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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 [39]; 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 $GABA_A$ from the insulin receptor signal transduction pathway cannot yet be dismissed.

Keywords

$GABA_A$; insulin; brain; diabetes; metabolic syndrome; competitive antagonist

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 [reviewed in 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].

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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 [reviewed in 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 minute 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 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.

Materials and Methods

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 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 2α:2β:1γ 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 0.5–2.5 mOhms. Currents were collected using the Warner TEV700 workstation/oocyte clamp with the HAI118 data acquisition systems using LabScribe Software, sampled at 100 samples/sec. Stable GABA-induced currents were established before continuing experiments. Currents were defined as

stable if the peak amount of current induced in 20–30 sec was within 5%. If GABA induced currents were stable, then GABA and a certain concentration of insulin were added *simultaneously* for 20–30 sec and that peak 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+insulin\Icontrol]x100. 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).

Results

Establishing an effect

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 ± 8.3 % n= 7; $p< 0.01$) (fig. 1a). At 1 μM GABA, a reduction of about −22 ± 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 ± 2.0 %) (fig. 1c). The high dose of insulin, 100 nM, did not cause any changes in current when added alone (data not shown.)

Dose response of insulin at 1 μM GABA

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 $Y= Bottom + (Top-Bottom)/1 + 10^{(logEC50-X)HillSlope}$ [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 ± 1.5%.

Effect of insulin on GABA dose response

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 ± 13% of maximal) (fig. 3). Significant decreases in GABA induced current occurred at submaximal concentrations of GABA ranging from 5 μM to 1 μM ($p = 0.03$).

Discussion

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 minutes, 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 nM to 3 nM depending on the tissues) [13,17,21]. 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 sec 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 minutes. 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 concentrations of insulin, a competitive nature for the insulin inhibition cannot be dismissed for $\alpha_1\beta_2\gamma_{2s}$ $GABA_A$ receptors. Another isoform ($\alpha_4\beta_3\gamma_1$) shows the mixed type inhibition [40] similar to β-carbolines, so as an overall mechanism of inhibition at $GABA_A$ receptors, insulin may be a non-competitive inhibitor; the effects of insulin would be dependent on the subunit composition of the $GABA_A$ receptor.

Insulin is a peptide hormone, which makes it unusual when compared to the many other ligands that interact at $GABA_A$ receptors. Most other GABA-acting ligands are small organic molecules, like propofol or BZs, or steroid hormones [22]. However at least one peptide is known to interact at $GABA_A$ receptors though its mechanism of action seems different from the inhibitory effect of insulin illustrated in this study. Diazepam Binding Inhibitor (DBI) is an endogenous inhibitor that binds the extracellular benzodiazepine site directly blocking BZ binding and allosterically inhibiting the $GABA_A$ receptor [8]. Though DBI unknown if the inhibitory mechanisms of insulin and DBI are similar, DBI provides corraborating evidence that a peptide, like insulin, could interact in the extracellular binding sites of the receptor.

The oocyte contains insulin receptors and is sensitive to insulin [31,12]. Therefore, a component of the insulin receptor signaling pathway could phosphorylate $GABA_A$ receptors when the oocyte is exposed to these nanomolar concentrations of insulin. Though the inhibitory effect of insulin on GABA-induced current seems to be a direct antagonism, the possibility that the effect could be from phosphorylation cannot be dismissed. Exposure of the oocyte to insulin would activate the insulin receptor signaling pathway. The insulin receptor signaling

pathway consists of many kinases. The PI3K/Akt kinase pathway seems to be the one activated for the insertion of $GABA_A$ receptors into the membrane [38]. Other kinases in the pathway could cause inhibition. Some studies indicate that activation of kinases in the "opposite" arm of the insulin receptor signal transduction pathway could have opposite effects on targets [rev in 36]. For example, in neuronal survival, the activation of the Akt branch increases neuronal survival (perhaps partly by the increase in cell surface $GABA_A$ receptors [27]) while activation of the ERK pathway contributes to neuronal death [36]. Therefore the other branch of the insulin receptor signal transduction pathway with ERK kinases could phosphorylate $GABA_A$ receptors and cause the inhibition in GABA-mediated current. Recent evidence indicates that ERK kinases do in fact inhibit GABA mediated current [7]. Phosphorylation acts on an intracellular site, so any immediate modifications there would be more allosteric in nature; longer term changes involve receptor insertion or degradation [3]. The relatively rapid nature of the inhibitory effect of insulin suggest a direct interaction on the receptor; the fact that insulin has a nanomolar affinity for the GABA receptor approximately equal to the EC_{50} of insulin for the insulin receptor suggests an interaction potentially involving the insulin receptor and phosphorylation. Further experiments will be necessary to determine which of these interactions is more important in the inhibitory effect of insulin on $GABA_A$ receptors.

Because at this putative neuronal isoform $\alpha_1\beta_2\gamma_{2s}$ of $GABA_A$ receptors, insulin only affects currents induced by lower amounts of GABA, the main effect of insulin could be on tonic GABA currents, especially if the inhibitory effect is extended to α_4 or α_6 containing receptors (there is some evidence for an effect at α_4 containing receptors [40]). At the $\alpha_1\beta_2\gamma_{2s}$ isoform studied here, the lower affinity for GABA caused by insulin could increase the deactivation or unbinding rate of GABA, reducing synaptic currents. Such an effect would be difficult to detect in the oocyte system used in this study due to slow solution exchange rates [42]. GABA currents are important in the overall excitability of the brain, and play a role in synaptic plasticity [32, 22,28]. Insulin, by inhibiting GABA currents, could therefore affect activities associated with GABA-mediated inhibition, especially dysfunctions associated with diabetes or improper insulin amounts. The $GABA_A$ isoform of this study, $\alpha_1\beta_2\gamma_{2s}$, is the most likely isoform expressed in most brain regions including cerebral cortex, hypothalamus, olfactory bulb and hippocampus, as well as many others [30,19]. These regions are ones where insulin could have opposing roles to GABA in different behaviors, including memory (hippocampus and cortex [43]); food intake and appetite (cortex [4], hypothalamus [9] and olfactory bulb [25]); and control of glucose concentrations (hypothalamus [20]). The premetabolic syndrome is characterized by increased levels of insulin [24]. Possible then, insulin inhibition of $GABA_A$ currents could contribute to the development metabolic syndrome including loss of control of glucose concentrations [20], and improper food intake [4,9,25] associated with the disorder [24].

In conclusion, I have found a novel action of insulin on neuronal type $\alpha_1\beta_2\gamma_{2s}$ $GABA_A$ receptors. This action is inhibitory and occurs simultaneously with the application of low concentrations of GABA; this action acts competitive in nature. The inhibitory effect of insulin on low concentration GABA-induced current could be important in the progress of metabolic syndrome to diabetes, and in some of the neurological side effects of diabetes.

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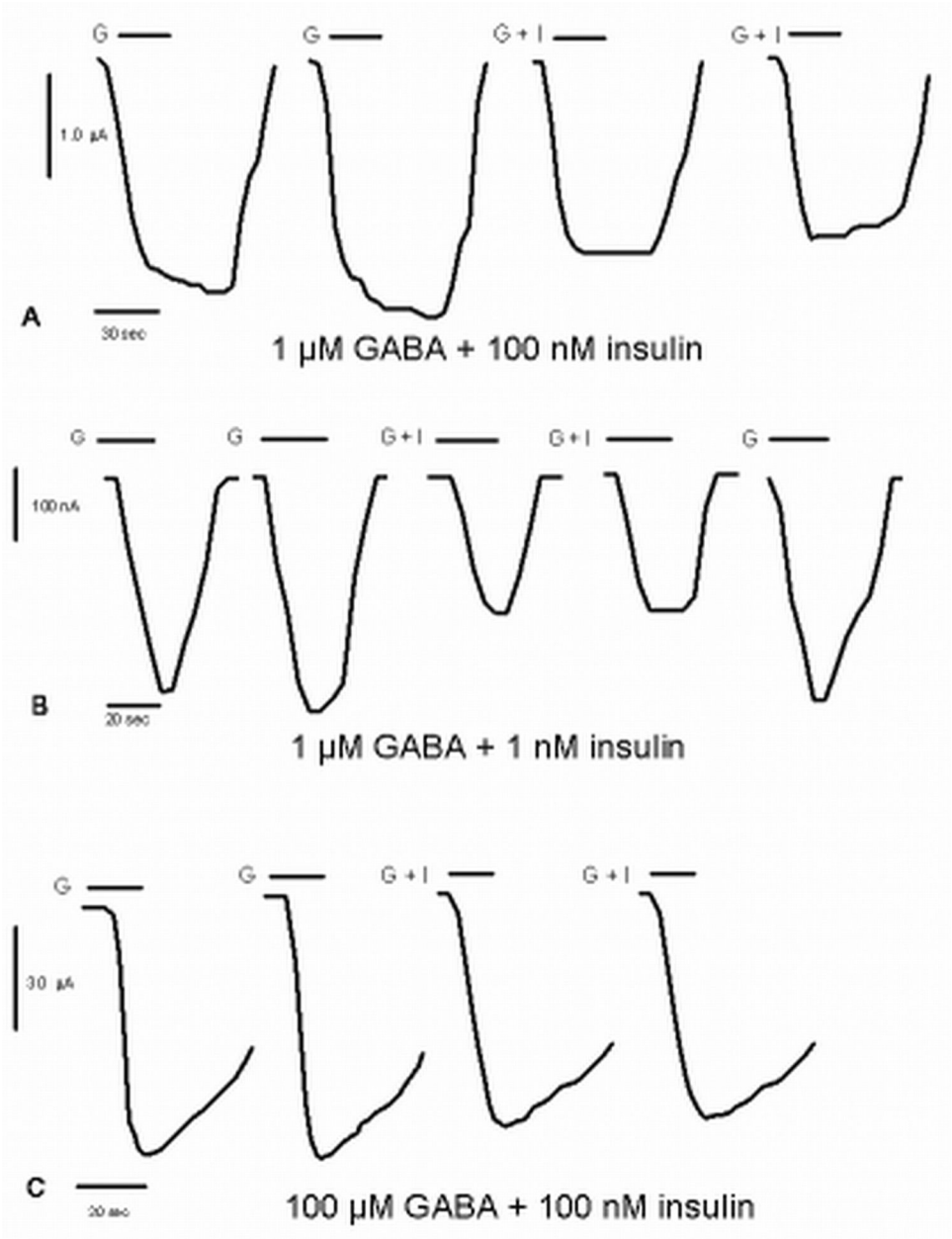

Figure 1. Effect of insulin on GABA-mediated currents at $\alpha_1\beta_2\gamma_{2S}$ receptors

All currents are from $\alpha_1\beta_2\gamma_{2s}$ $GABA_A$ receptors expressed in *Xenopus* oocytes clamped at −60 mV.

A. The first series of tracings shows that 100 nM insulin inhibits currents elicited by 1 μM GABA. The first two tracings are controls; the bar corresponds to the application of 1 μM GABA only (G). The next two are tracings of 1 μM GABA co-applied with 100 nM insulin with the bar corresponding to the time of co-application (G + I).

B. The second series of tracings shows that 1 nM insulin inhibits currents elicited by 1 μM GABA. The first two tracings are the controls; the bar corresponds to the application of 1 μM GABA only (G). The third and fourth tracings are the currents of GABA co-applied with 1 nM insulin with the bar corresponding to the time of co-application (G + I). The last trace shows

that the inhibitory effect of insulin is reversible with the bar corresponding to the application of 1 μM GABA only (G).

C. The third series of tracings shows no significant effect of 100 nM insulin in the presence of 100 μM GABA. The first two tracings the controls; the bar corresponds to the application of 1 μM GABA only (G). The last two tracings are the GABA currents in the presence of insulin with the bar corresponding to the time of co-application (G + I).

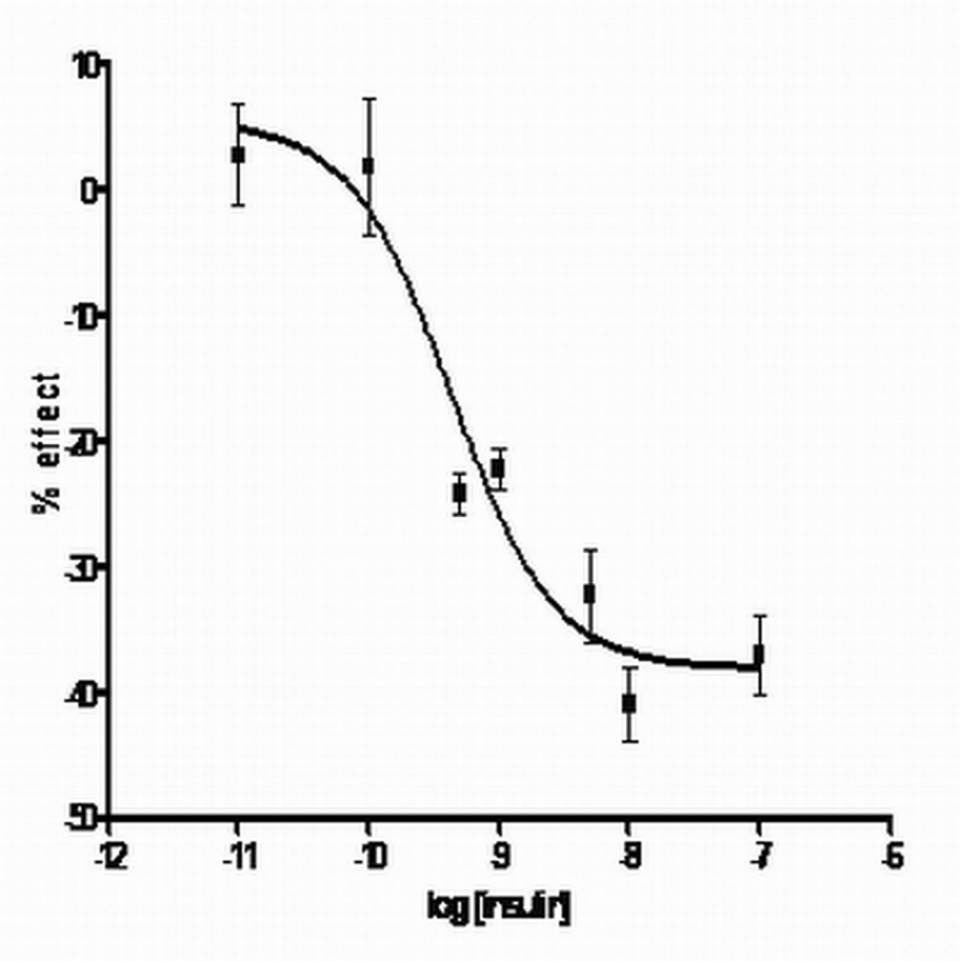

Figure 2. Insulin dose response at 1 μM GABA for $\alpha_1\beta_2\gamma_{2s}$ receptors

Various concentrations of insulin (100 nM to 0.01 nM) were added with 1 μM GABA. The percent effect is the change in current from control currents (no insulin). Points are the average and standard deviations of 3–6 experiments. The curve was fit using a two site model on GraphPad. Insulin effects on currents are statistically significant at 100 nM and 10 nM ($p < 0.05$). The maximal effect of insulin is approximately −38%, with an IC_{50} in the 4.3×10^{-10} range.

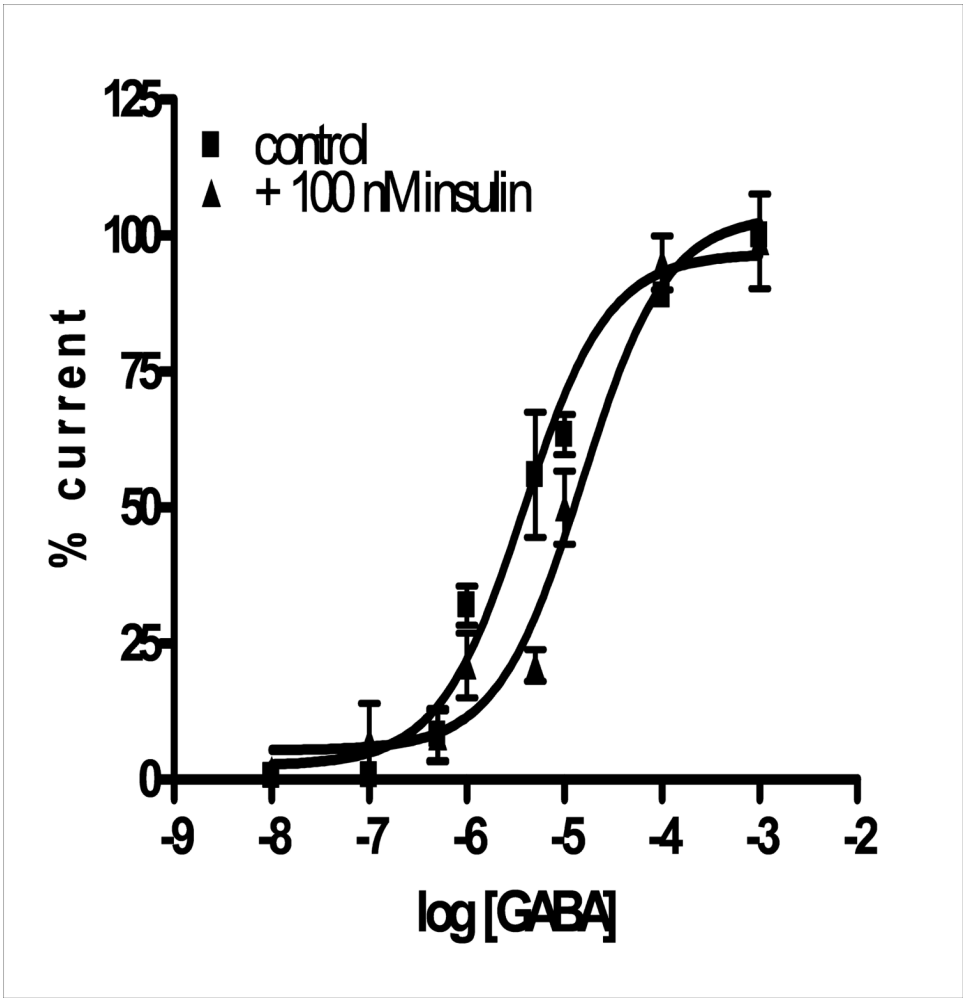

Figure 3. GABA current changes in the presence of 100 nM insulin at $\alpha_1\beta_2\gamma_{2s}$ receptors

The change in GABA-induced current due to the presence of 100 nM insulin is plotted as a function of GABA concentration. GABA EC_{50} is approximately 4 μM and shifts to 15 μM in the presence of insulin. Points are mean and standard deviations of 3–5 experiments.