Impact of progestins on estradiol potentiation of the glutamate calcium response

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One mechanism by which estrogen may modulate cognitive function is through potentiation of glutamate-mediated rises in intracellular calcium ($[Ca^{2+}]_i$) with resultant effects on neuronal morphology and signaling. Since progesterone is a component of hormone replacement therapy (HRT), we sought to determine whether therapeutically relevant progestins attenuated or blocked estrogen potentiation of glutamate-induced $[Ca^{2+}]_i$ rises. 17β-estradiol and progesterone, alone or in combination, significantly potentiated the rise in $[Ca^{2+}]_i$. When co-administered, progesterone attenuated the estrogen response to the level seen with progesterone alone. In contrast, medroxyprogesterone acetate (MPA) had no effect when administered alone and completely blocked the 17β-estradiol-induced potentiation when co-administered. These results may have important implications for effective use of HRT to maintain cognitive function during menopause and aging. *NeuroReport* 13:825–830 © 2002 Lippincott Williams & Wilkins.

Key Words: Calcium; Cognitive function; Estrogen; Glutamate; Hormone replacement therapy; Neuron; Progesterone

INTRODUCTION

Increasing evidence indicates that estrogen regulates cognitive function and associated biochemical and genomic mechanisms of learning and memory [1]. In young women, fluctuations in estrogen level due to the menstrual cycle are positively correlated with memory task performance with higher estrogen levels associated with better cognitive performance [2]. In postmenopausal woman, estrogen replacement therapy improves verbal and visual memory [3]. In addition to effects on cognition, estrogen replacement has further been implicated in reducing the risk of neurodegeneration and memory loss due to Alzheimer's disease (AD) [4,5]. The mechanism(s) underlying estrogen effects on cognition centers on the NMDA glutamate receptors, as blockade of NMDA receptors attenuates the effects of estrogen on neuronal correlates of memory [6,7].

Glutamate is the major excitatory neurotransmitter in the mammalian CNS. At least three pharmacologically distinct ionotropic receptors exist for glutamate: NMDA, kainate and AMPA receptors. Under normal physiological conditions, the NMDA receptor plays a vital role in the synaptic plasticity thought to underlie learning and memory [8], most likely through its permeability to calcium (Ca^{2+}) [9], which acts as a second messenger. NMDA receptors must be active in the hippocampal CA1 region in order to induce long-term potentiation (LTP) a model of information storage [10]. Blockade of NMDA receptors causes behavioral deficits similar to lesions of the hippocampus, as both spatial reference and working memory are impaired [10].

The case for an NMDA-receptor-dependent mechanism underlying estrogen effects on memory function is supported by morphological studies from both *in vitro* and *in vivo* preparations and by electrophysiological data from hippocampal slice preparations in both dissociated hippocampal neurons and hippocampal slices. A direct effect of estrogens on neuronal process outgrowth was observed in our laboratory using dissociated cortical and hippocampal neurons, which was blocked by an NMDA receptor antagonist [6]. Woolley and colleagues have shown that 17β-estradiol induces NMDA-receptor-dependent increases in dendritic spines associated with an increase in synapses [11]. The excitatory nature of these estrogen-induced synapses is supported by a parallel increase in NMDA receptor agonist binding sites and NMDAR1 subunit immunoreactivity [12]. The morphological and biochemical evidence which implicates a role of glutamate receptors in estrogen-induced neurotrophism is paralleled by electrophysiological evidence, as in slice preparations estradiol significantly increases both AMPA- and NMDA-generated EPSPs [7].

Although the receptors for estrogens and progestins belong to different nuclear receptor subfamilies and recognize distinct hormone response elements, there is considerable cross talk between the estrogen and progestin signaling pathways. Progestins have been shown to block the estrogen-stimulated expression of both c-fos and progesterone receptor mRNAs in uterine cells [13]. Furthermore, progestins and antiprogestins have been shown to

inhibit estrogen-stimulated uterine proliferation [13]. Thus, progestins have been added to hormone replacement therapy (HRT), in part, to reduce the risk of uterine cancers associated with unopposed estrogen [14]. In addition to prevention of endometrial hyperplasia, inclusion of progestins in HRT was thought to reduce estrogenic effects in breast that are associated with increased risk of breast cancer [14]. Contrary to this long-held belief, a recent study found that an estrogen–progestin regimen increased cancer risk beyond that associated with estrogen alone [15]. This emphasizes the necessity of studying the modulation of estrogenic effects by progestins in various systems. The purpose of this study was to determine the effects of two widely clinically used progesterones (progesterone and medroxyprogesterone acetate) on potentiation by estrogen of the glutamate response in hippocampal neurons.

MATERIALS AND METHODS

Animals: All studies were approved by the USC Institutional Review Board for animal care. Timed-pregnant Sprague–Dawley rats were purchased from Harlan (Indianapolis, IN). They were housed under controlled conditions of temperature (22°C), humidity and light (14:10 h light:dark) and water and food were available *ad lib*.

Chemicals: All culture materials were purchased from Gibco BRL (Rockville, MD). All chemicals were purchased from Sigma (St Louis, MO) unless otherwise noted. MPA was obtained from Pharmacia and Upjohn Company (Peapack, NJ). Steroids were dissolved in ethanol at 1 mg/ml and diluted in culture medium so that final ethanol concentration was $< 0.001\%$.

Neuronal culture: Primary cultures of dissociated hippocampal neurons were performed as described previously [6]. Briefly, hippocampi were dissected from the brains of E18 rat fetuses, treated with 0.02% trypsin in Hank's balanced salt solution (5.4 mM KCl, 0.4 mM KH_2PO_4, 137 mM NaCl, 0.34 mM $Na_2HPO_47H_2O$, 10 mM HEPES) 5 min at 37°C and dissociated by repeated passage through a series of fire-polished constricted Pasteur pipettes. Cells (1000/cc^2) were plated onto poly-D-lysine-coated coverslips (22 mm diametre). Nerve cells were grown in neurobasal medium (Gibco BRL) supplemented with 5 U/ml penicillin, 5 mg/ml streptomycin, and B27 supplement (Gibco BRL). Cultures were maintained at 37°C in a humidified 5% CO_2 atmosphere, and all experiments were performed after 12 days in culture. At 72 h prior to imaging cells were treated with indicated steroids or vehicle control.

[Ca^{2+}] microfluorimetry and imaging: [Ca^{2+}] in hippocampal neurons was determined by ratiometric imaging of the Ca^{2+}-sensitive fluorescent dye fura-2. Cells were loaded for 30 min at 37°C with 2 μM fura-2 acetoxymethyl ester (Molecular Probes; Eugene, OR) in Krebs–HEPES buffer (100 nM NaCl, 2.0 nM KCl, 1.0 nM $CaCl_2$, 1.0 nM $MgCl_2$, 1.0 nM NaH_2PO_4, 4.2 nM $NaHCO_3$, 12.5 nM HEPES and 10.0 nM glucose) then washed for a further 30 min in buffer to remove remaining fura-2 ester. The coverslip with loaded cells was then mounted in a perfusion chamber placed on an inverted microscope (Olympus IMT-2). The cells were perfused with Krebs–HEPES buffer at a flow rate of 2 ml/min. Baseline $[Ca^{2+}]_i$ was obtained for 1 min prior to initiation of perfusion with glutamate, which was maintained for the remaining duration of the imaging. Fura-2 was successively excited by a xenon light source at 340 and 380 nm by means of two narrow beam bandpass filters selected by a computer controlled filter wheel. The emitted fluorescence was filtered through a 520 nm filter, captured with an intensified CCD camera (COHU; San Diego, CA) and analyzed with InCyt Im2 software (Intracellular Imaging, Inc.; Cincinnati, OH). The concentration of Ca^{2+} was calculated by comparing the ratio of fluorescence at 340 and 380 nm against a standard curve of known [Ca^{2+}]. Data from regions of interest were displayed in real time and logged to hard disc. Data are presented as representative traces averaged from 10 cells per experiment. Responses to steroids were quantified by determining the difference between the average $[Ca^{2+}]_i$ for 1 min during glutamate exposure and the average $[Ca^{2+}]_i$ for 1 min prior to exposure. Changes in $[Ca^{2+}]_i$ are presented as mean ± s.e.m. from four independent experiments with ≥ 10 cells per experiment. Statistical comparisons utilized ANOVA followed by Student–Newman–Keul's *post hoc* analysis.

RESULTS

17β-Estradiol potentiates glutamate-induced increases in $[Ca^{2+}]_i$: Hippocampal neurons were exposed to 17β-estradiol (10 ng/ml; 37 nM) or vehicle control for 72 h prior to monitoring $[Ca^{2+}]_i$ in response to glutamate (25 μM) using the Ca^{2+}-sensitive fluorophore fura-2. Glutamate induced a rapid increase in $[Ca^{2+}]_i$ (Fig. 1a), with a mean change in $[Ca^{2+}]_i$ of 131 ± 16 nM ($n = 4$; Fig. 1b). In hippocampal neurons pretreated with 17β-estradiol, the glutamate-stimulated increase in $[Ca^{2+}]_i$ was significantly enhanced by ~70% (Fig. 1a), with a mean change in $[Ca^{2+}]_i$ of 223 ± 69 nM ($p < 0.01$; $n = 4$; Fig. 1b).

Progesterone potentiates glutamate-induced increases in $[Ca^{2+}]_i$: Hippocampal neurons were exposed to progesterone (10 ng/ml; 30 nM) or vehicle control for 72 h prior to monitoring $[Ca^{2+}]_i$ in response to glutamate (25 μM) using the Ca^{2+}-sensitive fluorophore fura-2. Glutamate induced rapid increases in $[Ca^{2+}]_i$ (Fig. 2a), with a mean change in $[Ca^{2+}]_i$ of 129 ± 14 nM ($n = 4$; Fig. 2). Hippocampal neurons pretreated with progesterone showed a significant enhancement (~32.5%) of glutamate-induced rise in $[Ca^{2+}]_i$ (Fig. 2a), with a mean change in $[Ca^{2+}]_i$ of 171 ± 33 nM ($p < 0.01$; $n = 4$; Fig. 2b). The progesterone-induced enhancement was less than that induced by 17β-estradiol.

Progesterone co-administration attenuates but does not fully block the 17β-estradiol-potentiation of glutamate-induced increases in $[Ca^{2+}]_i$: Hippocampal neurons were exposed to progesterone (10 ng/ml) or vehicle control in the presence and absence of 17β-estradiol (10 ng/ml) for 72 h prior to monitoring $[Ca^{2+}]_i$ in response to glutamate (25 μM). Glutamate induced a rapid increase in $[Ca^{2+}]_i$ (Fig. 2c), with a mean change in $[Ca^{2+}]_i$ of 129 ± 14 nM (Fig. 2d). Hippocampal neurons pretreated with 17β-estradiol again exhibited a significant enhancement of glutamate-

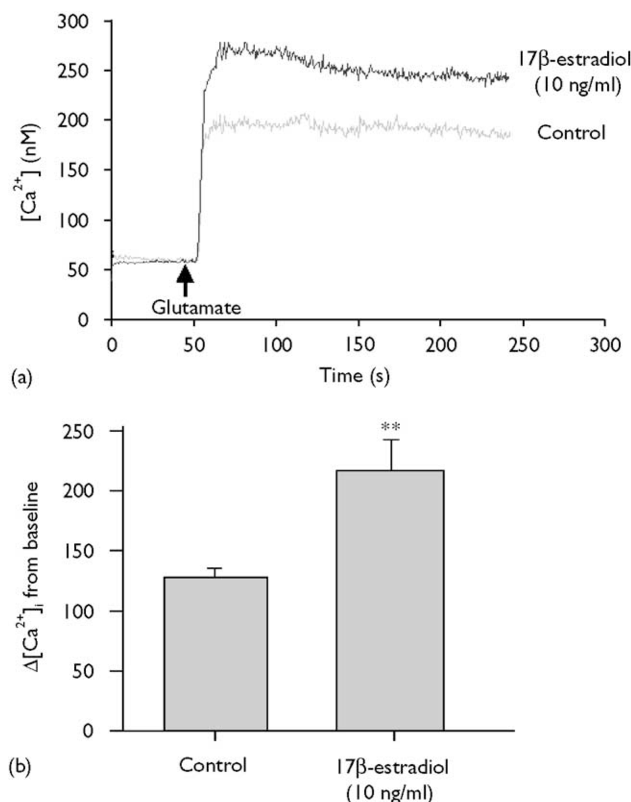

Fig. 1. Estrogen potentiates the glutamate-induced rise in $[Ca^{2+}]_i$. Hippocampal neurons pretreated with 17β-estradiol (10 ng/ml) exhibited a significantly greater response to glutamate (25 μM) than control neurons. (**a**) Representative tracings of the average $[Ca^{2+}]$ from 10 neurons over time in response to glutamate. (**b**) Quantitative changes in $[Ca^{2+}]_i$ in response to glutamate in the presence and absence of 17β-estradiol (*$p < 0.01$ *vs* control neurons; $n = 4$ independent experiments with ≥ 10 neurons/experiment).

stimulated increase in $[Ca^{2+}]_i$ (Fig. 3a), with a mean change in $[Ca^{2+}]_i$ of 213 ± 49 nM ($p < 0.01$; $n = 4$; Fig. 3b). As with estradiol pretreatment, when hippocampal neurons were pretreated with progesterone, the glutamate-induced rise in $[Ca^{2+}]_i$ was significantly enhanced (Fig. 2c), with a mean change in $[Ca^{2+}]_i$ of 171 ± 33 nM ($p < 0.01$; $n = 4$; Fig. 2d). The potentiation of the glutamate-induced rise in $[Ca^{2+}]_i$ was significantly less with progesterone treatment than with estrogen treatment ($p < 0.05$ *vs* estrogen alone; $n = 4$; Fig. 2d). When hippocampal neurons were pretreated with the progesterone (10 ng/ml) plus 17β-estradiol (10 ng/ml), the glutamate-induced rise in $[Ca^{2+}]_i$ was significantly enhanced (Fig. 2c), with a mean change in $[Ca^{2+}]_i$ of 156 ± 60 nM ($p < 0.01$ *vs* control; $n = 4$; Fig. 2d). However, the potentiation was significantly less when progesterone was co-administered with estradiol than with estrogen treatment alone ($p < 0.05$ *vs* estrogen alone; $n = 4$; Fig. 2d).

Medroxyprogesterone acetate does not potentiate glutamate-induced increases in $[Ca^{2+}]_i$: Hippocampal neurons were exposed to medroxyprogesterone acetate (MPA; 10 ng/ml; 25 nM) or vehicle control for 72 h prior to monitoring $[Ca^{2+}]_i$ in response to glutamate (25 μM) using the Ca^{2+}-sensitive fluorophore fura-2. Glutamate induced a rapid increase in $[Ca^{2+}]_i$ (Fig. 3a), with a mean change in $[Ca^{2+}]_i$ of 125 ± 44 nM ($n = 4$; Fig. 3b). In contrast to estradiol and progesterone pretreatment, hippocampal neurons pretreated with MPA exhibited no change in glutamate-stimulated rise in $[Ca^{2+}]_i$ ($n = 4$; Fig. 3a), with a mean change in $[Ca^{2+}]_i$ of 124 ± 61 nM ($n = 4$; Fig. 3b).

Medroxyprogesterone acetate co-administration inhibits 17β-estradiol enhancement of glutamate-induced increases in $[Ca^{2+}]_i$: Hippocampal neurons were exposed to MPA (10 ng/ml) or vehicle control in the presence and absence of 17β-estradiol (10 ng/ml) for 72 h prior to monitoring $[Ca^{2+}]_i$ in response to glutamate (25 μM). Glutamate induced a rapid increase in $[Ca^{2+}]_i$ (Fig. 3c), with a mean change in $[Ca^{2+}]_i$ of 134 ± 12 nM (Fig. 3d). Hippocampal neurons pretreated with 17β-estradiol again exhibited a significant enhancement of glutamate-stimulated increase in $[Ca^{2+}]_i$ (Fig. 3c), with a mean change in $[Ca^{2+}]_i$ of 223 ± 52 nM ($p < 0.01$; $n = 4$; Fig. 3d). Hippocampal neurons pretreated with MPA exhibited no change in glutamate-stimulated rise in $[Ca^{2+}]_i$ ($n = 4$; Fig. 3c), with a mean change in $[Ca^{2+}]_i$ of 124 ± 61 nM ($n = 4$; Fig. 3d). Co-administration of MPA with 17β-estradiol antagonized the 17β-estradiol-induced enhancement of the glutamate-stimulated rise in $[Ca^{2+}]_i$ (Fig. 3c), resulting in a mean change in $[Ca^{2+}]_i$ of 120 ± 32 nM ($p < 0.01$ *vs* 17β-estradiol-treated neurons; $n = 4$; Fig. 3d).

DISCUSSION

The present study demonstrates the divergent effects of different progesterones and their impact on estrogen potentiation of the glutamate-induced rise in $[Ca^{2+}]_i$. Progesterone, but not MPA, potentiated glutamate-induced rises in $[Ca^{2+}]_i$. Not only was MPA ineffective by itself, but also antagonized estrogen-induced potentiation of the glutamate response. These results suggest that the progestin used in HRT could impact estrogen regulation of mechanisms of cognitive function.

It is increasingly evident that fundamental cellular mechanisms involved in learning and memory include growth in the projections of, and synaptic connections between, neurons. Previously, we have shown that estrogens exert neurotrophic effects on cultures of dissociated neurons [1]. In addition to neurite sprouting, estradiol can induce an increase in both the basal and apical dendritic spines of CA1 pyramidal neurons concomitant with an increase in peak Ca^{2+} levels, an effect that was blocked by an NMDA receptor antagonist [16]. Such estrogen-modulated signaling would presumably lead to an increase in down-stream calcium-dependent responses such as an increase in phospho CREB [17]. In fact, Murphy and Segal found that long-term and constant estradiol exposure increases phosphorylation of CREB and that blockade of CREB phosphorylation blocked estradiol-induced spine formation in cultured hippocampal neurons [18].

In vivo work has shown that estrogen-induced spine formation in CA1 returns to basal density during estrous, coincident with falling levels of estrogen and progesterone [19]. Removal of circulating gonadal steroids by ovariectomy of adult rats resulted in a profound decline in dendritic

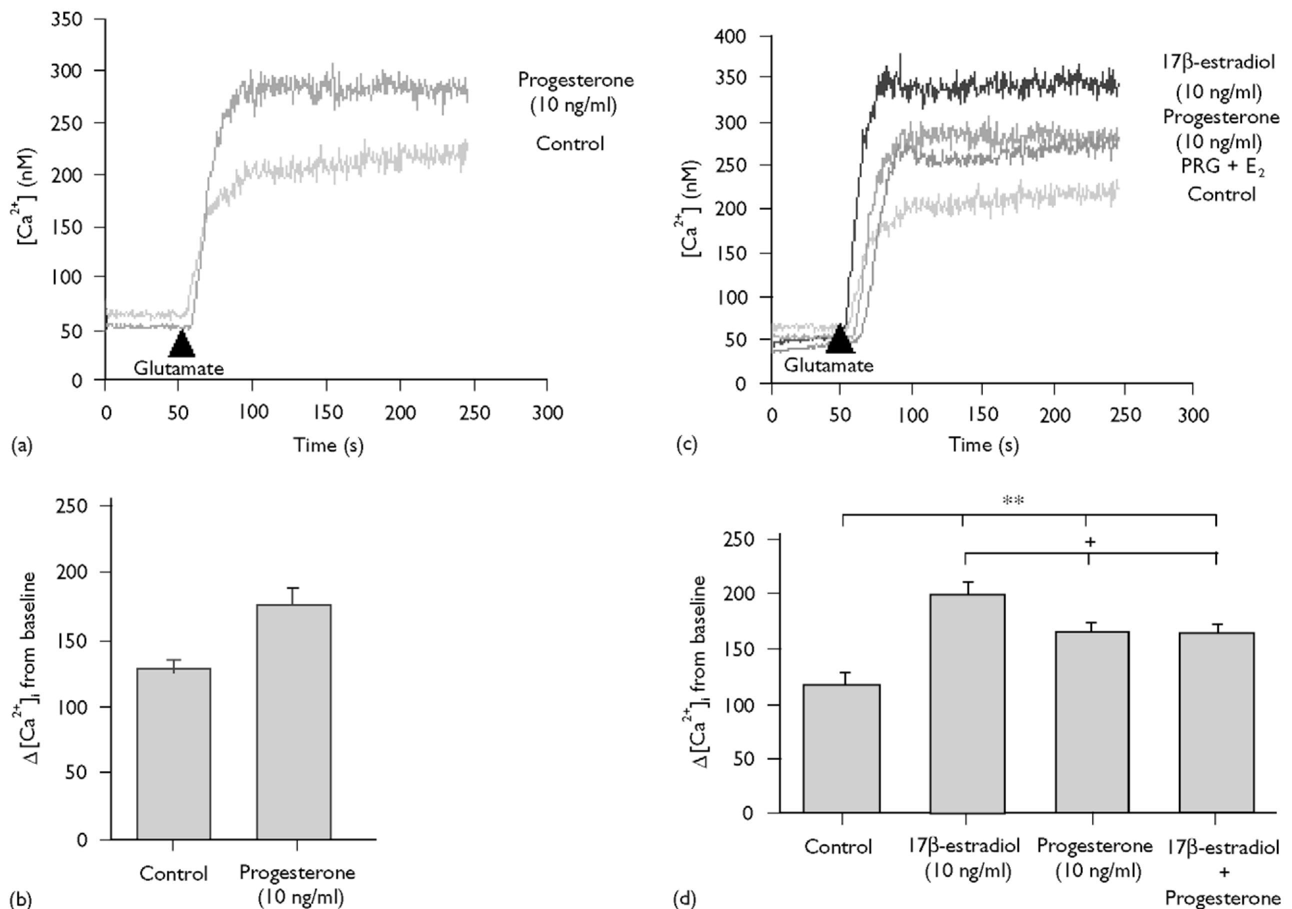

Fig. 2. Progesterone potentiates the glutamate-induced rise in $[Ca^{2+}]_i$. Hippocampal neurons pretreated with 17β-estradiol (10 ng/ml), progesterone (10 ng/ml) or both exhibited a significantly greater response to glutamate (25 μM) than control neurons. (**a**) Representative tracings of the average $[Ca^{2+}]$ from 10 neurons over time in response to glutamate. (**b**) Quantitative changes in $[Ca^{2+}]_i$ in response to glutamate in the presence or absence of progesterone. (**c**) Representative tracings of the average $[Ca^{2+}]$ from 10 neurons over time in response to glutamate. (**d**) Quantitative changes in $[Ca^{2+}]_i$ in response to glutamate in the presence or absence of steroids ($**p < 0.01$ *vs* control neurons; $+p < 0.05$ *vs* estradiol-treated neurons; $n = 4$ independent experiments with ≥ 10 neurons/experiment).

spine density that was transiently prevented by estrogen replacement [19]. Progesterone augmented the effect of estradiol for a short time period but resulted in a sharper decline than is observed following estradiol alone [19]. The effect of estradiol was mimicked *in vitro*, but in these studies high concentrations of progesterone were shown to block estradiol-induced dendritic spine formation in cultured hippocampal neurons [20]. This effect of progesterone was shown to be due to its conversion to tetrahydroprogesterone (THP), which at the high concentrations used enhances spontaneous GABAergic activity, offsetting the GABAergic inhibition associated with estradiol-induced spine formation [20]. Our data fit with a model of augmentation of the estradiol-induced spine formation by progesterone mediated by both steroids potentiating the NMDA response. The sharper decline of spine density *in vivo* may be the result of enhancement of GABAergic activity by THP [21] converted from progesterone without affecting estradiol or progesterone modulation of Ca^{2+} signaling. Further studies are needed to determine the effect of THP on estradiol-induced potentiation of the rise in $[Ca^{2+}]_i$. Due to steroidal metabolism by neurons in culture (although most steroidal conversion occurs in glial cells which are non-existent to exceedingly low in number in these cultures), it is possible that the potentiation by progesterone is mediated by one of its metabolites.

The 17β-estradiol enhancement of the physiological glutamate response agrees well with previous reports showing enhancement of NMDA receptor activation, LTP and memory by estrogens [3,7]. We showed that progesterone, alone or co-administered with 17β-estradiol, enhanced the glutamate-induced rise in $[Ca^{2+}]_i$. These results fit with the work of Cabrera and colleagues, who showed that progesterone increases the NMDA-induced dopamine release [22]. In addition, Vongher and Frye found that rats treated with progesterone, alone or in conjunction with estrogen, before neural insults had improved cognitive behavior [23]. Although, in the present study, the progesterone treatment resulted in a lower response than with estradiol alone, the response was still significantly potentiated relative to control. Taken together, the data indicate a complex interplay between progesterone and estrogen in the regulation of calcium signaling, neuron spine growth and cognitive function.

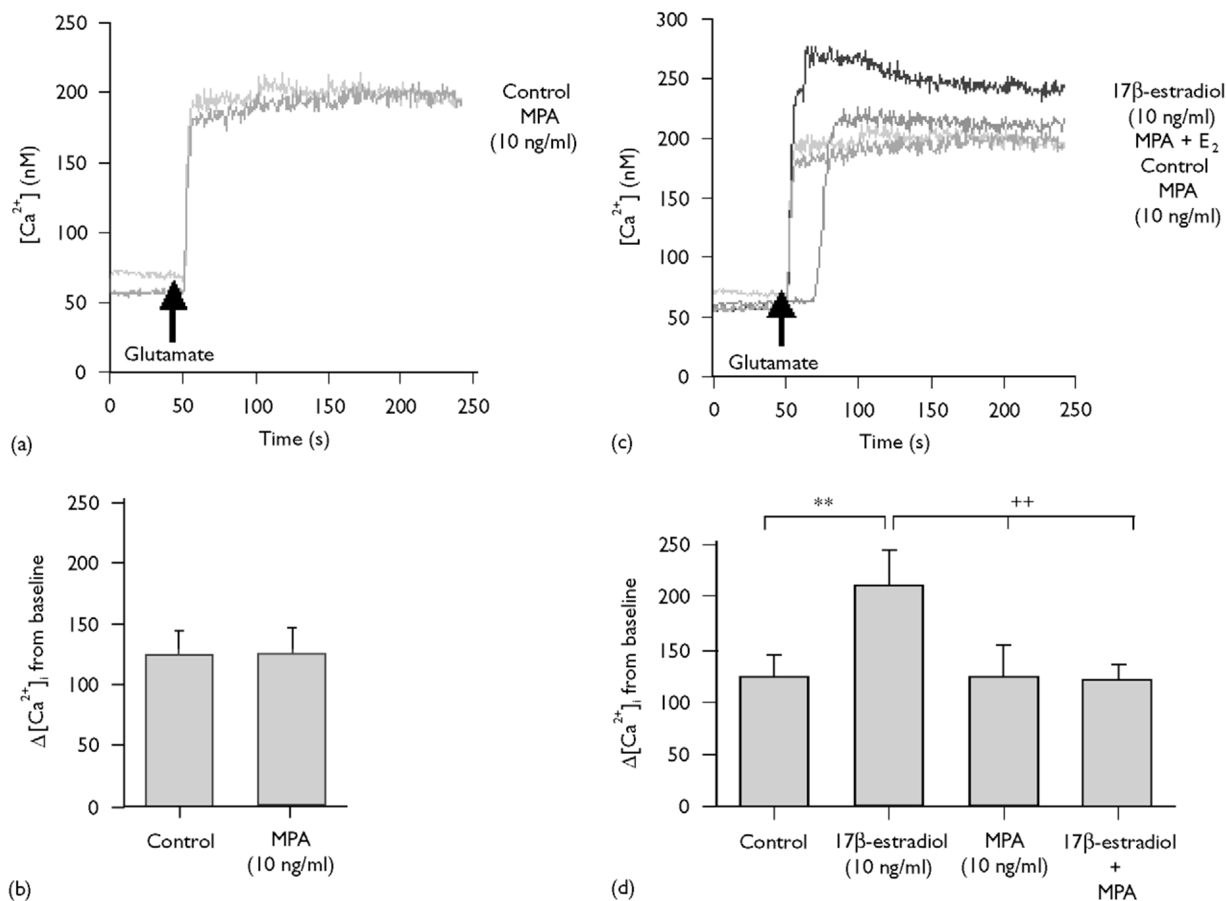

Fig. 3. Co-administration of medroxyprogesterone acetate inhibits the estrogen-induced potentiation of the glutamate-induced rise in $[Ca^{2+}]_i$. Hippocampal neurons pretreated with 17β-estradiol (10 ng/ml) exhibited a greater response to glutamate (25 μM) than control neurons. Hippocampal neurons pretreated with MPA (10 ng/ml), alone or in conjunction with estrogen, exhibited the same response to glutamate (25 μM) *vs* control neurons. (**a**) Representative tracings of the average $[Ca^{2+}]$ from 10 neurons over time in response to glutamate. (**b**) Quantitative changes in $[Ca^{2+}]_i$ in response to glutamate in the presence or absence of MPA. (**c**) Representative tracings of the average $[Ca^{2+}]$ from 10 neurons over time in response to glutamate. (**d**) Quantitative changes in $[Ca^{2+}]_i$ in response to glutamate in the presence or absence of steroids. $^{**}p < 0.01$ *vs* control neurons; $^{++}p < 0.01$ *vs* estradiol-treated neurons; $n = 4$ independent experiments with ≥ 10 neurons/experiment).

Although progesterone, alone or in combination with estradiol, potentiated the glutamate response, MPA blocked the estrogenic effect when co-administered with 17β-estradiol. This indicates a diverging effect of two widely used progesterones. We have previously reported similar effects of progesterones on estradiol-induced neuroprotection [24]. Progesterone and 19-norprogesterone, but not MPA, protected against glutamate excitotoxicity. Not only was MPA an ineffective neuroprotectant, but it also antagonized estrogen-induced neuroprotection. Considered together, these results are consistent with the emerging clinical data indicating divergent actions of different progesterones. Studies comparing the associations between unopposed estrogen use and combined estrogen–progestin therapy, including the Postmenopausal Estrogen/Progestin Interventions trial, have shown that MPA, but not micronized progesterone, blunted the beneficial association between estrogen and high-density lipoprotein cholesterol [25]. Likewise, even though progesterone can protect against increased risk of breast cancer associated with unopposed estrogen [14], the risk of breast cancer was higher with MPA plus estrogen than with estrogen alone [15]. The Kame project reported that the beneficial association between estrogen and cognitive change was opposed by the addition of MPA [3]. These results warrant further study into the mechanisms by which sex steroids modulate intracellular calcium levels and calcium signaling. Moreover, these data suggest that the type of progestin component of hormone replacement therapy can significantly affect its therapeutic efficacy for cognitive function.

CONCLUSION

The present study demonstrates the divergent effects of different progesterones and their impact on estrogen-potentiation of the glutamate-induced rise in $[Ca^{2+}]_i$. Progesterone, but not MPA, potentiated glutamate-induced rises in $[Ca^{2+}]_i$. Not only was MPA ineffective by itself, but also antagonized estrogen-induced potentiation of the glutamate response. Overall, these data point out the necessity of examining the composition of hormones used in HRT formulations, especially with regard to which type

of progesterone is included. This work belies a common underlying theme in endocrinology, that although structurally different ligands behave the same in some systems, they do not necessarily do so in all systems.

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