Rise in Intracellular Calcium via a Nongenomic Effect of Allopregnanolone in Fetal Rat Hypothalamic Neurons

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This study examines the early effects of 3α-hydroxy-5α-pregnan-20-one (allopregnanolone) on cytosolic free calcium concentration ($[Ca^{2+}]_i$) in primary cultures of fetal rat hypothalamic neurons. Microspectrofluorimetry of fluorescent Ca^{2+}-sensitive indicator Fura-2 was used to quantify these changes. Allopregnanolone (1 pM to 100 nM) increased $[Ca^{2+}]_i$ within 2–3 sec, in a dose-dependent manner, with an EC_{50} of 10 ± 4 nM. The stimulatory effect of allopregnanolone was attributable principally to a Ca^{2+} influx, as shown by the strong inhibition of external Ca^{2+} removal or by the calcium channel blocker nifedipine. The effect was stereospecific because the allopregnanolone isomer 3β-hydroxy-5α-pregnan-20-one had no effect on $[Ca^{2+}]_i$. Among two other steroids examined, progesterone had no effect on $[Ca^{2+}]_i$, but 17β-estradiol evoked a rise in $[Ca^{2+}]_i$, although to a lesser extent than allopregnanolone. The allopregnanolone-induced $[Ca^{2+}]_i$ rise was inhibited by picrotoxin and bicuculline but was unaffected by tetrodotoxin or by pretreatment of neurons with pertussis toxin. These results are consistent with a membrane site of action for allopregnanolone associated with $GABA_A$ receptors, leading to rapid changes in $[Ca^{2+}]_i$ in fetal rat hypothalamic neurons.

Key words: neurosteroids; calcium; hypothalamic neurons; Fura-2; allopregnanolone; nongenomic effect; $GABA_A$ receptor

The term "neurosteroids" was introduced by Baulieu (1981) to describe the steroids synthesized in brain tissue from cholesterol or from steroid hormone precursors (Robel and Baulieu, 1990, 1994; Baulieu, 1991; Orchinick and McEwen, 1993). According to their effects, they can be considered "neuroactive steroids." This term, coined by Paul and Purdy (1992), includes all steroids that are active on neuronal tissue regardless of whether they are synthesized in the brain.

Aside from the classical genomic actions of steroids (McEwen, 1991a), these substances may alter rapidly the excitability of neurons. In fact, the ability of certain steroids and their metabolites to influence brain activity, including firing rate of neurons, induction of sedation, anesthesia, neurosecretion, and behavioral changes, is well known (Schumacher, 1990; McEwen, 1991b). However, the mechanisms involved in these actions are not well understood. Recently, Ramirez et al. (1990) demonstrated that progesterone and its metabolites can modify neuronal secretion by acting through a nongenomic transmembrane-signaling mechanism. They postulated that progesterone could open Ca^{2+} channels of nerve terminals leading to a rapid neuronal secretion. Evidence suggesting a role of Ca^{2+} in this phenomenon was provided previously by Drouva et al. (1985), who showed that omission of Ca^{2+} in the medium or addition of D-600, a Ca^{2+} channel blocker, antagonized the stimulatory effect of progesterone on luteinizing hormone-releasing hormone release from mediobasal hypothalamic slices.

A mechanism involved in the nongenomic effect of steroids involves binding to a membrane-bound receptor complex, such as those for inhibitory, e.g., GABA (Majewska et al., 1986), and/or excitatory, e.g., NMDA neurotransmitters (Smith, 1987; Paul and Purdy, 1992; Mellon, 1994). Some steroids may potentiate or inhibit NMDA receptor-mediated responses (Smith et al., 1987; Wu et al., 1991; Irwin et al., 1992, 1994; Park-Chung et al., 1994), and they can also have agonistic or antagonist $GABA_A$ receptor-like activity (Paul and Purdy, 1992).

Interestingly, two of the neuroactive steroids that are the most potent negative modulators of $GABA_A$ receptors, 3α-hydroxy-5α-pregnan-20-one (allopregnanolone), which is the primary metabolite of progesterone, and allotetrahydrodeoxycorticosterone, derived from deoxycorticosterone, have been measured in brain and plasma, where their levels fluctuated in response to stress and during the estrus cycles of female rats (Paul and Purdy, 1992). In normal male rats, for example, allopregnanolone is detectable in the cerebral cortex and hypothalamus, its level being markedly and rapidly increased after acute swim stress (Purdy et al., 1991) or acute electroshock (Korneyev et al., 1993).

Taken together, these data strongly suggest that neurosteroids represent a new class of neuromodulators that can rapidly alter neuron excitability via nongenomic mechanisms. However, little information is available on the molecular mechanisms, particularly the excitatory ones, that may explain the rapid membrane effects of steroid hormones. Consequently, the aim of the present study was to examine the effect of allopregnanolone on intracellular calcium level ($[Ca^{2+}]_i$) in primary cultures of rat hypothalamic neurons by performing high-time resolution photometric measurements of $[Ca^{2+}]_i$ transients evoked by drug application.

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MATERIALS AND METHODS

Chemicals. Most of the standard chemicals, tetrodotoxin (TTX), EGTA, 17β-estradiol, progesterone, pertussis toxin (PTX), and nifedipine, were obtained from Sigma (St. Louis, MO). Fura-2 AM and Pluronic F-127 were purchased from Molecular Probes (Eugene, OR). Allopregnanolone (3α-hydroxy-5α-pregnan-20-one or 3α,5α-THP or RU 16709) was kindly provided by Roussel UCLAF (Romainville, France)

Cell culture. Primary cultures were prepared by mechanoenzymatic dissociation of fetal (day 17) Sprague–Dawley rat hypothalami as described previously (Tapia-Arancibia et al., 1988) with necessary modifications. Briefly, cells were plated onto chambered cover glass (Lab-Tek N° 178565, Nunc, Naperville, IL) coated previously with poly-D-lysine (10 μg/ml; M_r 220 kDa) and preincubated for 1 hr with 10% fetal calf serum in Minimum Essential Medium (MEM; Gibco, Grand Island, NY). Cells were seeded in growth medium composed of MEM supplemented with Nu serum (10%) (Collaborative Research, Lexington, MA), glucose (0.6%), glutamine (2 mM), and penicillin-streptomycin (2.5 U/ml), adjusted to pH 7.3. The cultures were maintained at 37°C in a humid atmosphere (95% air/5% CO_2). The proliferation of non-neuronal cells was inhibited by treatment with 10 μM cytosine-arabinoside for 48 hr between days 3 and 5 after plating. Three days before the experiments, a phenol red-free medium supplemented with 10% charcoal-treated Nu serum (devoid of steroids) was used. Experiments were performed after 8 d in culture.

Measurements of intracellular free calcium in single neurons. The $[Ca^{2+}]_i$ was determined from the intensity of the fluorescence signal with the Ca^{2+}-sensitive indicator Fura-2. Hypothalamic neurons in culture were washed with a serum-free medium (Locke's buffer) containing (in mM): NaCl 140, KCl 5, glucose 10, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 1.8, HEPES 10, adjusted to pH 7.4 with NaOH. The osmolarity of the buffer was 300–310 mOsm/kg. Cells were loaded with Fura-2 AM, which was dissolved first in water-free dimethyl sulfoxide (DMSO) and then diluted with Locke's buffer to a final concentration of 2.5 μM Fura-2 AM, which containing 0.02% (w/v) Pluronic F-127. The DMSO concentration in these mixtures was <0.01%. Dye loading was carried out for 30 min in a humidified O_2/CO_2 incubator at 37°C. Subsequently, loaded cells were washed with Locke's buffer and the cover glass was mounted on the microscope stage. The fluorescence measurements were performed with buffer and drug solutions that were maintained at 36–37°C throughout the measuring period.

Fast fluorescence photometer system. $[Ca^{2+}]_i$ in single cells was measured by the method described by Lambert et al. (1994) with necessary modifications. Briefly, the Zeiss (Oberkochen, Germany) photometer system was based on an inverted microscope (Axiovert 100) equipped for epifluorescence. Interference filters of 340/10 and 380/10 nm were mounted alternately (200 Hz) on the filter wheel, and excitation light was deflected through an oil-immersion objective (Zeiss-plan Neoflaur 100× 1.30, Ph 3). Fluorescence emission from individual cells was viewed through a diaphragm adjusted to the cell size. To minimize the background noise of the Fura-2 signals, successive values were averaged to a final resolution of 320 msec. With fluorescence values corrected for background and dark current, calculation of $[Ca^{2+}]_i$ was carried out from the ratio between 340 and 380 nm recordings, in accordance with the equation given by Grynkiewicz et al. (1985): $[Ca^{2+}]_i = K_d \times (R - R_{min})/(R_{max} - R) \times \beta$. To calculate $[Ca^{2+}]_i$ values from measured data, the parameters $K_d = 224$, $R_{min} = 0.203$, $R_{max} = 6.351$, and $\beta = 3.418$ were specified in the calibration menu.

Solutions and drug application. Stock solutions (10^{-2} M) of the steroids dissolved in ethanol were prepared daily and then diluted with Locke's buffer containing TTX (1 μM). All steroid solutions (10 μl) were applied within 5 sec directly in the proximity of the recorded neurons (10 μl in a total volume of 200 μl) using a motor-driven syringe (Hamilton-microlab 900, speed setting 9; Bonaduz, Switzerland). The final concentration of ethanol in these solutions was <0.01%. Ethanol (0.01%) has no effect by itself on basal $[Ca^{2+}]_i$. This method allowed reproducible changes in the medium surrounding a selected neuron and appeared to ensure fast concentration changes. Calcium-free medium (EGTA-buffer) contained (in mM): EGTA 2, NaCl 140, KCl 5, glucose 10, KH_2PO_4 1.2, $MgSO_4$ 1.2, and HEPES 10, pH 7.4. In this EGTA buffer, 100 nM free Ca^{2+} (which corresponds to the resting $[Ca^{2+}]_i$ as determined with Fura-2 measurements) was maintained.

Data analysis. Results were expressed as mean ± SEM. The statistical evaluation was performed by Student's *t* test. The estimations of EC_{50} and its standard error were determined with a Marquardt–Levenberg nonlinear least-squares curve-fitting algorithm (Origin, Microcal Software, Northampton, MA).

RESULTS

Effect of allopregnanolone on intracellular calcium

The basal level of $[Ca^{2+}]_i$ in hypothalamic neurons was 71 ± 8 nM (mean ± SEM, $n = 21$).

Applications (60–120 sec) of allopregnanolone (1 pM to 100 nM) evoked a marked increase of $[Ca^{2+}]_i$, reaching a peak rise within 2–3 sec in 85 of 113 neurons tested. Two distinct types of $[Ca^{2+}]_i$ responses were observed: (1) in a great majority of neurons (67/85, 79%), allopregnanolone induced a brief, single, transient $[Ca^{2+}]_i$ rise that decayed to near resting levels in <30 sec in the continued presence of the steroid (see, for example, 10 and 100 nM in Figs. 1*A*, 2, 3*B*, 4, and 6*B*); and (2) allopregnanolone triggered a single, sustained rise in $[Ca^{2+}]_i$ (18/85 neurons, 21%) with a plateau phase, decaying very slowly throughout the measuring period (see, for example, 1 nM in Figs. 1*A*, 3*A*, and 6*A*).

Figure 1*A* shows the increases in $[Ca^{2+}]_i$ induced by increasing concentrations of allopregnanolone. In six neurons tested, a significant rise in $[Ca^{2+}]_i$ is observed by steroid concentrations as low as 1 pM. Allopregnanolone elicits a concentration-dependent increase in $[Ca^{2+}]_i$ within the range of 1 pM to 100 nM. Maximal response is obtained at 100 nM, and the estimated EC_{50} value for allopregnanolone is 10 ± 4 nM (Fig. 1*B*).

Successive applications of allopregnanolone evoked a similar $[Ca^{2+}]_i$ rise. Figure 2 shows the $[Ca^{2+}]_i$ rise in response to two repetitive applications of 1 nM allopregnanolone. Four neurons tested displayed similar profiles. This result allowed us to use for some experiments the same neuron as its own control, thus diminishing the effect of individual variation among neurons.

Blockade of allopregnanolone-induced changes in $[Ca^{2+}]_i$

To investigate whether the allopregnanolone-induced $[Ca^{2+}]_i$ rise was dependent on the presence of external Ca^{2+}, allopregnanolone-sensitive neurons that showed a rise in $[Ca^{2+}]_i$ in the presence of 1.8 mM Ca^{2+} were treated with the steroid in EGTA buffer. Figure 3*A* shows that the allopregnanolone-induced $[Ca^{2+}]_i$ rise was reduced significantly in the presence of EGTA buffer. In the presence or absence of Ca^{2+} in the external medium, the $[Ca^{2+}]_i$ rise induced by 10 nM allopregnanolone was 466 ± 32 and 178 ± 10 nM, respectively ($p < 0.01$, $n = 9$). As shown in Figure 3*A* (*right*), there was still a small residual response to allopregnanolone in the absence of external Ca^{2+}, and this was observed consistently in all nine neurons tested. To examine whether the Ca^{2+} influx could involve voltage-sensitive Ca^{2+} channels, we tested the L-type, dihydropyridine-sensitive Ca^{2+} channel blocker nifedipine (Fig. 3*B*). Nifedipine (10 μM) applied 30 sec before and during the application of 10 nM allopregnanolone diminished by 75 ± 12% ($n = 8$) the calcium rise induced by allopregnanolone: 133 ± 23 vs 545 ± 43 nM (control) ($p < 0.01$, $n = 8$). These experiments, with or without nifedipine, were performed on the same neurons.

Specificity of allopregnanolone effect on $[Ca^{2+}]_i$

Two different approaches were used to define pharmacologically the steroid specificity. First, we investigated the ability of two other steroids (progesterone and 17β-estradiol) to increase $[Ca^{2+}]_i$ in the hypothalamic neurons. Progesterone (10 nM to 1 μM) had no significant effect on $[Ca^{2+}]_i$. In these experiments, the mean $[Ca^{2+}]_i$ values obtained in the absence or presence of 10 nM progesterone were 89

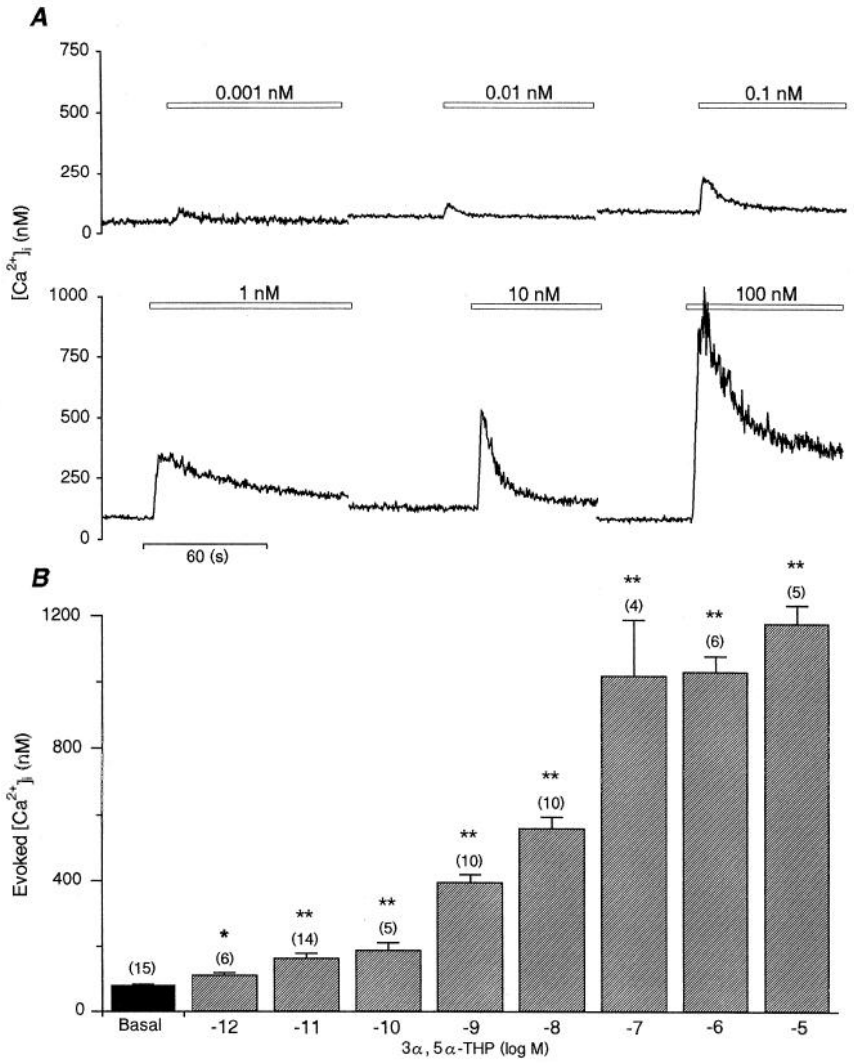

Figure 1. Dose-dependent effect of allopregnanolone (*3α,5α-THP*). *A*, *Traces* represent changes in $[Ca^{2+}]_i$ induced by increasing concentrations of allopregnanolone (0.001–100 nM) measured in a single neuron. Each stimulus was separated by 5 min intermission after washing twice in normal Locke's buffer. *B* shows the dose–response relationship of evoked $[Ca^{2+}]_i$ increase (mean ± SEM) as a function of allopregnanolone concentration. Data were obtained from several neurons (sample size is given in brackets) for a given concentration tested randomly. $^*p < 0.01$; $^{**}p < 0.001$ vs control without allopregnanolone.

± 9 and 106 ± 5 nM, respectively ($p > 0.05$, $n = 7$). Similarly, progesterone applied at higher concentration did not significantly affect the $[Ca^{2+}]_i$ levels ($p > 0.05$, $n = 6$), which ranged between 79 ± 16 nM (control) and 88 ± 13 nM (1 μM progesterone). Interestingly, these nonresponding neurons to progesterone exhibited a remarkable $[Ca^{2+}]_i$ rise to 10 nM allopregnanolone (mean peak value: 533 ± 96 nM, $n = 6$). In contrast, 17β-estradiol evoked $[Ca^{2+}]_i$ increase to a lesser extent than allopregnanolone: 17β-estradiol (100 nM)-induced $[Ca^{2+}]_i$ rise peaked at 288 ± 56 nM ($n = 10$) vs 1022 ± 20 nM ($n = 8$) ($p < 0.01$) induced by 100 nM allopregnanolone. The second paradigm consisted of examining the effect of the β-hydroxylisomer of allopregnanolone. Figure 4 shows that 3β-hydroxy-5α-pregnan-20-one used at the same concentration of that of the 3α-isomer (1 nM) had no effect on $[Ca^{2+}]_i$. However, after washing in Locke buffer the same neuron showed a marked response to allopregnanolone. The resting $[Ca^{2+}]_i$ was unchanged after addition of 3β-hydroxy-5α-pregnan-20-one (68 ± 7 nM), whereas in the same neurons 3α-hydroxy-5α-pregnan-20-one augmented $[Ca^{2+}]_i$ up to 327 ± 37 nM ($n = 7$).

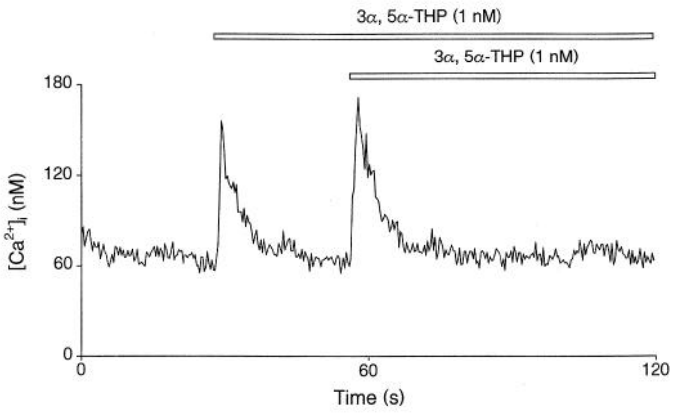

Figure 2. Successive allopregnanolone (*3α,5α-THP*) applications on $[Ca^{2+}]_i$ increase. A selected neuron was subjected to two successive applications of allopregnanolone (1 nM). The resting $[Ca^{2+}]_i$ had returned to prestimulation level before the second application. Note that the amplitude of $[Ca^{2+}]_i$ response induced by allopregnanolone is unchanged. Four neurons displayed similar profiles.

Lack of effect of PTX on allopregnanolone-induced $[Ca^{2+}]_i$

Hypothalamic neurons were incubated for 20 hr with 100 ng/ml PTX before loading with Fura-2 AM. $[Ca^{2+}]_i$ measurements also were carried out in the presence of PTX, which had no significant effect ($p > 0.05$) on allopregnanolone (10 nM)-induced $[Ca^{2+}]_i$ rise (Fig. 5).

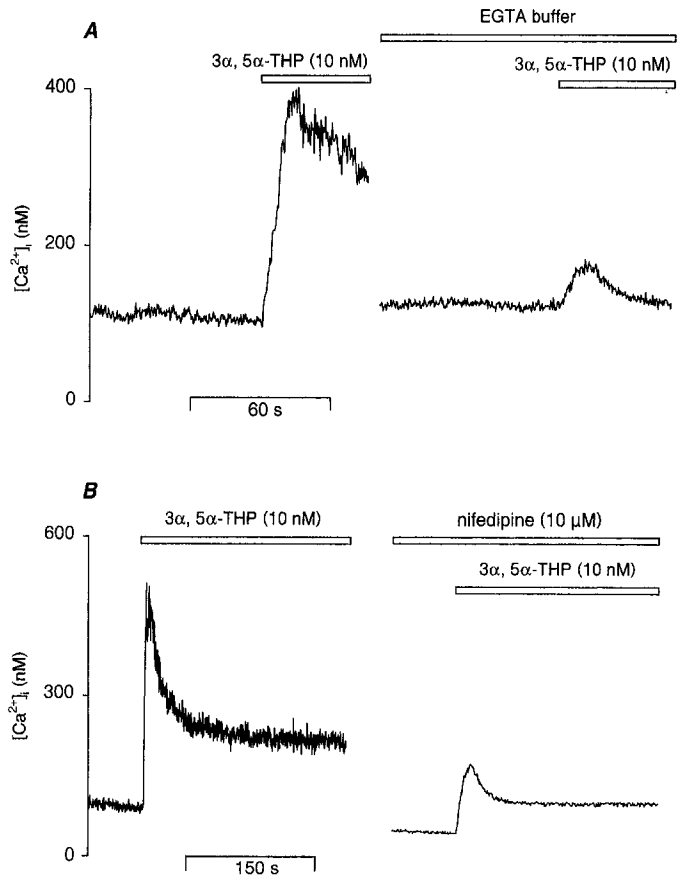

Figure 3. Effect of external Ca^{2+} on $[Ca^{2+}]_i$ increase induced by allopregnanolone (*3α,5α-THP*). *A*, The *left* panel represents the $[Ca^{2+}]_i$ response induced by 10 nM allopregnanolone in the presence of Locke's buffer containing 1.8 mM external Ca^{2+}. Preincubation of the same neuron in low Ca^{2+}-EGTA buffer (see Materials and Methods) significantly abolished $[Ca^{2+}]_i$ increase induced by allopregnanolone (*right*). Nine neurons displayed similar profiles when tested under similar conditions. *B*, Effect of L-type channel blocker, nifedipine, on $[Ca^{2+}]_i$ increase induced by allopregnanolone (*3α,5α-THP*). *Left*, Control $[Ca^{2+}]_i$ response induced by 10 nM allopregnanolone. *Right*, After washing, the same neuron was preincubated for 30 sec with 10 μM nifedipine and then subjected to 10 nM allopregnanolone. The $[Ca^{2+}]_i$ response induced by allopregnanolone is markedly decreased by the L-channel blocker. Nine neurons displayed similar profiles when tested under similar conditions.

Inhibitory effect of picrotoxin and bicuculline on allopregnanolone-induced $[Ca^{2+}]_i$

In an attempt to determine whether the $[Ca^{2+}]_i$ rise induced by allopregnanolone involved $GABA_A$ receptor participation, we performed a series of experiments with and without picrotoxin or bicuculline (Fig. 6) using the same neurons as their own control. Picrotoxin (10 μM) applied simultaneously with 10 nM allopregnanolone inhibited by 65 ± 7% the intracellular Ca^{2+} response to allopregnanolone: 422 ± 41 vs 150 ± 27 nM ($p < 0.01$, $n = 11$) (Fig. 6*A*). Bicuculline (50 μM) applied simultaneously with 10 nM allopregnanolone completely blocked (Fig. 6*B*) the peak of $[Ca^{2+}]_i$ rise induced by allopregnanolone: 434 ± 55 vs 47 ± 5 nM ($n = 8$) (Fig. 6*B*).

DISCUSSION

This is, to our knowledge, the first study showing very rapid stimulatory effect of a neurosteroid, allopregnanolone, on cytosolic free Ca^{2+} in central nervous system neurons. The peak was reached within 2–3 sec after allopregnanolone application and is one of the most rapid membrane effects of a steroid to be reported. It may be comparable with that observed for the activation of calcium channels by progesterone in plasma membranes of human sperm (Blackmore et al., 1990), or with the effect of steroids on electrical activity of nerve cells occurring when applied locally (Robel and Baulieu, 1990; McEwen, 1991a).

Our results suggest that allopregnanolone-induced $[Ca^{2+}]_i$ rise is dependent mainly on the presence of extracellular Ca^{2+}. Indeed, EGTA buffer strongly reduced the increased response to allopregnanolone. In addition, ~70% of the allopregnanolone-induced increase in $[Ca^{2+}]_i$ is inhibited by nifedipine, suggesting that part of the calcium influx occurs via voltage-gated calcium channels of L-type, which have been described in these cells (Tapia-Arancibia and Humbert, 1991; Desarménien et al., 1994; Dayanithi et al., 1995). These experiments also clearly show that there is still a residual $[Ca^{2+}]_i$ response to allopregnanolone. This response may be attributable to the involvement of intracellular Ca^{2+} stores (Simpson et al., 1995) or to the involvement of other voltage-activated Ca^{2+} channel types (Hofman et al., 1994). Further experiments are necessary to characterize this residual response to allopregnanolone. However, $GABA_A$ receptors seem to be involved in the $[Ca^{2+}]_i$ rise induced by allopregnanolone, as suggested by the inhibitory effect of picrotoxin and bicuculline.

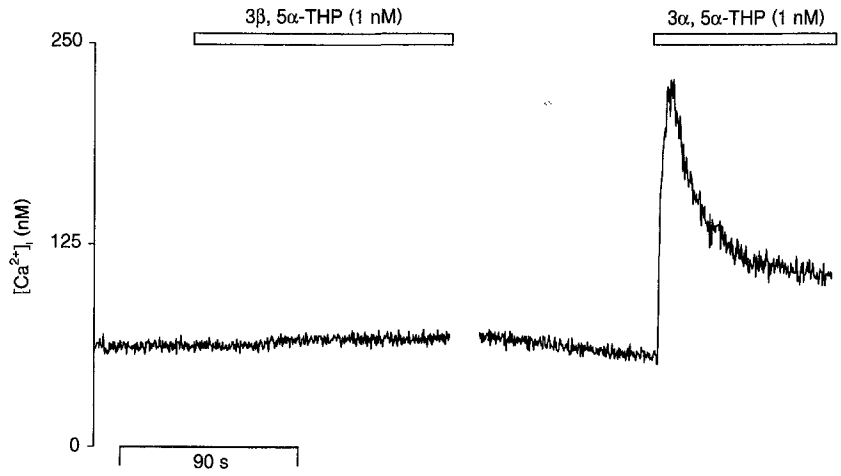

Figure 4. Specificity of allopregnanolone (*3α,5α-THP*) on $[Ca^{2+}]_i$ rise. *Left*, A selected neuron was subjected first to the isomer 3β,5α-THP (1 nM). *Right*, After washing, the same neuron exhibited a marked $[Ca^{2+}]_i$ increase when the isomer 3α,5α-THP (1 nM) was applied. Seven neurons displayed similar profiles.

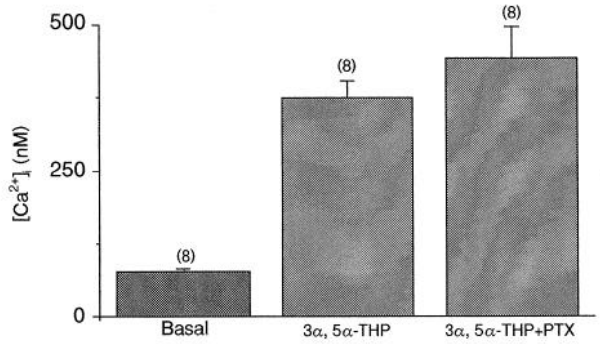

Figure 5. Lack of effect of PTX on 3α,5α-THP-induced $[Ca^{2+}]_i$ rise. The culture dishes were incubated for 20 hr with ($n = 8$) or without ($n = 8$) 100 ng/ml PTX and then were tested with 10 nM allopregnanolone. Diagram represents basal levels of $[Ca^{2+}]_i$ and allopregnanolone-evoked $[Ca^{2+}]_i$ increase in control neurons, and the response to allopregnanolone in the presence of PTX in PTX-treated neurons.

This is consistent with a potentiation by allopregnanolone of a $GABA_A$-mediated depolarization of neurons that secondarily may allow opening of voltage-sensitive calcium channels. We have shown previously, using the same experimental model, that GABA is released tonically in the incubation medium from hypothalamic neurons (Rage et al., 1992, 1993). According to this interpretation, it has been shown that in developing cortical neurons (Yuste and Katz, 1991), in cultured cerebellar granule cells (Connor et al., 1987), and in hippocampal neurons in the early postnatal life (Cherubini et al., 1991) GABA is able to depolarize cells.

The allopregnanolone-induced $[Ca^{2+}]_i$ rise is dose-dependent, saturable, stereoselective, and specific because it is not mimicked by the 3β-hydroxy isomer or by progesterone. A significant effect is produced by concentrations as low as 1 pM with a maximum at 100 nM, allowing us to envisage that such an effect might be physiologically relevant if it were corroborated in adult rats. In fact, during proestrus in female rats, plasma progesterone levels may rise to micromolar concentrations (Smith et al., 1975). Furthermore, allopregnanolone and allotetrahydrodeoxycorticosterone plasma levels rise during stress from concentrations lower than nanomolars to reach concentrations near of 10 nM (Purdy et al., 1991). Moreover, enzymes in the brain also can generate active metabolites from the parent steroids (i.e., progesterone), and local concentrations of the neuroactive steroids may reach high nanomolar concentrations because of the contribution from the local production. Thus, the progesterone concentration in the brain of normal rats can oscillate between 5 and 22 nM (Purdy et al., 1991; Korneyev et al., 1993) and can increase up to 55–65 nM after electroshock (Korneyev et al., 1993) and up to 20–42 nM after swimming stress (Purdy et al., 1991). Allopregnanolone concentration in normal rat brain is 2–4 nM and can increase up to 10–20 nM after swimming stress (Purdy et al., 1991) or up to 4–9 nM after acute electroshock (Korneyev et al., 1993). Because of the range of concentrations of progesterone and metabolites measured in plasma and brain, it seems reasonable to envisage that if the effect reported here is corroborated *in vivo* in adult rats, it could have a physiological relevance in some of these particular conditions.

It is well established that local target tissue metabolism is an important factor in the mechanism of action of steroid hormones. 5α-Dihydroprogesterone and allopregnanolone are the primary metabolites of progesterone accumulating in adult (Karavolas and Hodges, 1990) and fetal brain (Barnea et al., 1990). The site of formation of allopregnanolone is predominantly the brain (Corpéchot et al., 1993), and the enzyme activities that catalyze these

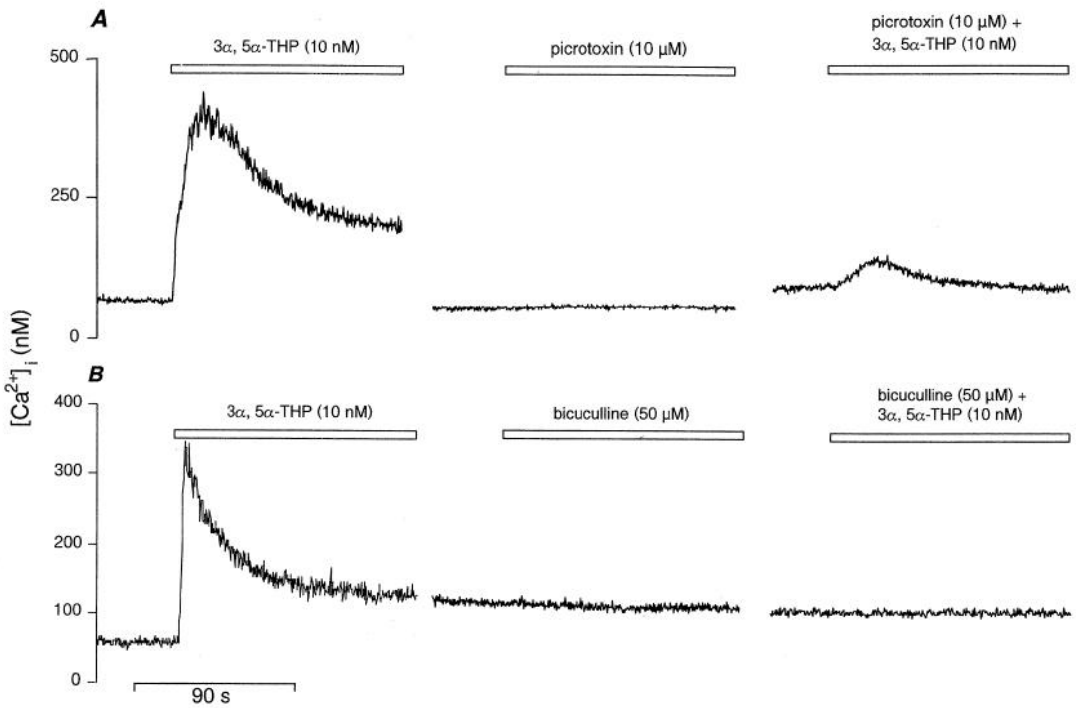

Figure 6. Effect of picrotoxin (*A*) and bicuculline (*B*) on 3α,5α-THP-induced $[Ca^{2+}]_i$ rise. *A*, *B*: *Left*, Control $[Ca^{2+}]_i$ responses induced by 10 nM allopregnanolone. *Middle*, When applied alone, 10 μM picrotoxin (*A*) and 50 μM bicuculline (*B*) did not significantly modify resting $[Ca^{2+}]_i$ level. *Right*, $[Ca^{2+}]_i$ responses evoked by simultaneous application of 10 μM picrotoxin plus 10 nM allopregnanolone (*A*) and 50 μM bicuculline plus 10 nM allopregnanolone (*B*).

conversions are expressed strongly in the hypothalamus and anterior pituitary (Karavolas and Hodges, 1990). Both 5α-reductase and 3α-reductase activities are affected by the estrus cycle with peak activities at proestrus, estrus, and metestrus (Karavolas and Hodges, 1990). In addition, the hypothalamus and the anterior pituitary specifically accumulate significant amounts of radiolabeled 5α-dihydroprogesterone and progesterone, the two substances being the precursors of allopregnanolone. [^{3}H]progesterone also is metabolized to 5α-dihydroprogesterone and allopregnanolone in male rat hypothalamic slices (Korneyev et al., 1993). These data clearly show that the adult rat hypothalamus is equipped with the enzymes and substrates for the synthesis of neurosteroids, and these also may be modulated *in vivo* by the ovarian cycle in the female.

Neurotransmitter and neurohormone release depend on calcium, and the modulation of calcium availability within the cell (either an increase in immature tissue or a decrease in adult tissue) may represent an important mechanism of action of active steroids at the cell surface. However, few reports are available on the regulatory effect of neurosteroids on calcium conductances or calcium concentrations in the central nervous system. Whole-cell patch-clamp studies in freshly isolated adult guinea pig hippocampal CA1 pyramidal neurons show that micromolar concentrations of allotetrahydrodeoxycorticosterone, dehydroepiandrosterone sulfate, and pregnanolone depress voltage-gated calcium currents (ffrench-Mullen and Spence, 1991). This effect also is reproduced by pregnenolone and pregnenolone sulfate but not by progesterone (Spence et al., 1991). This inhibition of calcium currents by the steroids seem to be mediated by a PTX-sensitive G-protein associated with the activation of PKC (ffrench-Mullen et al., 1994). But in cultured rat hippocampal neurons, high concentrations of pregnenolone sulfate failed to alter basal $[Ca^{2+}]_i$ or to modify the K^+-induced $[Ca^{2+}]_i$ rise, as measured by microspectrofluorimetry and the calcium-sensitive indicator Fura-2. However, at these concentrations (5–250 μM), the steroid potentiated the NMDA-induced rise of $[Ca^{2+}]_i$ in a dose-dependent manner (Irwin et al., 1992).

The possible functional significance that in developing neurons allopregnanolone may potentiate GABA-induced depolarization leading to an increased Ca^{2+} influx is not clear. It is possible that it is involved in the development or modification of inhibitory synapses in hypothalamic circuits.

In conclusion, the present data are consistent with the presence of a receptor or recognition site for allopregnanolone at or close to the cell surface, probably associated to $GABA_A$ receptors, leading to changes in $[Ca^{2+}]_i$ in hypothalamic neurons. These findings may open up entirely new areas of investigation, because rapid calcium changes induced by neurosteroids may represent a link of the metabolic cascade whereby hypothalamic neurons control the short-term process of release.

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