inhibited by PROG and NP, both of which had no effects when administered alone (Fig. 4C). On the other hand, oxytocin showed no antagonistic effects on DHEAS-induced nocifensive responses (Fig. 4D).

Discussion

The present study is the first to demonstrate that neurosteroids stimulate degranulation of mast cells through a $G_{q/11}$ 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 [^{35}S] GTP 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_i activity was regulated by these neurosteroids as well as by sigma ( ) ligands. These observations are supported by a recent report that neurosteroids increase the probability of glutamate release through a G_i-coupled receptor (Meyer et al., 2002). In *in vivo* experiments, both neurosteroids and agonists caused nociception through a common receptor (NS1/) 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 -HEX release from RBL-2H3 cells, was observed as early as 3 min (Fig. 1A). The degranulation induced by DHEAS or PREGS was abolished by PROG, E_2 or their membrane-impermeable BSA-conjugates (Fig. 1C). On the other hand, PROG-BSA-FITC binding was observed at the cell surface and this binding was abolished by PROG or DHEAS (Fig. 3). These

findings suggest that both DHEAS and PREGS behave as agonists, while PROG, E_2 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 $G_{q/11}$. These findings suggest that the neurosteroid-induced degranulation occurs through a $G_{q/11}$-coupled NS2 receptor in mast cells, in contrast to the neuronal G_i-coupled NS1/ 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 -HEX release (Fig. 2C). Moreover, Oxytocin also showed no inhibitory effect on PROG-BSA-FITC binding (supplemental data1). 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 -HEX release (Fig. 2D). Thus, these results all suggest that the $G_{q/11}$-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 previous 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 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_2 showed a potent antagonistic effect (Fig. 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 (Fig. 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 or 1.5 nM) appears to be higher than that required for *in vitro* degranulation (0.1 M), the possibility that the neurosteroid-induced degranulation and *in vivo* nocifensive responses are separate events can not 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., 2001). 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 nocifensive responses may be attributed to a local amplification mechanism, which was first proposed in the case of nociceptin/orphanin FQ (Ueda and 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 et al., 2000).

In conclusion, we have demonstrated that neurosteroids induce degranulation of mast cells through a $G_{q/11}$ protein-coupled membrane receptor. This observation is important for considering the physiological and toxicological significance of neurosteroids and EDCs, respectively.

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Supplementary information is available at the British Journal of Pharmacology website.

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Legends for Figures

Figure 1. Neurosteroid-induced -HEX release from RBL-2H3 cells and its blockade by PROG and EDCs. RBL-2H3 cells were incubated with 30 M quercetin for 48 h in normal growth medium. Data represent the mean ± S.E. from more than 3 separate experiments. *p<0.05, compared with the vehicle-treated group. (*A*) Time course of DHEAS-induced -HEX release. Confluent cultures in 24-well plates were stimulated with DHEAS (1 M) and -HEX release was evaluated using supernatants from the cells at the indicated periods. The results represent the ratio (%) of -HEX release to the total content. (B) Degranulation by several neurosteroids. The results represent the -HEX release (%) by various neurosteroids (1 M). (C) Antagonistic effects of PROG, E_2 and their BSA-conjugates (10 M) on DHEAS- and PREGS (1 M)-induced -HEX release. (D) Competitive blockade of DHEAS-induced degranulation by PROG-BSA (10 M). (E) Lack of -HEX release by EDCs (1 M). (F) Antagonism of DHEAS-induced -HEX release by EDCs (10 M).

Figure 2. Gq/11 and PLC activation-mediated -HEX release by DHEAS. The results represent the mean ± S.E. from more than 3 separate experiments. *p<0.05, compared with the DHEAS- or PREGS-treated group. (A) Effects of several inhibitors on DHEAS (1 M)-induced -HEX release. PTX, U-73343 and U-73122 were used at 10 M. Xestospongin C, wortmannin, RU-486 (RU) and ICI-182,734 (ICI) were used at 1 M. EGTA was used at 1 mM. (B) Effects of the $G_{q/11}$ AS-ODN on DHEAS (1 M)-induced -HEX release. Inset: Reduction in the $G_{q/11}$ level assessed by Western blotting analysis (2 g protein per lane, 12% acrylamide gel). Results represent

DHEAS-induced -HEX release (%) using RBL-2H3 cells pretreated with AS-ODN and MS-ODN for $G_{q/11}$. (C) No effect of oxytocine on DHEAS-induced -HEX release. Oxytocin (10 M) was added with or without DHEAS (1 M). (D) No effect of DHEAS (1 M)-induced -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 g protein per lane, 12% acrylamide gel). Veh: vehicle-, AS; AS-ODN-, MS; MS-ODN-treatment.

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

Figure 4. DHEAS-induced nocifensive responses. The results represent the mean ± S.E. of the total period (s) showing nocifensive biting and licking behaviors from more than 3 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.pl.)-induced nocifensive responses by co-administration of PROG-BSA (1-30 fmol, i.pl.). *p<0.05, compared with the DHEAS (10 fmol, i.pl.)-treated group. (C) Blockade of DHEAS-induced nocifensive responses by co-administration of Progesterone (PROG) or Nonyphenol (NP) at 10 fmol (i.pl.). † p<0.05, compared with the group without PROG-BSA. (D) No effect of oxytocien (10 fmol) on DHEAS (3 fmol)-induced nocifensive responses. *p<0.05, compared with the vehicle-treated group.

Fig.1

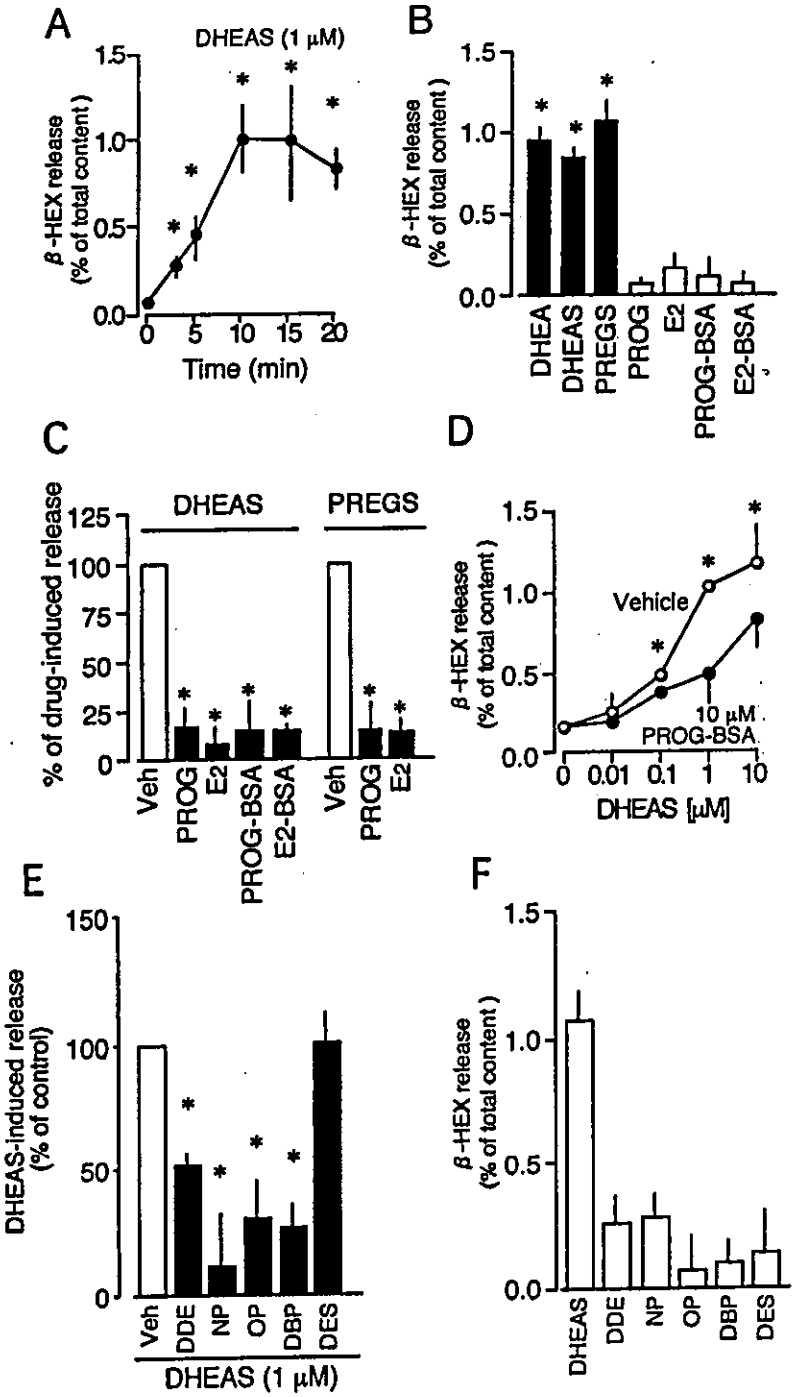

A
DHEAS (1 μM)
β-HEX release (% of total content)
Time (min)
B
DHEA
DHEAS
PREGS
PROG
E2
PROG-BSA
E2-BSA
C
DHEAS
PREGS
% of drug-induced release
Veh
PROG
E2
PROG-BSA
E2-BSA
D
Vehicle
10 μM PROG-BSA
DHEAS [μM]
E
DHEAS-induced release (% of control)
Veh
DDE
NP
OP
DBP
DES
DHEAS (1 μM)
F
DHEAS
DDE
NP
OP
DBP
DES

Fig.2

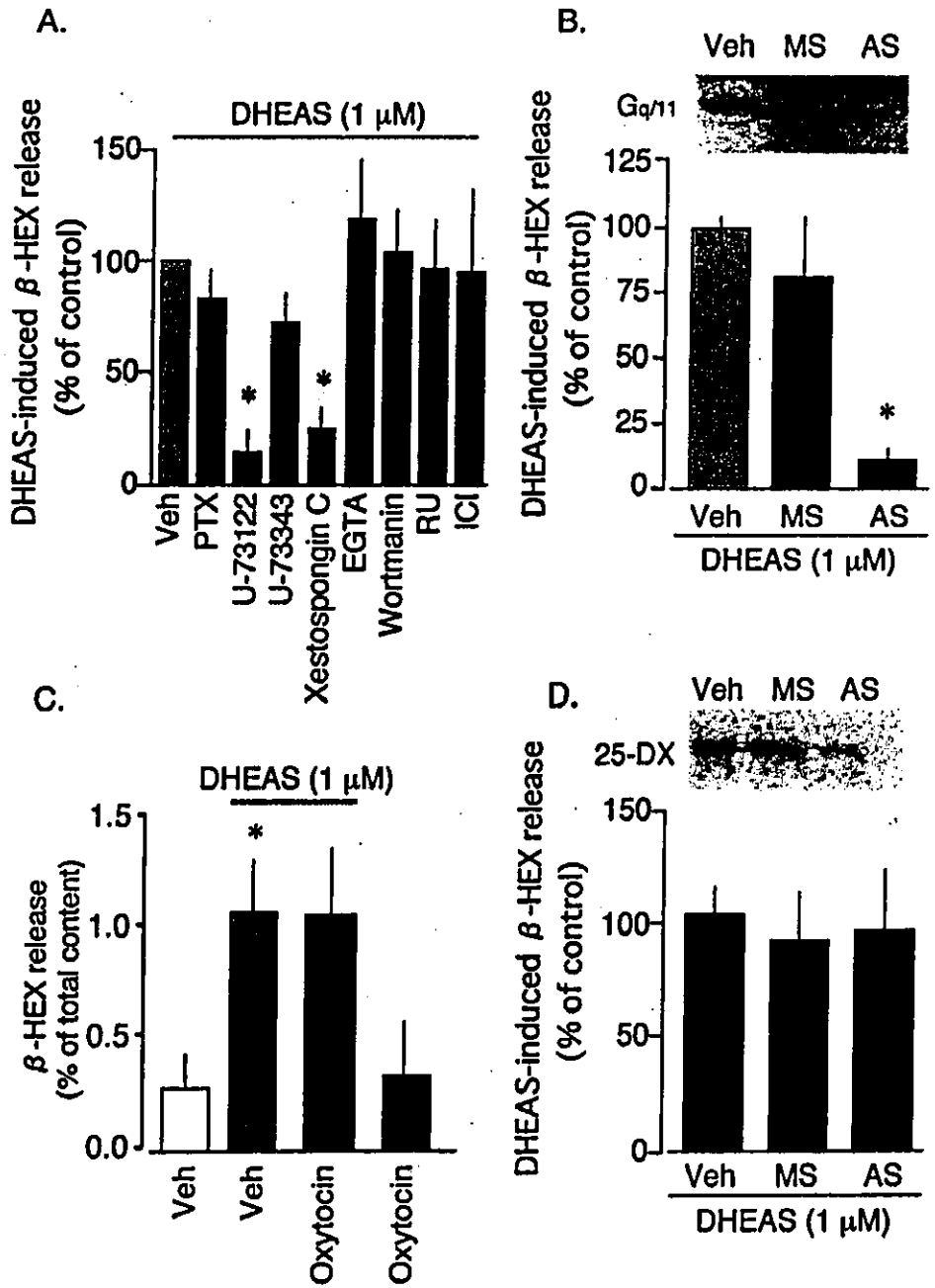

A.
DHEAS (1 μM)
DHEAS-induced β-HEX release (% of control)
150
100
50
0
*
*
Veh
PTX
U-73122
U-73343
Xestospongin C
EGTA
Wortmanin
RU
ICI
B.
Veh MS AS
Gq/11
DHEAS-induced β-HEX release (% of control)
125
100
75
50
25
0
*
Veh MS AS
DHEAS (1 μM)
C.
DHEAS (1 μM)
β-HEX release (% of total content)
1.5
1.0
0.5
0.0
*
Veh
Veh
Oxytocin
Oxytocin
D.
Veh MS AS
25-DX
DHEAS-induced β-HEX release (% of control)
150
100
50
0
Veh MS AS
DHEAS (1 μM)

Fig.3

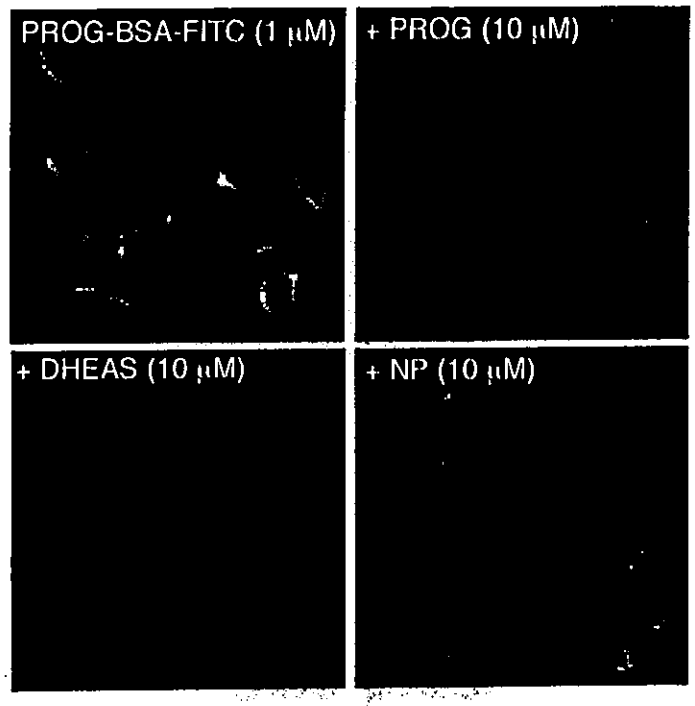
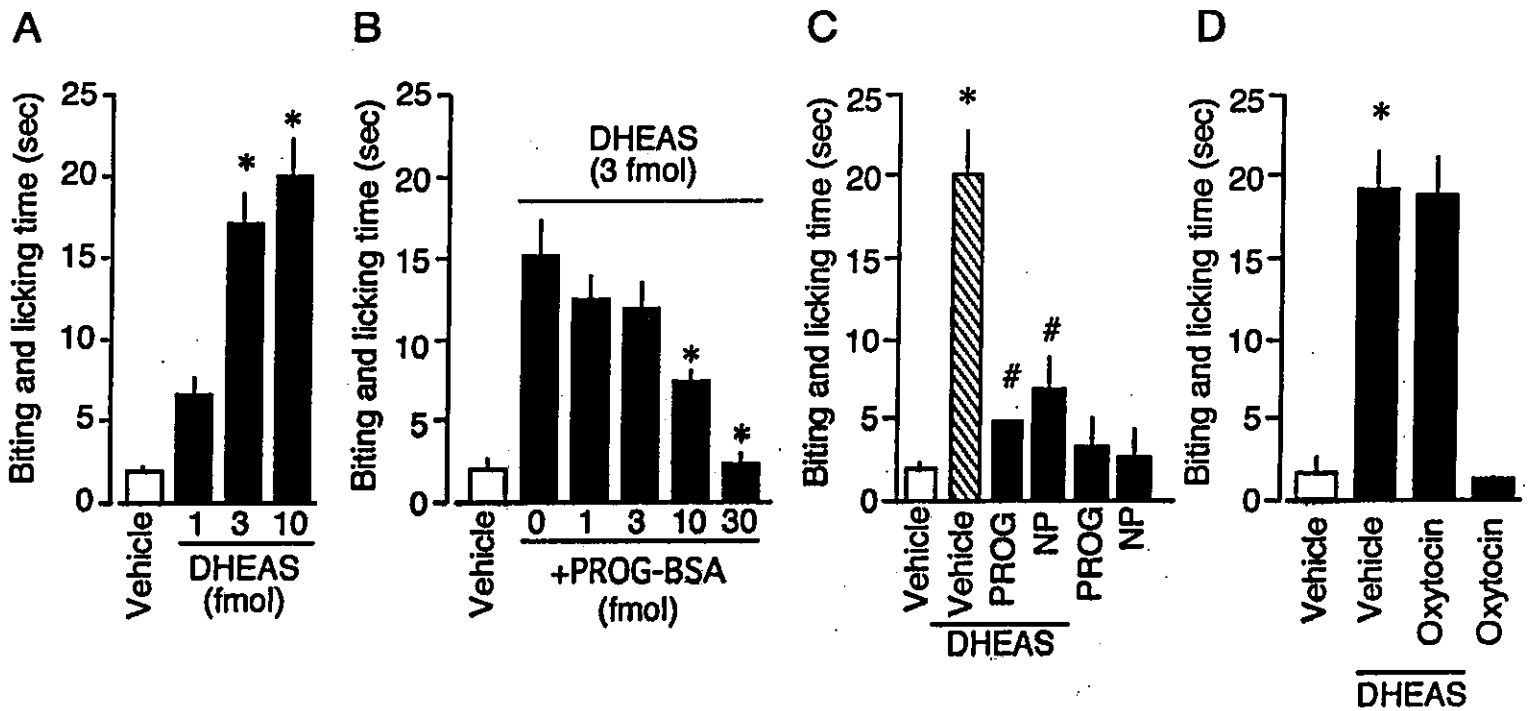


Fig.4


A
B
C
D
Biting and licking time (sec)
25
20
15
10
5
0
*
#
Vehicle
1 3 10
DHEAS
(fmol)
DHEAS
(3 fmol)
0 1 3 10 30
+PROG-BSA
(fmol)
Vehicle
PROG
NP
PROG
NP
DHEAS
Oxytocin
Oxytocin

Supplementary data