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ORIGINAL ARTICLE

Effects of 17beta-estradiol and IGF-1 on L-type voltage-activated and stretch-activated calcium currents in cultured rat cortical neurons

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Abstract

INTRODUCTION: Calcium transport pathways are key factors for understanding how changes in the cytoplasmic calcium concentration are associated with neuroprotection because calcium is involved in the onset of death signaling in neurons.
OBJECTIVES: This study characterized the effects of 17β-estradiol and IGF-1 on voltage-activated and stretch-activated calcium channels in rat cultured cortical neurons.
METHODS: The whole-cell patch-clamp technique, using a voltage steps protocol or by applying positive pressure into the micropipette, was used on 7–10 day cultured neurons from a Wistar rat cortex, and pharmacological characterization was performed on these neurons.
RESULTS: Both 17β-estradiol and IGF-1 inhibited the currents mediated by L-type voltage-activated calcium channels, although the IGF-1 effects were lower than those of 17β-estradiol. The effect of both hormones together was greater than the sum of the effects of the individual agents. Unlike IGF-1, 17β-estradiol decreased the current mediated by stretch-activated channels. The inhibition of the classical receptors of these hormones did not affect the results.
CONCLUSION: Both hormones regulate voltage-activated calcium channels in a synergistic way, but only 17β-estradiol has an inhibitory effect on stretch-activated calcium channels. These effects are not mediated by classical receptors and may be relevant to the neuroprotective effects of both hormones because they diminish calcium entry into the neuron and decrease the possibility for the onset of apoptotic signaling.

INTRODUCTION

Calcium homeostasis can be modified by several membrane transport mechanisms to maintain the cytoplasmic concentration of calcium, which exhibits a transient elevation pattern, a key factor for signaling processes (Lin *et al.* 2007). Additionally, this strict regulation guarantees an effective control of death processes (Dupont *et al.* 2007). However, moderate calcium increases have been associated with neuronal protection (Bickler & Fahlman 2004), making calcium transport regulation a key factor for explaining the mechanisms involved in neuroprotection and neurotoxicity (Sanchez *et al.* 2010).

17β-estradiol, (E2) considered as a neuroprotector (Amantea *et al.* 2005; Ba *et al.* 2004; Bagetta *et al.* 2004; Bains *et al.* 2007; Behl 2002a; Behl 2002b; Brann *et al.* 2007; Garcia-Segura *et al.* 2001; McCullough & Hurn 2003; Gonzalez *et al.* 2008), is synthesized mostly in the ovaries as well as locally at different tissues by the action of the aromatase enzyme on other steroids. E2 is a key factor in cell regulation processes such as growth, maturation and proliferation (Audesirk *et al.* 2003). Its classical receptors (Nilsson *et al.* 2001), ERα (*estrogen receptor α*) and ERβ, which belong to the ligand-activated transcription factor family (Carpenter & Korach 2006; Katzenellenbogen 1996), are expressed in neurons (Shughrue *et al.* 1997). In addition, E2 can act more rapidly through the activation of membrane receptors, which are also expressed on neurons (Beyer *et al.* 2003; Ronnekleiv *et al.* 2007) and are coupled to G proteins (Qiu *et al.* 2003).

Additionally, insulin-like growth factor I (IGF-1), a 70-amino acid peptide with high homology to insulin and a receptor that belongs to the tyrosine kinase receptor family (De Meyts & Whittaker 2002), has known neuroprotective action (Aperghis *et al.* 2004; Bilak & Kuncl 2001; Carro *et al.* 2001; Carro *et al.* 2003; Frago *et al.* 2002; Hung *et al.* 2007) in addition to its role in neural development (Russo *et al.* 2005). IGF-1 synthesis occurs mainly in the liver under the influence of growth hormone, but many other tissues also produce IGF-1 locally. IGF-1 is highly concentrated in the brain in the prenatal stage (Popken *et al.* 2004; Rotwein *et al.* 2002) and during times of aggression (Hodge *et al.* 2007; Holzenberger *et al.* 2000). The IGF-1 receptor is broadly expressed in neurons (El-Bakri *et al.* 2004; Eshet *et al.* 2004) and its activation can modify electrophysiological cell activity (Nunez *et al.* 2003).

The action of E2 and IGF-1 are related (Quesada & Micevych 2004), particularly with respect to their neuroprotective activity (Azcoitia *et al.* 1999; Cardona-Gomez *et al.* 2001; Mendez *et al.* 2005b); even more, both hormones are involved in neural plasticity and neural development stimulation (Aberg *et al.* 2006; Cardona-Gomez *et al.* 2000b; Garcia-Segura *et al.* 2000; Kipp *et al.* 2006; Yu *et al.* 2004) and may modify the cytoplasmic calcium concentration by direct or indirect actions on ion channels (Bence-Hanulec *et al.* 2000; Blair & Marshall 1997). Likewise, their effects potentiate and feed back into each other (Cardona-Gomez *et al.* 2002b; Mendez *et al.* 2003; Mendez *et al.* 2006; Topalli & Etgen 2004; Varea *et al.* 2010), their receptors coexist (Cardona-Gomez *et al.* 2000a) and regulate reciprocally (Cardona-Gomez *et al.* 2001) and the actions of both hormones converge on two intracellular transduction pathways, MAPK (ERK) and PI3K/PKB (Cardona-Gomez *et al.* 2002a; Cardona-Gomez *et al.* 2002b; Garcia-Segura *et al.* 2006; Mendez *et al.* 2005a; Garcia-Segura *et al.* 2010).

This study investigated the effect of these two hormones on two types of calcium ion channels, L-type voltage-activated calcium channels (LVACs) and stretch-activated channels (SACs), both expressed in neurons. Voltage-gated calcium channels mediate the influx of calcium in response to membrane depolarization; these channels have been extensively studied in excitable cells and are classified in different categories (L, N, P, Q, R and T) based on their electrophysiological characteristics (Catterall 2000). LVACs are most commonly expressed in neurons and can be identified by their dihydropyridine (DHP) sensitivity (Triggle 2006); the other types of high-voltage-activated channels are also expressed in rat neuron, but LVACs are responsible for the majority of the Ca^{2+} currents activated by voltage in these cells (Xiang *et al.* 2012) and they have been related to neuroprotective mechanisms (Hu *et al.* 2013; Ilijic *et al.* 2011; Wu *et al.* 2011b)..

On the other hand, SACs are stimulated by membrane stretching and have been found in every cell, including neurons (Gottlieb *et al.* 2004; Martinac 2004). They are classified into three categories according to their permeability: potassium permeable, chloride permeable and non-selectively cation permeable (Sackin 1995). The non-selective cation-permeable channels have variable permeability to calcium and could be important transport pathways for this ion in some cells, such as in cardiac myocytes (Wang *et al.* 2009). These channels are blocked by Gd^{3+} ions at low concentrations (Hamill & McBride 1996). LVACs and SACs have been associated with death signals (Lang *et al.* 2007), and this study shows evidence of their regulation by E2 and IGF-1.

MATERIALS AND METHODS

Animals and cell cultures

Neuronal cell cultures were obtained from female Wistar rats after 18 days of pregnancy, which were fecundated under controlled conditions. A minimum number of animals were used, and their management was in accordance with international ethics statutes and approved by the Bioethics Committee of the Universidad Tecnológica de Pereira. The rats were anesthetized with ketamine (800 mg/kg) and xylazine (5 mg/kg) and sacrificed by cervical dislocation. The fetuses were sur-

gically extracted and decapitated. The cerebral cortexes were dissected and cut in small pieces in Hank's solution and then digested with a 0.25% trypsin-EDTA solution for 15 minutes; the obtained material was mechanically disaggregated after a thorough washing. The cells were resuspended in DMEM (*Dulbecco's Modified Eagle's Medium*) supplemented with L-glutamine (2 mM) and 10% fetal bovine serum. Viable cells were counted on a hemocytometer, resuspended to a 250,000 cell/mL density and then seeded on plates previously covered with 0.1% poly-L-lysine. The next day, the medium was changed to Neurobasal medium (Gibco, Grand Island, New York) with a 2% B27 supplement (Gibco, Grand Island, New York), L-glutamine (2 mM) and antibiotics (penicillin 100 IU/mL, streptomycin 100 µg/mL and neomycin 200 µg/mL). All cultures were maintained under controlled conditions (37 °C, 95% air and 5% CO_2). Every 3 days, a third of the medium volume was changed to preserve cell adhesion.

Media and chemicals

All chemicals and solutions were obtained from Sigma-Aldrich (St. Louis, USA) unless otherwise stated. In the electrophysiological experiments, the standard external solution was composed of the following (mM): NaCl 140, CsCl 5, $CaCl_2$ 2, $MgCl_2$ 1, HEPES 15 and glucose 5, with a pH of 7.4 at 25° C adjusted with NaOH. The standard pipette solution was composed of the following (mM): CsCl 110, K^+-gluconate 20, NaCl 20, $CaCl_2$ 4, BAPTA 20 (free Ca^{2+} concentration of 102 nM, calculated using Maxchelator software), $MgCl_2$ 4, HEPES 15 and glucose 5, with a pH of 7.4 at 25 °C adjusted with NaOH. The following pharmacological agents were added to the external solution immediately before electrophysiological recordings when it was necessary: tetrodotoxin (TTX, 1 µM), tetraethylammonium (TEA, 5 mM), nifedipine (10 µM), $GdCl_3$ (10 µM), E2 (between 1 pM and 100 nM), IGF-1 (between 1 nM and 1 µM), ICI182780 (10 µM) and JB1 (10 nM). In experiments where the combined effects of these agents were evaluated, agents were added simultaneously to the solution at the same concentrations as when added individually. H89 (10 µM), PD98059 (10 µM), L-NAME (5 mM), wortmannin (100 nM) and chelerythrine (2.5 µM) were added 2 hours before the appropriate experiments.

Electrophysiological recording

The whole-cell patch-clamp technique was used in all recordings. The data acquisition, experimental control and signal analysis were performed using pClamp 10.2 software and an Axopatch 200B amplifier with a CV203BU headstage and the Digidata 1440A interface (Axon Instruments, Inc.). The process was visualized with an inverted microscope (TE2000U, Nikon, Tokyo, Japan). Selected neurons were bathed in the appropriate solution for each experiment. Micropipettes of borosilicate glass were made immediately before experiments using a pipette puller (P-97, Sutter Instruments, Novato, CA, USA); each pipette had a resistance between 5 and 10 MΩ.

The pipettes were immersed in the solutions and manipulated until the surface of a cell was reached. Each cell was selected for its morphological appearance and its degree of adhesion to the plate. After the pipette reached the cell, negative pressure was applied to make a high-resistance seal between the cell and the pipette. The cellular capacitance was measured before the experiments to normalize currents. Step protocols were used for the voltage clamp experiments; the steps were applied from a holding potential of –80 mV and increased by 10 mV for test potentials from –80 to +80 mV from for 300 ms at 0.5 Hz. To record the stretch-activated currents, an increase in intracellular pressure was created by applying a controlled positive pressure to a holding potential of –80 mV. All of the experiments were repeated at least six times.

Statistics

The data analysis was performed using the analysis tools available in pClamp 10.2 software and in SPSS software. If possible, an unpaired Student´s t-test analysis was made; otherwise, the correspondent non-parametric test was used. The results are shown as the mean ± standard error of the mean (SEM). All statistical tests were two-tailed and a p-value <0.05 was considered significant.

RESULTS

Figure 1 shows the recordings obtained from a neuron subjected to the conditions described above plus the addition of sodium channel (TTX, 1 µM) and voltage-activated potassium channels (TEA, 5 mM) inhibitors. The step protocol produced a depolarization-dependent calcium current (Figure 1A), which was completely inhibited by nifedipine, a dihydropyridine that selectively inhibits LVACs (El Beheiry *et al.* 2007; Stengel *et al.* 1998) at the concentration employed (10 µM) (Figure 1B); this effect was completely reversible (data not shown). Figure 1B shows the corresponding current-voltage relation curve, which displays the maximal current obtained at the different recorded voltages; the curve has the typical behavior of a LVAC: closed channels at potentials lower than –60 mV and reverse potentials around +40 mV.

To evaluate the responses, several doses of E2 (between 1 pM and 100 nM, Figure 1C) and IGF-1 (between 1 nM and 1 µM, figure 1E) were used within the range in which these agents have neuroprotective effects (Garcia-Segura *et al.* 2010; Mendez *et al.* 2006; Zhao & Brinton 2007). Figures 1B shows the typical effects and the I-V curves under the effect of E2 (20 nM, the lower concentration to elicit the maximal response); this hormone decreased the current magnitudes at every recorded voltage (Figure 1C,G). IGF-1 (250 nM) also had inhibitory effects as shown in figure

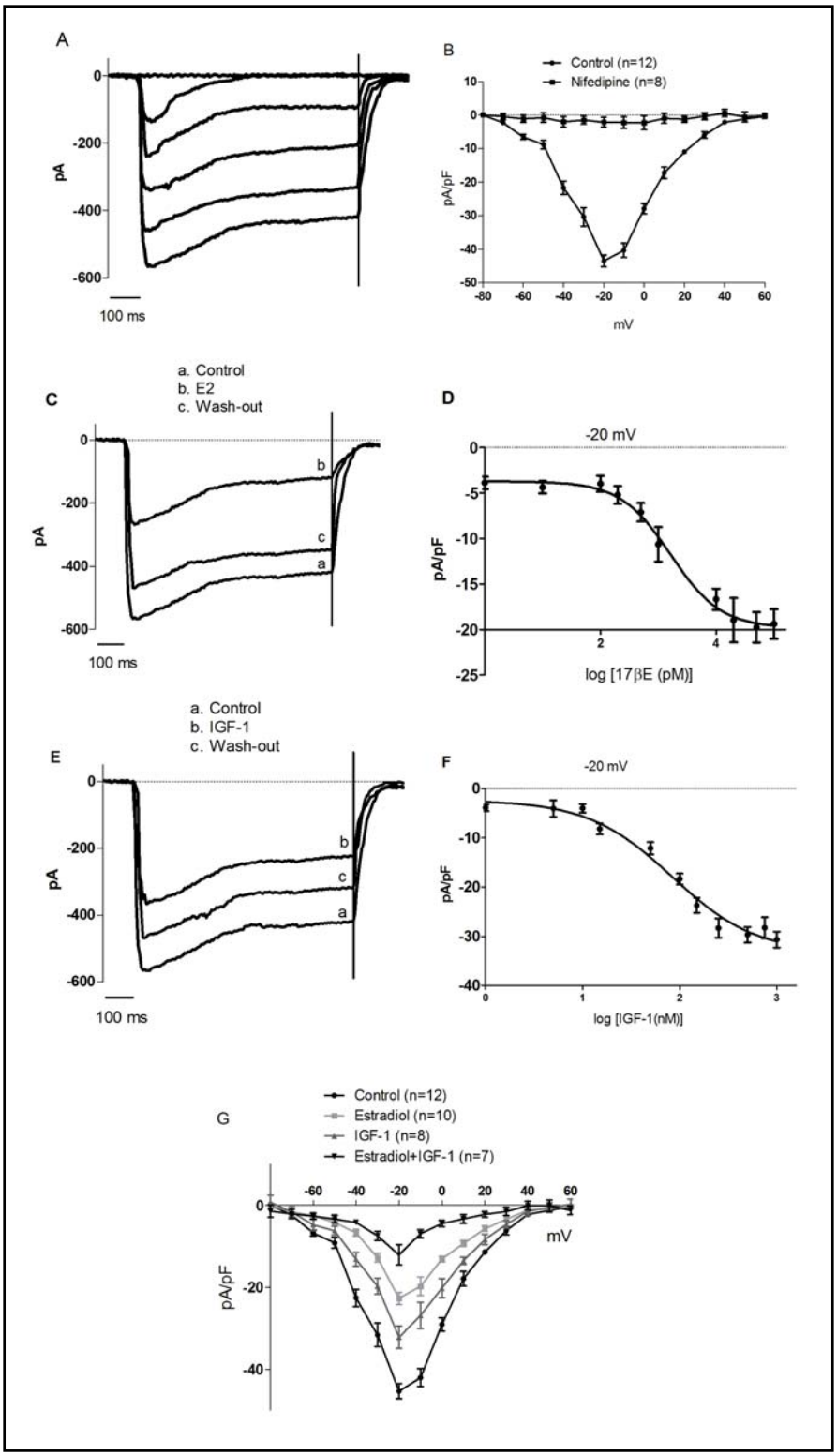

Fig. 1. A. Representative recordings of basal LVACs currents. Only the –80, –60, –50, –40, –30 and –20 (mV) recordings are shown. B. I–V relationship obtained from the recording of several cells (n is indicated in each case) in control conditions and under the effect of 10μM nifedipine; note the nearly complete inhibition of the currents. C. Recordings of the effect of 20 nM E2 on LVACs currents at –20 mV; note the nearly complete reversibility of the effect. D. E2 dose-response curve in which 20 nM was the minimal dose with the maximal response. E. Recordings of the effect of 250 nM IGF-1 on LVACs currents at –20 mV; the effect was almost completely reversible. F. IGF-1 dose-response curve, in which 250 nM was the minimal dose with the maximal response. G. I–V relationship obtained from the recordings of several cells at different voltages as indicated. The currents were normalized to capacitance (13.6 ± 2.4 pF). There are significant differences in all recordings between –40 mV and +20 mV ($p<0.05$).

1E, G; nevertheless, it had a significantly smaller effect than E2 at most voltages (Figure 1G). Simultaneous application of both hormones at the same concentrations, resulted in a stronger effect than the sum of the individual effects (Figure 1G). All the effects described above were reversible almost immediately following the clearance of the pharmacological agents as shown in Figure 1C and 1E.

Currents evoked by applying increased intrapipette pressure were recorded to evaluate the presence of SACs currents in neurons. The neurons were subjected to treatment with TTX and TEA at the previously mentioned doses and nifedipine at 100 μM, which were used to suppress voltage-activated sodium, potassium and calcium currents respectively, that might have interfered with the desired recordings. Figure 2A shows a typical recording of a neuron subjected to a pressure of 40 cm H_2O, the maximal response occurred without compromising cell vitality or the integrity of the patch-clamp. The pressure induced a current that was identical at different voltages (512 ± 34 pA) and was completely inhibited with 10 μM of $GdCl_3$, a feature of SAC currents (Figure 1A). Treatment with E2 attenuated the magnitude of the current (Figure 2C, D). 20 nM E2 was the lower concentration to elicit the maximal response (Figure 2D). On the other hand, IGF-1 had no effect on the positive pressure-induced currents (Figure 2E), even at concentrations as high as 1 μM.

All experiments were repeated in the presence of ICI182780 (10 μM) (Tocris, Bristol,UK), a classical estrogen receptor inhibitor; JB1 (10 nM), a competitive inhibitor of the binding of IGF-1 with its classical receptor; H89 (10 μM), a PKA (*protein kinase A*) inhibitor; PD98059 (10 μM), a MAPK

(*mitogen-activated protein kinase*) inhibitor; L-NAME (5 mM), a NOS (*nitric oxide synthase*) inhibitor; wortmannin (100 nM), a PI_3K (*phosphatidyl inositol 3 kinase*) inhibitor; or chelerythrine (2.5 µM), a PKC (*protein kinase C*) inhibitor. No significant differences in the recorded currents were observed under these different conditions (Table 1).

DISCUSSION

This study evaluated the effects of two neuroprotective hormones, E2 and IGF-1, on two calcium pathways in rat primary cultured neurons.

The results show that both hormones have an inhibitory effect on LVACs, but not on other high-voltage-activated calcium currents. IGF-1 had a significantly lower effect. These findings are consistent with previous reports, which have shown that IGF-1 (Blair & Marshall 1997) has inhibitory effects on high-voltage-gated calcium channels from rat hippocampal neurons and that E2 inhibits LVACs from sensory neurons (Lee *et al.* 2002), hippocampal neurons (Brewer *et al.* 2009) and smooth myocytes (Ullrich *et al.* 2007). In contrast, other studies have shown stimulating effects of E2 on LVACs from hippocampal neurons (Wu *et al.* 2005; Sarkar *et al.* 2008). These effects contrast our findings, which can be explained by different experimental circumstances, different doses or specific effects in a cell type. The doses employed here were those derived from the dose-response curves performed for each agent and they are in the range of those employed in other studies (Di Liberto *et al.* 2012; Hernandez-Fonseca *et al.* 2012; Hilton *et al.* 2006; Huang *et al.* 2013; Nixon & Simpkins 2012; Smejkalova & Woolley 2010; Sribnick *et al.* 2009; Wu *et al.* 2011a; Yu *et al.* 2012). There was an additive effect on LVACs when both hormones were applied simultaneously, which suggests the existence of cross-talk mechanisms between the two signaling pathways; this synergistic method of action has been shown in other studies (Cardona-Gomez *et al.* 2001; Cardona-Gomez *et al.* 2002b; Garcia-Segura *et al.* 2006; Mendez *et al.* 2005a; Sanchez *et al.* 2011).

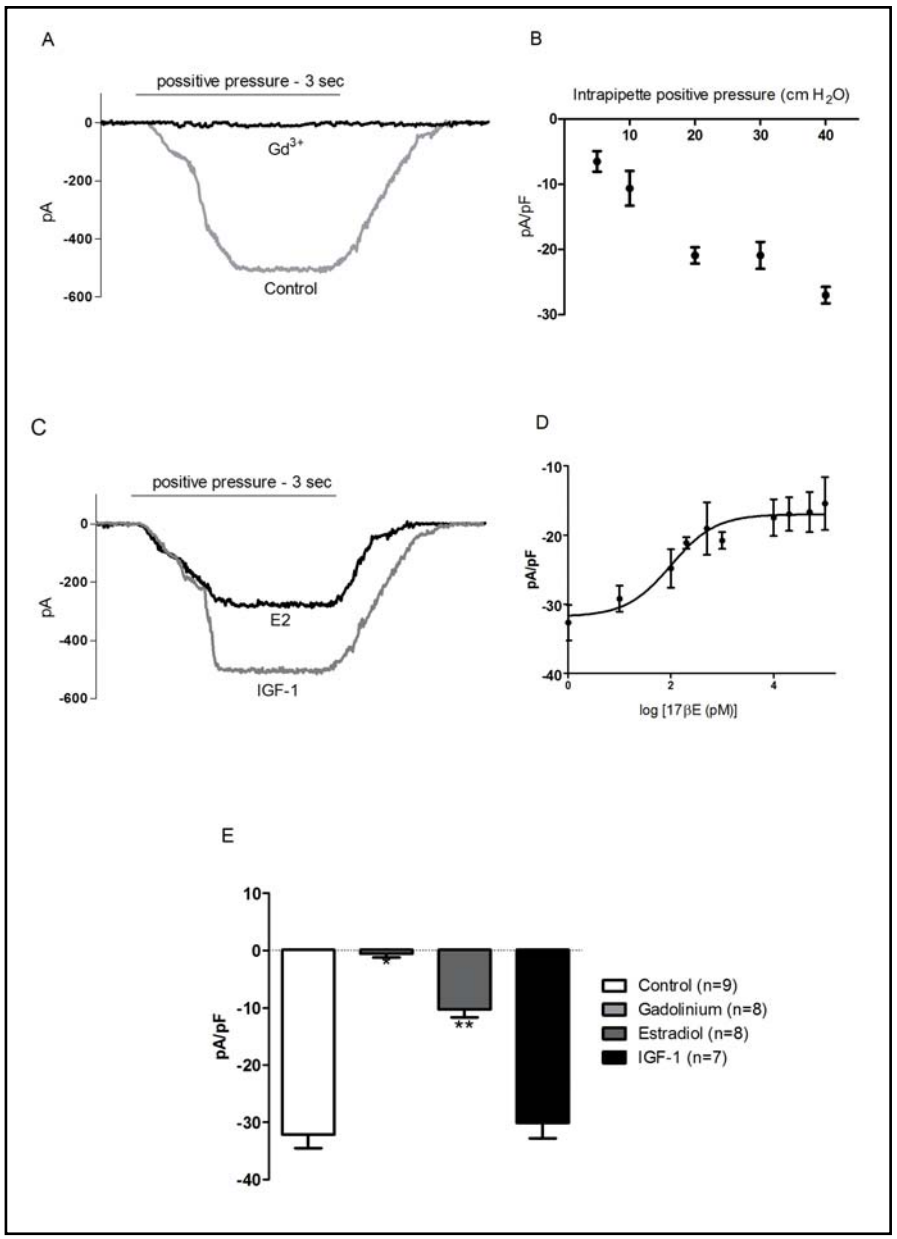

Fig. 2. A. Recording of 40 cmH_2O intrapipette positive pressure-induced calcium currents at –60 mV under control conditions and under the effect of 10 µM Gd^{3+}; note the complete abolition of the current. B. Pressure-response relationship; pressures larger than 40 cmH2O disrupted the membrane patch. C. Recording of SACs currents under the effect of 20 µM E2 and 1 µM IGF-1; note the nearly complete reversibility of the effect of E2 and the absence of effects of IGF-1. D. E2 dose-response curve in which 20 nM was the minimal dose with the maximal response. E. Comparison of the effects of E2 and IGF-1 on SACs currents. All of the currents were normalized to capacitance (13.6 pF ± 2.4 pF). * denotes significant differences with control ($p<0.05$) and ** denotes significant differences ($p<0.05$) with control and the * marked bar.

Only E2 had significant inhibitory effects on SACs, which were identified in this study because of their activation after membrane stretching (induced by the application of positive pressure to the micropipette) and because of their sensitivity to low concentrations of Gd^{3+}. Moreover, IGF-1 did not affect SAC current, even at high concentrations, but the reasons are unclear. It is

Tab. 1. Effects of several inhibitors on LVACs currents normalized at –20 mV, potential in which the maximal current was obtained, and on SACs currents, at –60 mV. Values are shown as the mean ± SEM. There are no significant differences in any of the comparative data groups with controls ($p>0.05$). LVAC: L-voltage-activated currents recorded at –20 mV and shown as pA/pF; SAC: stretch-activated currents recorded at –60 mV, shown as pA/pF; E2: 17β-estradiol; IGF-1: insulin-like growth factor 1. $n = 6$ in all cases.

		Control	ICI182780	JB1	H89	PD98059	L-NAME	Wortmannin	Chelerythrine
E2	LVAC	–18.5±3.1	–20.7±4.6	–17.7±3.4	–19.6±4.5	–21.3±6.1	–20.1±3.9	–20.6±2.1	–18.2±4.6
	SAC	–12.9±2.7	–11±2.8	–9.9±4.6	–13±1.9	–12.3±3.9	–9.6±3.8	–11.9±4.9	–10.1±3.6
IGF-1	LVAC	–26.6±4.8	–28.5±5.3	–29.6±4.6	–26.2±6.3	–36.1±4.7	–29.1±5.9	–30.8±4.8	–27.3±6.2
	SAC	–30.9±4.3	–29.6±6.6	–33.5±5.4	–30.9±4.5	–27±3.6	–31.5±3.7	–27.1±4.8	–34.4±2.6
E2 + IGF-1	LVAC	–10.7±5.6	–9.5±2.1	–6.6±2.7	–8.9±4.6	–10.4±3.7	–12.8±4.9	–9.7±3.2	–10.2±3.1
	SAC	–11.5±5.2	–14.9±4.7	–13.7±3.5	–12.7±5.7	–10.8±2.2	–12.6±2.2	–13.6±6.3	–11.8±5.5

important to consider these channels when evaluating the neuroprotective and neurotoxic effects of certain drugsbecause of their expression in neurons (Takahashi & Gotoh 2000) and their potentially high conductance (Sackin 1995; Martinac 2004), which may be responsible for significant calcium currents.

All the previously described effects occurred in a very short period of time, suggesting that both hormones utilize a mechanism of action other than a genomic pathway, which requires a longer period of time. The fact that the effects did not change even in the presence of inhibitors of classical receptors suggests a direct action on the carrier protein or through a different type of receptor. Fast, non-genomic actions have been described for E2 through a membrane receptor (Beyer *et al.* 2003; Ronnekleiv *et al.* 2007), and it is possible that both hormones can act directly on ion channels. Because non-genomic actions on neurons, neuroprotection included,have been associated with PKA, PKC and NOS activation (Segars & Driggers 2002; Vasudevan & Pfaff 2008) and because MAPK and IP_3K are the classical pathways involved in IGF-1 intracellular effects and in the synergistic effects of both hormones (Bondy & Cheng 2004; Blair & Marshall 1997; Fernandez *et al.* 2007; Mendez *et al.* 2006), the cells were pretreated with specific inhibitors for PKA, PKC, MAPK, IP_3K and NOS. None of these agents modified the effect of E2 or IGF-1 on LVACs or SACs, which eliminates these signaling pathwaysas a mechanism that explains the findings in the present study. Moreover, some authors have found that estrogens may activate glutamate receptors, in particular mGLUR2/3, which in turn can block LVACs in rat hippocampal neurons (Boulware *et al.* 2005) and rat retinal ganglion cells (Robbins *et al.* 2003). However, this effect is mediated by PKA and the inhibition of the PKA pathway did not affect the action of E2 on LVACs.

The effects found here could be mediated by another transduction mechanism, but the possibility that these two hormones act directly on the channels can not be ruled out; , since there is evidence of E2 acting directly on calcium channels(Ullrich *et al.* 2007), although at higher concentrations. Previously, our laboratory reported synergistic effects of E2 and IGF-1on the sodium calcium exchanger in rat cortical neurons (Sanchez *et al.* 2011); these effects were not mediated by classical receptors and promoted an intracellular calcium decrease, in a similar manner as the effects on LVACs and SACs that were found in this study.

If these hormones affect calcium transport pathways, this mechanism could provide neuronal survival capacity against harmful agents, by inhibiting calcium influx and leading to a decrease in intracellular levels of calcium, avoiding the activation of the enzymatic cascades associated with cell death. (Sanchez *et al.* 2010).

Pharmacological neuroprotection requires knowledge about the regulation of cytoplasmic calcium levels and, in a similar manner, it is also essential to understand calcium transportation mechanisms (Sanchez *et al.* 2010). Both LVACs and SACs are plausible targets of several neuroprotective agents according to the findings of the present study, and therefore, the modulation of these channels can be a useful strategy for the development of new perspectives on neuroprotection. Although the doses employed in the present study were higher than the plasma concentration for both hormones, the effects described here are still relevant given that local and systemic concentrations are not necessarily the same and the effects on neurons may be modified by the local environment. Further research needs to be conducted to clarify the significance of these effects in clinical neuroprotection.

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