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DIRECT BINDING OF ESTRADIOL ENHANCES *SLACK* (SEQUENCE LIKE A CALCIUM-ACTIVATED POTASSIUM CHANNEL) CHANNELS' ACTIVITY

L. ZHANG,[d]* M. SUKHAREVA,[b] J. L. BARKER,[b] D. MARIC,[b] Y. HAO,[a] Y. H. CHANG,[b] W. MA,[c] T. O'SHAUGHNESSY[c] AND D. R. RUBINOW[d]

[a]*Laboratory of Molecular Pathophysiology, National Institute of Mental Health, National Institutes of Health, Bethesda, MD 20892, USA*

[b]*Laboratory of Neurophysiology, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, MD 20892, USA*

[c]*Center for Bio/Molecular Science and Engineering, Naval Research Laboratory, Washington, DC 20375, USA*

[d]*Behavioral Endocrinology Branch, NIMH, NIH, Building 10, Room 65340, MSC 1276, Bethesda, MD 20892-1276, USA*

Abstract—17β-Estradiol (E2) is a major neuroregulator, exerting both genomic and non-genomic actions. E2 regulation of *Slack* (sequence like a calcium-activated potassium channel) potassium channels has not been identified in the CNS. We demonstrate E2-induced activation of *Slack* channels, which display a unitary conductance of about 60 pS, are inhibited by intracellular calcium, and are abundantly expressed in the nervous system. In lipid bilayers derived from rat cortical neuronal membranes, E2 increases *Slack* open probability and appears to decrease channel inactivation. Additionally, E2 binds to the *Slack* channel and activates outward currents in human embryonic kidney-293 cells that express *Slack* channels but not classical estrogen receptors (i.e. ERα or ERβ). Neither E2-induced activation nor the binding intensity of E2 to the *Slack* channel is blocked by tamoxifen, an ER antagonist/agonist. Thus, E2 activates a potassium channel, *Slack*, through a non-traditional membrane binding site, adding to known non-genomic mechanisms by which E2 exerts pharmacological and toxicological effects in the CNS. Published by Elsevier Ltd on behalf of IBRO.

Key words: estrogen, ion channel, neuron, electrophysiology, non-genomic, estrogen receptor.

Studies over the last decade have demonstrated that estrogen plays an important role not only in reproduction, but also in regulation of brain structure and function (McEwen and Alves, 1999; Schechter, 1999). Estrogen regulates neuronal survival (Zhang et al., 2001; Harms et al., 2001; Green and Simpkins, 2000; Pike, 1999), growth (Zhang et al., 2000; Granholm, 2000), differentiation (Jakab et al., 2001; Cambiasso et al., 2000), synaptic plasticity (Brake et al., 2001), neurotransmitter receptor expression (Jackson and Etgen, 2001), as well as behavior (Wood and Williams, 2001; Bardi et al., 2001). Estrogen may also play a beneficial role in several CNS disorders commonly seen in women, including depression (Schmidt et al., 2000; Pfaff et al., 2000). Compatible with the broad range of actions of E2 is the complexity of identified cellular effects, comprising both long term (genomic) and short term (non-genomic) actions (Watters et al., 1997; Toran-Allerand et al., 1999; Collins and Webb, 1999; Singh et al., 2000; Kato, 2001; Karkanias and Etgen, 1993). In the long-term actions, E2 freely diffuses across the cell membrane and binds to a cytoplasmic/nuclear receptor. The activated hormone receptor undergoes a conformational change that permits binding to both co-regulatory proteins and to DNA response elements, resulting in the regulation of RNA-dependent protein synthesis (McEwen and Alves, 1999; Schechter, 1999; Meda et al., 2000; Miller et al., 1989). This process takes minutes to hours to days and can be blocked by either antagonists of the estrogen receptor (ER) or inhibitors of transcription or protein synthesis (Nabekura et al., 1986). In contrast, the short term actions are not dependent upon mRNA transcription or protein synthesis (Nabekura et al., 1986), often can be initiated by binding to the cell membrane (i.e. do not require entry into the cell), and occur with a time course of milliseconds to minutes (Watters et al., 1997; Singh et al., 1999, 2000; Collins and Webb, 1999; Kato, 2001; Karkanias and Etgen, 1993).

Although the rapid, presumed non-genomic actions of hormones (McEwen and Alves, 1999; Schechter, 1999) have been described since Selye (1941), a number of very basic questions remain largely unanswered: Where are the binding sites for the non-genomic actions of E2. Are the observed non-genomic actions direct or secondary to modulation of cell signaling pathways?

Several lines of evidence indicate that the rapid effects of E2 are not likely to be the consequence of nuclear events, but rather must be related to events occurring at the cell surface (Watters et al., 1997; Toran-Allerand et al., 1999; Collins and Webb, 1999; Singh et al., 2000; Kato, 2001; Karkanias and Etgen, 1993; Razandi et al., 2002; Wyckoff et al., 2001; Qiu et al., 2003; Kelly and Wagner, 1999). Both classical and unique ERs exist in the caveolae or caveolar-like microdomains of membranes where they link to scaffolding proteins (e.g. caveolin-1, flotillin; Toran-

*Corresponding author. Tel: +1-301-451-8452; fax: +1-301-480-0123.
E-mail address: lezhang@usuhs.mil (L. Zhang).

Abbreviations: BSA, bovine serum albumin; cDNA, complementary deoxyribonucleic acid; CNS, central nervous system; E2, 17β-estradiol; EGTA, ethylene glycol-bis-(*b*-aminoethyl ether) *N*,*N*,*N*′, *N*′-tetraacetic acid; ER, estrogen receptor; FITC, fluorescein isothiocyanate; HEK, human embryonic kidney; HEPES, *U*-(2-hydroxyethyl) piperaxine-1-ethane sulfonic acid; I/V, current/voltage; mRNA, messenger ribonucleic acid; PKA, protein kinase A; Po, open probability; pS, picosiemens; SK, small-conductance calcium-activated K^+ channel; *Slack*, sequence like a calcium-activated potassium channel.

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Allerand et al., 2002) and multiple signaling molecules, particularly the G-proteins, which then activate a wide array of signal transduction systems, including adenylate cyclase/protein kinase A (PKA; Szego and Davis, 1967; Aronica et al., 1994; Gu and Moss, 1996; Qiu et al., 2003), phospholipase C/phosphlotidylinositol/diacylglycerol (Qiu et al., 2003; Le Mellay et al., 1997; Simoncini et al., 2000), nitric oxide synthase (Simoncini et al., 2000), protein kinase B (Akt) (Zhang et al., 2001) and mitogen activated protein kinase (Watters et al., 1997; Toran-Allerand et al., 1999; Collins and Webb, 1999; Singh et al., 2000; Kato, 2001; Razandi et al., 2003). Through alterations in signal transduction, E2 can also regulate ion channel activity (and hence cellular activation) by modulating the linkage between agonist stimulated G-protein coupled receptors and the channels that mediate their effects (Razandi et al., 2003; Qiu et al., 2003). In addition to modulation of cell signaling pathways, direct effects of E2 on channel conductance have recently been demonstrated. For example, through a G-protein linkage, E2 can directly inhibit calcium channel ion conductance (Mermelstein et al., 1996). Further, E2 activates large-conductance calcium-activated potassium channels (Maxi-K, 45–280 pS) in oocytes injected with Maxi-K mRNA by direct binding to β-subunits of the channels (Valverde et al., 1999). E2, then, can alter cell excitability by directly binding Maxi-K at the cell membrane and changing potassium channel gating (Moss and Law, 1971; Bueno and Pfaff, 1976).

In addition to the large-conductance channels, another calcium-dependent potassium channel, called *Slack* (sequence like a calcium-activated K channel), has been identified and is expressed in many brain regions, including cerebral cortex, hippocampus and thalamus (Joiner et al., 1998; Bhattacharjee et al., 2002). *Slack* potassium channels rectify outwardly with a unitary conductance of about 25–65 pS and, unlike Maxi-K channels, are inhibited by Ca^{2+} (Joiner et al., 1998). The effect of E2 on *Slack* channels has not previously been ascertained.

We attempted to answer the following questions: 1) do rat cortical neuronal membranes contain *Slack* channels? 2) if present, are the channels regulated by E2? and 3) do the effects of E2 occur through ER?

EXPERIMENTAL PROCEDURES

All experimentation involving animals described below was conducted in accord with accepted Association for Assessment and Accreditation of Laboratory Animal Care standards of humane animal care. The protocol was designed to minimize the number of animals used and to minimize any suffering.

Single channel recording

Neuronal membranes from cerebral cortex of postnatal 1 day rats were incorporated into lipid bilayers made of phosphatidylcholine: phosphatidylethanolamine in a ratio of 2:8 in decane (20 mg/ml). Solutions contained 100 mM KCL and 10 mM morpholinepropanesulfonic acid, pH 7. The internal Ca^{2+} concentration was 100 nM-20 μM. E2 (10 nM, 100 nM, 10 μM) was added to the external side of the channel at the indicated concentrations. Po, the open probability for the full conductance-state, was calculated using Pclamp software (Axon Instruments, Union City, CA, USA).

Slack plasmid preparation

Slack plasmid was kindly provided by Drs. W. J. Joiner and L. K. Kaczmarek of Yale University School of Medicine (New Haven, CT, USA) (Joiner et al., 1998). For studies in HEK-293 (human embryonic kidney) cells (ATCC, Rockville, MD, USA), *Slack* was subcloned between the *Not*I and *Xho*I sites of pcDA3 (Invitrogen, Carlsbad, CA, USA) and then between the *Not*I and *Apa*I sites of pTRACER (Invitrogen) before transient transfection.

HEK-293 cell recordings and *Slack* transfection

In preparation for transfection, HEK-293 cells were cultured in Phenol Red-free Dulbecco's modified Eagle's medium containing 10% fetal calf serum (all from Life Technologies) in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. Before transient transfection of HEK-293 cells with the *Slack*/pTRACER construct, cells were grown to approximately 50% confluence on glass bottom dishes. Cells were transfected for 5 h with 6 μl lipofectamine (Life Technologies) premixed with 1 μg cDNA. Conventional whole cell patch recordings were made from fluorescent cells 1–2 days later. Electrodes had a resistance of 3–5 mΩ for whole cell recordings. The bath solution consisted of 140 mM NaCl, 1.0 mM $CaCl_2$, 3 mM KCl, 29 mM glucose and 25 mM HEPES (pH 7.4). The pipette (intracellular) solution contained 3.25 mM KCl, 97.5 mM potassium gluconate, 5 mM EGTA and 10 mM HEPES (pH 7.2). Data were acquired on-line at 5–20 kHz, filtered at 1–2 kHz, and analyzed using pClamp 8.2 (Axon Instruments; Joiner et al., 1998). Current/voltage (I/V) curves were calculated on the basis of recordings made following voltage steps from −80 mV to +60 mV in 20 mV increments, with a holding potential of −70 mV.

Labeling with E2–bovine serum albumin (BSA)–fluorescein isothiocyanate (FITC)

HEK-293 cells were cultured as described above. HEK-293 cells stably expressing *Slack* channels (Joiner et al., 1998) or not were cultured for 24 h in glass bottom dishes, then the cells were incubated with 10 μM 17-β estradiol 6-(*O*-carboxymethyl) oxime–BSA–FITC conjugate (Sigma, St. Louis, MO, USA) for 1 h at 37 °C. Cells were washed twice with phosphate-buffered saline and viewed with a Leica DMIL inverted microscope. Fluorescence densities were quantified by NIH image software. Fluorescence data presented are representative of three independent experiments. To confirm the specificity of E2 binding, competition experiments were performed: *Slack*-transfected cells were preincubated for 15 s with either unlabeled competitor E2–BSA (0–100 μM), tamoxifen (20 μM) or ICI-182,780 (100 nM), then incubated with the competitor or receptor antagonist plus E2–BSA–FITC (10 μM) or BSA–FITC for 1 h. After labeling, the cells were fixed for 10 min in 4% paraformaldehyde and mounted as described above. Intensity measurements were obtained by outlining entire cells from three randomly chosen fields for each (concentration of E2–BSA) group and by calculating the average brightness value over the entire surface area of the cell with NIH imaging. The intensity was measured on an arbitrary gray scale from 0 to 255. Three independent experiments were conducted. For each experiment, the background intensity was subtracted from the intensity scores for each group. As a negative control for the experiment, a plasmid expressing potassium small-conductance calcium-activated K^+ channels (SK) was used to transfect HEK-293 cells. The transfected cells were incubated with E2–BSA–FITC for 1 h at 37 °C, and signals were detected by a fluorescence microscope.

E2 binding assay

Cell membranes of HEK-293 cells transfected either with or without *Slack* were incubated for 60 min at 4 °C with 0.3 mM [2,4,6,7,16,17–^3H(*N*)]-estradiol in a medium containing 50 mM Tris–HCl, 120 mM