Estradiol Reduces Calcium Currents in Rat Neostriatal Neurons via a Membrane Receptor

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Until recently, steroid hormones were believed to act only on cells containing intracellular receptors. However, recent evidence suggests that steroids have specific and rapid effects at the cellular membrane. Using whole-cell patch-clamp techniques, 17β-estradiol was found to reduce Ba^{2+} entry reversibly via Ca^{2+} channels in acutely dissociated and cultured neostriatal neurons. The effects were sex-specific, i.e., the reduction of Ba^{2+} currents was greater in neurons taken from female rats. 17β-Estradiol primarily targeted L-type currents, and their inhibition was detected reliably within seconds of administration. The maximum reduction by 17β-estradiol occurred at picomolar concentrations. 17β-Estradiol conjugated to bovine serum albumin also reduced Ba^{2+} currents, suggesting that the effect occurs at the membrane surface. Dialysis with GTPγS prevented reversal of the modulation, suggesting that 17β-estradiol acts via G-protein activation. 17α-Estradiol also reduced Ba^{2+} currents but was significantly less effective than 17β-estradiol. Estriol and 4-hydroxyestradiol were found to reduce Ba^{2+} currents with similar efficacy to 17β-estradiol, whereas estrone and 2-methoxyestriol were less effective. Tamoxifen also reduced Ba^{2+} currents but did not occlude the effect of 17β-estradiol. These results suggest that at physiological concentrations, 17β-estradiol can have immediate actions on neostriatal neurons via nongenomic signaling pathways.

Key words: estradiol; neostriatum; patch clamp; Ca^{2+} current; estrogen receptor; neuromodulation; steroid; nongenomic

Estradiol, as well as other steroid hormones, is known to alter gene transcription via intracellular receptors (Pfaff, 1980; Pfaff and Schwartz-Giblin, 1988; Carson-Jurica et al., 1990). As expected, estrogen receptors are found in regions often associated with reproduction, such as the mammalian hypothalamus and uterus (Stancel et al., 1973; Stumpf and Sar, 1978; McEwen, 1979). However, steroid hormones also act in regions that do not contain intracellular steroid receptors. These effects often are too fast to be attributed to genomic activation and are believed to occur via stimulation of membrane receptors.

One region in which a membrane receptor may be important is the brain, where estradiol has rapid effects that are not readily attributable to traditional steroid receptors. For example, estradiol decreases the spontaneous firing of medial preoptic area neurons (Kelly et al., 1977) and increases the firing rate of pituitary cells (Dufy et al., 1979). Estradiol also potentiates EPSPs in the hippocampus (Wong and Moss, 1991, 1992) and Glu responses in the cerebellum (Smith et al., 1987, 1989) while hyperpolarizing medial amygdala neurons (Nabekura et al., 1986; Minami et al., 1990). In the guinea pig, estrogen suppresses μ-opioid- and $GABA_B$-based hyperpolarization of

hypothalamic arcuate neurons (Kelly et al., 1992). Moreover, estrogen directly potentiates K^+-stimulated dopamine release in rat nucleus accumbens (Thompson and Moss, 1994).

Another target of estrogen that lacks intracellular estrogen receptors is the adult neostriatum (Pfaff and Keiner, 1973; Stumpf and Sar, 1978; Simerly et al., 1990). Administration of estradiol in vivo potentiates the induced release of dopamine and rotational behavior (Becker, 1990a,b; Castner et al., 1993). Pulsatile administration of estradiol in vitro rapidly stimulates neostriatal dopamine release (Becker et al., 1990a). Estrogen pretreatment increases the firing rate of neostriatal neurons in response to dopamine (Arnauld et al., 1981), possibly via changes in D_1/D_2 dopamine-receptor coupling (Demotes-Mainard et al., 1990) or dopamine-receptor binding (Gordon and Perry, 1983; Clopton and Gordon, 1986; Levesque and Di Paolo, 1988; Tonnaer et al., 1989; Bazzett and Becker, 1994). Moreover, estrogen implanted directly into the neostriatum enhances sensorimotor performance (Becker et al., 1987). Many of these effects of estradiol on the neostriatum are rapid, with the earliest measured changes occurring within 15 min of steroid administration (Levesque and Di Paolo, 1988).

Although the actions of estradiol on neostriatal functioning are widespread, the mechanism by which estradiol acts is unclear. Intracellular estrogen receptors have been reported in the neostriatum of newborn rat pups (Toran-Allerand et al., 1992), but they have not been reported in the adult. Moreover, the effects of estradiol within the neostriatum are too fast to be attributed to genome activation. The most parsimonious hypothesis is that estradiol affects the neostriatum via a membrane receptor. To test this, estradiol was applied to isolated neostriatal neurons while measuring voltage-gated Ba^{2+} currents. Ba^{2+} currents through Ca^{2+} channels were chosen as an assay because of
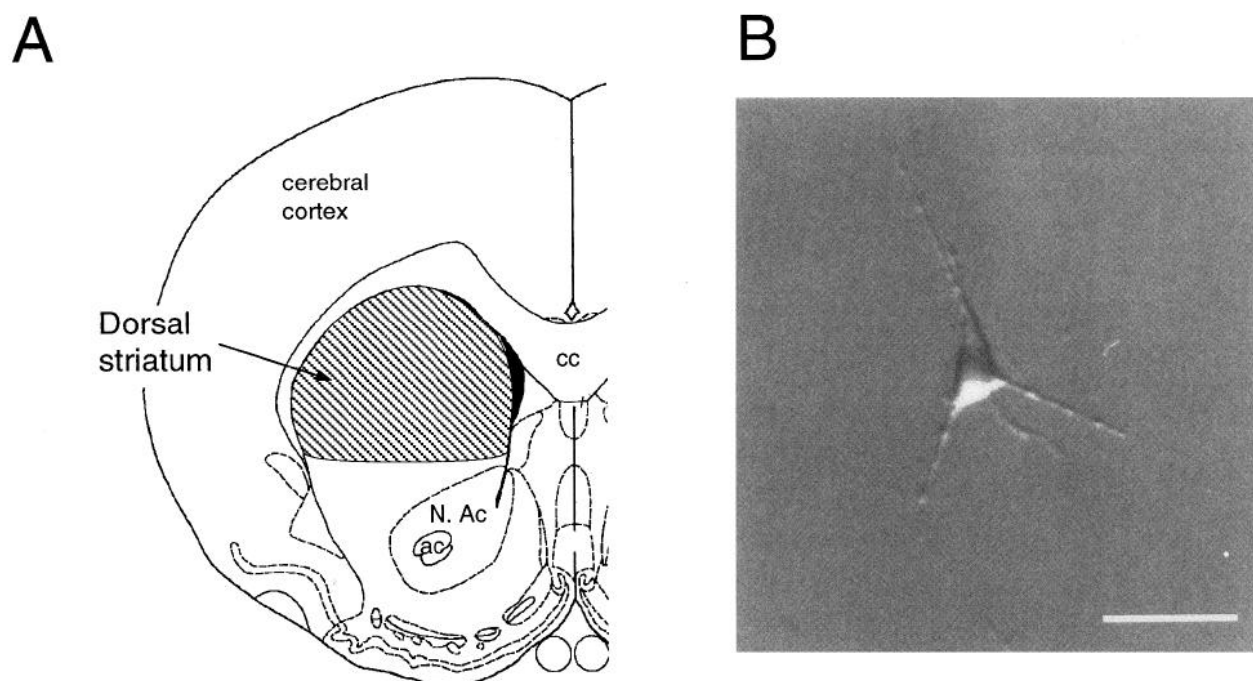
Figure 1. Recordings were taken from acutely isolated neurons of the dorsal neostriatum. *A*, Diagram of a coronal section through adult rat brain illustrating the neostriatal region isolated and dissociated for electrophysiological recording (adapted from Paxinos and Watson, 1986). *B*, Photomicrograph of a typical medium spiny neuron acquired via acute dissociation. Scale bar, 50 μm.
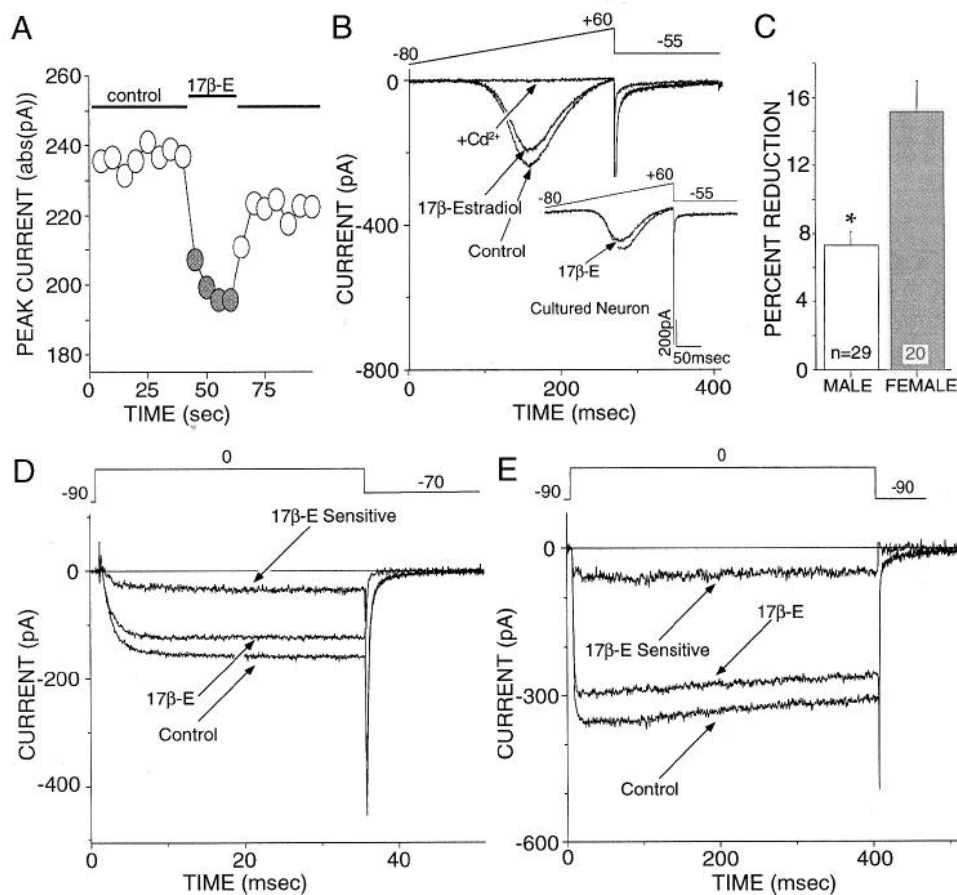
Figure 2. 17β-Estradiol inhibited Ba^{2+} currents in rat neostriatal neurons. *A*, Peak Ba^{2+} current measured in a neostriatal neuron taken from an 8-week-old female rat. 17β-Estradiol (*17β-E*; 100 pM) reduced peak current within seconds of administration. Episodes were obtained from a voltage-ramp protocol repeated once every 5 sec. *B*, Example traces taken from the time course illustrated in *A*. Cadmium (*+Cd^{2+}*; 200 μM) inhibits all current, demonstrating that only Ca^{2+} currents are being measured. *Inset*, 17β-Estradiol (1 pM) also was found to reduce the Ba^{2+} current in primary cultured neostriatal neurons (10 mM Ba^{2+} used as the charge carrier), suggesting that the steroid effect is not a function of the acute dissociation. *C*, Estradiol causes a larger inhibition of Ba^{2+} currents in 4-week-old females than in male rat neostriatal neurons ($p < 0.0001$; concentrations of 17β-estradiol were ≥1 pM). *D, E*, The current sensitive to 17β-estradiol (1 pM) activated relatively rapidly and inactivated slowly.
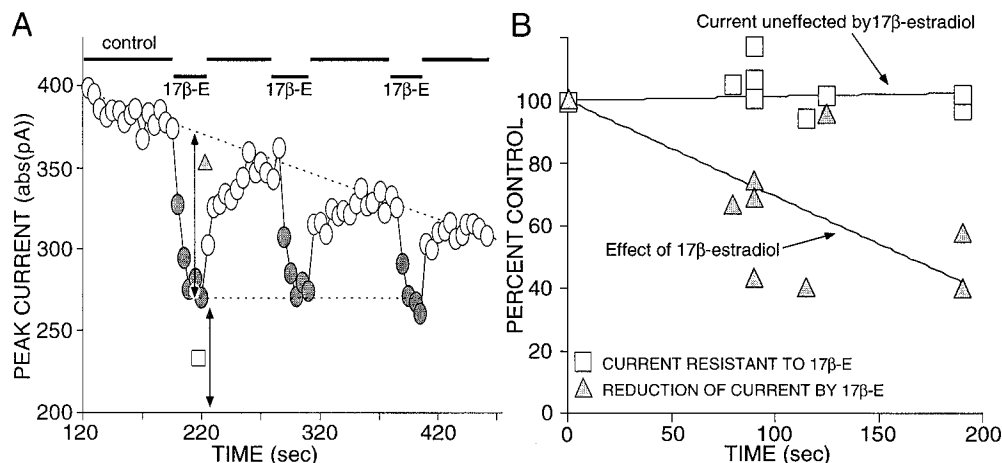
Figure 3. The modulation by 17β-estradiol diminished with repeated application as a consequence of rundown of the estradiol-sensitive current. *A,* Plot of peak current as a function of time. 17β-Estradiol was applied successively. The magnitude of the modulation (*filled triangles*) was estimated from a line fit to the control records (*dotted line*). The "resistant" current amplitude was defined as the amplitude of the current remaining in the presence of estradiol (*open squares*). *B,* Plot of modulated (*filled triangles*) and resistant current (*open squares*) for the time course in *A* and for five other neurons. Note that the resistant current did not change with time, suggesting that the loss of the responsiveness to estradiol was a consequence of rundown in the estradiol-sensitive current.

the importance of these channels in several cellular functions shown to be regulated by estrogen. The experiments reported in this study show that at physiologically relevant concentrations, 17β-estradiol acts in a steroid-specific manner to activate a G-protein-coupled membrane receptor that reduces L-type currents in rat neostriatal neurons. Furthermore, the effects were sex-specific, being larger in neurons taken from females than males.

MATERIALS AND METHODS

Acute-dissociation procedure. In most experiments, neostriatal neurons from prepubertal Sprague–Dawley female rats (4 weeks) were acutely dissociated using methods similar to those described previously (Surmeier et al., 1991, 1992). Neostriatal neurons also were taken from male rats of the same age and adult female ~8-week-old rats. Neostriatal neurons cultured from neonatal tissue also were studied. Results from neurons taken from one of these other preparations are so noted. For the acute dissociation, rats were anesthetized deeply with methoxyflurane and then decapitated. Brains were removed rapidly, blocked, sliced, and placed into a 4°C sucrose solution (in mM): 250 sucrose, 2.5 KCl, 1 NaHPO$_4$ (7 H$_2$0), 11 glucose, 2 MgSO$_4$, 2 CaCl$_2$, 15 HEPES, pH 7.4, 300 mOsm/l. Tissue was cut (400 μm slices) with a Vibroslice (Campden Instruments, London, UK) while immersed in a 4°C low-Ca^{2+} (100 μM) HEPES-buffered salt solution (in mM): 140 Na-isothionate, 2 KCl, 4 MgCl$_2$, 0.1 CaCl$_2$, 23 glucose, 15 HEPES, pH 7.4, 300 mOsm/l. Slices were incubated in a Cell-Stir chamber (Wheaton, Millville, NJ) from 1 to 4 hr in a Earle's balanced salt solution (Sigma, St. Louis, MO) containing 100 μM ascorbic acid (Sigma) and 100 μM N^G-n-L-arginine (Sigma) while bubbled with 95% O$_2$/5% CO$_2$ at room temperature. After removing slices from the first

holding chamber, regions of the dorsal neostriatum were isolated under a dissecting microscope. Dissections were limited to regions rostral to the decussation of the anterior commissure, avoiding contamination from the globus pallidus (Fig. 1*A*). The dissected regions of the neostriatum then were placed into a second holding chamber containing 1.5 mg/ml protease (type XIV, Sigma) in HBSS (Sigma) at 35°C. The enzyme chamber also contained 100 μM ascorbic acid and 100 μM N^G-n-L-arginine and was bubbled with 100% O$_2$. After 30 min of enzyme treatment, tissue was washed in low-Ca^{2+} HEPES-buffered saline and mechanically dissociated with a graded series of fire-polished Pasteur pipettes. The cell suspension (~2 ml) then was plated into a 35 mm Lux Petri dish (Nunc, Naperville, IL), mounted on the stage of an inverted microscope, and allowed to settle.

Primary culture. For dissociation and subsequent culture of embryonic day 17 neostriatal neurons, we followed procedures described previously (Surmeier et al., 1988). Briefly, timed-pregnant female rats were anesthetized with methoxyflurane and cervically dislocated. The brains of pups were removed and placed into a 4°C Ca^{2+}-, Mg^{2+}-free HBSS solution. Striata were removed under a dissecting microscope, washed, and placed into culture media (1:1 Ham's F12/EMEM, Gibco, Grand Island, NY) supplemented with fetal calf serum (10%, HyClone, Logan, UT), insulin (5 mg/ml), penicillin (50 U/ml), and streptomycin (50 mg/ml; latter three from Sigma). The striata were triturated using a graded series of fire-polished Pasteur pipettes and then filtered to remove debris. Viability was determined with a trypan blue exclusion test. The cells were plated into Lux dishes at a density of 1–2 × 10^6 cells/dish. Cultures then were incubated in a 95% humidity/5% CO$_2$ atmosphere and fed twice/week with a basal media supplemented with 5% fetal calf serum and 5% horse serum (HyClone) until recording (~1 week after plating). Before recording, the culture dishes were washed for 20 min with steroid-free bath solution. The same recording
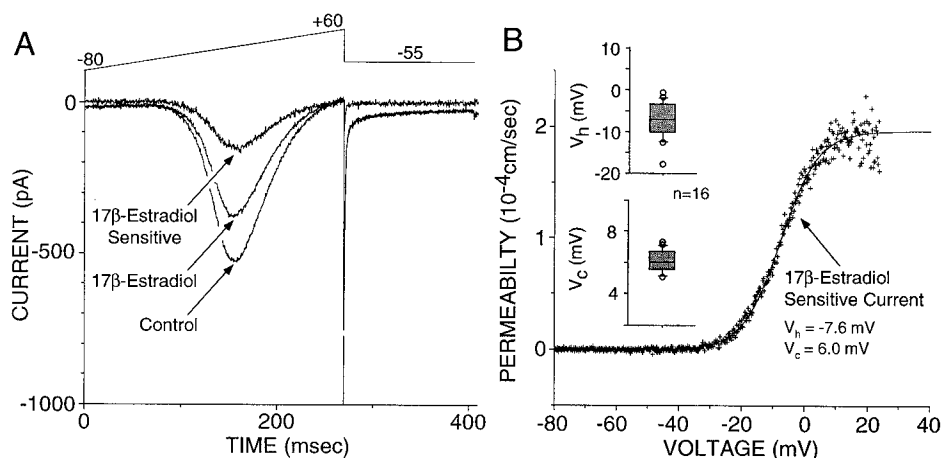
Figure 4. The voltage dependence of the Ba^{2+} current sensitive to 17β-estradiol indicated that the steroid inhibited a high-voltage-activated current. *A,* Administration of 17β-estradiol (100 pM) caused a reduction of Ba^{2+} current. The current sensitive to 17β-estradiol modulation was used to derive the permeability estimates shown in *B. B,* Permeability estimates were fit with a Boltzmann function. The variation in slope factor and half-maximal activation observed for the 16 neurons examined are shown in the *inset box plots.*

protocols were used for cultured neurons as for neurons obtained via acute dissociation.

Whole-cell recordings. Whole-cell recordings were performed using standard techniques (Hamill et al., 1981; Surmeier et al., 1995). Electrodes were pulled from Corning 7052 glass (Corning, NY) and fire-polished just before use. The internal recording solution typically consisted of (in mM): 190 N-methyl-D-glucamine (NMG), 40 HEPES, 4 MgCl$_2$, 5 BAPTA, 12 phosphocreatine, 2 Na$_2$ATP, 0.2 Na$_3$-GTP or 0.4 Na$_3$-GTPγS, 0.1 leupeptin, pH-adjusted to 7.2–7.3 with H$_3$SO$_4$, 265–275 mOsm/l [all obtained from Sigma except H$_3$SO$_4$, which was obtained from Fluka (Ronkonkoma, NY)]. Intracellular solutions of 0.1 and 10 mM BAPTA also were used occasionally with no apparent effect on the response by 17β-estradiol. The external recording solution consisted of (in mM): 135 NaCl, 20 CsCl, 1 MgCl$_2$, 10 HEPES, 0.001 tetrodotoxin (TTX), 5 BaCl$_2$, pH-adjusted to 7.35 with NaOH, 300–309 mOsm/l. Drugs were applied with a gravity-fed "sewer-pipe" system. The six application capillaries (~150 μm inner diameter) that made up the drug array were positioned a few hundred micrometers from the recorded cell. Solution changes were effected by altering the position of the array with a DC-drive system, manipulated by a PC-based controller (Newport-Klinger, La Jolla, CA). Solution changes were complete within 1–2 sec, as measured in (our) previous experiments by changes in reversal potential. Potentials were not corrected for the liquid-junction potential with the NMG-SO$_4$ internal, which was ~7 mV. Recordings were performed at room temperature.

17β-Estradiol (Sigma or Research Biochemicals, Natick, MA) was diluted in dimethylsulfoxide (DMSO; Sigma) as a concentrated stock (1 mM) before each experiment and then serially diluted to its final concentration. Final concentration of DMSO typically was ≤10^{-6}% (final concentration when using 100 pM estradiol). We have found DMSO concentrations up to 0.01% to have no effect on neostriatal Ba^{2+} currents but, as a precaution, external media (including all control solutions) in initial experiments contained matched DMSO concentrations; solutions always were matched when final concentrations of DMSO were ≥10^{-5}%. In later experiments (including the occlusion studies performed with the Ca^{2+}-channel blockers), extracellular recording solutions also contained 0.01% cytochrome c. Cytochrome c was found to help keep the steroids in solution, resulting in a greater inhibition of Ba^{2+} current. Similar procedures were used when using 17α-estradiol, tamoxifen, estrone, estriol, 2-methoxyestriol, and 4-hydroxyestradiol (Sigma). Comparisons between 17α- and 17β-estradiol were performed between neostriatal neurons; all other comparisons were done within the same cell with the order of steroid treatment randomly assigned. Phenol-red usually was added to several of the recording solutions as a pH indicator. Although phenol-red can have weak estrogenic effects via activation of the intracellular estrogen receptor in cell cultures (Berthois et al., 1986), removal of phenol-red in our solutions had no effect on estradiol reduction of Ba^{2+} currents. Estradiol conjugated to bovine serum albumin (E-BSA; Sigma) was made up as a concentrated stock (52 mM) in a standard Tris-buffer, aliquoted, and frozen (−20°C) until use. The concentration of BSA was matched in all drug solutions when using E-BSA. For occlusion experiments, ω-agatoxin-IVA (ω-AgTx-IVA; Peptides International, Louisville, KY), ω-conotoxin-GVIA (ω-CTx-GVIA; Peninsula Labs, Belmont, CA), and ω-conotoxin-MVIIC (ω-CTx-MVIIC; Neurex, Menlo Park, CA) were made up as a concentrated stock (0.100, 1, and 200 mM, respectively), aliquoted, and then diluted into external media. Nifedipine (Research Biochemicals) was dissolved as a concentrated stock (1 mM) in 95% ethanol and then diluted. In experiments with nifedipine, final ethanol concentrations never exceeded 0.1%. Ethanol concentrations were matched in all drug solutions.

Electrode resistances were ~3–6 MΩ in bath. Recordings were obtained with an Axon Instruments 200 voltage-clamp controlled and monitored with pClamp (version 5.0 or 6.0, Axon Instruments, Foster City, CA) running on a 486 PC clone with a 125 kHz interface (Axon). After formation of a >1 GΩ seal, the membrane was ruptured. The electrode series resistance (8–20 MΩ) was compensated (80–85%) electronically and monitored periodically. Neostriatal recordings were from medium spiny neurons (6–12 μm diameter, whole-cell capacitance 3–9 pF) that had short (<50 μm) proximal dendrites (see Fig. 1B). Voltage control was assessed by examining the tail currents after strong depolarizations. Cells in which the tail current did not decay rapidly and smoothly were discarded.

Electrophysiological data typically were gathered using voltage ramps (Bargas et al., 1994). With ramps, cells were held at −80 mV and

depolarized (0.5 mV/msec) to +60 mV, at which point the cell was rapidly repolarized to −55 mV (for ~100 msec). Ramp episodes were repeated once every 5 sec. Long (~400 msec) and short (~35 msec) steps also were used occasionally. Data analysis was performed on a Macintosh Quadra with either Axograph (version 2.0, Axon) or KaleidaGraph (version 3.0, Synergy Software, Reading, PA) software.

Statistics. Maximal reductions in Ba^{2+} currents in male and female neostriatal neurons were compared using a between-subjects *t* test. Similar
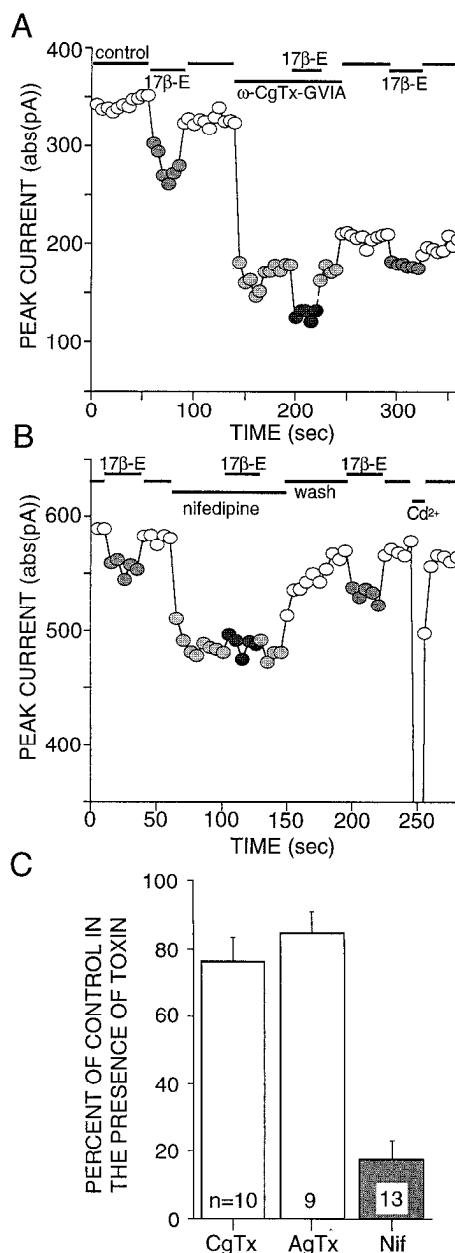

Figure 5. 17β-Estradiol (1–100 pM) inhibited primarily nifedipine-sensitive (L-type) neostriatal Ca^{2+} channels. *A,* In the presence of 1 μM ω-CTx-GVIA, estradiol was still effective in reducing Ba^{2+} current, suggesting that N-type currents were not inhibited prominently by estradiol. Similar results were observed in the presence of 100 nM ω-AgTx-IVA and 1 μM ω-CTx-MVIIC (data not shown), suggesting that P- and Q-type currents also were not the principal targets of 17β-estradiol. *B,* Nifedipine (5 μM), however, typically occluded the response of estradiol, suggesting that estradiol targeted primarily L-type Ca^{2+} channels. *C,* Statistical summary illustrating the ability of ω-CTx-GVIA (*CgTx*), ω-AgTx-IVA (*AgTx*), or nifedipine (*Nif*) to occlude the effect of 17β-estradiol. The toxin data suggest that ~80% of the current inhibited by estradiol was sensitive to nifedipine.

statistics were used to compare the effectiveness of 17α- and 17β-estradiol. The ability of estrone, estriol, 2-methoxyestriol, 4-hydroxyestradiol, and tamoxifen to reduce Ba^{2+} currents was compared with 17β-estradiol using a within-subjects t test. The percent recovery of estradiol block in the presence of GTPγS was compared with neurons dialyzed with normal GTP using a between-subjects t test; n values indicate the number of neurons tested.

RESULTS

17β-Estradiol reduces neostriatal Ba^{2+} currents

When applied directly to neostriatal neurons, 17β-estradiol decreased Ba^{2+} currents evoked by depolarization within seconds of administration but did not alter "resting" conductances at −80 mV. The modulation was readily reversible by washing. The effects of 17β-estradiol occurred at physiological concentrations (Fig. 2A). The reduction by 17β-estradiol on Ba^{2+} currents was observed in both acutely dissociated and primary cultured neostriatal neurons, suggesting that the enzymatic treatment of the acutely isolated cells was not responsible for the actions of the hormone (Fig. 2B). The magnitude of the Ba^{2+} current reduction by 17β-estradiol was similar in size to that observed when neostriatal dopamine or acetylcholine receptors are stimulated (Howe and Surmeier, 1995; Surmeier et al., 1995) and was in the range of effects observed when other neuromodulators are applied to neurons excised from central nervous system tissue.

The effects of 17β-estradiol were sex-dependent. Although neostriatal neurons taken from both male and female rats were affected by 17β-estradiol, the modulation was significantly greater ($p < 0.0001$) in tissue taken from female rats (15.11 ± 1.9%, n = 20 from 4 females vs 7.28 ± 0.8%, n = 29, 8 males; Fig. 2C). The current sensitive to 17β-estradiol activated rapidly (~3 msec) and displayed little to no inactivation with Ba^{2+} as the charge carrier (Fig. 2D,E).

Often, the reduction in peak current by 17β-estradiol diminished with repeated application. There are two potential explanations for this result: desensitization of the estrogen-signaling pathway, or nonspecific rundown of estrogen-sensitive currents. To help determine which mechanism was responsible, 17β-estradiol was applied at periodic intervals and the modulation was monitored. Shown in Figure 3A is a plot of peak current as a function of time in a neuron in which the modulation by estradiol diminished with repeated application. Two measurements were taken from this time course: the amplitude of the estradiol modulation and the amplitude of the "resistant" current (i.e., that portion of the current left in the

presence of 17β-estradiol). The measurements from this cell and from five others are plotted as a function of time in Figure 3B. This plot shows that although the 17β-estradiol tended to diminish with time, the resistant current did not, suggesting that rundown of the 17β-estradiol-sensitive current, rather than desensitization, was responsible for the change. We cannot exclude completely, however, the possibility that desensitization occurred in parallel with rundown. Of the currents expressed by neostriatal neurons, L-type currents are particularly susceptible to rundown, suggesting that these currents are targeted by the 17β-estradiol modulation.

17β-Estradiol modulates L-type Ca^{2+} channels

To characterize better the currents affected by estradiol, membrane-permeability estimates were generated using the Goldman–Hodgkin–Katz constant-current equation (Hille, 1994). The permeability estimates of the 17β-estradiol-sensitive current then were fit with a Boltzmann function (Fig. 4A,B). The steady-state voltage dependence of the estradiol-sensitive current was consistent with it being composed of N-, P-, or L-type currents (Bargas et al., 1994). To determine which of these currents was being modulated, occlusion experiments with channel-specific antagonists were performed. The P-type- and N-type-channel toxins ω-AgTx-IVA (100 nM) and ω-CTx-GVIA (1 μM) only partially occluded the effects of 17β-estradiol (Fig. 5A,C). Experiments using the mixed N- and Q-type Ca^{2+}-channel toxin ω-CTx-MVIIC (1 μM) yielded similar results (n = 4; data not shown). However, nifedipine (5 μM), an L-type-channel antagonist, produced a much stronger occlusion of the response to 17β-estradiol, suggesting that 17β-estradiol targeted primarily L-type currents (Fig. 5B,C).

The actions of estradiol are steroid-specific

The effects of 17β-estradiol were dose-dependent, with a maximal reduction of Ba^{2+} currents occurring at 100 pM (concentrations of 10 nM and 1 μM did not reduce the current further). 17α-Estradiol, the stereoisomer of the endogenous 17β-estradiol (considered to be physiologically inactive at the intracellular estrogen receptor), also was found to reduce Ba^{2+} currents. However, the reduction by 17α-estradiol was significantly less ($p < 0.002$) than that of 17β-estradiol (Fig. 6A). The ability of other estrogenic compounds to reduce neostriatal Ba^{2+} currents is summarized in Figure 6B (steroids were tested at 100 pM). The hydroxylated analog of 17β-estradiol, estriol
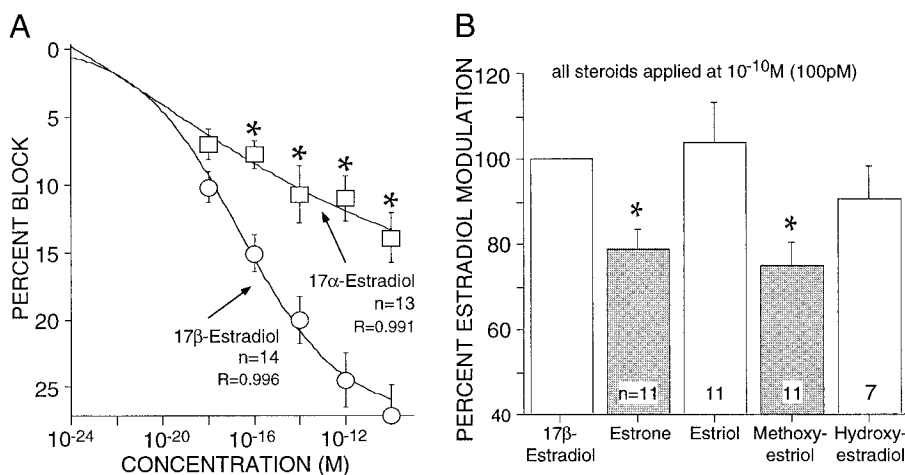
Figure 6. Inhibition of Ba^{2+} currents by 17β-estradiol was steroid-specific and dose-dependent. *A*, 17α-Estradiol reduced Ca^{2+} currents but was significantly less effective than 17β-estradiol ($p < 0.002$). The effect of 17β-estradiol was dose-dependent, with maximal responses occurring at 100 pM. A Hill plot derived from the dose–response data of 17β-estradiol indicated a slope factor of 0.17 ($R > 0.999$; data not shown). *B*, Compared with 17β-estradiol, estriol and 4-hydroxyestradiol were equally effective in reducing Ba^{2+} currents; estrone and 2-methoxyestriol were significantly less effective ($p < 0.002$). Each steroid was applied at a concentration of 10^{-10} M (100 pM).

(103.8 ± 9.4% as effective as 17β-estradiol; n = 11), and 4-hydroxyestradiol (90.6 ± 7.6%; n = 7) were equally effective in reducing Ba^{2+} currents. Estrone (78.7 ± 4.8%; n = 11) and 2-methoxyestriol (74.8 ± 5.7%; n = 11), on the other hand, were significantly less effective (p < 0.002). Tamoxifen (100 pM), considered to be an antagonist at the intracellular estrogen receptor, was found to reduce Ba^{2+} currents as well (11.7 ± 1.3% reduction of maximal current; n = 17), although significantly less (p < 0.005) than 17β-estradiol. Moreover, tamoxifen only slightly occluded the inhibition of 17β-estradiol on Ba^{2+} currents (18.0 ± 8.2%; n = 10).

17β-Estradiol acts at a surface receptor

17β-Estradiol appears to reduce Ba^{2+} currents by acting at the membrane surface, not on an intracellular receptor. E-BSA, which cannot penetrate the cellular membrane, also was found to inhibit Ba^{2+} currents (Fig. 7A,B). In this E-BSA, estradiol was conjugated on the 17th carbon. Because the inhibition of 17α-estradiol on Ba^{2+} currents was significantly less than that of 17β-estradiol (Fig. 6A), it was unclear whether the efficacy of E-BSA was caused, at least in part, by the presence of free estradiol. To verify that 17β-estradiol acts at the membrane surface, a subset of cells was intracellularly perfused with 17β-estradiol. 17β-Estradiol was allowed to diffuse into the cell for at least 5 min before we applied 17β-estradiol externally (time between membrane rupture and recording). Based on estimates of dialysis times for similar compounds with cells similar in size to ours (Pusch and Neher, 1988), the concentration of estradiol in the cell interior and the electrode should have equilibrated in this period. After dialysis, 17β-estradiol (1 pM) applied extracellularly still inhibited Ba^{2+} currents (Fig. 7C).

The inhibition of Ba^{2+} currents by 17β-estradiol appeared to depend on activation of a G-protein. Under normal conditions, the 17β-estradiol modulation readily reversed when the hormone was removed (70.4 ± 10.0% of the current blocked by 17β-estradiol reversed after return to the control solution; n = 7, Fig. 8B). However, when the cell was dialyzed with GTPγS (400 μM) recovery after 17β-estradiol removal was significantly (p < 0.05) reduced (40.5 ± 10.9%, n = 8; Fig. 8A,B). This pattern suggests that 17β-estradiol activates a G-protein in a signaling pathway targeting Ca^{2+} channels.

G-proteins are capable of modulating Ca^{2+} channels either via cytosolic second-messenger pathways or via direct interaction with the channel or an associated protein (Hille, 1992a). This latter signaling pathway often is referred to as membrane-delimited. A common characteristic of membrane-delimited inhibition of Ba^{2+} currents is the ability of strong membrane depolarization to reverse the modulation temporarily. To test whether the pathway mediated by the effects of 17β-estradiol shared this property, agonist was applied with and without a brief (30 msec) depolarizing (100 mV) prepulse. The modulation of Ba^{2+} currents by 17β-estradiol was unaffected by the strong prepulse [the modulation after a prepulse was 100.6 ± 4.6% (n = 22) of the control; Fig. 9A–C], suggesting that inhibition of Ba^{2+} currents by 17β-estradiol was not mediated by a membrane-delimited G-protein interaction.

The impact of intracellular Ca^{2+} on the effects of 17β-estradiol was examined by varying the concentration of the Ca^{2+} chelator BAPTA in the patch-pipette solution. Typically, neostriatal neurons were studied with the intracellular Ca^{2+} concentration at low nanomolar levels by recording with an
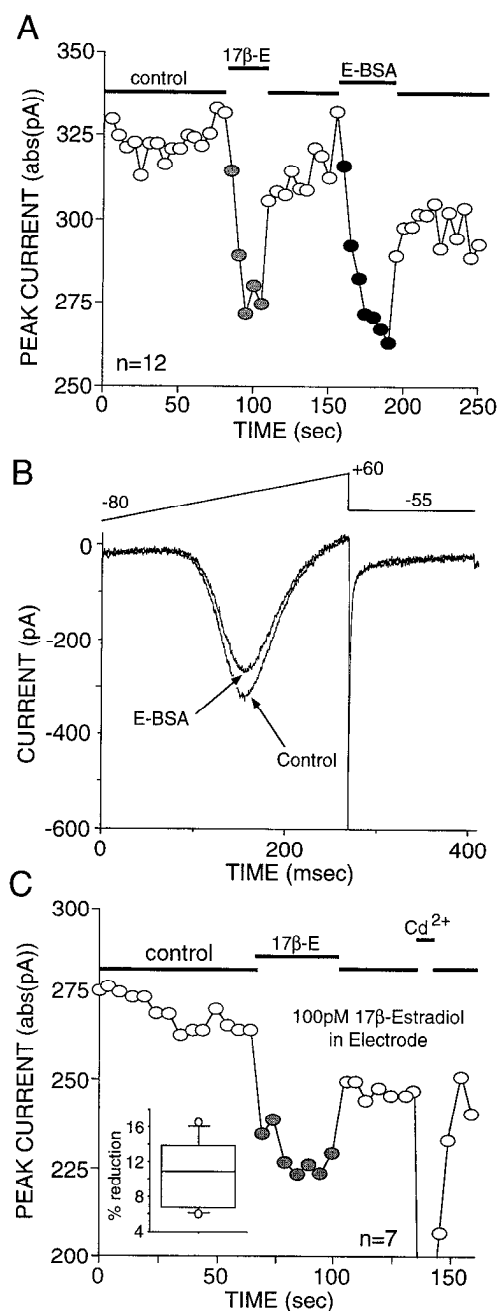
Figure 7. The effects of 17β-estradiol occur at the cellular membrane. A, E-BSA (52 pM) also was found to inhibit neostriatal Ba^{2+} currents, suggesting that the immediate actions of 17β-estradiol occur at the membrane surface. The administration of 17β-estradiol (52 pM) and E-BSA produced a similar reduction in current. B, Individual traces taken from the time course shown in A. C, Dialyzing the inside of the cell with 17β-estradiol (100 pM) did not influence the response to 17β-estradiol (1 pM) administered externally, suggesting an effect at the extracellular face of the membrane. *Inset*, Statistical summary of the percent reduction of the whole-cell Ba^{2+} current inhibited by 1 pM 17β-estradiol after intracellular dialysis.

internal solution containing 5–10 mM BAPTA and no added Ca^{2+}. When the BAPTA concentration was lowered to 0.1 mM to allow fluctuations in intracellular Ca^{2+}, no change in the magnitude of the modulation by 17β-estradiol was observed [15.5 ± 2.7% (n = 12) reduction of the whole-cell currents by
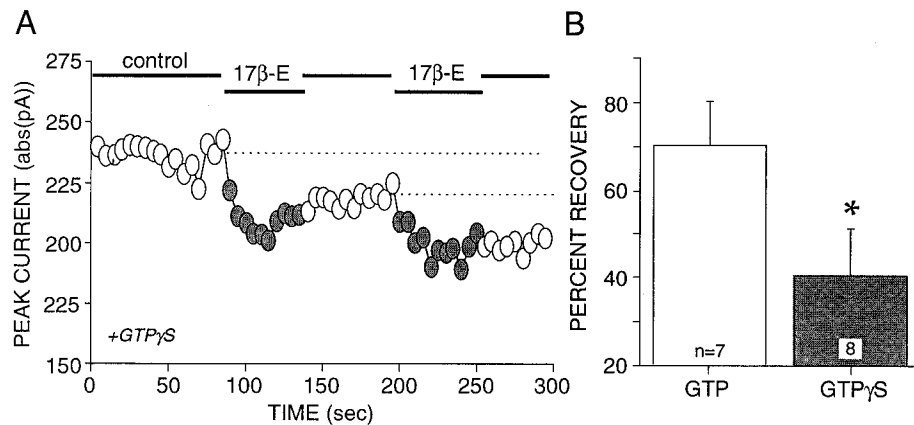
Figure 8. 17β-Estradiol inhibition of neostriatal Ba^{2+} currents appeared to occur via G-protein activation. *A*, Time course of 17β-estradiol (100 pM) modulation in a neostriatal neuron dialyzed with GTPγS. In the presence of GTPγS, the effect of 17β-estradiol did not reverse readily with removal of agonist, in contrast to the situation with GTP dialysis. *B*, Statistical summary of the percent recovery when the neuron was dialyzed with either GTP (200 μM) or GTPγS (400 μM). The effects of estradiol were less likely to reverse in neurons perfused with GTPγS ($p < 0.05$).

estradiol with 0.1 mM BAPTA vs 14.9 $\pm$ 2.8% ($n = 8$) with 10 mM BAPTA]. This finding suggests that 17β-estradiol does not require an elevation of intracellular Ca^{2+} to inhibit voltage-activated currents (Fig. 7D).

DISCUSSION

These results demonstrate that physiological concentrations of 17β-estradiol rapidly reduce L-type currents in neostriatal neurons. This modulation was sex-specific in that 17β-estradiol was more effective in neurons acquired from female rats than those from males. The effect was also steroid-specific: 17α-estradiol, estrone, and 2-methoxyestriol were less effective in modulating Ba^{2+} currents than 17β-estradiol. Finally, these effects appeared to be mediated by a G-protein-coupled receptor at the membrane surface.

One difference between our results and previous reports is the steroid concentrations required to elicit maximal effects. In hippocampal neurons, micromolar concentrations of corticosteroids are required to maximally reduce Ba^{2+} currents (ffrench-Mullen and Spence, 1991; ffrench-Mullen et al., 1994; ffrench-Mullen, 1995). Although some steroids may be synthesized within the brain and reach micromolar concentrations (Corpechot et al., 1983; Lanthier and Patwardham, 1986; Baulieu and Robel, 1990), there is no evidence that this is the case for estrogen. We found that 17β-estradiol was maximally effective in inhibiting Ca^{2+} channels at picomolar concentrations, close to those concentrations found in the blood of late proestrus rats (Smith et al., 1975). Although the effects of 17β-estradiol were dose-dependent, extremely low concentrations of the hormone also produced reduction of Ba^{2+} currents. The ability of lower concentrations of 17β-estradiol to modulate currents may be attributable to the lipophilic properties of the steroid and the experimental paradigm. The membrane of a dissociated neuron may act as an "estrogen sink," raising the concentration in the membrane several log units above that in the aqueous phase (Stein, 1986). How partitioning is regulated *in situ* is a question of obvious importance for determining the physiological impact of circulating estrogen. However, the dose response also is complicated by the fact that 17β-estradiol appears to reduce Ba^{2+} currents via G-protein activation.

Although it appears 17β-estradiol reduces Ba^{2+} currents via a receptor embedded in the cellular membrane, the steroid-binding site need not be on the external face. The binding site may be within the outer membrane layer of the cell. Externally applied 17β-estradiol should enter and diffuse quickly within this external lipid leaflet, only slowly "flipping" into the inter-

nal lipid layer. The fact that dialyzing neostriatal neurons intracellularly with 17β-estradiol has no apparent influence on the effectiveness of externally applied 17β-estradiol is consistent with this model. Although the magnitude of the modulation by 17β-estradiol was somewhat less when the cell was intracellularly dialyzed with this steroid, this phenomenon likely is caused by our waiting at least 5 min between cell rupture and recording. As shown in Figure 3, the effect of 17β-estradiol decreases over time.

The ability to reverse rapidly the effects of 17β-estradiol with washing also suggests that the binding site is not energetically remote from the external medium. By analogy, nifedipine, also a lipophilic compound, blocks L-type Ca^{2+} channels by binding to a channel site accessible from within the membrane, rather than a site approached from the mouth of the pore (Hille, 1992b). The effects of nifedipine, like those of 17β-estradiol, can be rapidly reversed with our perfusion system after drug removal (see Fig. 3).

An argument for the effect of 17β-estradiol being mediated by a specific estrogen receptor is that the modulation was both stereospecific and steroid-specific. 17α-Estradiol was considerably less effective than 17β-estradiol. Estrone and 2-methoxyestriol also were less effective than 17β-estradiol. However, estriol and 4-hydroxyestradiol, two steroids with an additional hydroxyl group, were equally effective. Apparently, there is more than one site on the estrogen molecule important for receptor activation. Estriol is hydroxylated at carbon 16, whereas 4-hydroxyestradiol is hydroxylated at carbon 4. Neither of these alterations appeared to affect the efficacy of the steroid. Moreover, estrone differs from 17β-estradiol at carbon 17, and 2-methoxyestriol differs from estriol at carbon 2; yet both were less effective than 17β-estradiol in reducing currents. The data suggest that if 17β-estradiol is binding to a membrane receptor, then it can interact with both sides of the molecule. The binding of steroids to the intracellular estrogen receptor exhibits similar properties (i.e., lack of site specificity) (Anderson et al., 1975; Davies et al., 1975; Martucci and Fishman, 1979; Kirchhoff et al., 1984). However, it should be noted that binding cannot be equated readily to receptor activation.

At equimolar concentrations, tamoxifen did not antagonize the effects of 17β-estradiol effectively. Although tamoxifen and its metabolites generally are considered to be antagonists at the intracellular receptor, tamoxifen also can induce estrogenic effects (Jordan, 1984; Jordan and Murphy, 1990). It has been reported also that tamoxifen has cellular actions independent
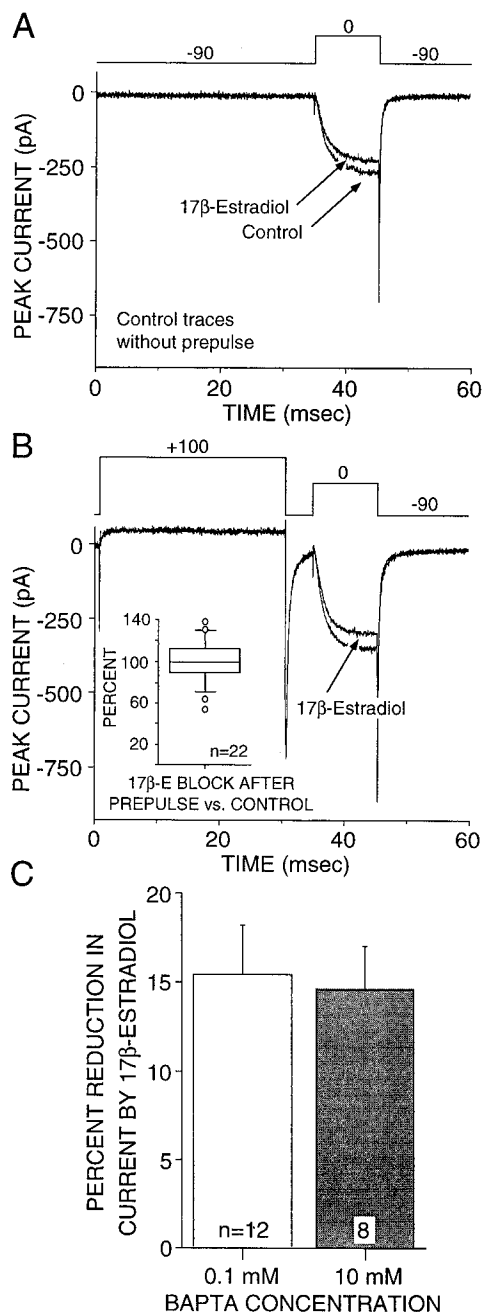
Figure 9. The modulation by 17β-estradiol was neither voltage-dependent nor intracellular Ca^{2+} chelation-dependent. *A*, Ba^{2+} currents evoked by a step to 0 mV from a holding potential of -90 mV in the presence and absence of estradiol (1 pM). *B*, Ba^{2+} currents evoked by a step to 0 mV when preceded by a 30 msec prepulse to $+100$ mV. Note that the estradiol modulation is unaffected by the prepulse. *Inset*, Box-plot summary of the percent modulation after the depolarizing prepulse (compared with control modulation). Cd^{2+}-insensitive currents were subtracted from all traces. *C*, Variation in the intracellular concentration of the Ca^{2+} chelator BAPTA did not alter the magnitude of the modulation by 17β-estradiol, suggesting that the second-messenger pathway by which 17β-estradiol inhibits Ca^{2+} channels is Ca^{2+}-independent. Shown are the percent reductions in peak current produced by 17β-estradiol (at least 1 pM) in recordings with 0.1 and 10 mM BAPTA dialysates (no added Ca^{2+}). *Bars* are SEM.

of estrogen receptors. For example, there are specific, high-affinity binding sites for tamoxifen in rat brain (Sudo et al., 1983; Gray and Ziemian, 1992). Also, tamoxifen inhibits pro-

tein kinase C (O'Brian et al., 1985; Su et al., 1985; Issandou et al., 1990) and, at high concentrations, competes for neostriatal dopamine receptors (Hiemke and Ghraf, 1984). These observations are consistent with the partially additive effects of tamoxifen and 17β-estradiol in modulating Ba^{2+} currents observed in this study.

Often, it is assumed that activation of intracellular steroid receptors results in long-term changes in gene expression. Nongenomic actions, on the other hand, are believed to be rapid in onset but fleeting in duration. However, this may not necessarily be true. L-Type Ca^{2+} channels are found primarily in somatic and proximal dendritic membrane (Westenbroek et al., 1990; Hell et al., 1993). By decreasing L-type currents, 17β-estradiol may affect the spike generation and patterning of the neuron, but also gene transcription (Murphy et al., 1991; Bading et al., 1993). Moreover, G-protein activation may induce long-term intracellular changes via phosphorylation. Therefore, the nongenomic actions of 17β-estradiol, although rapid in onset, also may have long-term effects on neostriatal function. Previous reports studying the nongenomic actions of 17β-estradiol within the neostriatum often have examined potentiated dopamine release, alterations in dopamine-receptor binding, and enhancement of sensorimotor responses. Future experiments will examine whether activation of the G-protein, second-messenger cascade by 17β-estradiol reported in this study is related to these other effects.

Of interest, the reduction of Ba^{2+} currents by 17β-estradiol was somewhat faster (maximal effects occurred after several seconds) when applied directly to neostriatal neurons than that reported previously when 17β-estradiol was applied to slices (several minutes) (Nabekura et al., 1986; Becker, 1990a). Moreover, changes that occur when estradiol is administered *in vivo* take somewhat longer (15 minutes to several hours) (Becker et al., 1987; Levesque and Di Paolo, 1988; Bazzett and Becker, 1994). The onset of the effects from 17β-estradiol likely is a reflection of the delivery mechanism used in each experiment.

17β-Estradiol was effective in reducing Ba^{2+} currents in nearly all neurons tested. Recorded cells displayed the morphology and surface area typical of medium spiny projection neurons. Projection neurons in the neostriatum, however, are not homogeneous: one group projects to the globus pallidus, whereas the other projects to both the globus pallidus and the substantia nigra (Kawaguchi et al., 1990; Gerfen, 1992). The neostriatum can be subdivided further with anatomical markers into patch and matrix compartments (Pert et al., 1976; Gerfen et al., 1985; Bolam et al., 1988; Gerfen, 1989). Because a vast majority of neurons were affected by 17β-estradiol, it is likely that neurons from each of these anatomical groupings are estrogen-sensitive. Future studies will determine whether the response to 17β-estradiol differs between these different types of neurons, an important consideration when attempting to understand how 17β-estradiol affects basal ganglia functioning.

Aside from the involvement of a G-protein, the signaling mechanism by which estrogen reduces Ba^{2+} currents must await further study. L-Type channels can be modulated via several discrete mechanisms in neostriatal neurons. D_1 dopamine agonists, for example, enhance L-type currents via a protein kinase A cascade involving a pertussis toxin (PTX)-insensitive G-protein and adenylyl cyclase (Surmeier et al., 1995). On the other hand, muscarinic agonists inhibit L-type currents via a pathway involving a PTX-insensitive G-protein (Howe and Surmeier, 1995). This pathway appears to depend on the release of inositol triphos-

phates (IP_3) and the release of intracellular Ca^{2+}. Of interest, estrogen also has been shown to increase IP_3 synthesis in other cell types (Morley et al., 1992). However, 17β-estradiol was effective in reducing Ba^{2+} currents through Ca^{2+} channels when the intracellular Ca^{2+} was chelated to low nanomolar levels, suggesting that 17β-estradiol is acting via a second-messenger system not involved with the release of intracellular Ca^{2+}.

It is important to determine the relationship between the reduction by 17β-estradiol on Ca^{2+} currents and other estrogen-mediated changes in rat neostriatum. The role of neostriatal dopamine neurotransmission on locomotor behavior is well documented. Because Ca^{2+} is crucial for neurotransmitter release and modifies intracellular changes induced by receptor activation, the question remains whether the reduction of Ca^{2+} currents by 17β-estradiol is responsible for the effects observed previously on neostriatal dopaminergic systems and behaviors. Future studies will determine whether these phenomena are causally related.

In summary, 17β-estradiol reduces neostriatal Ba^{2+} currents through Ca^{2+} channels via activation of a G-protein-signaling pathway. The effect is greater in female neostriatal neurons than in cells taken from male rats, and the magnitude of the reduction is steroid-specific. These effects occur at physiological concentrations and may play an important role in alterations in motor performance during different stages of the estrus cycle.

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