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NOVEL CALCIUM-RELATED TARGETS OF INSULIN IN HIPPOCAMPAL NEURONS

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Abstract—Both insulin signaling disruption and Ca^{2+} dysregulation are closely related to memory loss during aging and increase the vulnerability to Alzheimer's disease (AD). In hippocampal neurons, aging-related changes in calcium regulatory pathways have been shown to lead to higher intracellular calcium levels and an increase in the Ca^{2+}-dependent afterhyperpolarization (AHP), which is associated with cognitive decline. Recent studies suggest that insulin reduces the Ca^{2+}-dependent AHP. Given the sensitivity of neurons to insulin and evidence that brain insulin signaling is reduced with age, insulin-mediated alterations in calcium homeostasis may underlie the beneficial actions of insulin in the brain. Indeed, increasing insulin signaling in the brain via intranasal delivery has yielded promising results such as improving memory in both clinical and animal studies. However, while several mechanisms have been proposed, few have focused on regulation on intracellular Ca^{2+}. In the present study, we further examined the effects of acute insulin on calcium pathways in primary hippocampal neurons in culture. Using the whole-cell patch-clamp technique, we found that acute insulin delivery reduced voltage-gated calcium currents. Fura-2 imaging was used to also address acute insulin effects on spontaneous and depolarization-mediated Ca^{2+} transients. Results indicate that insulin reduced Ca^{2+} transients, which appears to have involved a reduction in ryanodine receptor function. Together, these results suggest insulin regulates pathways that control intracellular Ca^{2+} which may reduce the AHP and improve memory. This may be one mechanism contributing to improved memory recall in response to intranasal insulin therapy in the clinic.

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Abbreviations: AD, Alzheimer's disease; AHP, afterhyperpolarization; AUC, area-under-the-curve; CICR, calcium-induced calcium-release; DIV, days *in vitro*; KCl, potassium chloride; L-VGCC, L-type voltage-gated Ca^{2+} current; ROI, region-of-interest; Rya, ryanodine; RyR, ryanodine receptor; SEM, standard error of the mean; SERCA, sarcoplasmic-endoplasmic Ca^{2+}-ATPase; SOCCs, store-operated calcium channels; TTX, tetrodotoxin; VGCC, voltage-gated Ca^{2+} channel.

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INTRODUCTION

Aging is a major risk factor for Alzheimer's disease (AD), and both brain aging and AD are characterized by a progressive decline in cognitive and memory function. A promising approach for the treatment of cognitive impairment in AD seeks to maintain or enhance insulin signaling in the brain. This approach is based on nearly two decades of research that provides support for strong associations between diabetes, cognitive decline, and AD (Ott et al., 1996; Leibson et al., 1997; Luchsinger, 2010; Bosco et al., 2011; De Felice, 2013). Other support comes from studies showing that insulin can act as a cognitive and neural modulator (Baura et al., 1993; Cholerton et al., 2011; Heni et al., 2014a; Sartorius et al., 2015). Insulin acting in the brain is synthesized by pancreatic β-cells and gains access to the brain via the blood brain barrier. In the periphery and in the brain, insulin has been shown to bind to insulin receptors and to regulate glucose uptake by inducing translocation of glucose transporters to the plasma membrane (McNay et al., 2013).

In aging or AD, declining insulin levels, insulin receptor numbers, and/or glucose transporters can lead to dysregulation in glucose uptake which may underlie cognitive decline (Schioth et al., 2012a). Indeed, there is evidence that insulin receptor numbers and their functions are decreased in aging and AD animal models (Zaia and Piantanelli, 1996; Frolich et al., 1998; Zhao et al., 2004, 2008). At the cellular level, brain insulin deficiency and reduction in insulin signaling, perhaps mediated by insulin resistance, could represent one of the altered pathways linked to altered memory function or synaptic communication during aging and AD (Craft et al., 1996; Rasgon & Jarvik, 2004; de la Monte et al., 2006; Talbot et al., 2012; De Felice, 2013; Sasaoka et al., 2014; Maimaiti et al., 2016). One approach to combat this reduction in insulin signaling that has received much interest clinically is the use of intranasal insulin delivery to selectively increase insulin concentration in the brain (Born et al., 2002).

The intranasal route of insulin administration raises insulin acutely in the central nervous system without much risk of peripheral hypoglycemia (Kern et al., 1999; Ott et al., 2014; Lochhead et al., 2015; Anderson et al.,

2016; Maimaiti et al., 2016). Intranasal insulin therapy has been shown to improve memory function in AD patients and in healthy individuals (Benedict et al., 2004; Reger et al., 2006, 2008; Hanson and Frey, 2008; Craft et al., 2012; Schioth et al., 2012b; Freiherr et al., 2013; Craft et al., 2017). Further, animal models of aging and AD also show the positive impact of intranasal insulin in combating cognitive decline (Francis et al., 2008; Marks et al., 2009; Apostolatos et al., 2012; de la Monte, 2013; Adzovic et al., 2015; Salameh et al., 2015; Anderson et al., 2016; Maimaiti et al., 2016). Despite these promising outcomes, the mechanisms of action in the brain, and specifically on neurons of the hippocampus where insulin plays a recognizable role in learning and memory (Zhao et al., 1999, 2004), remain largely unknown.

Because there is compelling evidence that aspects of cognitive aging may be due to calcium dysregulation that results in enhanced calcium levels (Porter et al., 1997; Thibault et al., 2001; Clodfelter et al., 2002; Hemond and Jaffe, 2005; Gant et al., 2006; Oh et al., 2013) and voltage-gated calcium channel (VGCC) activity (Moyer et al., 1992; Thibault and Landfield, 1996; Thibault et al., 2001; Nunez-Santana et al., 2014), we focused on studying two well-characterized calcium sources in hippocampal neurons. The rationale for this approach was based on recent evidence from our lab that insulin acutely reduces the calcium-dependent afterhyperpolarization (AHP) in neurons recorded from hippocampal slices (Pancani et al., 2013; Maimaiti et al., 2016). The AHP is a hyperpolarization potential that is enhanced in aging, limits neuronal firing, and is associated with cognitive decline (Disterhoft et al., 1996; Tombaugh et al., 2005; Kadish et al., 2009; Pancani et al., 2013). The larger AHP seen in aging, is mediated in part, by an increase in the density of L-VGCCs (Thibault and Landfield, 1996) and by calcium-induced calcium-release (CICR) through activation of ryanodine receptors (RyRs) (Kumar and Foster, 2005; Gant et al., 2006, 2013). Because we have also previously characterized these two calcium sources using neuronal depolarization protocols that evoke increases in intracellular calcium in hippocampal neurons (Attucci et al., 2002; Clodfelter et al., 2002; Gant et al., 2011), we sought to quantify their sensitivity to treatment with acute insulin. Here we used KCl-mediated long-lasting neuronal depolarization to activate large amplitude calcium transients under imaging conditions and also directly measure calcium influx in patch clamping experiments.

We tested the hypothesis that acute insulin (glulisine, zinc-free and fast acting) would reduce calcium levels, or promote calcium homeostasis by altering VGCC and/ or RyR function in cultured neurons. To address these questions, we used patch-clamp recording of VGCCs with rapid drug delivery, as well as Fura-2 imaging during KCl-mediated depolarization in the presence or absence of a RyR blocker. Our results indicate that insulin is able to reduce intracellular calcium levels during periods of neuronal depolarization and that this is mediated, at least in part, by reductions in VGCC and RyR function. These two aging-sensitive neuronal Ca^{2+} targets, therefore, are sensitive to the actions of insulin and could represent novel therapeutic targets for cognitive decline in aging and/ or AD. Re-establishing Ca^{2+} homeostasis represents a mechanism by which insulin and by extension, its targeted delivery to the brain (*i.e.*, intranasal insulin), may offset learning and memory dysregulation *in vivo*.

EXPERIMENTAL PROCEDURES

Cell culture

Hippocampal mixed (neuron/glia) cultures were prepared as described previously (Porter et al., 1997; Pancani et al., 2009, 2011) and established from (E18) Sprague–Dawley rats. E18 pups and hippocampi were dissected under a microscope in ice-cold Hank's balanced salt solution (Thermo Fisher Scientific Inc., MA, USA) supplemented with 4.2 mM $NaHCO_3$, 10 mg/L gentamicin and 12 mM HEPES (pH 7.3). Hippocampi were transferred to a 37 °C 0.25% Trypsin EDTA solution (Thermo Fisher) and left at room temperature for 11 min. Trypsin was removed and the hippocampi were washed three times with Minimum Essential Medium (MEM). Hippocampi were then titrated, and diluted with MEM to the desired final concentration ($5–7 \times 10^5$ neurons/mL) before being plated onto 35-mm poly-L-lysine coated dishes. Cultured neurons were incubated (36 °C, 5% CO_2, 95% O_2) for 24 h before the first medium exchange. At this time, half of the medium was replaced with 90% SMEM supplemented with 10% Horse serum. After three days *in vitro* (DIV), half of the medium was replaced with SMEM, horse serum, 5-Fluoro-2-Dioxyuridine and uridine to stop glial cell growth. At DIV 10, a sodium bicarbonate solution (200 μL) was added to help maintain pH and limit evaporation.

For whole-cell recording experiments, plastic culture dishes were used (Corning Inc., Corning, NY, USA) and for calcium imaging experiments, glass bottom culture dishes (Mattek Crop., Ashland MA, USA) were used. All data presented were collected between DIV 13 and 17, when neuronal connections, the density of VGCCs, and measures of cell survival are relatively stable (Porter et al., 1997; Blalock et al., 1999). All experiments were conducted following 24-h exposure to a lower glucose-containing MEM (5.5 mM; MEM with no added glucose). This was done to maintain relatively normal glucose oxidation rates and insulin sensitivity (Pancani et al., 2011). All data presented were obtained at room temperature.

VGCC recording solution

External recording solution of VGCC currents was as follows (in mM): 111 NaCl, 5 $BaCl{\cdot}H_2O$, 5 CsCl, 2 $MgCl_2$, 10 glucose, 10 HEPES, 20 $TEA{\cdot}Cl{\cdot}H_2O$, pH 7.35 with NaOH, and 500 nM tetrodotoxin (TTX) was added before recording to inhibit Na^+channels. The internal pipette solution (in mM): 145 CH_4O_3S-methanesulfonic acid, 10 HEPES, 3 $MgCl_2$, 11 EGTA, 1 $CaCl_2$, 13 $TEA{\cdot}Cl{\cdot}H_2O$, 14 phosphocreatine Tris-salt, 4 Tris-ATP, 0.3 Tris-GTP, pH 7.3 with CsOH. All solutions were sterile filtered.