Estrogens directly potentiate neuronal L-type Ca^{2+} channels

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L-type voltage-gated Ca^{2+}channels (VGCC) play an important role in dendritic development, neuronal survival, and synaptic plasticity. Recent studies have demonstrated that the gonadal steroid estrogen rapidly induces Ca^{2+} influx in hippocampal neurons, which is required for neuroprotection and potentiation of LTP. The mechanism by which estrogen rapidly induces this Ca^{2+} influx is not clearly understood. We show by electrophysiological studies that extremely low concentrations of estrogens acutely potentiate VGCC in hippocampal neurons, hippocampal slices, and HEK-293 cells transfected with neuronal L-type VGCC, in a manner that was estrogen receptor (ER)-independent. Equilibrium, competitive, and whole-cell binding assays indicate that estrogen directly interacts with the VGCC. Furthermore, a L-type VGCC antagonist to the dihydropyridine site displaced estrogen binding to neuronal membranes, and the effects of estrogen were markedly attenuated in a mutant, dihydropyridine-insensitive L-type VGCC, demonstrating a direct interaction of estrogens with L-type VGCC. Thus, estrogen-induced potentiation of calcium influx via L-type VGCC may link electrical events with rapid intracellular signaling seen with estrogen exposure leading to modulation of synaptic plasticity, neuroprotection, and memory formation.

estrogen receptors | signaling | estradiol | memory

A large body of evidence shows that estrogens exert multiple rapid effects on the structure and function of neurons in a variety of brain regions, including the hippocampus (1). For example, estrogens rapidly potentiate kainite-induced currents in hippocampal neurons from wild-type (2) as well as from estrogen-receptor (ER)-α knockout (3) mice and induce rapid spine synapse formation in the CA1 hippocampus of ovariectomized (OVX) rats (4). Furthermore, acute application of estrogens to hippocampal slices increases NMDA and AMPA receptor transmission (5), induces long-term potentiation (LTP) and long-term depression (LTD) (6), and rapidly modulates neuronal excitability in rat medial amygdala (7) and hippocampus(8).

It is well known that estrogens interact with cell membrane components and initiate signaling events leading to a rise in intracellular Ca^{2+}, and activation of Src kinase, G protein-coupled receptor (GPCR), MAPK, PI3K/AKT, PKA, and adenylyl cyclase (9). The mechanism(s) by which estrogens induce these rapid and diverse effects remains largely unknown. Ca^{2+} is a second messenger that can trigger the modification of synaptic efficacy. A plasticity-induction protocol like repetitive low-frequency synaptic stimulation (10) induces the elevation of postsynaptic intracellular Ca^{2+}. The level of intracellular Ca^{2+} concentration can activate numerous kinases like CAMK, PKA, PKC, MAPK, PI3K, or phosphatases (11–15), which, respectively, phosphorylate or dephosphorylate ion channels, transcription factors, and other proteins that are involved in synaptic plasticity and memory formation. Because voltage-gated Ca^{2+}channels (VGCC)-mediated extracellular Ca^{2+} influx in neurons initiates the activation of these same signaling cascades (16–20), we hypothesized that estrogens potentiate VGCC. Here, we report that estrogen facilitates L-type VGCC in hippocampal neurons via an ER-independent mechanism, through direct binding with a domain that overlaps the dihydropyridine-binding site. Moreover, the capacity of estrogen to potentiate specifically L-type Cav1.2 as shown here may impart a distinctive role of estrogen in modification of synaptic efficacy.

Results

Estrogen Potentiates L-Type VGCC. Initial reports have shown that 17β-estradiol induces rapid rise of intracellular Ca^{2+} concentration ($[Ca^{2+}]i$) in hippocampal neurons that was inhibited by an L-type calcium channel blocker (21). Whereas the authors indicated estrogen-mediated activation of signaling events was responsible for the effect, an alternative possibility is that estrogen directly binds to and enhances the activity of the channel.

To test this hypothesis, we measured the effects of estrogens on whole-cell Ba^{2+}/Ca^{2+} currents in embryonic day (E)18 primary cultured rat hippocampal neurons. Ca^{2+} channel currents were isolated by inhibiting Na^{+} currents with extracellular tetrodotoxin and K^{+} channels with intracellular Cs^{+} and extracellular 4-AP, and either Ba^{2+} or Ca^{2+} was used as the charge carrier. Fig. 1*A* shows depolarization-activated Ba^{2+}currents (I_{Ba2+}) recorded from this hippocampal neuron in the absence (control) or presence of 100 pM 17β-E2. The average amplitude of Ba^{2+} current was 93 ± 13 pA in the control condition from a single hippocampal neuron. In the presence of 100 pM 17β-E2, the Ba^{2+} currents were increased to 192 ± 18% of the control (Fig. 1*A*). The potentiating effect persisted even after washout of estrogen. In a number of experiments, we observed that the stimulatory effect of a 5-min exposure to estrogen persisted for >20 minutes (data not shown). In addition, we observed a similar effect of estrogen in a hippocampal slice preparation [supporting information (SI) Fig. S1].

To determine whether the effect of estrogens on Ca^{2+} channels is concentration dependent, hippocampal neurons were exposed to various concentrations of 17β-E2 (Fig. 1*B*). Mean peak I_{Ba2+} revealed a dose-dependent increase in Ba^{2+} currents with as little as 10 pM 17β-E2 (Fig. 1 *B* and *C*). Effects of 17β-E2 were also very rapid. The onset of estrogen action was estimated to be <550 ms (Fig. 1*D* and *Methods*).

We sought to determine which Ca^{2+} channel subtype was being modulated by estrogens. Approximately 1/3 of the elicited Ba^{2+} current was due to activation of L-type Ca^{2+} channels, as evidenced by the inhibitory effects of the L-type inhibitor nifedipine. We thus assessed whether this channel may be a target of 17β-E2. As illustrated in Fig. 1 *E* and *F*, nifedipine (10 μM) nearly completely abolished the 17β-E2-induced potentiation of Ba^{2+} current, indi-

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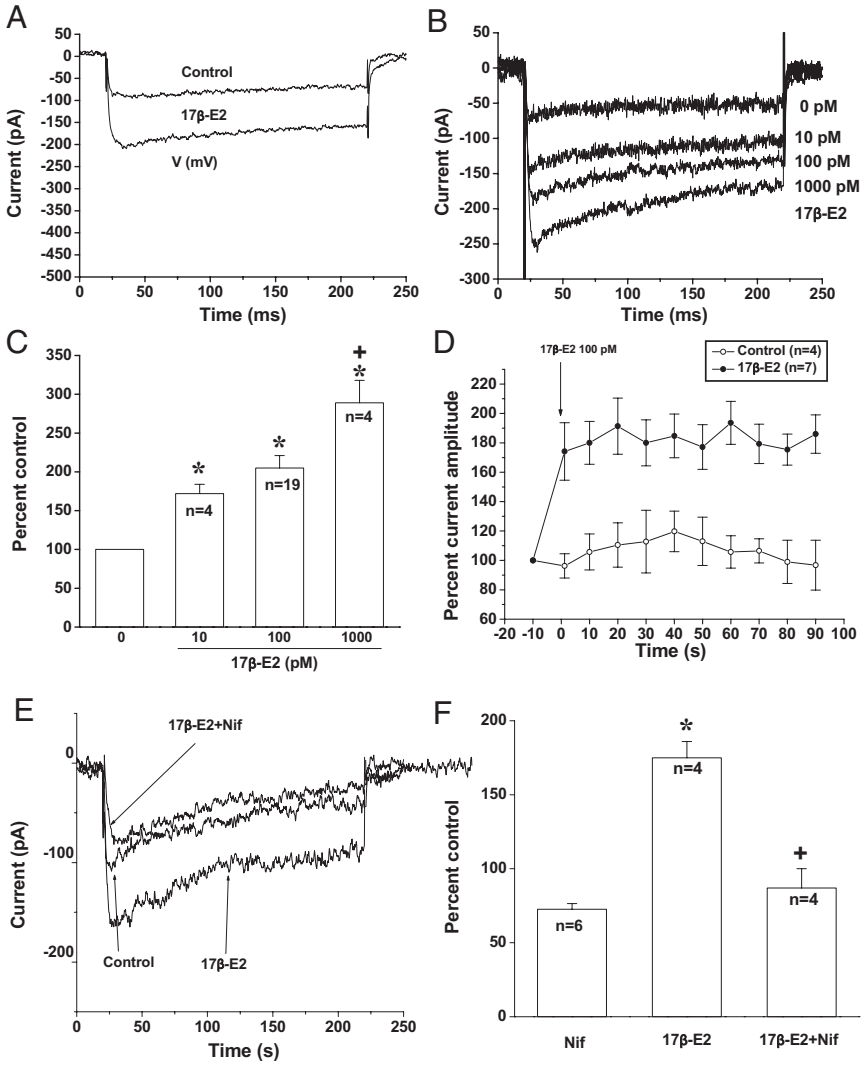

Fig. 1. The modulatory effects of 17β-E2 on VGCC currents recorded in hippocampal neurons. (*A*) Whole-cell Ba^{2+} current (I_{Ba2+}) recorded from a primary hippocampal cultured neuron in the presence or absence of 17β-E2. I_{Ba2+} (5 mM Ba^{2+} used as the charge carrier) was elicited by pulses to 0 mV from a holding potential of −90 mV. (*B*) Typical concentration-dependent effect of 17β-E2 on Ca^{2+} current recorded from a hippocampal neuron (*C*). Mean concentration dependence of 17β-E2 on hippocampal neuron Ca^{2+} currents. All currents were normalized to the control (assigned as 100%). Data are mean ± SEM. *, $P < 0.05$, one-way ANOVA test; +, $P < 0.05$, compared with 10, 100 nM, Newman–Keuls multiple comparison test. (*D*) Rapid effect of estrogen on VGCC. The arrow indicates the time of application and initiation of trains of I_{Ba2+} elicited by depolarization from −90 mV to 0 mV (set at 0 min). The current amplitudes were normalized to the control before application with 17β-E2 ($n = 7$) or saline ($n = 4$). Note that 17β-E2 caused an immediate enhancement of I_{Ba2+}, whereas the saline control had no effect. Furthermore, the estrogen effect was sustained even after application. (*E*) Effect of L-type Ca^{2+} channel inhibitor, nifedipine (Nif), on 17β-E2-induced potentiation of Ba^{2+} current. Bath application of 10 μM Nif completely blocked the potentiation of Ba^{2+} current by 17β-E2. (*F*) Summary of Nif blockade of 17β-E2-induced potentiation of Ba^{2+} currents. Nif blocked ≈1/3 of Ba^{2+} current, demonstrating the presence of L-type VGCCs. In the presence of Nif, the stimulatory effects of 17β-E2 were blocked. Current amplitudes were normalized to the control. Each data point represents four cells. *, $P < 0.05$, compared with the control; +, $P < 0.05$ compared with 17β-E2 alone, paired *t* test.

cating that 17β-E2's ability to enhance Ca^{2+} current is due fully to potentiation of L-type channels.

Estrogen Action on L-Type VGCC Does Not Require Classical Estrogen Receptor. Next, we asked whether the potentiation of VGCC is mediated by an estrogen-generated intracellular signal or by binding to a plasma membrane component. That 17β-E2 appears to increase Ba^{2+} currents by acting at the membrane surface rather than through an intracellular receptor was shown by the fact that membrane-impermeable 17β-E2-BSA was found to potentiate VGCC, although to a lesser extent than 17β-E2 alone (Fig. 2 *A* and *B*). To determine the role of ER in this response, we administered the ER antagonist, ICI-82,780, which did not antagonize the 17β-E2 potentiation of Ca^{2+} currents (Fig. 2 *C* and *E*). Furthermore, we tested the synthetic derivative of estrogen, ZYC-26 (2-adamantyl-estrone), which does not bind to either ERα or ERβ at concentrations ranging from 1 pM to 10 μM (22) and does not stimulate uterine growth in ovariectomized rats (23). As shown in Fig. 2 *D* and *E*, 100 pM ZYC-26 efficiently potentiated VGCC in hippocampal neurons.

Estrogen Potentiates Recombinant Cav1.2 VGCC in the Absence of Estrogen Receptors. Of the L-type VGCC in the hippocampus, the predominant isoform is Cav1.2 (24–26). Synaptic and extrasynaptic localizations of Cav1.2 L-type VGCC correspond to putative roles of L-type calcium currents in synaptic modulation and in the propagation of dendritic Ca^{2+} spikes (27–29). To investigate the direct action of estrogen on L-type Cav1.2, we assessed the action of 17β-E2 in HEK293 cells transiently cotransfected with the pore-forming subunit Cav1.2 and the accessory β1b and α2δ subunits and GFP expression plasmids. HEK293 cells do not endogenously express either L-type VGCC or estrogen receptors (30, 31). Transfected HEK cells (GFP positive) showed the expected Ca^{2+} current activated in response to the same depolarizing protocol (Fig. S2). However, in the absence of transfection of the neuronal L-type Ca^{2+} channel, no current could be elicited in these cells in response to depolarization, thus providing a model to assess the dependence of the observed 17β-E2 response on these two entities. As we observed in neurons, exposure of recombinant L-type Ca^{2+} channels to 17β-E2 resulted in a significantly enhanced Ca^{2+} current (Fig. S2). These data support our contention that the 17β-E2-potentiated Ba^{2+}/Ca^{2+} current in hippocampal neurons is through the L-type Cav1.2 Ca^{2+} channel via a mechanism independent of ER.

Estrogen Induces Extracellular Calcium Influx Through L-Type VGCC. To quantify the 17β-E2-induced potentiation of extracellular Ca^{2+} influx via VGCC, we measured intracellular Ca^{2+} transients induced by high-K^+-mediated membrane depolarization using the Fura-2 dye and digital imaging microfluorometry. Fig. S3 shows that high K^+-induced depolarization was able to activate VGCC. The 17β-E2 potentiated the extracellular Ca^{2+} influx in hippocam-

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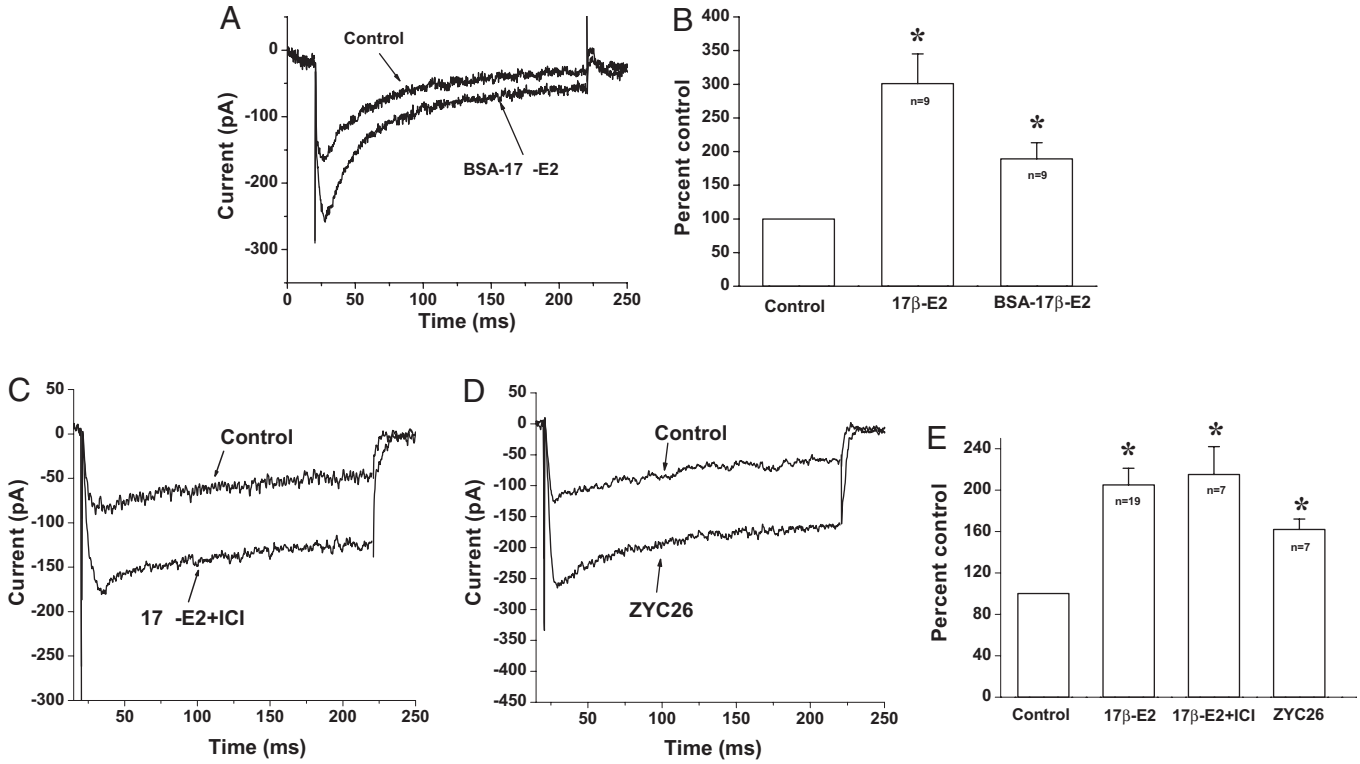

Fig. 2. The modulatory effects of 17β-E2 occur at the neuronal membrane and are ER independent. (*A*) Ca^{2+} current traces, shown in the presence or absence of 250 pM BSA-conjugated 17β-E2 in the bath solution. (*B*) Summary data: Data are expressed as mean ± SEM. *, $P < 0.05$, paired *t* test, compared with the control; +, $P < 0.05$, unpaired *t* test, compared with 17β-E2 group. (*C*) Lack of role of ERs in estrogen-induced potentiation of L-type Ca^{2+} currents. The ability of estrogen to potentiate Ba^{2+} current in hippocampal neurons was not attenuated by 1 μM ER antagonist, ICI 182,780. (*D*) Potentiation of L-type Ca^{2+} currents by ZYC-26, a non-ER-binding estrogen analogue. Whole-cell hippocampal neuronal Ba^{2+} current was potentiated by 100 pM ZYC-26. (*E*) Summary of effect of ZYC-26 and ICI 182,780 on hippocampal Ba^{2+} current. For direct comparison, the 17β-E2 data from Fig. 1 *C* and *D* are replotted. *, $P < 0.05$, paired *t* test, compared with the control.

pal neurons cultured *in vitro*, and nifedipine inhibited the potentiation of Ca^{2+} influx elicited by 17β-E2.

Estrogen-Induced L-Type VGCC Potentiation Mechanism. The mechanism by which estrogens potentiate L-type Ca^{2+} channel was also addressed. One potential mechanism involves estrogen-induced rapid activation of L-type associated kinases such as PKA, PKC, and/or CaMKII. These kinases are known to facilitate L-type Ca^{2+} channels (32, 33). To assess this possibility, we used the pharmacological inhibitors of phospholipase C (PLC)/PKC and CaMKII. As shown in Fig. S4, neither PLC nor CaMKII inhibitors had an effect on estrogen-induced activation of L-type VGCC.

Next, we explored the possibility that potentiation of VGCC occurs as a result of direct binding of estrogen with the channel. It has been shown that estrogen and the estrogen-like compound F90927 directly potentiate Maxi-K (34) and L-type Ca^{2+} channels (35), respectively. Electrophysiological studies using charged dihydropyridines (DHPs) demonstrate that the binding site is accessible exclusively from the outside of the plasma membrane (36, 37). We assessed whole-cell binding of 17β-E2 to antagonist-binding sites on L-type Ca^{2+} channels. As shown in Fig. 3, an L-type Ca^{2+} channel antibody directed against a non-dihydropyridine-binding site region of the channel (Fig. 3, red) and 17β-E2-FITC-BSA (Fig. 3, green) showed membrane localization in HT-22 cells, an immortalized

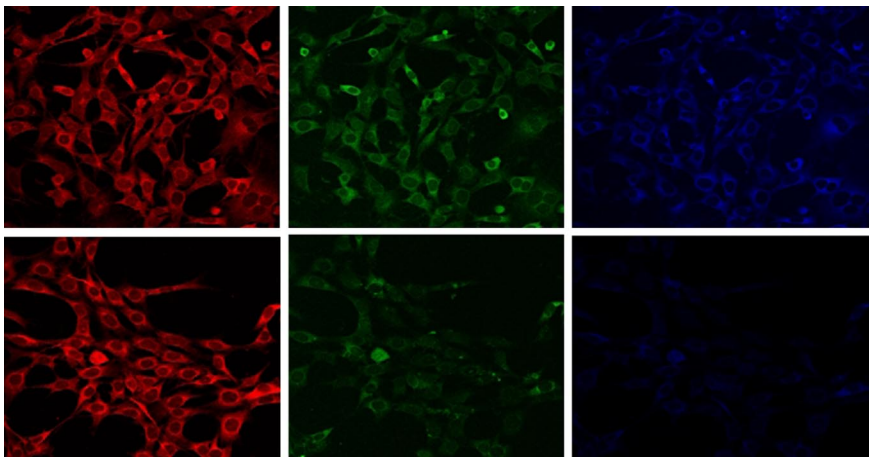

Fig. 3. Bay K 8644 and 17β-E2 compete for the same fluorescent dihydropyridine (DHP)-binding sites in HT-22, a hippocampal cell line. (*Upper*) Confocal microscopy imaging from left to right: L-type Ca^{2+} channel α1C specific antibody staining of HT-22 cells (red), BSA-FITC conjugated 17 β-E2 (green), (−) ST-BODIPY-DHP, (4,4-difluro-7 steryl-4-bora-3a,4a-diaza)-3-(s-indacene) propionic acid, high-affinity enantiomer (blue). (*Lower*) Both fluorescent BSA-FITC-E2 (1 nM) and fluorescent DHP (1 nM) binding was competed out in the presence of excess nonfluorescent DHP (Bay K 8644, 1 μM) as visualized by lesser fluorescent intensity compared with the control (*Upper*).

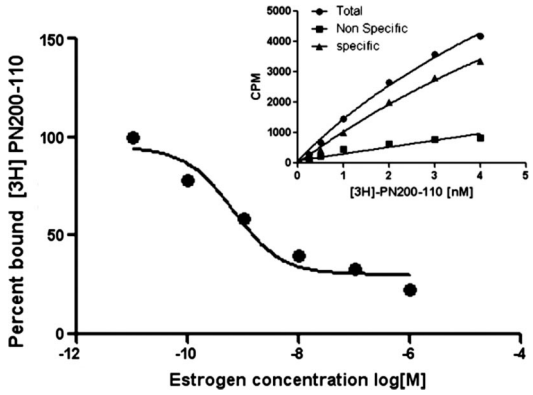

Fig. 4. Competition binding curves for the displacement of 1 nM [3H] PN200–110 by varying concentrations of 17β-E2 in membranes from transiently transfected neuronal α1C and the auxiliary subunits-β1b and α2δ expression plasmids of L-type calcium channel in HEK293 cells. (*Inset*) Typical equilibrium binding curves for [3H] PN200–110 alone.

murine hippocampal neuronal cell line. This binding pattern resembled that of binding fluorescent (−) ST-BODIPY-DHP (Fig. 3, blue), an antagonist for L-type Ca^{2+} channels (35). Additionally, treatment of cells with an excess of nonfluorescent Bay K (1 μM), an L-type Ca^{2+} channel agonist, reduced both 17β-E2-FITC-BSA and DHP binding but did not affect L-type Ca^{2+} channel antibody binding (Fig. 3), suggesting that 17β E2 may bind to the same DHP agonist/antagonist-binding region of L-type calcium channels. We confirmed this idea by launching a two-pronged experimental approach. A competitive binding assay using membrane preparation from HEK 293 cells transiently transfected with wild-type α1c and the accessory subunits showed that estrogen competes with the radioligand [3H] PN200–110 for α1c binding with an IC_{50} of 0.67 nM (Fig. 4). Because competitive binding is done on membranes that are electrically neutral, and electrophysiological effects are voltage and channel state dependent, it is critical to study the coupling between estrogen binding and potentiation of calcium current. Thus, we used a specific mutant of α1c channel that is insensitive to both DHP agonists and antagonists and tested the effects of estrogen on potentiation of calcium current. As shown previously (38), mutant channels composed of Q1070M and T1066Y amino acid residues in motif III S5 of rabbit heart α1c subunit of L-type VGCC, are insensitive to DHP agonist and antagonist. Motif IIIS5 of rabbit heart α1c is 100% homologous with the rat brain α1c used in our experiment (Fig. S5). We therefore chose to test the effects of estrogen on the neuronal α1c mutant T1066Y channel. We confirmed that the T1066Y-expressing channel was insensitive to nifedipine (data not shown). Interestingly, the ability of estrogen to potentiate the T1066Y mutant channel was greatly attenuated (Fig. 5 *B* and *C*). Furthermore, whole-cell binding assays of transiently expressed wild-type α1C channels in HEK 293 cells (Fig. 6) show expression of α1C channels as evidenced by binding of a channel-specific antibody (Fig. 6*A*), binding of FITC-BSA-Estrogen (Fig. 6*B*), and binding of fluorescently labeled DHP antagonist (Fig. 6*C*). There was a markedly reduced binding of both FITC-BSA-Estrogen (Fig. 6*F*) and fluorescently labeled DHP antagonist (Fig. 6*G*) but not an antibody that recognizes both wild-type and mutant α1C channel protein (Fig. 6*E*), compared with the wild-type channel. This effect, coupled with our finding that estrogen competes with both a radiolabeled and a fluorescently tagged DHP, suggest that the binding domains overlap.

Discussion

The rapid interaction of estrogens with L-type Ca^{2+} channels and the resulting potentiation of voltage-induced Ca^{2+} currents could explain the observation that multiple and diverse signaling pathways are rapidly activated by estrogens. Calcium transients, as a result of entry through L-type Ca^{2+} channels, are known to activate Src kinase, GPCR, MAPK, PI3K/AKT, PKA, and adenylyl cyclase signaling pathways (16–20).

These rapid actions of estrogens appear to explain the observation that in pre-, peri-, and postmenopausal women, estrogens affect neuronal activity measured by fMRI in a variety of brain regions during the performance of cognitive (39) and sustained attentional (40) tasks. Furthermore, estrogens enhance visual and place memory (41) and working memory performances in rats (42) and facilitate cholinergic neurotransmission in the septal–hippocampal pathways(43). Several mechanisms have been reported by which estrogen acutely and more chronically potentiates memory-related synaptic plasticity in the hippocampus. Estradiol has been shown to increase dendritic spinogenesis in the hippocampus (44, 45), increase the expression of NMDA receptor (NMDAR) subunit NR2B (46), and potentiate NMDAR-mediated synaptic activity, including LTP (47, 48). The identity of the ER involved in potentiation of synaptic plasticity and memory has not yet been fully confirmed. For example, in one report, ER-α but not ER-β (49), yet in another report ER-β but not ER-α (50) regulates hippocampal synaptic plasticity and enhances cognitive ability. Our data suggest a possible mechanism by which estrogen, via L-type Ca^{2+} channel potentiation, modulates memory-related synaptic plasticity. Recently, it has been shown that activity of L-type Ca^{2+} channels is essential for generating persistent neural firing in a neural circuit that is involved in working memory (51). Also, theoretical studies have shown that L-type Ca^{2+} channels enhance persistent firing to variations in synaptic strength or neuronal excitability (52, 53). Therefore, we envisage that estrogen by augmenting persistent neural activity may enhance working memory performance seen in various experimental settings.

The lack of involvement of ERs in the observed potentiation by 17β-E2 of whole-cell hippocampal Ca^{2+} currents is supported by several observations. First, the potentiation was seen at 10 pM 17β-E2, a concentration that is 500-fold lower than the EC_{50} of 17β-E2 for either ERα or ERβ. Second, the potentiation was seen with ZYC-26, a nonfeminizing estrogen that does not interact *in vitro* or *in vivo* with either Ers (22, 23). Third, the 17β-E2 effects were not antagonized by concentrations of ICI-182,780 that are 35-fold higher than the IC_{50} for the ERs. Finally, HEK-293 cells transfected with the essential components of the L-type Ca^{2+} channel but lacking Ers (30, 31) also responded potently to 17β-E2.

We also studied the mechanism underlying the estrogen modulation of VGCC. The lack of effect of CaMK II or PLC/PKC inhibitors does not support that the observed estrogenic action is initiated by these intracellular signaling pathways. However, the very rapid onset of estrogen action, which was estimated to be less than a second, supports a direct interaction of estrogen with the channel protein. We confirmed this idea by (*i*) whole-cell binding assay using fluorescent ligand in hippocampal-derived neuronal cell line, HT22, where α1C channel expresses endogeneously; (*ii*) whole-cell binding in HEK293 cells transiently expressed mutant and wild-type α1C channel; (*iii*) competitive binding assay using radioligand; and (*iv*) electrophysiological studies using wild-type and dihydropyridine-insensitive channels. The displacement of a L-type Ca^{2+} channel agonist Bay K 8644 with estrogen also provides evidence for this direct mechanism. Furthermore, it is noteworthy that a structurally similar estrogen-like compound, F90927, has recently been shown to directly modulate L-type Ca^{2+} channels in myocytes (35). Our studies indicate that estrogen itself binds with high affinity to the L-type VGCC at a domain that overlaps with the dihydropyridine site.

Functional consequences of estrogen-induced potentiation of L-type Ca^{2+} channels in hippocampal neurons are now becoming clear. Neuronal activity-dependent potentiation of L-type Ca^{2+} channels has an important role in synaptic plasticity and in memory

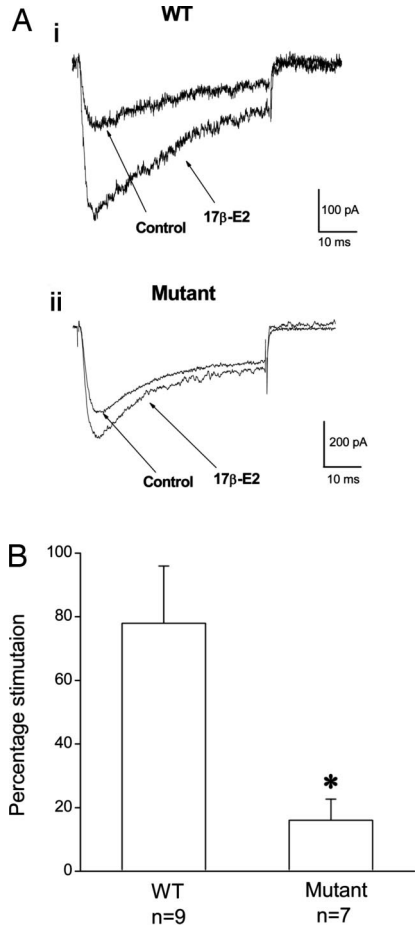

Fig. 5. 17β-E2 modulation is attenuated in dihydropyridin e-insensitive channels. (*A*) Modulation of L-type VGCC by 17β-E2 in wild-type and mutant α1C channels transiently expressed in HEK 293 cells. Whole-cell Ca^{2+} currents were recorded from the wild-type or mutant α1CT1066Y with a 55-ms depolarization pulse from holding potential of -90 to 0 mV. The 17 β-E2 (100 pM) was applied in the bath for 3 min. The ability of 17β-E2 to enhance Ca^{2+} currents was greatly attenuated in the dihydopyridine-insensitive L-type channel. (*B*) Mean results for these studies. The currents are normalized to the control (assigned as 100%). n = at least 4 cells. Note the sensitivity to 17β-E2. *, $P < 0.05$; compared with the wild type, unpaired t test.

(27, 54, 55). Recently, the function of L-type Ca^{2+}channels in spatial learning, synaptic plasticity, and triggering of learning associated biochemical processes were evaluated in a transgenic mouse with an inactivated gene that encodes the Cav1.2 gene in the hippocampus and neocortex (56). This study showed selective loss of protein synthesis-dependent but NMDAR-independent LTP, a severe impairment of hippocampus-dependent spatial memory, loss of activation of MAPK pathway, and repressed cAMP response element-dependent transcription in hippocampal neurons. Also, very recently, it has been shown that the activity of L-type calcium channels is important for spike timing-dependent LTP that is absent in Fragile X syndrome (57). Therefore, we speculate that estrogen-induced, direct potentiation of L-type Ca^{2+} channels could have implications in modulating synaptic plasticity and memory formation. Inasmuch as the L-type Ca^{2+} channel potentiation occurs at estradiol concentrations (10 to 1,000 pM) seen in reproductively competent rodents and women, we believe that these observations are relevant to neuronal regulation in both animals and women.

Materials and Methods

Brain Slice Preparation. Transverse hippocampal brain slices (200 μm) were preferred for patch clamp recording. Details of the slice preparation procedure are provided in *SI Text*.

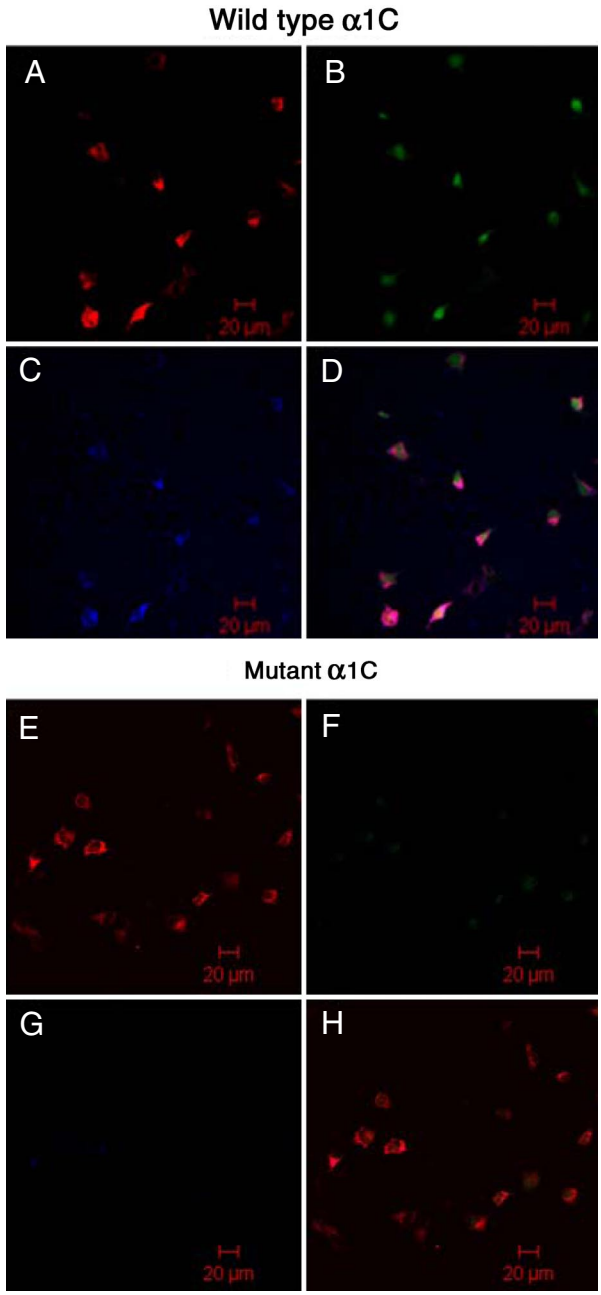

Fig. 6. DHP and 17β-E2 binding characteristics of wild-type α1C and mutant α1C (T1066Y) L-type VGCC transiently expressed in HEK-293 cell. (*Upper*) Confocal microscopy imaging of wild-type α1C channels stained for α1C specific antibody (red) (*A*), BSA-FITC-E2 (1 nM) binding (green) (*B*), (−) ST-BODIPY-DHP binding (blue) (*C*), and merge (*D*). (*Lower*) Confocal microscopy imaging of mutant (T1066Y) α1C channels stained for α1C-specific antibody (red) (*E*), BSA-FITC-E2 (1 nM) binding (green) (*F*), (−) ST-BODIPY-DHP binding (blue) (*G*), and merge (*H*).

Primary Neuronal Cultures. We also studied regulation of VGCCs by 17β-E2 in primary hippocampal cultures. Details of the preparation of the cultures are provided in *SI Text*.

Whole-Cell Recording. Macroscopic Ca^{2+} or Ba^{2+} currents were measured by using whole-cell patch clamp (58). Recording solutions, protocols, and other details are provided in *SI Text*.

Construction and Properties of Mutant Calcium Channels. We used a mutant α1c subunit containing a threonine-to-tyrosine point mutation at position 1036 of rat brain coding sequence, which was constructed by Michael E. Greenberg [Harvard

University (19)]. This mutant is homologous to rabbit heart α1c mutant, as shown in Fig. S2. Electrophysiological studies of rabbit heart α1c mutant [T1066Y (38)] indicated that this mutant is insensitive to both agonist and antagonist without effecting the basal channel activity.

Expression of Ca^{2+} Channels. HEK-293 cell were transfected by using TransIT-293 transfection reagent (Mirus). Cells were transfected with a 2:1:1 ratio of plasmid DNA composed of neuronal, wild-type, or mutant α1C (Cav1.2), β1b, and α2δ L-type subunits [gift from M. E. Greenberg, Harvard University (18)] and a GFP expression plasmid, pGFP-C1 (Clontech), with a ratio of 10:1 channel subunits to GFP. For whole-cell binding experiments, HEK-293 cells were transfected with a 2:1:1 ratio of plasmid DNA composed of either wild-type or mutant α1C (T1066Y) and β1b and α2δ L-type subunits.

Measurement of $[Ca^{2+}]_i$. The measurement of Ca^{2+} influx into neuronal culture is described in detail in *SI Text*.

Whole-Cell Ligand Binding Assay. Whole-cell ligand binding to HT-22 cells culture is described in detail in *SI Text*.

Preparation of Membranes. Transfected HEK 293 cells were washed, scraped, and homogenized by using a glass–Teflon homogenizer in buffer X containing 50 mM Tris, 100 μM PNSF, 100 μM benzamidine, 1 μM pepstatin A, 1 μg/ml leupeptin, and 2 μg/ml aprotinin (pH 8.0). The homogenate was centrifuged at 1,000 $\times$ *g* for 5 min. The supernatant was collected and centrifuged at 100,000 $\times$ *g* in a Beckman ultra centrifuge using a SW 41 rotor for 1 h at 4°C. The membrane pellet was washed and resuspended in buffer X.

Radioligand Binding. Detailed methods for the equilibrium binding assays culture is described in *SI Text*.

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