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RAPID EFFECTS OF GONADAL STEROIDS UPON HYPOTHALAMIC NEURONAL MEMBRANE ULTRASTRUCTURE

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Summary—Freeze-fracture methodology was used to study rat hypothalamic arcuate nucleus (AN) neuronal plasma membrane organization following *in vitro* perfusion of brain slices with 17-β-estradiol ($17\beta E_2$) or other test compounds. Physiological levels (10^{-10} M) of $17\beta E_2$ caused an increase in neuronal membrane exo-endocytotic pits within 1 min of perfusion. The increased density of pits was dose related, sustained at a constant rate during 10 min of perfusion, reverted to control values after perfusion with estradiol-free medium for 1 h, and was accompanied by an increased uptake of horseradish peroxidase by the arcuate nucleus in brain slices. The $17\beta E_2$-induced increase in exo-endocytotic pit density was blocked by tamoxifen (10^{-8} M). Cholesterol (10^{-10} M), 17-α-estradiol (10^{-6} M) or dihydrotestosterone (10^{-6} M) had no effect on exo-endocytotic pit density. Testosterone had about 50% the potency of $17\beta E_2$ in increasing exo-endocytotic pit density. These results indicate that physiological levels of $17\beta E_2$ can have rapid effects upon arcuate nucleus neuronal membrane ultrastructure.

INTRODUCTION

Numerous studies have suggested that plasma membranes are involved in biological effects of steroid hormones. The demonstration of rapid effects of gonadal steroids on neuronal excitability [1–6] raises, but does not answer, the question of possible direct actions of gonadal steroids on neuronal membranes. For this reason we began analyzing neuronal plasma membrane ultrastructure in steroid-sensitive hypothalamic nuclei exposed to gonadal steroids in protocols designed to determine the timing and control of these actions. For these studies we employed freeze-fracture because it is the method of choice to study plasma membrane organization. This technique displays the internal structure of membranes and has proven valuable in revealing hormonal and trophic influences on neuronal membranes [7–11]. We have concentrated upon the arcuate nucleus because it is a well-characterized sex-steroid-sensitive area of the rat hypothalamus that shows sex differences in synaptic connectivity [12] contains estradiol-producing [13] and -binding [14, 15] cells and shows predictable effects of estradiol administration on neurons and glial cells [12, 16–19]. Using freeze-fracture we found consistent sex differences in intramembrane protein particle content in developing and adult arcuate nucleus neuronal membranes [11]. We also found that chronic estrogen treatment of the type which causes hypothalamic failure and remodelling of arcuate nucleus synapses [18] in female rats results in a long-term reorganization of IMP distribution in neuronal membranes (in preparation). Therefore, in order to determine the speed and character of estrogen's actions on neuronal membranes, we have extended our freeze-fracture studies to include acute estrogen agonist–antagonist exposure. The results of this study indicate that physiological levels of estrogen can have rapid and specific effects upon arcuate nucleus neuronal membrane ultrastructure.

EXPERIMENTAL

Preparation of tissue slices

Wistar albino female rats (200 g body wt) were ovariectomized under 2,2,2-tribromoethanol (i.p., 1 cm^3/100 g body wt of a 2.5% solution in 0.9% NaCl) 3 months prior to sacrifice and maintained on a 12 h : 12 h light–dark cycle with tap-water and rat chow available, *ad libitum*. Following decapitation, the whole brain was quickly removed and placed on ice. Coronal brain slices (400 μm thick) containing arcuate nucleus were cut on a McIlwain tissue chopper and stored at 35 ± 1°C in artificial cerebrospinal fluid (ACSF) for at least 1 h before experimentation. The ACSF was composed of (in mM): NaCl, 127; KCl, 1.9; KH_2PO_4, 1.2; $MgSO_4$, 1.3; $CaCl_2$, 2.4; $NaHCO_3$, 26; dextrose, 10; and was saturated with 95% O_2 and 5% CO_2. The pH of the oxygenated ACSF was 7.2–7.3.

Slice perfusions

Slices were laid on nylon nets suspended in a 1.0-ml flow-through chamber and perfused with

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Table 1. Summary of the experimental conditions studied with freeze-fracture

Test compounds	Duration of slice perfusion
Cholesterol 10^{-10} M	1–10 min
17-β-Estradiol 10^{-10} M	1–10 min
17-α-Estradiol 10^{-6} M	1–10 min
17-β-estradiol 10^{-12}, 10^{-10} and 10^{-6} M	5 min
Tamoxifen 10^{-8} M	5 min
Tamoxifen 10^{-8} M + 17-β-Estradiol 10^{-10} M	5 min
Testosterone 10^{-10} and 10^{-6} M	5 min
Dihydrotestosterone 10^{-6} M	5 min
17-β-Estradiol 10^{-10} M	30 min, followed by 1 h more with ACSF free of steroids

oxygenated ACSF (35 ± 1°C) at a rate of 1.5–2.0 ml/min. The brain slice preparation and conditions of perfusion were in keeping with electrophysiological studies [20, 21], assuring good maintenance of arcuate neurons during the study period. Electron microscopic studies on tissue slices maintained for 6 h in the perfusion chamber indicated retention of good morphology of the neuronal membranes.

Test compounds were dissolved in ethanol and added to the perfusion media while control slices received medium containing ethanol alone at a final concentration of 10^{-4}%. The test compounds and the perfusion protocols used for study with freeze-fracture are shown in Table 1.

In a second set of experiments, slices were perfused with ACSF containing 1 mg/ml of horseradish peroxidase (HRP) in the presence or absence of test steroids, after which the HRP-exposed slices were removed and rinsed in untreated ACSF. Then the posteroventral part of the arcuate nucleus was microdissected [22], homogenized and assayed for protein [23] and HRP [24]. Corrections for non-specific HRP uptake at each time point were made by subtracting the amount of HRP taken up by the arcuate nucleus at 0°C.

Freeze-fracture

After timed perfusion with test compounds the slices were quickly immersed in a fixative solution of 1% glutaraldehyde and 1% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The slices were kept for 1 h in this fixative and then washed overnight in 0.1 M phosphate buffer. The posteroventral part of the left arcuate nucleus was punched out from the slices [22], soaked for 2 h in 0.1 M phosphate buffer, pH 7.4, containing 20% glycerol, and then coated with polyvinyl alcohol, frozen in Freon 22 cooled with liquid nitrogen, fractured and replicated [25] in a Balzers BAF 400D apparatus (Balzers, Liechtenstein). The platinum–carbon replicas were cleaned by sequential treatment with sodium hypochlorite, chloroform–methanol (2:1) and distilled water.

Quantitative evaluation of freeze-fracture replicas

Replicated fracture faces of membranes from arcuate nucleus dendrites identified by their cytoplasmic organization [11] (Fig. 1) were photographed at 30,000 magnification (calibrated with a Fullam reference grid: 2160 lines/mm). Photographic prints were coded and evaluated without knowledge of the experimental protocol. The number of exo-endocytotic pits in the inner leaflet of the plasma membrane (P-face) was recorded within a test square grid superimposed on photographic prints. To avoid errors in counting due to variable radius of curvature of the plasma membrane, the calculation of adequate size of the test square grid was carried out as described previously [26]. Five or more separate experiments were performed for each experimental condition (Table 1). Each experiment utilized 5 animals, with one slice from each animal studied by freeze-fracture. At least 10 pictures were evaluated for each slice. Statistical comparisons were carried out using the Mann–Whitney *U*-test [27] and the Student's unpaired *t*-test.

RESULTS

Extensive views were observed of neuronal membrane faces in freeze-fracture replicas from arcuate nucleus slices (Fig. 1). The general morphology was similar to that observed when arcuate nucleus was fixed *in situ* and then freeze-fractured [11]. During freeze-fracture, the plasma membrane is split in the middle of its phospholipid matrix and yields two complementary membrane leaflets; the P and E fracture faces. The P-face corresponds to the inner or protoplasmic leaflet of the membrane and the E-face represents the outer leaflet. Both faces appear as smooth surfaces in which are imbedded particles that represent, at least in part, membrane proteins (Fig. 2). In control slices perfused with steroid-hormone-free or cholesterol-containing (10^{-10} M) ACSF, neuronal perikarya and dendrites showed only occasional exo-endocytotic pits in the P-face of the plasma membrane. The density of pits was unchanged after perfusion for

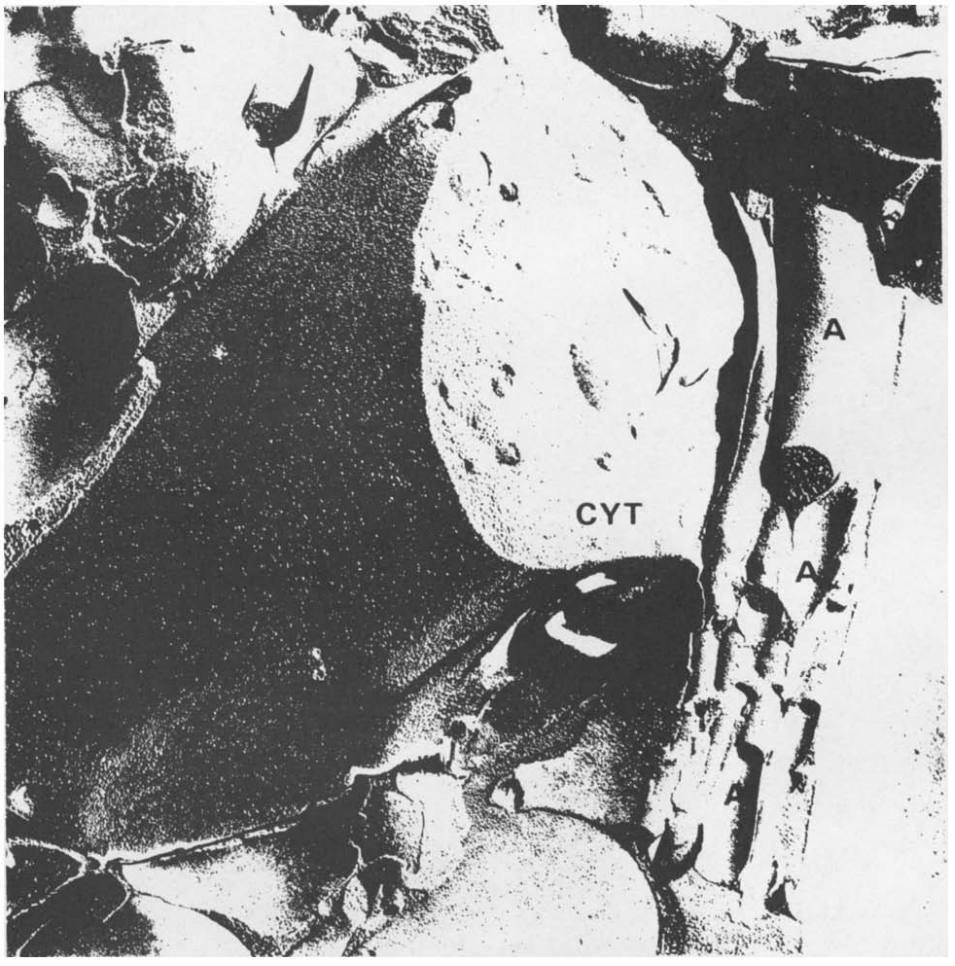

Fig. 1. Freeze-fracture replica from the posteroventral part of the arcuate nucleus of an ovariectomized rat. The replica was prepared from a nucleus microdissected from a hypothalamic slice that had been perfused with control artificial cerebrospinal fluid (ACSF) for 2 h, followed by 5 min with ACSF containing 10^{-10} M 17-β-estradiol. The replica shows the characteristic freeze-fracture appearance of an arcuate nucleus dendrite. The inner leaflet or P-face (PF) of the plasma membrane and the cross-fractured cytoplasm (CYT) can be identified. In addition, the field of the replica was also crossed by some axonal profiles (A). 30,000×.

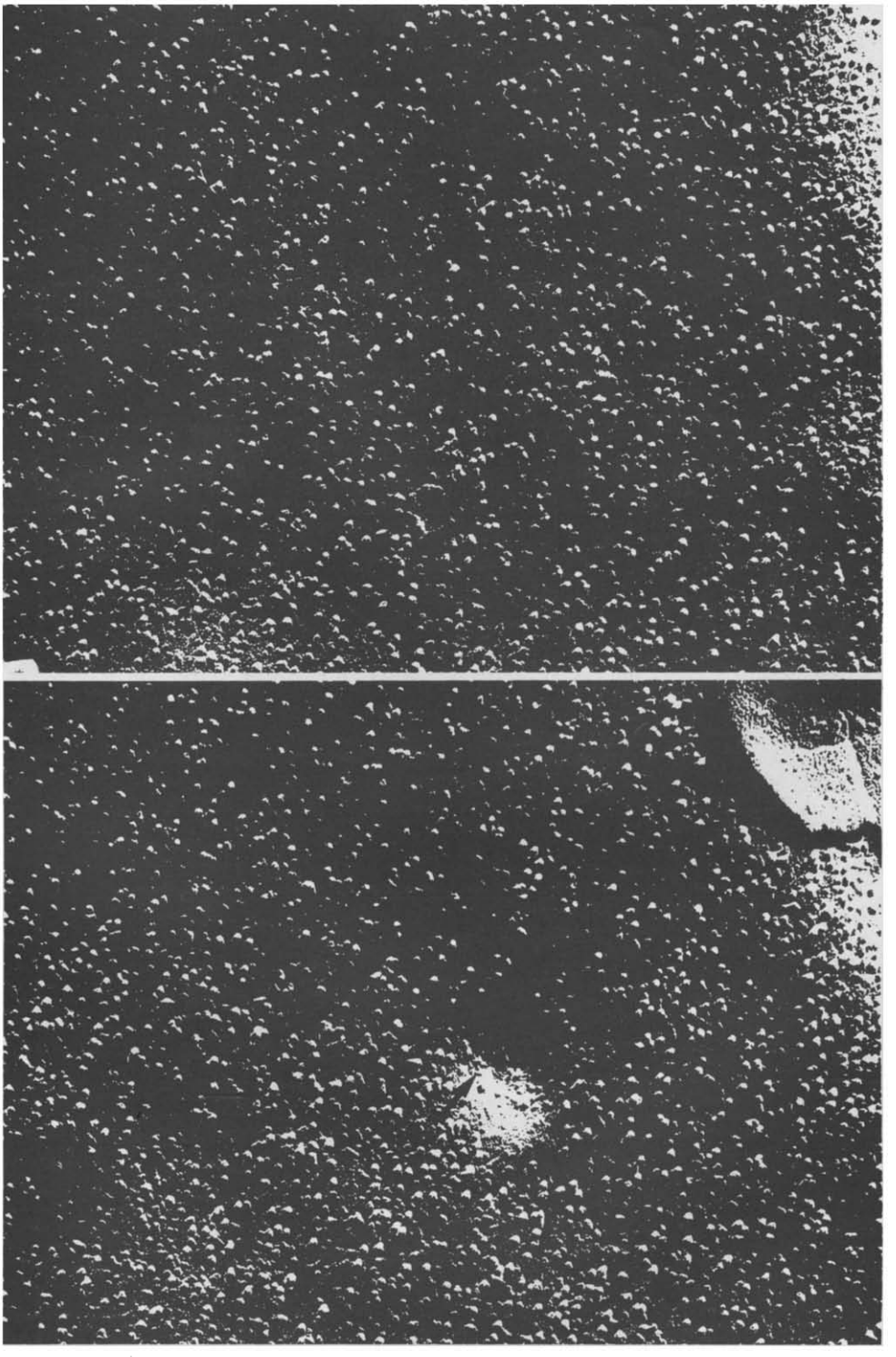

Fig. 2. Examples of freeze-fracture replicas from arcuate nucleus dendrites. (A) From a hypothalamic slice exposed to control steroid-free ACSF. (B) From a hypothalamic slice exposed to 10^{-10} M 17-β-estradiol for 1 min. An exo-endocytotic pit is observed in the membrane face in B (arrow). Both figures: 86,000×.

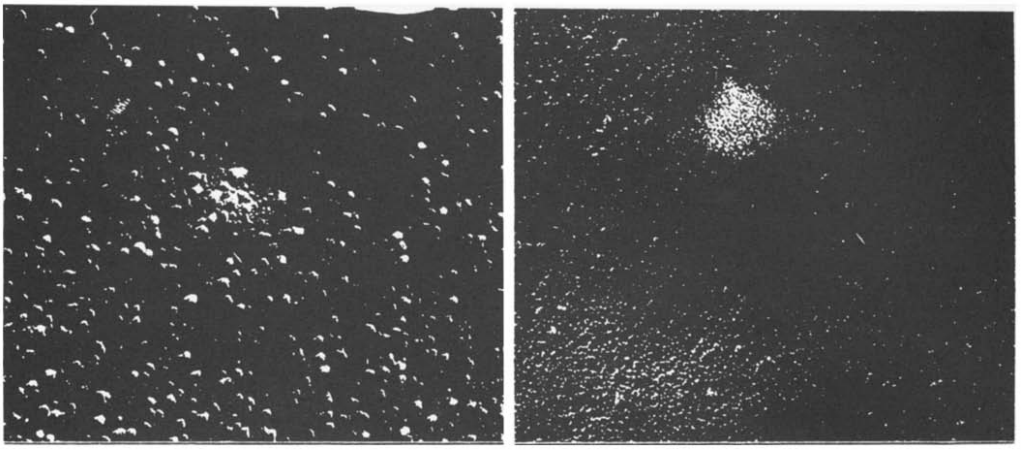

Fig. 4. Replicas of arcuate nucleus dendrites showing the characteristic freeze-fracture appearance of exo-endocytotic pits in the inner (P-face) and outer (E-face) leaflets of the membrane. (A) P-face. (B) E-face. Exo-endocytotic pits appear as depressions in the P-face and as domes in the E-face. Both figures: 120,000×.

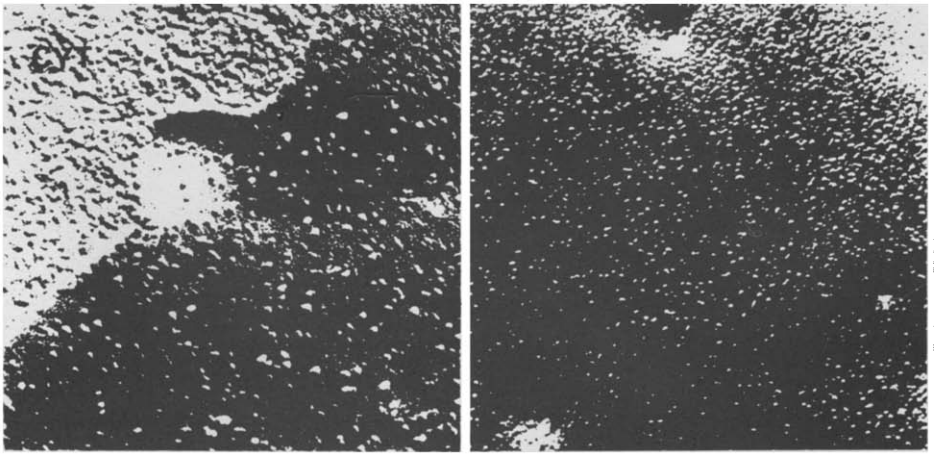

Fig. 5. Freeze-fracture replicas from arcuate nucleus dendrites. (A) Example of a cross-fractured exoendocytotic pit invaginating into the cytoplasm (CYT). (B) Example of a dendrite exposed to 10^{-6} M 17-β-estradiol for 5 min. Several pits can be observed in the P-face. (A) 81,000×. (B) 58,000×.

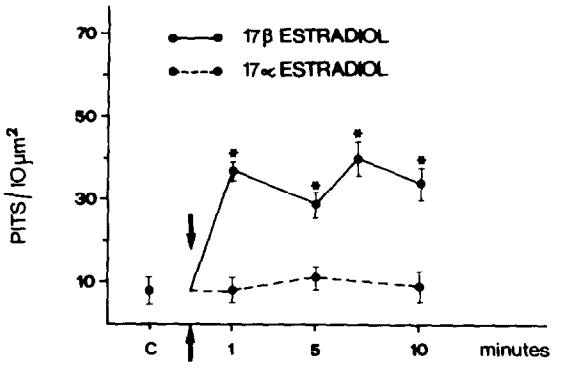

Fig. 3. Density of exo-endocytotic pits in the P-face of arcuate nucleus dendrite membranes, after perfusion with ACSF containing 10^{-10} M 17-α-estradiol or 10^{-10} M 17-β-estradiol. The density of pits was increased after 1-min exposure to 17-β-estradiol. In contrast, 17-α-estradiol was without effect, compared with control slices unexposed to steroids (C). The arrows indicate the time when steroids were applied. Values represent the means ± SEM of 10 separate experiments. *Value significantly different ($P < 0.001$) when compared with control slices (C).

several hours in the steroid-free ACSF medium (5 ± 3, 6 ± 2 and 4 ± 1 pits/10 μm^2 at 1, 4 and 6 h, respectively). In contrast, the density of pits was significantly increased within 1 min after perfusion with ACSF medium containing a physiological concentration (10^{-10} M) of 17-β-estradiol and did not increase further during the 10 min of continued exposure (Figs 2 and 3). Pits observed in the P-face of the neuronal membrane after estradiol exposure appeared as complementary domes in the E-face (Fig. 4); their diameter in the P-face was 128 ± 9 nm, and in the E-face 138 ± 8 nm (means ± SEM). In favorable replicas, it was observed that these structures correspond to invaginations of the plasma membrane (Fig. 5). No effect on membrane pits was observed when 17-α-estradiol was used, even at 4 orders of magnitude greater concentration than βE_2 (Figs 3 and 6). Addition of ethanol, with or without cholesterol (10^{-10} M), did not change the number of pits. In some experiments polyvinylpyrrolidone was used instead of ethanol to dissolve the estradiol, but this did not modify the results: 17-β-estradiol (10^{-10} M) increased the density of pits, whereas 17-α-estradiol (10^{-6} M) did not (data not shown).

Having established that a plateau of E_2 action was present between 1 and 10 min of perfusion, we investigated the effects at 5 min of increasing concentrations of 17-β-estradiol. Figure 6 illustrates the lack of effect of 10^{-12} M 17-β-estradiol, while the significant increase in the density of exo-endocytotic pits observed at a concentration of 10^{-10} M was further enhanced at 10^{-6} M (Figs 5 and 6). The potent estrogen antagonist tamoxifen, at 10^{-8} M, had no effect on the density of pits, but tamoxifen at 10^{-8} M blocked the effect of 17-β-estradiol (10^{-10} M) on pit density (Fig. 6). Other steroids were tested at 5 min; testosterone had about half the effect of 17-β-estradiol at 10^{-10} M and at 10^{-6} M; 5-α-dihydrotestosterone at up to 10^{-6} M was without effect on the density of membrane pits (Fig. 6).

The possible reversibility of the effect of 17-β-

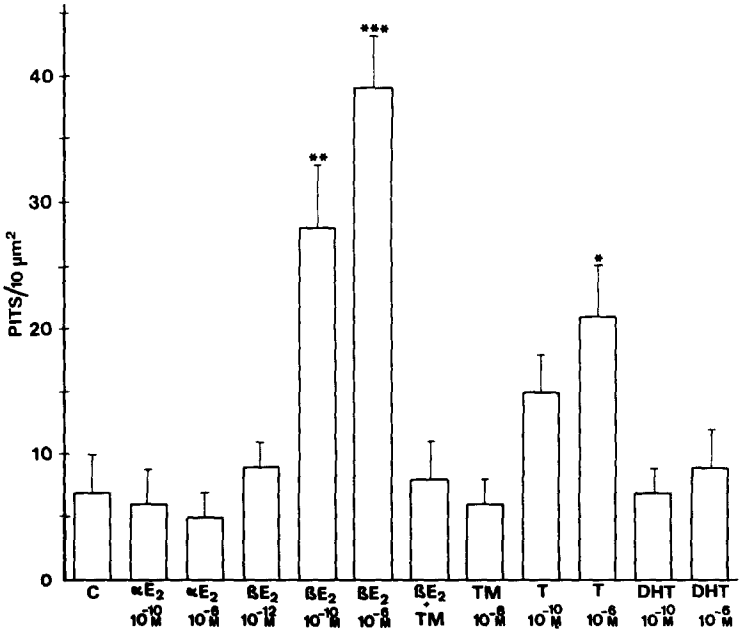

Fig. 6. Number of exo-endocytotic pits in the P-face of the plasma membrane of arcuate nucleus dendrites. Hypothalamic slices were perfused for 2 h with control ACSF and then for 5 min with ACSF containing: 17-α-estradiol (αE_2), 17-β-estradiol (βE_2), 10^{-10} M 17-β-estradiol and 10^{-8} M tamoxifen (βE_2 + TM), tamoxifen (TM), testosterone (T), 5-α-dihydrotestosterone (DHT) or ethanol (C). Values represent the means ± SEM of 5 separate experiments. Asterisks indicate values significantly different (*$P < 0.05$, **$P < 0.02$, ***$P < 0.001$) when compared with the control slices (C).

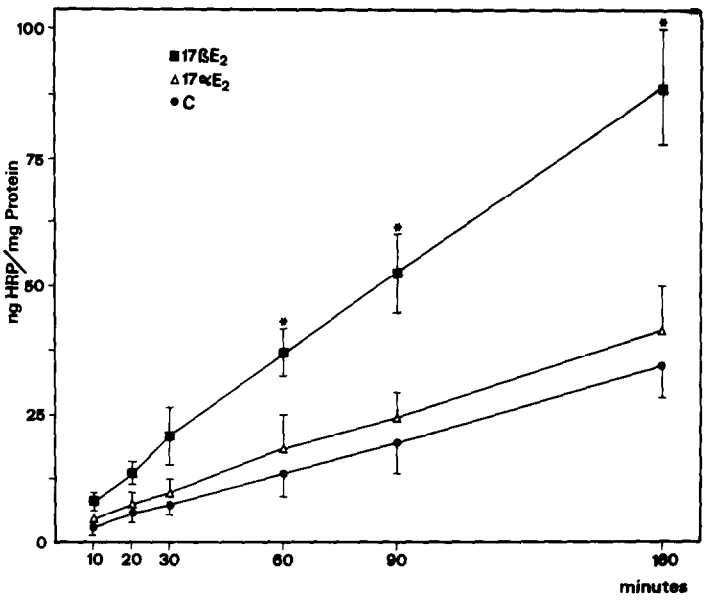

Fig. 7. Effect of estradiol on the time-course of horseradish peroxidase (HRP) uptake by arcuate nucleus in brain slices. Slices were first perfused with control ACSF for 2 h and then, at 0 time, with ACSF containing 1 mg/ml HRP and either 10^{-10} M 17-β-estradiol (17βE_2), 10^{-6} M 17-α-estradiol (17αE_2) or ethanol (C). Tissue slices were removed and microdissected for study at each time point and assayed for HRP. Data were corrected for nonspecific HRP uptake at 0°C. Values represent the mean ± SEM of 5 separate experiments. *Value significantly different ($P < 0.001$) when compared with the control slices (C).

estradiol on neuronal membranes was studied by perfusing some hypothalamic slices with ACSF containing 10^{-10} M 17-β-estradiol for 30 min and then switching to control ACSF medium for 1 h. Slices were then fixed and freeze-fractured. After this experimental sequence the density of exo-endocytotic pits was no different from that observed in control slices which had been incubated in steroid-free ACSF for the same 1 h 30 min period (6 ± 3 vs 5 ± 2 pits/10 μm^2 in estradiol-treated and control slices, respectively).

In order to detect possible functional correlations between increased numbers of neuronal membrane exo-endocytotic pits and changes in arcuate nucleus endocytotic activity, we perfused mediobasal hypothalamic slices with ACSF medium containing 1 mg/ml of horseradish peroxidase (HRP) in the presence of 17-β-estradiol (10^{-10} M), 17-α-estradiol (10^{-6} M) or ethanol alone. Slices perfused with 17-β-estradiol showed an increased uptake of HRP over slices perfused with hormone-free ACSF. In contrast, 17-α-estradiol had no effect on HRP uptake (Fig. 7).

DISCUSSION

Quantitative evaluation of freeze-fracture replicas from the rat arcuate nucleus indicated that within 1 min physiological doses of 17-β-estradiol increased the density of exo-endocytotic pits in the neuronal plasma membranes. There is a similarity between the exo-endocytotic pits in neuronal membranes induced by sex steroids in these experiments and pits involved in receptor-mediated endocytosis in other cell types [28]. That these plasmalemmal ultrastructural changes are associated with increased endocytotic activity is suggested by the increased uptake of HRP elicited by 17-β-estradiol in arcuate nucleus slices. These findings fit with previous experiments showing rapid stimulation by testosterone of endocytosis in mouse kidney cortex slices [29–31]. Rapid ultrastructural effects of estradiol on plasma membrane have also been detected upon the luminal surface of the uterine endometrium where the number of microvilli was increased 30 s after the i.v. injection of a physiological dose of 17-β-estradiol in ovariectomized rats [32].

In our study, the 17-β-estradiol action appears specific, since it was not elicited by tamoxifen or 17-α-estradiol, but was blocked by tamoxifen. Since both tamoxifen and 17-α-estradiol can form catechol estrogens as rapidly as 17-β-estradiol [33], it seems unlikely that catechol formation is critical to this action. The action of testosterone is interesting since dihydrotestosterone had no effect at 10^{-6} M. In the mouse kidney, where androgens are rapidly active and specific in increasing membrane transport, dihydrotestosterone is about as active as testosterone [31]. It seems therefore possible that in our experiments, where testosterone was 50% as effective as 17-β-estradiol, the testosterone action

was via conversion to 17-β-estradiol, as we have described in other cases [13].

Under the conditions studied, the 17-β-estradiol action was rapid, dose-dependent, blocked by tamoxifen and reversed by estrogen deprivation within 1 h. While the general results are compatible with steroid-receptor-mediated actions, the rapidity of these *in vitro* steroid hormone effects—within 1 min—is a far shorter time than is considered necessary for cell nuclear sex hormone receptor-mediated induction of protein synthesis [34, 35]. Therefore, our results raise the question of the possible non-genomic or post-transcriptional effects of 17-β-estradiol. There is much biochemical and electrophysiological evidence for rapid effects of steroid hormones on neurons [1–5, 36]. Rapid neuronal actions of steroids could be mediated by effects at the level of the plasma membrane and some have supposed that they require the presence of specific membrane receptors. Although specific binding sites for sex steroids have been reported on the plasma membrane of neurons [37] and other target cells [38], it is not necessary to require the proof of such receptors to explain rapid steroid actions on membrane. For instance, sex steroids may interact with membrane phospholipids [39] and produce alterations in membrane fluidity or in other membrane properties [31, 40]. Further experiments using preparations in which transcriptional elements will be removed from the experimental model are necessary to clarify whether the effects observed in this study can be attributed to a non-genomic action of steroid hormones.

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