

Activation of InsP₃ receptors is sufficient for inducing graded intrinsic plasticity in rat hippocampal pyramidal neurons

Sufyan Ashhad,¹ Daniel Johnston,² and Rishikesh Narayanan¹

¹Cellular Neurophysiology Laboratory, Molecular Biophysics Unit, Indian Institute of Science, Bangalore, India; and ²Center for Learning and Memory, The University of Texas at Austin, Austin, Texas

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Ashhad S, Johnston D, Narayanan R. Activation of InsP₃ receptors is sufficient for inducing graded intrinsic plasticity in rat hippocampal pyramidal neurons. *J Neurophysiol* 113: 2002–2013, 2015. First published December 31, 2014; doi:10.1152/jn.00833.2014.—The synaptic plasticity literature has focused on establishing necessity and sufficiency as two essential and distinct features in causally relating a signaling molecule to plasticity induction, an approach that has been surprisingly lacking in the intrinsic plasticity literature. In this study, we complemented the recently established necessity of inositol trisphosphate (InsP₃) receptors (InsP₃R) in a form of intrinsic plasticity by asking if InsP₃R activation was sufficient to induce intrinsic plasticity in hippocampal neurons. Specifically, incorporation of D-myo-InsP₃ in the recording pipette reduced input resistance, maximal impedance amplitude, and temporal summation but increased resonance frequency, resonance strength, sag ratio, and impedance phase lead. Strikingly, the magnitude of plasticity in all these measurements was dependent on InsP₃ concentration, emphasizing the graded dependence of such plasticity on InsP₃R activation. Mechanistically, we found that this InsP₃-induced plasticity depended on hyperpolarization-activated cyclic nucleotide-gated channels. Moreover, this calcium-dependent form of plasticity was critically reliant on the release of calcium through InsP₃Rs, the influx of calcium through N-methyl-D-aspartate receptors and voltage-gated calcium channels, and on the protein kinase A pathway. Our results delineate a causal role for InsP₃Rs in graded adaptation of neuronal response dynamics, revealing novel regulatory roles for the endoplasmic reticulum in neural coding and homeostasis.

endoplasmic reticulum; HCN channels; hippocampus; inositol trisphosphate receptors; intrinsic plasticity

INOSITOL 1,4,5-TRISPHOSPHATE (InsP₃) receptors (InsP₃R), activated by their endogenous agonist InsP₃, are calcium release channels that critically contribute to the excitable nature of neuronal endoplasmic reticulum (ER) (Berridge 2009; Verkhratsky 2005). Cytosolic mobilization of InsP₃ and the consequent activation of InsP₃Rs play important roles in several physiological processes including synaptic plasticity, calcium waves, calcium homeostasis, gene transcription, and integration of intraneuronal biochemical signals (Berridge 2002; Berridge et al. 2000; Choe and Ehrlich 2006; Foskett et al. 2007; Patterson et al. 2004; Rose and Konnerth 2001; Ross 2012; Taylor and Tovey 2010; Verkhratsky 2005). InsP₃R-related signaling mechanisms are extremely complex, with several upstream activators, subtype-dependent modulation by cytosolic calcium, structural and functional interactions involving numerous calcium sources and ion channels, state-depend

ent spatial spread of calcium release through these receptors, and a diversity of downstream signaling targets contributing to this complexity (Ashhad and Narayanan 2013; Choe and Ehrlich 2006; Clapham 2007; Fagni et al. 2000; Foskett 2010; Foskett et al. 2007; Kato et al. 2012; Taylor and Tovey 2010). Consequent to this complexity and the inaccessibility of ER receptors to direct electrophysiological recordings, study of InsP₃Rs has predominantly been through direct pharmacological activation of upstream receptors, including muscarinic acetylcholine receptors and group 1 metabotropic glutamate receptors (mGluR), or indirect activation of InsP₃Rs through store-depletion agents (Berridge 2009; Brager and Johnston 2007; Brager et al. 2013; Clemens and Johnston 2014; Fernandez de Sevilla et al. 2008; Huber et al. 2000; Narayanan et al. 2010; Pin and Duvoisin 1995; Verkhratsky 2005; Watabe et al. 2002). However, these metabotropic receptors and store-depletion agents regulate several downstream signaling cascades that are independent of InsP₃Rs (Pin and Duvoisin 1995; Thiele 2013; Wang et al. 2007), and there are nonspecificities associated with the associated pharmacological agents (Charpak et al. 1990; Chuang et al. 2000; Ugolini et al. 1999), implying that such analysis is not specific to the activation of InsP₃Rs alone. Furthermore, the recent discovery of intracellular mGluRs (Purgert et al. 2014) also complicates the interpretation of such experiments, together leading to ambiguities on the specific roles of cytosolic InsP₃ in neurophysiology.

The synaptic plasticity literature has focused on establishing necessity and sufficiency as two essential features in causally relating a signaling molecule to a form of plasticity (Bliss and Collingridge 1993; Lledo et al. 1995; Lynch 2004; Malenka and Bear 2004; Malenka et al. 1988; Martin et al. 2000; Neves et al. 2008). For instance, whereas the necessity of postsynaptic calcium elevation for the induction of long-term potentiation (LTP) was established using calcium chelators (Lynch et al. 1983), the sufficiency of calcium for inducing LTP was later demonstrated using postsynaptic uncaging of calcium (Malenka et al. 1988). Similarly, turning to the involvement of calcium/calmodulin-activated kinase II (CaMKII) in LTP, specific CaMKII inhibitors were employed to show that CaMKII was necessary for the induction of LTP (Otmakhov et al. 1997), whereas the sufficiency of CaMKII in inducing LTP was revealed by injecting constitutively active CaMKII using patch micropipettes (Lledo et al. 1995). In this context, it was recently demonstrated that InsP₃Rs are necessary for the induction of plasticity of intrinsic response dynamics that is consequent to calcium store depletion (Narayanan et al. 2010). In this study, the authors demonstrated that calcium store depletion, a pathologically critical phenomenon that is known

Address for reprint requests and other correspondence: R. Narayanan, Molecular Biophysics Unit, Indian Institute of Science, Bangalore 560 012, India (e-mail: rishi@mbu.iisc.ernet.in).

to activate numerous intracellular signaling pathways (Berridge 2002; Cahalan 2009; Lefkimiatis et al. 2009; Mattson et al. 2000; Verkhatsky 2005), activates a putative neuroprotective response by reducing neuronal excitability. Although this study demonstrated the necessity of InsP₃R in inducing a form of intrinsic plasticity, the sufficiency of InsP₃R activation for altering neuronal intrinsic properties has remained unexplored. More generally, especially compared with the more extensive synaptic plasticity literature, the causal delineation of signaling components associated with intrinsic plasticity, by employing necessity and sufficiency as two distinct and essential tools, has been surprisingly lacking.

To fill this lacuna, and to disambiguate the precise roles of cytosolic InsP₃ in neurophysiology, we asked if direct injection of InsP₃ into hippocampal pyramidal neurons was sufficient to alter intrinsic response properties. Our results demonstrate that direct activation of InsP₃Rs is sufficient to induce long-lasting plasticity in neuronal intrinsic response dynamics and uncover the signaling mechanisms underlying this plasticity. Importantly, we also show that this InsP₃-induced form of plasticity is graded, whereby lesser activation of InsP₃Rs led to lesser amount of plasticity, suggesting potential roles for this form of plasticity under physiological conditions. Together with and complementary to the earlier demonstration of the necessity of InsP₃Rs in inducing a form of intrinsic plasticity (Narayanan et al. 2010), our demonstration that the specific activation of InsP₃Rs is sufficient to induce graded intrinsic plasticity provides direct causal evidence for novel roles of InsP₃ and intracellular stores in regulating neuronal integration, neural coding, and homeostasis through changes in intrinsic neuronal properties.

MATERIALS AND METHODS

Ethical approval. All experiments reported in this study were performed in strict adherence to the protocols cleared by the Institute Animal Ethics Committee of the Indian Institute of Science (Bangalore, India) and The University of Texas at Austin Institutional Animal Care and Use Committee. Experimental procedures were similar to previously established protocols (Narayanan et al. 2010; Narayanan and Johnston 2007, 2008) and are detailed below.

Surgery and slice preparation. Male Sprague-Dawley rats (4–10 wk old) were anesthetized by intraperitoneal injection of a combination of ketamine and xylazine. After onset of deep anesthesia, as determined by cessation of toe-pinch reflex, rats were transcardially perfused with ice-cold cutting solution containing (in mM) 210 sucrose, 2.5 KCl, 1.25 NaH₂PO₄, 25 NaHCO₃, 0.5 CaCl₂, 7 MgCl₂, 7 dextrose, and 3 sodium pyruvate (all from Sigma Aldrich). They were then decapitated, and the brain was removed quickly in the presence of ice-cold cutting solution. Near-horizontal slices (350 μm) were prepared from the hippocampus, using a VT1000P vibratome (Leica), while submerged in oxygenated ice-cold cutting solution. The slices were incubated for 15–25 min at 34°C in a holding chamber containing (in mM) 125 NaCl, 2.5 KCl, 1.25 NaH₂PO₄, 25 NaHCO₃, 2 CaCl₂, 2 MgCl₂, 10 dextrose, and 3 sodium pyruvate and then at room temperature for at least 1 h before recording. The holding chamber was continuously carbogenated with a mixture of 95% O₂ and 5% CO₂ gas.

Electrophysiology. Slices were visualized under a ×63 water-immersion lens through a Dodt contrast microscope (Carl Zeiss Axioexaminer). Somatic whole cell current-clamp recordings were made from CA1 pyramidal neurons using a Dagan BVC-700A amplifier. Data acquisition was done using custom-written software in the Igor Pro environment (Wavemetrics), with signals sampled at 10

kHz. During the entire course of experiments the slices were perfused with carbogenated artificial cerebrospinal fluid (ACSF) at ~34°C containing (in mM) 125 NaCl, 3 KCl, 1.25 NaH₂PO₄, 25 NaHCO₃, 2 CaCl₂, 1 MgCl₂, and 10 dextrose. Borosilicate glass electrodes, pulled (P-97 Flaming/Brown micropipette puller; Sutter) from capillaries of 1.5-mm outer diameter and 0.86-mm inner diameter (Sutter), with resistance of 3–7 MΩ were used for patch-clamp recordings. The intracellular pipette solution contained (in mM) 120 K-gluconate, 20 KCl, 10 HEPES, 4 NaCl, 4 MgATP, 0.3 Na₂-GTP, and 7 K₂-phosphocreatine, pH 7.3 with KOH. Series resistance was monitored and compensated online using the bridge-balance circuit of the amplifier. Experiments were discarded only if the initial resting membrane potential was more depolarized than –60 mV, if series resistance rose above 30 MΩ, or if there were fluctuations in temperature during the course of the experiment. Unless otherwise stated, experiments were performed at the initial resting membrane potential of the cell. Voltages have not been corrected for the liquid junction potential, which was experimentally measured to be ~8 mV. Control recordings were performed at regular intervals to ensure that there were no time-dependent changes (over a 45-min period) in any of the intrinsic properties when electrophysiological recordings were performed in the absence of any pharmacological agent.

Pharmacological agents. Drugs used in the experiments were D-myo-InsP₃ (InsP₃; Sigma Aldrich or Tocris Bioscience), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), 10 μM (+)bicuculline, 10 μM picrotoxin, 50 μM D,L-2-amino-5-phosphonovaleric acid (D,L-APV), and 2 μM CGP55845 (all synaptic blockers from Allied Scientific), 50 μM NiCl₂ (Sigma-Aldrich), 10 μM nimodipine (Tocris Bioscience), 1 mg/ml heparin (20,000–25,000 molecular weight; Calbiochem), 20 μM ZD7288 (Tocris Bioscience), 20 mM BAPTA (Life Technologies), 20 μM PKA inhibitor (PKI) (14-22) amide, myristoylated (PKAi peptide; Tocris Bioscience), and 500 nM KT5720 (Tocris Bioscience). For experiments with ZD7288, slices were first incubated in ACSF containing 100 μM ZD7288 for 10 min before the start of the experiment. For the experiments with KT5720, slices were also pretreated with 500 nM KT5720 for at least 45 min before the start of recordings. Necessary care was taken and appropriate controls performed for each of the drugs used to ensure that there were no time-dependent changes initiated by just the presence of the drug in the bath or pipette. Through the course of the study, experiments with only 10 μM InsP₃ were interleaved with experiments where any of the other pharmacological agents were also applied. This was to ensure that the InsP₃ stock was not degraded and elicited the same levels of plasticity in the absence of the drug.

Data analysis. Physiologically relevant measurements from the recordings were computed by employing well-established analysis procedures (Narayanan et al. 2010; Narayanan and Johnston 2007, 2008). Specifically, for measuring input resistance (R_{in}), the response of neurons to pulse-current injection was analyzed and its steady-state voltage deflection plotted against the injected current to get the current-voltage (I - V) curve. It was fitted with a straight line whose slope formed the R_{in} of that neuron. α -Excitatory postsynaptic potentials (α -EPSPs) were evoked by current injections of the form $I_{\alpha} = I_{\max} t \exp(-\alpha t)$, with $\alpha = 0.1 \text{ ms}^{-1}$. Temporal summation (S_{α}) was analyzed as the ratio of the first α -EPSP amplitude to the fifth α -EPSP amplitude from the voltage response to five α -excitatory postsynaptic current injections at 20 Hz. A linear chirp current (see Fig. 1B) spanning 15 Hz in 15 s was employed to estimate local impedance, an important measure of frequency-dependent neuronal excitability (Narayanan and Johnston 2008). The choice of the chirp rate at 1 Hz/s (15 Hz in 15 s) was dictated by experiments that demonstrated that a further increase in the chirp rate did not enhance the accuracy of the impedance-based measurements (Narayanan and Johnston 2007). To get the impedance amplitude profile (ZAP), the Fourier transform of voltage response of the neuron to the chirp stimulus was divided by the Fourier transform of injected current (Fig. 1F). The frequency at which this impedance amplitude profile reached

its maximum ($|Z|_{\max}$) formed the resonance frequency (f_R). Resonance strength (Q) was defined as the ratio of $|Z|_{\max}$ to the impedance magnitude at 0.5 Hz (Fig. 1*F*). The impedance phase profile (ZPP) was computed as the phase of the Fourier transform of voltage response to Fourier transform of the chirp stimulus. Total inductive phase (Φ_L) was defined as the area under the inductive part of the ZPP. All data analyses were performed using custom-written software in Igor Pro (Wavemetrics), and statistical analyses were performed using the R computing package (<http://www.r-project.org/>).

RESULTS

The main objective of this study was to assess the specific role of InsP₃ in altering intrinsic response dynamics (IRD) of CA1 pyramidal neurons. To fulfill this, somatic whole cell current-clamp recordings from these neurons were performed with different concentrations of D-myo-InsP₃ in the recording pipette (Fig. 1, *A* and *B*). At the start and the end of the experiment, current pulses ranging from -50 to $+50$ pA (for

700-ms duration), with an increment of 10 pA, were injected to obtain the steady-state voltage response of the neuron. The response of the neurons to five α -excitatory postsynaptic currents (α -EPSCs) was also measured at these time points. For 45 min after the initial measurements, the response of the neuron to a chirp stimulus spanning 15 Hz in 15 s was continuously (2 per min) monitored (Fig. 1*B*). A hyperpolarizing test pulse of 100 pA was appended to the chirp stimulus to obtain an estimate of the input resistance. The neuron was maintained in subthreshold voltages through the entire course of the experiment to avoid synergistic interaction between backpropagating action potentials and InsP₃ in the pipette (Ross 2012).

Inclusion of InsP₃ was sufficient to induce persistent plasticity in neuronal intrinsic response dynamics. We recorded several measurements of neuronal IRD through the course of our experiment with 10 μ M D-myo-InsP₃ included in the pipette (Fig. 1). We found that the inclusion of InsP₃ in the

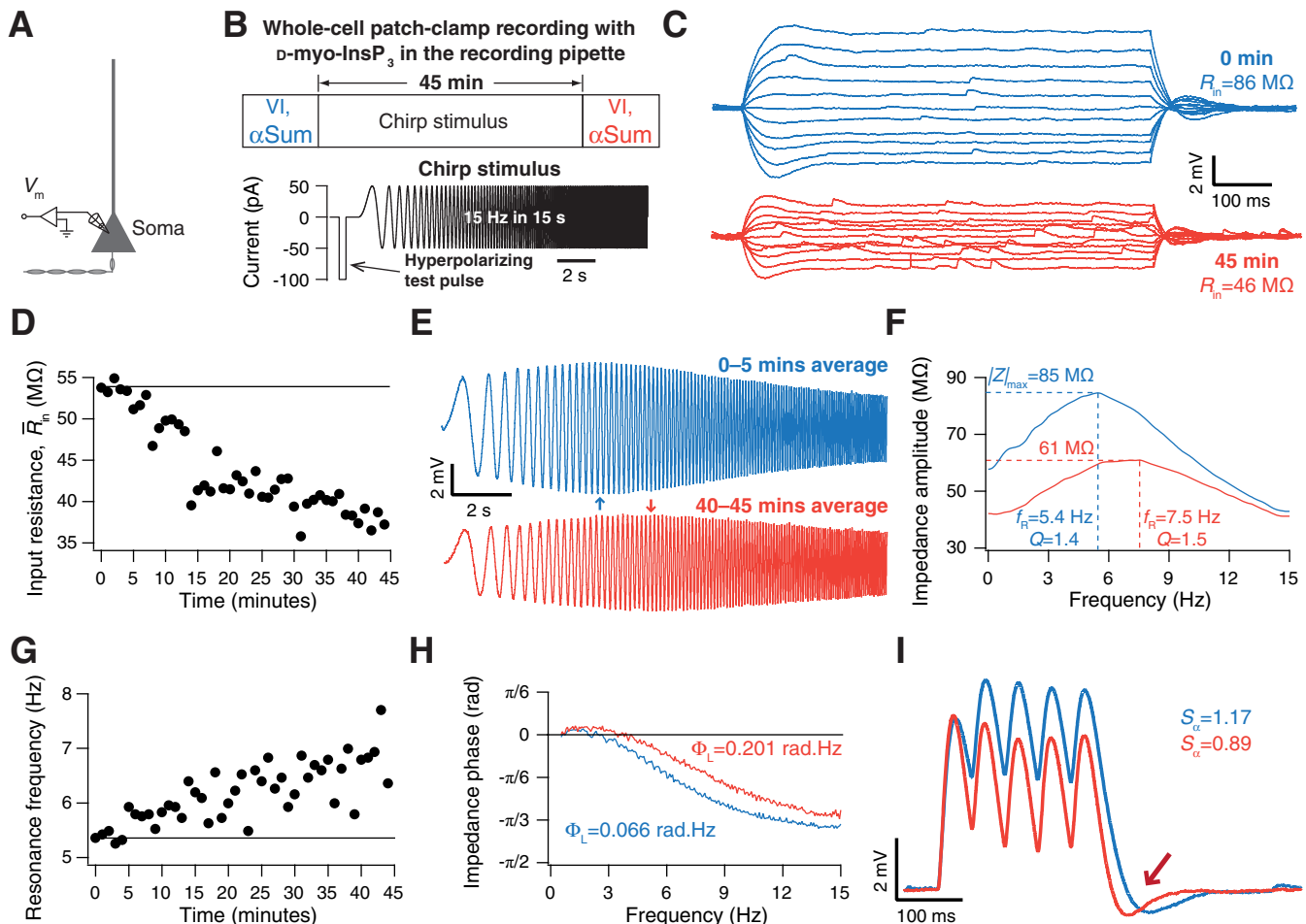


Fig. 1. Inclusion of inositol trisphosphate (InsP₃) in the recording pipette was sufficient to induce plasticity in intrinsic response dynamics of a hippocampal pyramidal neuron. *A* and *B*: schematic diagram depicting the whole cell current-clamp recording setup (*A*) and experimental protocol (*B*, top) used to study InsP₃-induced plasticity of intrinsic response dynamics (IRD). Whole cell current-clamp recording was performed with 10 μ M D-myo-InsP₃ in the recording pipette. The color code for all traces (*B*, top) and the chirp stimulus (*B*, bottom) are also depicted. *V_m*, membrane potential. *C*: voltage response of a representative neuron to the pulse-current injections (*VI* in *B*), at the beginning (blue) and after 45 min (orange) of the experiment. Input resistance (R_{in}) values obtained from these traces are also shown. *D*: time course of an estimate of input resistance, R_{in} (computed from steady-state voltage response of the neuron to the hyperpolarizing pulse part of the chirp stimulus shown in *B*). *E*: voltage response of the neuron to the chirp stimulus at the beginning (blue; 0–5 min average) and the end (orange; 40–45 min average) of the experiment. *F*: impedance amplitude profiles obtained from traces shown in *E*, also depicting the measurements obtained from these profiles: resonance frequency (f_R), maximal impedance amplitude ($|Z|_{\max}$), and resonance strength (Q). *G*: time course of f_R . *H*: impedance phase profiles obtained from traces shown in *E*, also depicting the values of total inductive phase (Φ_L) obtained from these profiles. *I*: voltage response of the neuron to the injection of a sequence of 5 alpha currents (20 Hz) at the beginning (blue) and end (orange) of the experiment. The values of summation ratio (S_α) are shown, and the red arrow indicates an increase in the rebound potential after 45 min. All traces and analyses presented are from the same neuron.

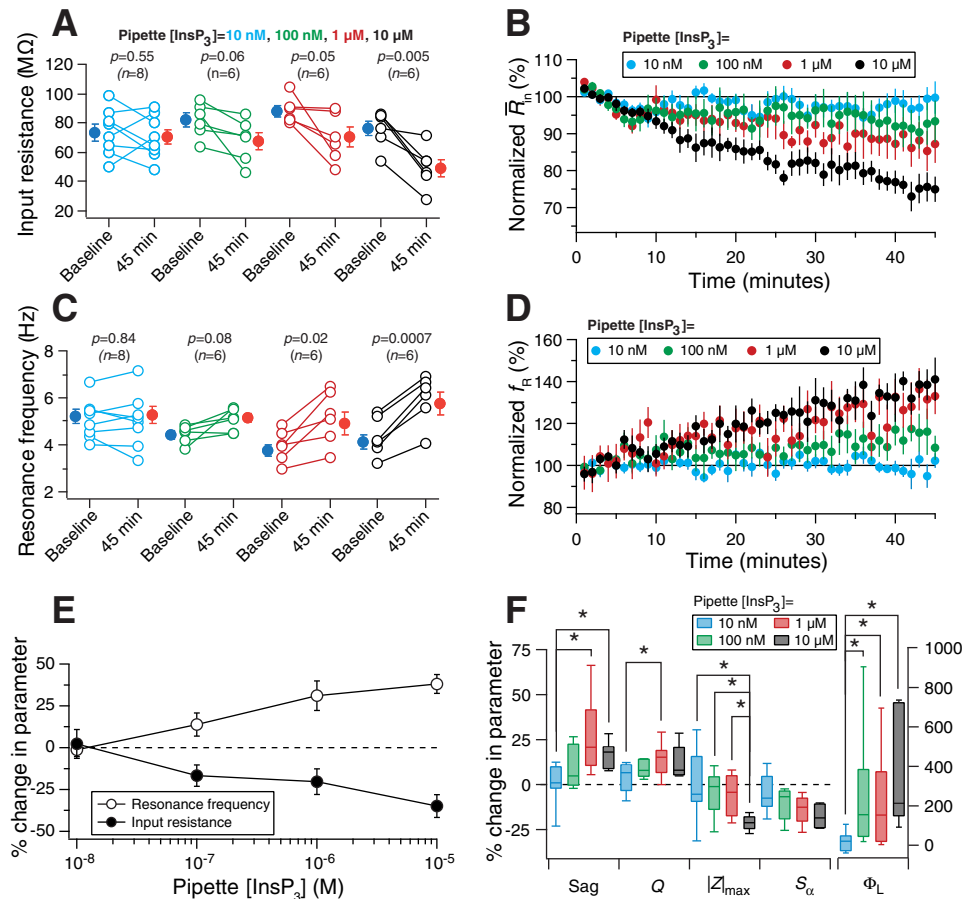


Fig. 2. InsP₃-induced plasticity in intrinsic response dynamics was graded, with the gradation dependent on InsP₃ concentration ([InsP₃]) in the recording pipette. *A*: population plots of R_{in} measured at the beginning (blue) and the end (orange) of experiments where different [InsP₃] were included in the recording pipette. The color codes for [InsP₃] are as follows: cyan, 10 nM ($n = 8$); green, 100 nM ($n = 6$); red, 1 μ M ($n = 6$); and black, 10 μ M ($n = 6$). *B*: time courses of normalized \bar{R}_{in} when experiments were performed with various [InsP₃] in the recording pipette. *C*: population plots of f_R measured at the beginning (blue) and the end (orange) of experiments where different [InsP₃] were included in the recording pipette. *D*: time courses of normalized f_R when experiments were performed with various [InsP₃] in the recording pipette. *E*: %change in R_{in} and f_R (at the end of the experiment, with reference to the beginning) plotted as a function of [InsP₃] in the recording pipette. For *A–E*, data are means \pm SE and P values (when presented) are from paired Student's t -tests. *F*: summary plot of %change in various measurements (from their respective baseline values) after 45 min into the recording with various [InsP₃]. Data are medians and quartiles. * $P < 0.05$, Mann-Whitney test.

pipette induced a persistent and significant reduction in the input resistance (R_{in}) of these neurons (Fig. 1, *C* and *D*). InsP₃-induced plasticity was also reflected in impedance-related measurements as an increase in resonance frequency (f_R ; Fig. 1, *E–G*), resonance strength (Q ; Fig. 1, *E* and *F*), and total inductive phase (Φ_L ; Fig. 1*H*), accompanied by a reduction in maximal impedance amplitude ($|Z|_{max}$; Fig. 1, *E* and *F*) and temporal summation (S_α ; Fig. 1*I*). The temporal progression of InsP₃-induced changes revealed a monotonic reduction in R_{in} (Fig. 1*D*) and a corresponding increase in f_R (Fig. 1*G*), persistent for the 45-min time course of the experiment. We repeated the experiment with 10 μ M D-myo-InsP₃ included in the pipette for several neuronal recordings and found significant plasticity across all these measurements to be consistent across neurons (Fig. 2 and Table 1). Note that although InsP₃ was present in the pipette through the course of the experiment, it is expected that cytosolic enzymes metabolize exogenously applied InsP₃ (Berridge and Irvine 1989; Irvine and Schell 2001; Shears 1989), thereby restricting the period of InsP₃R activation by exogenously applied InsP₃.

InsP₃-induced plasticity of IRD was graded and was dependent on InsP₃ concentration. Although the results with 10 μ M D-myo-InsP₃ report its sufficiency to induce intrinsic plasticity in hippocampal neurons, we did not know if this form of plasticity was a graded or an all-or-none phenomenon. To address this, we repeated our experiment with various concentrations of InsP₃ in the recording pipette and found that the magnitude of InsP₃-induced plasticity in all IRD measurements was dependent on the concentration of InsP₃ ([InsP₃]). Specif-

ically, increasing [InsP₃] in the recording pipette significantly increased the percentage changes in R_{in} (Fig. 2, *A*, *B*, and *E*) and f_R (Fig. 2, *C–E*), apart from imparting similar increases across other IRD-related measurements including sag ratio (Fig. 2*F*). These results suggested that InsP₃-induced plasticity was graded, thereby pointing toward a potential role for InsP₃ mobilization under physiological conditions in altering neuronal response properties.

InsP₃-induced plasticity was expressed through changes in HCN channel properties. Which ion channel mediated the expression of InsP₃-induced plasticity of IRD? The direction of

Table 1. Measurements sensitive to changes in HCN channels

| Measurement | Initial | 45 Min | P Value |
|--------------------------|-----------------|-----------------|----------------------|
| RMP, mV | 60.9 \pm 0.4 | 56.3 \pm 1.3 | 3.4 $\times 10^{-3}$ |
| R_{in} , M Ω | 76.0 \pm 5.0 | 49.5 \pm 5.8 | 5.1 $\times 10^{-3}$ |
| Sag, % | 19.7 \pm 2.2 | 22.9 \pm 2.5 | 2.6 $\times 10^{-3}$ |
| f_R , Hz | 4.13 \pm 0.3 | 5.77 \pm 0.4 | 6.9 $\times 10^{-4}$ |
| Q | 1.2 \pm 0.05 | 1.4 \pm 0.09 | 0.035 |
| $ Z _{max}$, M Ω | 80.6 \pm 5.0 | 63.7 \pm 4.6 | 8.9 $\times 10^{-5}$ |
| Φ_L , rad-Hz | 0.04 \pm 0.02 | 0.12 \pm 0.05 | 0.024 |
| S_α | 1.14 \pm 0.04 | 0.94 \pm 0.05 | 1.4 $\times 10^{-3}$ |

Values are means \pm SE of measurements sensitive to changes in HCN channels, determined at the beginning of (initial) and after 45 min of experiments where 10 μ M inositol trisphosphate was included in the recording pipette. P values are reported for the paired Student's t -test. RMP, resting membrane potential; R_{in} , input resistance; f_R , resonance frequency; Q , resonance strength; $|Z|_{max}$, maximum impedance amplitude; Φ_L , total inductive phase; and S_α , summation of α -excitatory postsynaptic potentials.

plasticity in the physiological measurements that we employed for characterizing neuronal response dynamics, along with their well-established sensitivity to HCN channels (Brager and Johnston 2007; Brager et al. 2013; Clemens and Johnston 2014; Fan et al. 2005; Magee 1998; 2000; Narayanan et al. 2010; Narayanan and Johnston 2007, 2008), pointed us to the hypothesis that the expressed form of plasticity is dependent on HCN channels. Furthermore, depletion of intracellular calcium stores is known to induce plasticity in HCN channels, through an InsP₃R-dependent mechanism (Clemens and Johnston 2014; Narayanan et al. 2010). Against this background, we tested our hypothesis that changes in HCN channels mediate the expression of InsP₃-induced plasticity by repeating our plasticity experiments (10 μ M InsP₃) in the presence of the HCN-channel blocker ZD7288 (Gasparini and DiFrancesco 1997;

Magee 1998; Narayanan et al. 2010; Narayanan and Johnston 2007). We found that the InsP₃-induced plasticity of IRD was abolished when recordings were performed in the presence of 20 μ M ZD7288 in the recording pipette (Fig. 3). Specifically, there was no significant change in R_{in} in the presence ZD7288, and it remained close to its initial value through the course of the experiment (Fig. 3, A–C). Additionally, there were no significant changes in S_{α} (Fig. 3D) and the impedance amplitude and phase profiles (Fig. 3, E–G), consequently abolishing plasticity in f_R , Q , and $|Z|_{max}$, as well (Fig. 3, H–J). When we compared the percentage changes in various measurements in the presence of ZD7288, we found that they were significantly different from measurements in the control experiments where ZD7288 was absent (Fig. 3J). Together, plasticity in physiologically relevant measurements sensitive to changes in HCN

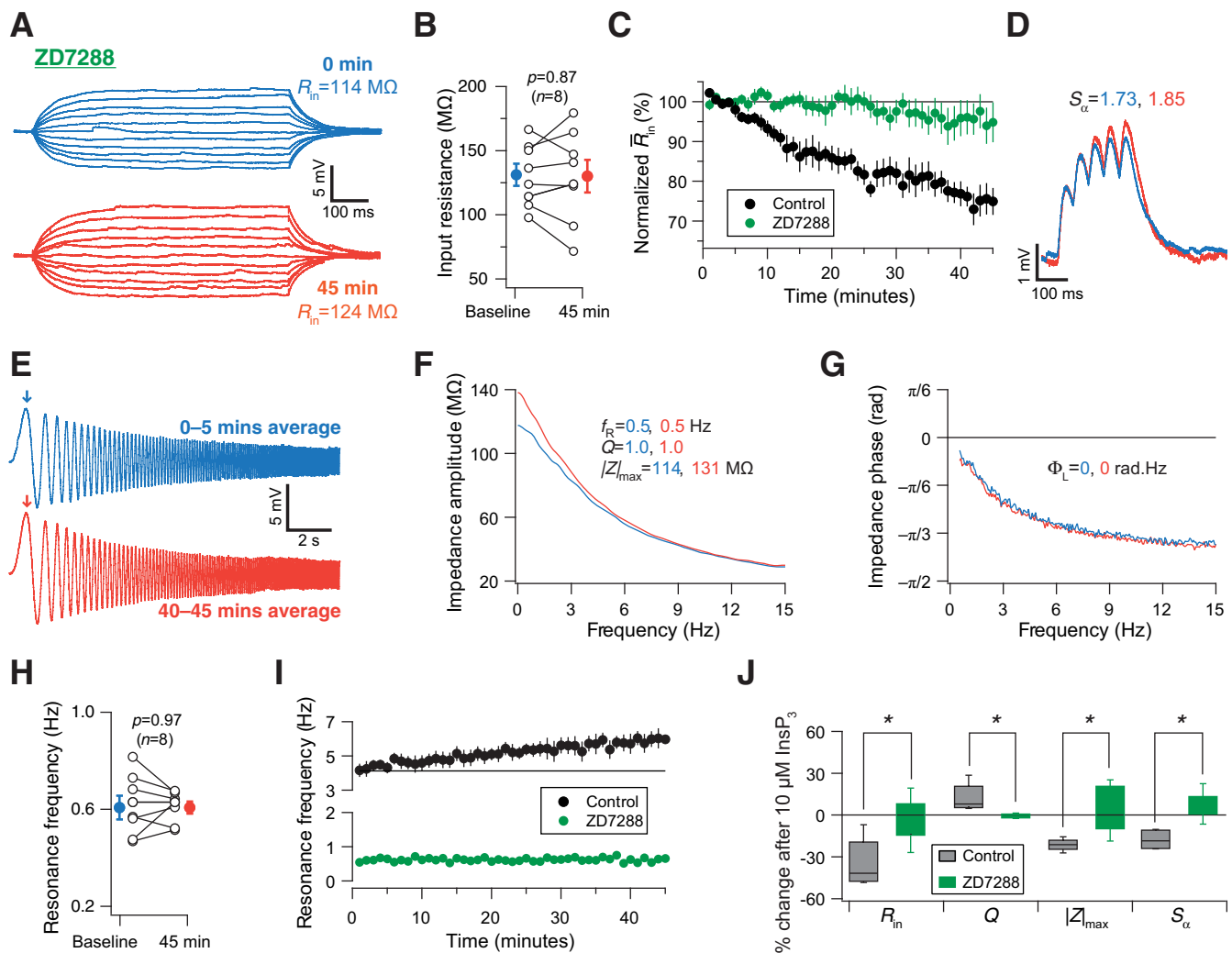


Fig. 3. InsP₃-induced plasticity was abolished in the presence of ZD7288. Experiments depicted were performed with 10 μ M InsP₃ and 20 μ M ZD7288 ($n = 8$) in the recording pipette. **A**: voltage response of a representative neuron to the pulse-current injections at the beginning (blue) and after 45 min (orange) of the experiment. R_{in} values obtained from these traces are also shown. **B**: population plots of R_{in} measured at the beginning (blue) and the end (orange) of experiments performed in the presence of ZD7288. **C**: time course of normalized R_{in} in the presence (green) and absence (black) of ZD7288. **D**: voltage response of the neuron to the injection of a sequence of 5 alpha currents (20 Hz) at the beginning (blue) and end (orange) of the experiment. The S_{α} values are shown. **E**: voltage response of the neuron to the chirp stimulus at the beginning (blue; 0–5 min average) and the end (orange; 40–45 min average) of the experiment. **F** and **G**: impedance amplitude (**F**) and phase (**G**) profiles obtained from traces shown in **E**, also depicting the measurements obtained from these profiles: f_R , $|Z|_{max}$, Q , and Φ_L . **H**: population plots of f_R measured at the beginning (blue) and the end (orange) of experiments performed in the presence of ZD7288. **I**: time course of f_R in the absence (black; top) and presence of ZD7288 (green; bottom). For **B** and **C** and for **H** and **I**, data are means \pm SE and P values (when presented) are from paired Student's t -tests. **J**: summary plot of %change in various measurements (from their respective baseline values) after 45 min into the recording in the presence of ZD7288. Data are medians and quartiles. * $P < 0.05$, Mann-Whitney test.

channels (Fig. 2), coupled with pharmacological evidence through the use of ZD7288 (Fig. 3), provide clear lines of evidence in favor of our hypothesis that InsP₃-induced plasticity in IRD is mediated by changes in HCN channels.

InsP₃-induced plasticity of IRD was dependent on the elevation in cytosolic calcium concentration. Cytosolic InsP₃ is metabolized into different phosphate derivatives by a variety of cytosolic enzymes (Berridge and Irvine 1989; Irvine and Schell 2001), and there are several structural interactions between InsP₃ receptors and other signaling molecules (Fagni et al. 2000; Kato et al. 2012; Kennedy 2000). Furthermore, given the fast degradation of InsP₃ within the cell and the similarity of the time course of changes with depletion-induced plasticity in HCN channels (Brager et al. 2013; Clemens and Johnston 2014; Narayanan et al. 2010), we postulated that InsP₃-induced changes in the intrinsic response dynamics was plasticity consequent to an initial surge of calcium. Against this, is plasticity in IRD a consequence of InsP₃R-induced elevation in cytosolic calcium levels, or is it a consequence of some structural interactions or due to activation of calcium-independent biochemical signaling pathways such as those associated

with phosphate derivatives of InsP₃ (Harwood 2005)? To answer this, we repeated our plasticity protocol (Fig. 1B) in the presence of 20 mM BAPTA in the recording pipette and found that InsP₃-induced plasticity of IRD was abolished when cytosolic calcium was chelated by BAPTA (Fig. 4). Specifically, there was no significant change in either R_{in} or f_R , which also reflected in their temporal progression (Fig. 4, A–D), and in other IRD measurements (sag, Q , $|Z|_{max}$, S_{α} , and Φ_L ; Fig. 4E). Together, these observations established that InsP₃-induced plasticity of IRD required an elevation in the cytosolic calcium concentration and was not due to some calcium-independent nonspecific effects of introducing InsP₃.

Plasticity in IRD was mediated by cytosolic influx of calcium through InsP₃Rs, with contributions from NMDA receptors and voltage-gated calcium channels. What sources contributed to the cytosolic calcium influx that resulted in InsP₃-induced plasticity in IRD? Apart from InsP₃Rs being the obvious candidate, synergistic interactions between several calcium sources (Berridge 2002; Berridge et al. 2000; Choe and Ehrlich 2006; Clemens and Johnston 2014; Narayanan et al. 2010; Ross 2012; Verkhratsky 2005) coupled with structural interac-

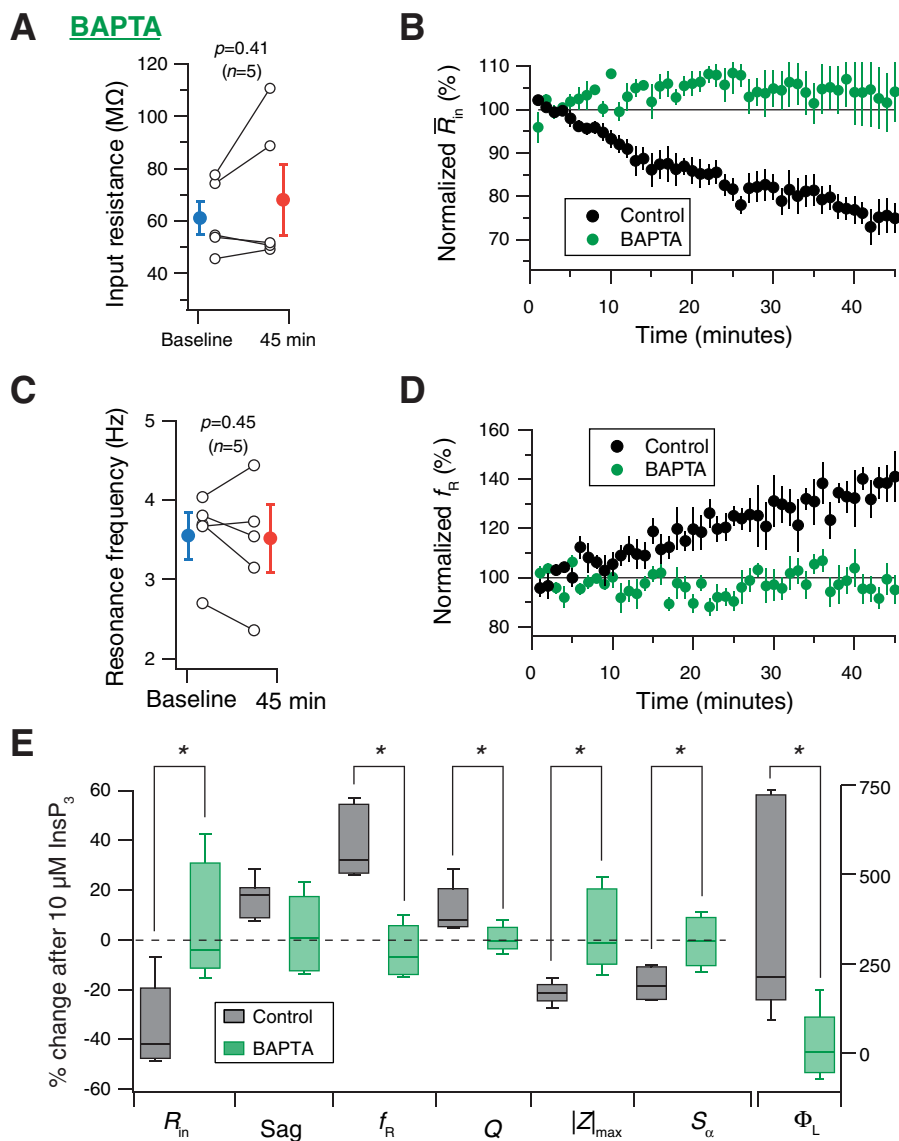


Fig. 4. InsP₃-induced plasticity was abolished in presence of BAPTA. Experiments depicted were performed with 10 μ M InsP₃ and 20 mM BAPTA ($n = 5$) in the recording pipette. *A*: population plots of R_{in} measured at the beginning (blue) and the end (orange) of experiments performed in the presence of BAPTA. *B*: time course of normalized R_{in} in the presence (green) and absence (black; control) of BAPTA. *C*: population plots of f_R measured at the beginning (blue) and the end (orange) of experiments performed in the presence of BAPTA. *D*: time course of normalized f_R in the absence (black; top) and presence of BAPTA (green; bottom). For A–D, data are means \pm SE and P values (when presented) are from paired Student's t -tests. *E*: summary plot of %change in various measurements (from their respective baseline values) after 45 min into the recording in the presence of BAPTA. Data are medians and quartiles. * $P < 0.05$, Mann-Whitney test.

tions between InsP₃Rs and other signaling molecules provide further routes for cytosolic calcium influx. From the perspective of interactions, InsP₃Rs are linked to PSD-95 and NMDA receptors (NMDARs) through various scaffolding proteins, and structural coupling and functional interactions between InsP₃Rs and voltage-gated calcium channels (VGCC) apart from several other signaling molecules are well established (Choe and Ehrlich 2006; Fagni et al. 2000; Foskett 2010; Foskett et al. 2007; Kato et al. 2012; Kennedy 2000; Patterson et al. 2004). Therefore, we systematically tested the role of several calcium sources in mediating InsP₃-induced plasticity in IRD.

First, to assess the role of InsP₃Rs in mediating the plasticity, we repeated our experiments in the presence of 1 mg/ml heparin, a selective blocker of InsP₃R. Incorporation of heparin in the recording pipette completely abolished the InsP₃ (10 μM)-induced plasticity in these neurons (Fig. 5, A–F), establishing that InsP₃-induced plasticity was a consequence of calcium release through InsP₃Rs. Whereas the demonstration of the dependence of depletion-induced intrinsic plasticity by blocking InsP₃Rs revealed the necessity of InsP₃Rs for inducing intrinsic plasticity (Narayanan et al. 2010), this abolishment of InsP₃-induced plasticity by InsP₃R blockers unveiled the sufficiency of InsP₃R activation for inducing intrinsic

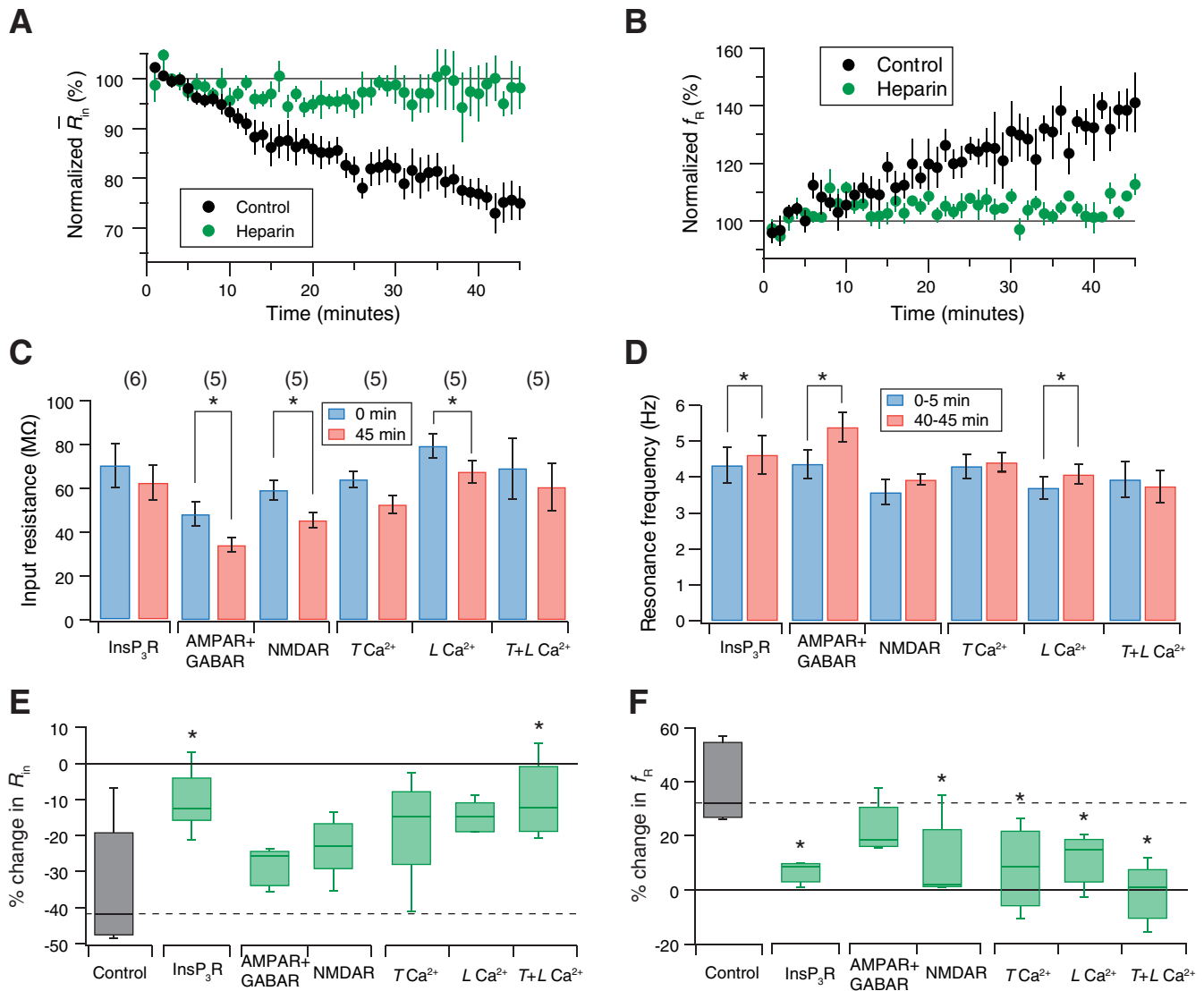


Fig. 5. InsP₃ receptors (InsP₃R), NMDA receptors (NMDAR), and voltage-gated calcium channels (VGCCs) contribute as calcium sources for InsP₃-induced plasticity. All experiments depicted were performed with 10 μM InsP₃ in the recording pipette. *A* and *B*: time courses of normalized R_{in} (*A*) and f_R (*B*) in the presence (green) and absence of 1 mg/ml heparin (black; control) in the recording pipette. *C* and *D*: summary plots of values of R_{in} (*C*) and f_R (*D*) at the beginning (blue) and at the end (orange) of experiments when various channels/receptors were blocked using specific pharmacological agents. For *A–D*, data are means \pm SE. * $P < 0.05$, paired Student's *t*-test. *E* and *F*: summary plots of InsP₃-induced changes (%) in R_{in} (*E*) and f_R (*F*) obtained when various channels/receptors were blocked using specific pharmacological agents (green) compared with the control (black) where no other pharmacological agent was employed. Data are medians and quartiles. * $P < 0.05$, Mann-Whitney test. Pharmacological agents indicated in *C–F* are defined as follows: InsP₃R, 1 mg/ml heparin in recording pipette ($n = 6$); AMPAR+GABAR, 10 μM (+)bicuculline, 10 μM picrotoxin, 10 μM 6-cyano-7-nitroquinoxaline-2,3-dione, and 2 μM CGP55485 in extracellular recording solution ($n = 5$); NMDAR, 50 μM 2-amino-5-phosphonovaleric acid (D,L-APV) in extracellular recording solution ($n = 5$); TCa²⁺ (T-type calcium channels), 50 μM NiCl₂ in extracellular recording solution ($n = 5$); LCa²⁺ (L-type calcium channel), 10 μM nimodipine in extracellular recording solution ($n = 5$); T+L Ca²⁺, 50 μM NiCl₂ and 10 μM nimodipine in extracellular recording solution ($n = 5$). See text for definitions.

plasticity. Next, we established that blocking AMPA and GABA receptors using a cocktail of pharmacological agents [10 μ M CNQX, 10 μ M (+)bicuculline, 2 μ M CGP55845, and 10 μ M picrotoxin] did not have any effect on the InsP₃-induced plasticity, suggesting that baseline synaptic activity did not affect this form of IRD plasticity. However, blocking NMDARs using 50 μ M D,L-APV, in the extracellular recording solution resulted in a differential effect on different IRD measurements, with a significant reduction in the magnitude of InsP₃-induced plasticity in f_R but not in R_{in} (Fig. 5, C–F). Although blocking L-type calcium channels (10 μ M nimodipine in the extracellular recording solution) did not have any significant effect on the IRD plasticity, blocking T-type calcium channels (50 μ M NiCl₂ in the extracellular recording solution) or both L- and T-type calcium channels resulted in a significant reduction of InsP₃-induced plasticity (Fig. 5, C–F). Together, these results suggested that InsP₃-induced plasticity was mediated by calcium influx through InsP₃R, with NMDARs and VGCCs providing additional modulatory effects on the plasticity.

InsP₃-induced plasticity was dependent on the PKA signaling pathway. Which downstream signaling pathway was responsible for the expression of InsP₃-induced plasticity? It has been previously reported that depletion of internal stores can activate the PKA pathway (Lefkimmiatis et al. 2009) and induce an InsP₃R-dependent form of plasticity in HCN channels (Narayanan et al. 2010). Motivated by these, and to assess the role of the PKA pathway on InsP₃-induced plasticity in IRD, we repeated our protocol (Fig. 1B; 10 μ M InsP₃) with two distinct PKA inhibitors. We found that inhibiting PKA, either by incorporating 20 μ M PKAi in the recording pipette or by adding 500 nM KT5720 in the extracellular recording solution, significantly diminished the magnitude of plasticity in f_R and R_{in} (Fig. 6). These results underline a critical role for the PKA pathway in InsP₃-induced plasticity of IRD.

In summary, converging signaling mechanisms and similar plasticity in equivalent intrinsic measurements of depletion-induced (Narayanan et al. 2010) and InsP₃-induced forms of plasticity (Figs. 2–6) respectively establish necessity and sufficiency of InsP₃Rs for inducing intrinsic plasticity. Together, these results causally delineate specific roles for InsP₃Rs in inducing changes in neuronal intrinsic properties.

DISCUSSION

In this study, we demonstrated that specific activation of InsP₃Rs through their endogenous agonist InsP₃ is sufficient to induce long-lasting changes in neuronal intrinsic properties. Together with the earlier complementary demonstration of the necessity of InsP₃Rs for a form of intrinsic plasticity (Narayanan et al. 2010), this demonstration of sufficiency clearly demarcates a causal role of InsP₃Rs in regulating neuronal intrinsic properties, differentiating them from the activation of upstream signaling pathways that contribute to the mobilization of InsP₃. This is essential because the upstream signaling events, such as the activation of G protein-coupled receptors or store depletion, typically target several downstream mechanisms apart from the mobilization of InsP₃. Additionally, in establishing the graded nature of the plasticity by employing different concentrations of InsP₃, we also show that this InsP₃-induced form of plasticity is expressed through changes in

HCN channels and is critically dependent on calcium release through InsP₃Rs and on the PKA pathway.

Graded plasticity. An important finding in the synaptic plasticity literature is that synaptic plasticity is not an all-or-none phenomenon but, instead, is graded (Berridge and Irvine 1989; Enoki et al. 2009; Montgomery and Madison 2002; O'Connor et al. 2005). Such activity- and state-dependent synaptic plasticity mechanisms allow neurons to operate over a large dynamic range, thereby increasing their information storage capacity (Montgomery and Madison 2002, 2004). In this context, a crucial finding from our study is that the InsP₃-induced plasticity in HCN channel properties is graded (Fig. 2). Plasticity in ion channels alters intrinsic response properties of a neuron and spatiotemporal integration of a neuron (Frick and Johnston 2005; Johnston and Narayanan 2008; Magee and Johnston 2005; Narayanan and Johnston 2012; Remy et al. 2010; Shah et al. 2010). Thus graded plasticity in HCN channels, in conjunction with graded synaptic plasticity, provides a neuron with multiple plasticity mechanisms, thereby immensely increasing the computational and storage capability of these neurons. Furthermore, because intracellular mobilization of InsP₃ can occur through several upstream signaling pathways, this diversity could contribute to differential mobilization of cytosolic InsP₃, leading to graded intrinsic plasticity under different physiological and pathological conditions.

Several theoretical and experimental studies have explored the effect of specific levels of cytosolic calcium concentration on the polarity and magnitude of synaptic plasticity (Lisman 1989; Nishiyama et al. 2000; Shouval et al. 2002). In this work, we demonstrate that different concentrations of cytosolic InsP₃ and subsequent release of store calcium can induce graded plasticity in voltage-gated ion channels. Whereas graded synaptic plasticity is postulated to exhibit a biphasic dependence on cytosolic calcium, in striking contrast, our results show that intrinsic plasticity induced by different concentrations of cytosolic InsP₃ is not biphasic albeit being graded. Concurrent plasticity in synaptic and intrinsic properties has been hypothesized to play critical roles in neural coding, learning, memory, and homeostasis (Narayanan and Johnston 2012; Turrigiano 2011). With some forms of homeostatic mechanisms pointing toward modulation of intrinsic properties, it is imperative that graded forms of synaptic plasticity would require graded forms of intrinsic plasticity (Honnuraiah and Narayanan 2013; Narayanan and Johnston 2012, 2010; Turrigiano 2011). Our study suggests that such fine-tuning in intrinsic neuronal properties could be achieved by differential mobilization of store calcium through graded activation of InsP₃Rs. Whereas our results explore one form of calcium-dependent plasticity in intrinsic properties, other forms of calcium-dependent graded plasticity through changes in HCN channels through other pathways (Biel et al. 2009; Brager and Johnston 2007; Fan et al. 2005; Narayanan et al. 2010; Shah et al. 2010) and/or in other ion channels (Frick and Johnston 2005; Lujan et al. 2009; Magee and Johnston 2005; Remy et al. 2010; Shah et al. 2010; Turrigiano 2011) could play critical roles in maintaining homeostasis of physiological properties through collective forms of channelostasis (O'Leary et al. 2013; Rathour and Narayanan 2014; 2012a).

Mechanisms. Depletion of calcium stores by blocking sarco-(endo)plasmic reticulum Ca²⁺-ATPase (SERCA) pumps results

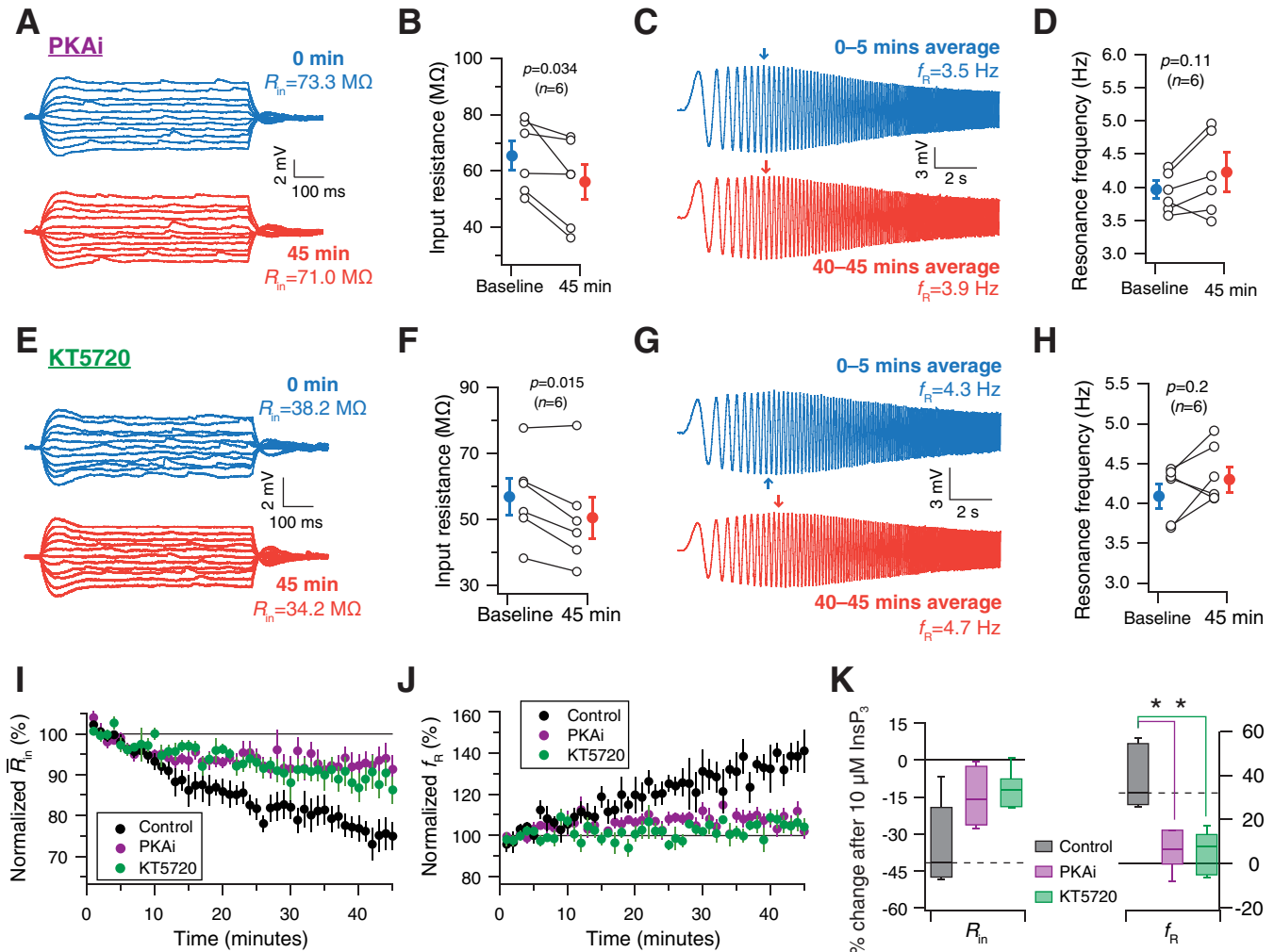


Fig. 6. InsP₃-induced plasticity was dependent on the PKA signaling pathway. All experiments depicted were performed with 10 μM InsP₃ in the recording pipette. **A:** voltage response of a representative neuron to the pulse-current injections at the beginning (blue) and after 45 min (orange) of an experiment where 20 μM PKA inhibitor (PKAi) peptide was included in the recording pipette. R_{in} values obtained from these traces are also shown. **B:** population plots of R_{in} measured at the beginning (blue) and the end (orange) of experiments performed in the presence of PKAi peptide ($n = 6$). **C:** voltage response of the neuron to the chirp stimulus at the beginning (blue; 0–5 min average) and the end (orange; 40–45 min average) of an experiment where 20 μM PKAi peptide was included in the recording pipette. f_R values obtained from these traces are also shown. **D:** population plots of f_R measured at the beginning (blue) and the end (orange) of experiments performed in the presence of PKAi peptide. **E–H:** same as **A–D**, but the experiments were performed in the presence of 500 nM KT5720 in the recording solution, instead of PKAi. For **A–H**, data are means \pm SE and P values correspond to paired Student's t -test. **I** and **J:** time courses of normalized R_{in} (**I**) and f_R (**J**) with 10 μM InsP₃ and 20 μM PKAi peptide ($n = 6$) in the recording pipette (green), 10 μM InsP₃ in the recording pipette and 500 nM KT5720 ($n = 6$) in the bath (purple), or only 10 μM InsP₃ (black; control) in the recording pipette. **K:** summary plots of %change in R_{in} and f_R obtained from experiments performed with the 2 different PKA inhibitors and their comparison with the control experiments. Color codes are the same as in **I** and **J**. Data are medians and quartiles. * $P < 0.05$, Mann-Whitney test.

in changes in HCN channel properties (Brager et al. 2013; Clemens and Johnston 2014; Narayanan et al. 2010) mediated by calcium release through InsP₃Rs. Assessing the complementary sufficiency counterpart to these results on the necessity of InsP₃Rs for intrinsic plasticity, our study shows that calcium release through InsP₃R is sufficient to induce plasticity in HCN channels (Figs. 4 and 5). Whereas store depletion-induced plasticity could be regarded as a neuroprotective mechanism that expresses under pathophysiological conditions (Brager et al. 2013; Clemens and Johnston 2014; Narayanan et al. 2010), our study demonstrates graded release of calcium through InsP₃Rs, a physiologically plausible scenario (Berridge 2002; Choe and Ehrlich 2006; Clapham 2007; Foskett et al. 2007; Park et al. 2008; Patterson et al. 2004; Rose and Konnerth 2001; Taylor and Tovey 2010; Verkhratsky 2002), could result in graded plasticity of intrinsic

properties. Furthermore, we found that calcium flux through NMDARs and VGCCs could modulate InsP₃-induced plasticity, apart from the critical role for InsP₃Rs (Fig. 5). This is consistent with findings that calcium influx through several channels can synergistically contribute to a large elevation in cytosolic calcium under several physiological instances (Berridge et al. 2000; Clemens and Johnston 2014; Fagni et al. 2000; Kato et al. 2012; Narayanan et al. 2010; Ross 2012), potentially recruiting well-established structural interactions among InsP₃Rs, mGluRs, NMDARs, and VGCCs in this process (Fagni et al. 2000; Foskett et al. 2007; Kato et al. 2012; Naisbitt et al. 1999; Sala et al. 2001; Taylor and Tovey 2010; Tu et al. 1999; Tu et al. 1998; Xiao et al. 2000). Finally, InsP₃-induced plasticity was dependent on PKA activation (Fig. 6), similar to depletion-induced plasticity (Lefkimmatis et al. 2009; Narayanan et al. 2010), suggesting a

convergent set of signaling pathways that contribute to depletion- and InsP₃-induced plasticity.

Systematic investigations in the future should explore the diversity of signaling mechanisms involved in differential plasticity of HCN channels (Brager and Johnston 2007; Brager et al. 2013; Clemens and Johnston 2014; Fan et al. 2005; Narayanan et al. 2010; Narayanan and Johnston 2010; Shah et al. 2010), also accounting for specific localization of the various signaling molecules and their kinetics and binding interactions (Choe and Ehrlich 2006; Foskett et al. 2007; Kennedy et al. 2005; Koteleski and Blackwell 2010; Patterson et al. 2004; Rose and Konnerth 2001). In the context of our results, especially the differential signaling dependence of the different physiological measurements (Fig. 5–6), such investigations should also explore the specific impact of local dendritic release of calcium through InsP₃ receptors on localized plasticity of dendritic response properties (e.g., Frick et al. 2004; Losonczy et al. 2008), apart from systematically investigating the InsP₃R subunits involved in this form of plasticity (Hertle and Yeckel 2007; Nishiyama et al. 2000). Such analyses would provide a more holistic understanding on how intracellular stores alter neuronal response properties, on how localized ion channel plasticity could differentially alter measurements at various somatodendritic locations (Clemens and Johnston 2014; Narayanan et al. 2010; Rathour and Narayanan 2012b), and on how such plasticity mechanisms could synergistically contribute toward the maintenance of physiological homeostasis across the somatodendritic arbor (O'Leary et al. 2013, 2014; Rathour and Narayanan 2014; Turrigiano 2011).

Implications. Our results provide direct evidence for an InsP₃-induced reduction in neuronal gain, an increase in the optimal response frequency of the neuron, and a reduction in temporal summation of postsynaptic potentials (Fig. 2). Whereas these are consistent with established roles for HCN channels, there are other physiological implications for such plasticity in HCN channels. For instance, alterations in HCN channels would change the coupling across compartments through changes to transfer impedance (Cook et al. 2007; Hu et al. 2009; Kole et al. 2007; Ulrich 2002; Vaidya and Johnston 2013) and would change the intraneuronal synchronization frequency (Vaidya and Johnston 2013). Furthermore, changes in HCN channels can alter spike initiation dynamics, thereby allowing neurons to behave as coincidence detectors or as integrators (Das and Narayanan 2014). Finally, metaplasticity through HCN channels is well established (Honnuraiah and Narayanan 2013; Narayanan and Johnston 2010; Nolan et al. 2004) and provides a link between HCN plasticity and synaptic plasticity. Therefore, synaptic plasticity that is dependent on InsP₃Rs (Bortolotto et al. 1999; Nishiyama et al. 2000) could synergistically interact with the metaplasticity introduced by HCN channel plasticity to play significant roles in neural coding and homeostasis (Honnuraiah and Narayanan 2013; Narayanan and Johnston 2012; O'Leary et al. 2013; Rathour and Narayanan 2014; Turrigiano 2011). From this standpoint, it would also be important to explore if the release of presynaptic store calcium (Verkhatsky 2005) would alter HCN channels that are known to express in presynaptic terminals, as well (Bender et al. 2007; Huang et al. 2011), thereby providing additional roles for InsP₃-induced plasticity in regulating presynaptic release probability and synaptic maturation (Bender et al. 2007; Huang et al. 2011). Finally, although our focus has

been limited to the hippocampus and to HCN channels, future studies should investigate the role of InsP₃ and its receptors in plasticity of other channels and in other neurons under physiological and pathological conditions.

In summary, our results further emphasize the critical role for InsP₃ and intracellular stores as synergistic integrators of several biochemical signals across the neuronal arbor (Berridge 1998; Berridge et al. 2000; Park et al. 2008; Patterson et al. 2004). These findings also constitute a novel addition to the several existing forms of interactions between the endoplasmic reticulum and the plasma membrane (Ashhad and Narayanan 2013; Berridge 2002; Choe and Ehrlich 2006; Clapham 2007; Foskett et al. 2007; Kato et al. 2012; Ross 2012) by providing direct evidence for the role of cytosolic InsP₃ in altering neuronal excitability and intrinsic response dynamics. This InsP₃-induced form of intrinsic plasticity underscores the necessity for an expansive reassessment of the already extensive roles of InsP₃ and intracellular stores in cell signaling, neuronal integration, neural plasticity, learning, memory, neural coding, and homeostasis.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

S.A., D.J., and R.N. conception and design of research; S.A. and R.N. performed experiments; S.A. and R.N. analyzed data; S.A., D.J., and R.N. interpreted results of experiments; S.A. and R.N. prepared figures; S.A. and R.N. drafted manuscript; S.A., D.J., and R.N. edited and revised manuscript; S.A., D.J., and R.N. approved final version of manuscript.

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