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A novel, rapid, inhibitory effect of insulin on $\alpha_1\beta_2\gamma_{2s}$ γ-aminobutyric acid type A receptors

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ABSTRACT

In the CNS, GABA and insulin seem to contribute to similar processes, including neuronal survival; learning and reward; and energy balance and food intake. It is likely then that insulin and GABA may interact, perhaps at the $GABA_A$ receptor. One such interaction has already been described [Q. Wan, Z.G. Xiong, H.Y. Man, C.A. Ackerley, J. Braunton, W.Y. Lu, L.E. Becker, J.F. MacDonald, Y.T. Wang, Recruitment of functional GABA(A) receptors to postsynaptic domains by insulin, Nature 388 (1997) 686–690]; in it a micromolar concentration of insulin causes the insertion of $GABA_A$ receptors into the cell membrane, increasing GABA current. I have discovered another effect of insulin on $GABA_A$ currents. Using a receptor isoform, $\alpha_1\beta_2\gamma_{2s}$ that is the likely main neuronal $GABA_A$ isoform expressed recombinantly in *Xenopus* oocytes, insulin inhibits GABA-induced current when applied simultaneously with low concentrations of GABA. Insulin will significantly inhibit currents induced by EC_{30-50} concentrations of GABA by about 38%. Insulin is potent in this effect; IC_{50} of insulin was found to be about 4.3×10^{-10} M. The insulin effect on the GABA dose responses looked like that of an antagonist similar to bicuculline or β-carbolines. However, an effect of phosphorylation on the $GABA_A$ receptor from the insulin receptor signal transduction pathway cannot yet be dismissed.

The pancreatic hormone insulin can cross the blood–brain barrier and become concentrated in the brain [6,36]. This neuronal insulin has many potential functions in the brain and individual. Changes in neuronal insulin levels or sensitivity, including in diabetes, can affect many different neurological functions. Many are long term, such as in neuronal survival, including the development of Alzheimer disease [36]. Insulin signaling pathways are involved in glucose regulation, body energy homeostasis [43], and food intake of organisms [33,9]. Insulin too may block some of the reward pathways in the ventral striatum and prefrontal cortex; the decrease feeling of reward from glucose in these areas may also be part of satiation [4].

Neuronal insulin and the neurotransmitter γ-aminobutyric acid (GABA) may both contribute significant roles in some neural diseases and activities. In many cases these contributions are opposing in nature. These activities include neurodegeneration/neuronal survival [5,37]; pathology or depressive symptoms associated with Alzheimer's disease [23,16]; and synaptic plasticity [15].

Since GABA and insulin overlap and usually have opposite effects in many neural activities, it is reasonable to hypothesize that insulin and GABA may intimately interact. One place would be at the $GABA_A$ receptor. The $GABA_A$ receptor is a reasonable target for insulin–GABA interactions because the $GABA_A$ receptor is already a target for many different ligands including hormones, and the $GABA_A$ receptor can be phosphorylated by kinases in the insulin receptor signal transduction pathway. The $GABA_A$ receptor is a GABA gated chloride channel. Upon binding of GABA, the channel allows Cl-ions to flow into the cell, causing hyperpolarization. Many different ligands can positively or negatively affect the amount of GABA-induced current by binding a site on the receptor. Positive modulators include benzodiazepines (BZs), ethanol, anesthetics, and some pregnane-derived steroids. Negative modulators include bicuculline, picrotoxin, and some steroid derivatives. The sites for these ligands are somewhere within the pentamer of the receptor; the pentamer usually consists of 2 α, 2 β and 1 γ subunit drawn from a family of 6 α, 3 β and 3 γ [22,17]. The subunits expressed in the highest levels in most brain areas as demonstrated by both *in situ* hybridization and RT-PCR are the α_1, β_2, and γ_{2s} [30,22,28].

Evidence for a $GABA_A$–insulin interaction already exists. Previous research has shown that a 10 min exposure to 0.5 μM insulin will increase the number of cell surface $GABA_A$ receptors [39]. This effect is likely due to phosphorylation of the $GABA_A$ receptors by kinases such as phosphoinositide 3-kinase (PI3-K) that are in the insulin receptor signaling pathway [38].

I hypothesized that there could be a quicker, potentially direct, and more potent effect of insulin on $GABA_A$ receptors, one that

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could potentially explain any opposing actions of insulin and GABA. These studies demonstrate such a quicker, more potent inhibitory effect of insulin on $GABA_A$ receptors does exist.

Oocytes (Stage IV–V) from *Xenopus laevis* were isolated and defolliculated by mechanical separation and incubation in 0.05% collagenase. Oocytes were washed extensively in OR-3 media (70% Leibovitz' L15/Gibco). All animal care, use and surgeries are standard protocols and were approved by the WSSU IACUC Committee. Insulin was the bovine form (cat I-5500) from Sigma (St. Louis, MO). Insulin was dissolved in 0.1% acetic acid and diluted in perfusion buffer. No change in pH was detected in the dilutions (not shown). All other chemicals are from commercial sources.

Rat $GABA_A$ subunit cDNAs are cloned into the pGEMHE vector. Wild type α_1, β_2, and γ_{2S} subunits were transcribed *in vitro* using T7 kits from Ambion/Applied Biosciences and diluted to 200 ng/μl using nuclease-free water. RNAs were injected into the oocytes at a 1:1:1 ratio of subunits in 50 nl total volume. Oocytes were incubated at 18 °C for 2–3 days in OR-3 media to allow for surface expression of the receptors. By using the 1:1:1 ratio for the subunits, we assume the surface receptors will be the typical $\alpha_1\beta_2\gamma_{2s}$ in a $2\alpha{:}2\beta{:}1\gamma$ ratio [41]. Though the insulin is bovine, and the receptor subunits from rat, insulin is well conserved. Between bovine and rat forms of insulin, there are only 4 amino acid differences, 2 on each the α and β chains, out of a total of 54 residues (NCBI data base).

Electrophysiology was performed by the two-electrode voltage clamp technique. Oocytes were perfused with Calcium Free Frog Ringer's (CFFR) (115 mM NaCl, 2.5 mM KCl, 1.8 mM Mg_2Cl, 10 mM HEPES, pH 7.5) at a rate of 5 ml/min and clamped at −60 mV at room temperature. Electrodes filled with 3 M KCl had a resistance between 0.5 and 2.5 mOhms. Currents were collected using the Warner TEV700 workstation/oocyte clamp with the HAI118 data acquisition systems using LabScribe Software, sampled at 100 samples/s. Stable GABA-induced currents were established before continuing experiments. Currents were defined as stable if the peak amount of current induced in 20–30 s was within 5%. If GABA-induced currents were stable, then GABA and a certain concentration of insulin were added *simultaneously* for 20–30 s and peak was recorded. The GABA–insulin co-application was repeated. GABA was then applied alone to be sure insulin washed out, or had no other slightly longer effects on subsequent currents. To do the insulin dose response curve a constant concentration of GABA (1 μM, approximate EC_{30}) was applied in the presence of varying amounts of insulin. To do the GABA dose response curves various concentrations of GABA were applied in the absence or presence of a constant concentration of insulin, 100 nM. The large dose of insulin was used to ensure a significant effect. Percent changes from control currents were calculated as $[I\text{+insulin}/I_{\text{control}}] \times 100$. Significance between control (no insulin) and experimental (with insulin) GABA-induced currents for a single concentration was determined by *t*-test. In the dose responses, any significance between concentrations was determined by one-way ANOVA (Instat, GraphPad, San Diego, CA).

After stable GABA-induced currents were established 100 nM insulin was added simultaneously with a submaximal concentration of GABA (EC_{30}; 1 μM). A significant decrease in GABA-induced current was seen at 1 μM GABA ($-38 \pm 8.3\%$ $n = 7$; $p < 0.01$) (Fig. 1a). At 1 μM GABA, a reduction of about $-22 \pm 4.0\%$ ($n = 6$; $p < 0.01$) occurs when only 1 nM insulin is co-applied (Fig. 1b). Near saturating GABA (100 μM) currents were not significantly affected by simultaneous application of 100 nM insulin ($-0.33 \pm 2.0\%$) (Fig. 1c). The high dose of insulin, 100 nM, did not cause any changes in current when added alone (data not shown.).

Using 1 μM GABA, where the largest percent decrease in current seemed to occur, a dose response curve for the inhibitory effect of insulin was done (Fig. 2). Insulin in ranges from 0.01 nM to 100 nM was added in the presence of 1 μM GABA. The data was plotted percent change in current *v*. the concentration of insulin. The best fit curve is a one site model with variable slope using the equation $Y = \text{Bottom} + (\text{Top} - \text{Bottom})/1 + 10^{(\log EC_{50} - X)\text{Hill Slope}}$ [26]; the IC_{50} was 0.43 nM and the Hill number was 0.2 (Graphpad; $r = 0.95$). The maximal effect was calculated to be $-38 \pm 1.5\%$.

Using 100 nM insulin, changes in the GABA dose response were investigated. Various concentrations of GABA ranging from 0.1 μM to 1 mM were added alone and then simultaneously with 100 nM insulin. The dose responses show a significant shift in the mean EC_{50} of GABA from 3.8 ± 1.1 μM (control) to 15 ± 1.1 μM (with insulin) ($p = 0.0003$) with no effect on maximal current ($99 \pm 13\%$ of maximal) (Fig. 3). Significant decreases in GABA-induced current occurred at submaximal concentrations of GABA ranging from 5 μM to 1 μM ($p = 0.03$).

The data presented suggest that insulin has a rapid inhibitory effect on $GABA_A$ receptor current. The term rapid is used to differentiate the effect from the decreases of GABA current seen by Wan et al. [39], in which insulin is incubated with $GABA_A$ receptors for 10 min, not seconds. The effect seems potent with an IC_{50} around 0.43 nM. Serum insulin concentrations are approximately 49 pmol/l for a population of fasting men [21], and 50 pmol/l for women [1]. Insulin can cross the blood–brain barrier [6] and become concentrated in the brain; brain levels are reported to be 10–100 times higher than that of serum, depending on the brain area [18]. This higher neuronal insulin concentration compares favorably with the IC_{50} of insulin for the $GABA_A$ inhibitory effect. The IC_{50} for insulin at the $\alpha_1\beta_2\gamma_{2s}$ receptor (0.43 nM) also compares favorably with the EC_{50} for insulin for the insulin receptor (about 0.05–3 nM depending on the tissues) [13,17,29]. The effect of these concentrations of insulin, when co-applied with low concentrations of GABA, is to inhibit GABA-induced current at neuronal type $\alpha_1\beta_2\gamma_{2s}$ receptor isoforms by approximately 38%.

This rapid inhibitory effect of insulin is different from the described effect of an increase in current due to receptor insertion into the plasma membrane. Both this inhibitory effect and the previously described potentiating effect occur at $\alpha_1\beta_2\gamma_{2s}$ isoforms. This inhibitory effect is more rapid; it occurs *simultaneously* with a 20–30 s application of GABA. It is more potent: the IC_{50} is in the 10^{-10} M range with 100 nM insulin at or near saturating. The potentiating effect described by Wan et al., [39] is much different; it requires 500 nM insulin and incubation times of at least 10 min. Therefore, the simultaneous, rapid inhibition of GABA-induced currents by nanomolar amounts of insulin represents a novel, separate effect of insulin on $GABA_A$ receptors. This effect may be important in some of the roles of insulin in brain.

The effect of insulin on the $\alpha_1\beta_2\gamma_{2s}$ isoform of $GABA_A$ receptors is clearly antagonistic. The type of antagonism, whether competitive or non-competitive is less clear. The effect of insulin: a rightward shift in the GABA EC_{50} and no significant effect on maximal current looks like a typical competitive inhibitor, bicuculline [2]. Other known competitive inhibitors of the $GABA_A$ receptor, such as pitrazepin [14] and thiocolchicoside [11] result in similar effects and shifts in GABA dose response curves [2,11,14]. However, β-carboline non-competitive (inverse agonist) inhibitors sometimes have similar effects on GABA dose response curves, reducing the affinity of GABA [10], so an antagonistic effect similar to β-carbolines by insulin cannot be totally eliminated. More typically though, β-carbolines show a mixed type inhibition, with significant changes in GABA EC_{50} and maximal response [34,35], which is not seen for GABA inhibition by insulin at this $\alpha_1\beta_2\gamma_{2s}$ isoform (Fig. 3). Also, activation of the receptor is not seen by large amounts of insulin as can occur with the β-carbolines [34]. With significant effects only on GABA EC_{50} and no induced current by high