Mechanism of the Rapid Effect of 17β-Estradiol on Medial Amygdala Neurons

JUNICHI NABEKURA, YUTAKA OOMURA, TAKETSUGU MINAMI, YUJI MIZUNO, ATSUO FUKUDA

The mechanism by which sex steroids rapidly modulate the excitability of neurons was investigated by intracellular recording of neurons in rat medial amygdala brain slices. Brief hyperpolarization and increased potassium conductance were produced by 17β-estradiol. This effect persisted after elimination of synaptic input and after suppression of protein synthesis. Thus, 17β-estradiol directly changes the ionic conductance of the postsynaptic membrane of medial amygdala neurons. In addition, a greater proportion of the neurons from females than from males responded to 17β-estradiol.

ESTRADIOL CAN BE CONCENTRATED in the medial amygdala (Med-AMG), the medial preoptic area, the ventromedial hypothalamus, and the arcuate nucleus (*1*). When implanted in the Med-AMG, estradiol modulates feeding behavior (*2*), gonadotropin release (*3*), and ovulation (*4*). Thus, the Med-AMG is an estrogen-sensitive target tissue that is involved in the integration of chemosensory information.

In many tissues estrogen binds to an intracellular receptor and triggers RNA-dependent protein synthesis (*5*). Recently, it has also been suggested that estrogen influences neural electrical activity by a different, short-latency effect (*6*). Within a few minutes of systemic administration, estradiol briefly increases the activity of medial preoptic and anterior hypothalamic neurons (*7*). Rapid changes in neural activity also occur in vivo after electrophoretic administration of estradiol to medial preoptic neurons (*8*) and in vitro after bath application to hippocampal (*9*), parvicellular ventromedial hypothalamic, and arcuate (*10*) neurons. We report here the use of intracellular recording to investigate the rapid effect of estradiol on neurons in the Med-AMG and to define the mechanism of its effect on the neural membrane.

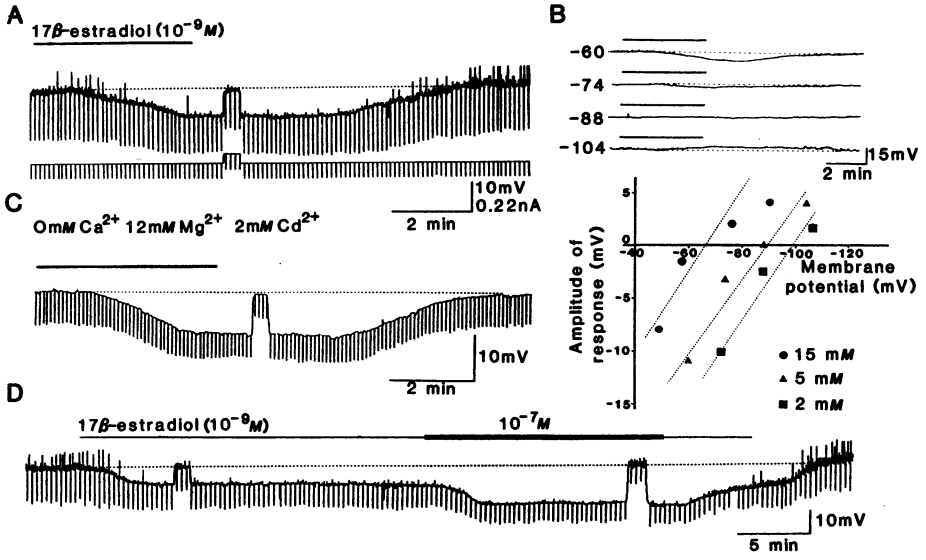

Fig. 1. Hyperpolarization induced by 17β-estradiol in typical female Med-AMG neurons. (A) Superfusion with $10^{-9}M$ 17β-estradiol (indicated by overbar) caused an 8-mV hyperpolarization with about a 28% decrease in input membrane resistance (hyperpolarization, 8.8 ± 2.9 mV; decrease in the input membrane resistance, 29.3 ± 6.8% at $10^{-9}M$ 17β-estradiol, mean ± SD, n = 14; no difference between female and male). Input membrane resistances were measured by short downward directed displacements induced by hyperpolarized current pulses (0.1 nA, 250 msec). When the hyperpolarization had stabilized, membrane was restored to resting potential (−62 mV) by a manually controlled depolarizing current; the decrease in membrane resistance was unchanged. Volume of preheating system, 3 ml; flow rate of perfusion medium, 2 to 3 ml/min. (B, top) Response to 17β-estradiol ($10^{-9}M$) as a function of change in membrane potential. Reversal potential was −88 mV at 5 mM external K^+. (B, bottom) Shift of reversal potential for 17β-estradiol ($10^{-9}M$) response by change in external K^+ concentration (same neuron as in top). Amplitude of response to 17β-estradiol as function of membrane potential. Reversal potentials were −65, −88, and −98 mV at 15, 5, and 2 mM external K^+, respectively. (C) 17β-Estradiol ($10^{-9}M$)–induced hyperpolarization after elimination of synaptic inputs. Superfusion by medium containing 12 mM Mg^{2+} and 2 mM Cd^{2+}, but no Ca^{2+}, for more than 15 minutes nearly eliminated spontaneous and locally stimulated evoked synaptic inputs, but did not alter 17β-estradiol–induced hyperpolarization (−11 mV) or the accompanying 32% decrease in input membrane resistance. Resting membrane potential, −58 mV. (D) Test for desensitization of 17β-estradiol hyperpolarization. Original resting membrane potential, −60 mV. During application of $10^{-9}M$ 17β-estradiol (thin bar) for 20 minutes, membrane potential (7-mV hyperpolarization) and input membrane resistance (26% decrease) maintained initial values. After altering 17β-estradiol concentration to $10^{-7}M$ (thick bar), the membrane hyperpolarized further (15 mV from resting membrane level) with a further decrease in input membrane resistance (42%). Both values remained stable throughout treatment and recovered after treatment. Each figure was obtained from a different neuron.

Coronal brain slices (450 μm thick) of the amygdala, including the medial nucleus, were obtained (two slices from each rat) from 42 adult male (8 to 10 weeks old; 190 to 250 g) and 82 female Wistar rats (*11*), obtained from Seiwa Animal Company and housed under standardized conditions of temperature (23° ± 1°C) and light (7:00 a.m. to 7:00 p.m.) in our facility for 2 weeks. Female rats (8 to 9 weeks old; 170 to 200 g) were ovariectomized under Ketalar anesthesia (100 mg per kilogram of body weight). Two weeks after ovariectomy, animals were injected subcutaneously with a single priming injection of estradiol benzoate (70 μg per kilogram of body weight). Two days later, brain slices were incubated for 90 minutes in Krebs-Ringer solution at room temperature. The slice was transferred to a recording chamber and submerged in a continuously flowing solution equilibrated with 95 percent O_2 and 5 percent CO_2 at 36°C (*12*). For morphological analysis of electrophysiologically identified neurons, horseradish peroxidase (4 percent) was dissolved in 0.5M KCl-tris buffer and injected intracellularly through the recording electrode with positive current pulses. Stained neurons were reconstructed by three-dimensional computer analysis (*11*).

Superfusion with 17β-estradiol hyperpolarized 19 of 70 neurons (27 percent) from females and 3 of 37 neurons (8 percent) from males. Each neuron was obtained from a different rat (*13*). There was a significant difference between males and females in the number of responding neurons in the Med-AMG ($P < 0.05$, χ^2 test). No response was observed when 17β-estradiol was applied to neurons (male, 15; female, 21) of the basolateral amygdala. Hyperpolarization of Med-AMG neurons by 17β-estradiol was associated with increased membrane conductance and with decreased neural excitability as indicated by the reduction of spon-

Department of Physiology, Faculty of Medicine, Kyushu University, 60, Fukuoka 812, Japan.

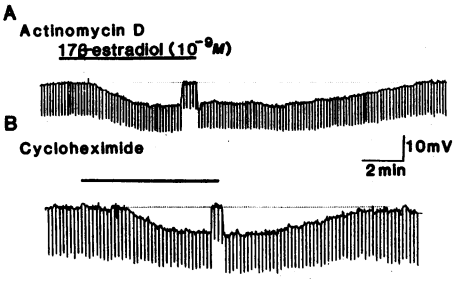

Fig. 2. 17β-Estradiol hyperpolarization in presence of protein synthesis inhibitors. Actinomycin D (10 μg/ml) (A) and cycloheximide (30 μg/ml) (B) were added to the preincubation and perfusion medium. Slices were incubated with protein synthesis inhibitors for at least 4 hours before use. Although membrane potential fluctuated somewhat, 17β-estradiol (10^{-9}M) hyperpolarized the membrane [(A) 8 mV; (B) 10 mV] and decreased input membrane resistance [(A) 24%; (B) 32%] with a time course and amplitude similar to those in normal medium. Resting potential: (A) −56; (B) −60 mV. (A) and (B) were obtained from different slices.

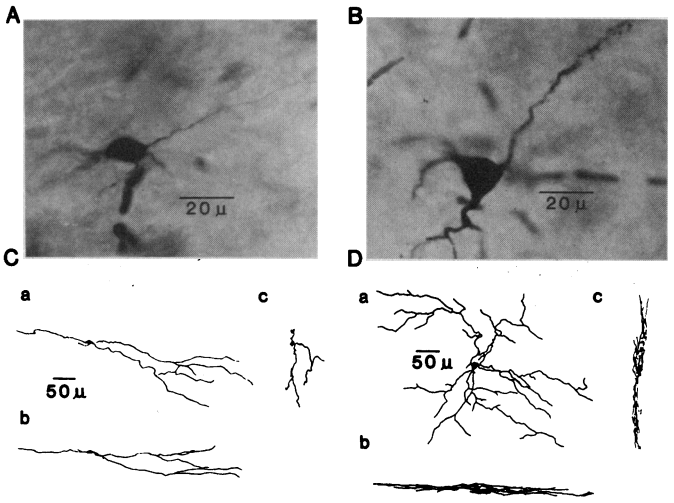

Fig. 3. Morphological differences between representative 17β-estradiol–responding (A and C) and nonresponding (B and D) neurons. (A) and (B) were reconstructed by computer for the three-dimensional views in (C) and (D). The right side of each figure is toward the ventral surface of amygdala. a, Coronal view; b, lateral view; c, ventral view.

taneous firing (Fig. 1A). Each application of testosterone (10^{-7} to 10^{-9}M) and 17α-estradiol (10^{-7} to 10^{-9}M), an inactive form of estradiol (*14*), to a separate group of four 17β-estradiol–responding neurons, did not change the membrane potential. When the membrane was hyperpolarized to various potentials with inward current and the effect of estradiol was tested, the amplitude of the hyperpolarization gradually decreased and was completely eliminated at −88 mV. At more hyperpolarized membrane potentials, the response to estradiol was reversed and became a depolarization (Fig. 1B, top). This established the reversal potential of the estradiol effect at approximately −88 mV, which is similar to the reversal potential of the afterhyperpolarization (−92 mV) that follows the action potential elicited by direct intracellular stimulation. The reversal potentials of estradiol hyperpolarization were approximately −98, −88, and −65 mV for external K^+ levels of 2.0, 5.0, and 15.0 m*M*, respectively (Fig. 1B, bottom). A shift of 53 mV for each tenfold change in the external K^+ concentration agrees with the Nernst equation. Because the hyperpolarization was not reversed when Cl^--containing electrodes were used, we concluded that the estradiol hyperpolarization was caused by an increase in the K^+ permeability of the Med-AMG neuron.

Perfusion with a solution containing 12 m*M* Mg^{2+} and 2 m*M* Cd^{2+}, but no Ca^{2+}, eliminated the spontaneous and evoked synaptic responses of the Med-AMG neurons. However, 17β-estradiol still induced a hyperpolarization accompanied by increased membrane conductance (Fig. 1C). This indicated that the hyperpolarization was not caused by an increase or a decrease in the release of transmitter from presynaptic terminals, but was a direct effect on the postsynaptic neurons of the Med-AMG. It was therefore unlikely that this was an effect of catechol estrogens, which are metabolites of estradiol formed in brain tissue and which decrease production of the neurotransmitters norepinephrine and dopamine (*15*). In addition, the effect of estradiol was dose-dependent and did not desensitize (Fig. 1D). In another experiment, different concentrations of 17β-estradiol (10^{-8}, 10^{-7}, and 10^{-6}M) were applied and the responses measured. These three doses of estradiol caused 33, 42, and 40 percent decreases in the input membrane resistance (11 mV, 14 mV, and 14 mV hyperpolarization), respectively, suggesting that the concentration that produced the maximum response was between 10^{-8}M and 10^{-7}M. This value was similar to the biochemical concentration that saturates the estrogen binding sites on synaptic plasma membranes from brain (*16*).

To test whether protein synthesis is required for the estradiol effect, we used perfusate containing actinomycin D (*17*) or cycloheximide (*18*). Application of 17β-estradiol still produced hyperpolarization and decreased the input membrane resistance (Fig. 2, A and B) with a time course and amplitude similar to those in the normal solution (Fig. 1A). Thus, 17β-estradiol seems to act directly on the neural membrane rather than indirectly by an effect on RNA-dependent protein synthesis.

After the estradiol effect was characterized, we injected horseradish peroxidase intracellularly into 7 estradiol-responding and 16 nonresponding neurons. Estradiol-responding neurons (female, 6, male, 1) were mainly small oval or fusiform neurons (13.8 ± 3.8 μm in diameter; mean ± SD) with a few dendrites (less than 5) and a few spinelike appendages (Fig. 3, A and C). Almost all processes of estradiol-responding neurons ran to the stria terminalis and the ventral surface of the amygdaloid body. Twelve (female, 10; male, 2) of the 16 nonresponding neurons were pyramidal-shaped (17.1 ± 3.9, μm in diameter; mean ± SD), had more than four dendrites that extended in various directions, and had dense spinelike appendages (Fig. 3, B and D). The other four nonresponding neurons (female, 2; male, 2) were similar morphologically to estradiol-responding neurons. Similar morphological features of estradiol-responding neurons were demonstrated in ventromedial hypothalamic and arcuate nuclei (*10*).

Our results indicate a direct effect of estradiol on K^+ permeability in the postsynaptic membrane of the Med-AMG neurons. This rapid change might be regulated by 17β-estradiol by its interaction with membrane binding sites of estrogen, as demonstrated in synaptic plasma membrane of the brain (*16*).

Med-AMG neurons differ morphologically between the sexes (*19*) and the Med-AMG, but not the basolateral amygdala, is an estrogenic feedback site for gonadotropin and prolactin release in the female but not the male (*20*). Thus, Med-AMG neurons may monitor estradiol levels in the blood or cerebrospinal fluid and may send information about the stage of the estrous cycle to other brain areas. This information could modulate gonadotropin release and other functions, such as feeding.

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12. The composition of solution was 124 m*M* NaCl, 5 m*M* KCl, 1.24 m*M* NaH_2PO_4, 2.4 m*M* $CaCl_2$, 1.3 m*M* $MgSO_4 \cdot 7H_2O$, 26 m*M* $NaHCO_3$, and 10 m*M* glucose (*p*H 7.4). In all experiments, drugs and solutions of different ionic components were applied in the bath and dissolved in perfusion medium. Recording glass pipettes were filled with 3*M* potassium acetate (d-c resistance, 60 to 100 MΩ).
13. Only neurons that had membrane potentials that were more hyperpolarized than −50 mV and were stable for more than 30 minutes (up to 6 hours) were used. We employed two slices including the middle portion of the Med-AMG from each rat. The responsiveness to the drugs was approximately the same in each slice. To ensure that the results from each neuron were independent, duplicate data obtained from the same rat were eliminated.
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Technical Comments

GABA Receptor–Mediated Chloride Transport in a "Cell-Free" Membrane Preparation from Brain

Harris and Allan (*1*) present evidence that they say demonstrates functional coupling of γ-aminobutyric acid (GABA) receptors to chloride (Cl^-) channels in isolated "cell-free" membranes prepared from brain. However, characterization (*2*) of the "cell-free" membrane preparation used by Harris and Allan (*1*) [initially described by Chasin *et al.* (*3*) and modified by Daly *et al.* (*4*)] has revealed the presence of many intact cells, including neurons. Thus, it is incorrect to conclude that the technique they describe (*1*) measures GABA-barbiturate receptor-effector coupling in "cell-free" brain membranes.

Table 1. Distribution of visible elements in filtered and unfiltered preparations from guinea pig cerebral cortex. The unfiltered particulate material was prepared as described by Harris and Allan (*1*) according to the methods of Chasin *et al.* (*3*) and Daly *et al.* (*4*) from guinea pig cerebral cortex. The filtered particulate material was prepared according to Hollingsworth *et al.* (*2*) by filtering a suspension of unfiltered elements through 100-mesh nylon and then through a 10-μm filter (Millipore). A suspension of both preparations [protein (~1.0 mg/ml) in Krebs-Ringer bicarbonate-glucose buffer] was examined by light microscopy (magnification ×6900) with a reflected-light differential-contrast photomicroscope (Zeiss II) and a video image recorder (*5*). Visible elements were identified as described (*2*) and quantified by counting elements in 100 frames.

Element	Diameter (μm)	Unfiltered	Filtered
Vesicles			
Small	0.5–2.5	671	460
Large	5–30	131	10
Erythrocytes	6–7	18	7
Nonneuronal cells	11–13	44	8
Neuronal cells	(15 × 22)	22	0
Unidentified debris		24	6

Using a modification of the reflected-light differential-interference contrast system (*5*) with a video image recorder (*2*), we have examined and quantified the visible elements present in the crude membranes prepared according to the method used by Harris and Allan (*1*, *4*). While both conventional light and electron microscopy are insufficient for visualizing whole cells, reflected-light differential-interference microscopy reveals that this "membrane" preparation contains, in addition to vesicles of various diameters, intact neurons, glia, erythrocytes, unidentified whole cells, and large clumps of unidentifiable "debris" (Table 1). Recently, Hollingsworth *et al.* (*2*) have shown that this crude membrane preparation can be purified by filtration through nylon mesh and a 10-μm filter to yield a relatively "cell-free" preparation of pre- and postsynaptic vesicles. The resulting preparation has been shown to be enriched in "snowman-shaped" entities called "synaptoneurosomes" and to be devoid of intact neurons (Table 1) (*2*). We have reported barbiturate-, muscimol-, and picrotoxin-sensitive $^{36}Cl^-$ flux in this filtered synaptoneurosome preparation (*6*). Figure 1 demonstrates the stimulation of $^{36}Cl^-$ uptake and efflux induced by the GABA receptor agonist muscimol in filtered synaptoneurosomes.

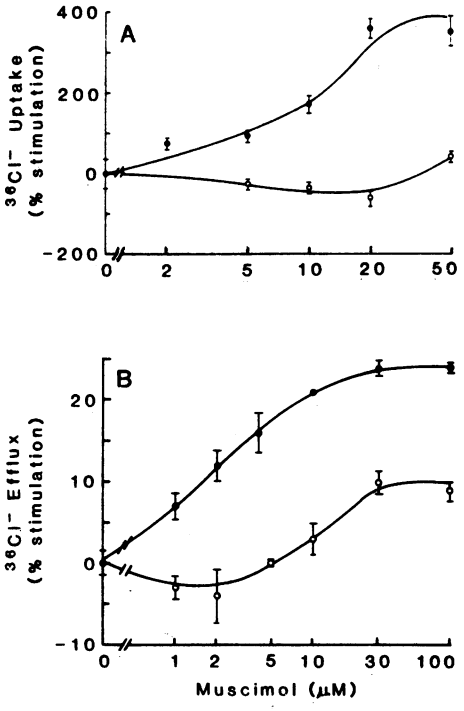

Fig. 1. Muscimol-induced stimulation of $^{36}Cl^-$ uptake (A) and efflux (B) in cerebral cortical synaptoneurosomes. For uptake, synaptoneurosomes prepared from rat cerebral cortex (*2*) were incubated for 5 seconds (30°C) with $^{36}Cl^-$ and various concentrations of muscimol (*6*). The GABA antagonist bicuculline methiodide (100 μ*M*) was added 3 minutes prior to the $^{36}Cl^-$. The reaction was terminated by dilution with ice-cold buffer containing 20 m*M* Hepes/9 m*M* Tris, 118 m*M* NaCl, 4.7 m*M* KCl, 1.18 m*M* $MgSO_4$, and 2.5 m*M* $CaCl_2$ (*p*H 7.4) and by rapid filtration through GF/C Whatman filters. $^{36}Cl^-$ efflux was determined 2 minutes after dilution of preloaded synaptoneurosomes as previously described (*6*). The EC_{50} for muscimol-stimulated $^{36}Cl^-$ uptake and efflux is approximately 3 to 8 μ*M*. Data are the means ± SEM of quadruplicate determination and representative of at least three such experiments.