Chronic stimulation induces adaptive potassium channel activity that restores calcium oscillations in pancreatic islets in vitro

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Abbreviated Title: Adaptive K^+ channels restore islet oscillations (50 characters)

Key terms: islets, beta cells, oscillations, pulsatility, intracellular calcium, tolbutamide, KATP-channel, potassium, persistent hyperinsulinemic hypoglycemia of infancy (PHHI), congenital hyperinsulinemia.

Number of Figures and Tables: 7

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Disclosure Statement: The authors have nothing to disclose.

Abstract

Insulin pulsatility is important to hepatic response in regulating blood glucose. Growing evidence suggests that insulin-secreting pancreatic β cells can adapt to chronic disruptions of pulsatility to rescue this physiologically important behavior. We determined the time scale for adaptation and examined potential ion channels underlying it. We induced the adaptation both by chronic application of the ATP-sensitive K^+ (K(ATP)) channel blocker tolbutamide and by application of the depolarizing agent potassium chloride (KCl). Acute application of tolbutamide without pretreatment results in elevated Ca^{2+} as measured by fura-2AM and the loss of endogenous pulsatility. We show that after chronic exposure to tolbutamide (12-24 h), Ca^{2+} oscillations occur with subsequent acute tolbutamide application. The same experiment was conducted with potassium chloride (KCl) to directly depolarize the β cells. Once again, following chronic exposure to the cell stimulator, the islets produced Ca^{2+} oscillations when subsequently exposed to tolbutamide. These experiments suggest that it is the chronic stimulation, and not tolbutamide desensitization, that is responsible for the adaptation that rescues oscillatory β-cell activity. This compensatory response also causes islet glucose sensitivity to shift rightward following chronic tolbutamide treatment. Mathematical modeling shows that a small increase in the number of K(ATP) channels in the membrane is one adaptation mechanism that is compatible with the data. To examine other compensatory mechanisms, pharmacological studies provide support that Kir2.1 and TEA-sensitive channels play some role. Overall, this investigation demonstrates β-cell adaptability to overstimulation, which is likely an important mechanism for maintaining glucose homeostasis in the face of chronic stimulation.

Introduction

Tight regulation of blood glucose and the body's energy demands requires precise insulin secretion that reflects the blood glucose concentration. This is achieved by secreting periodic large pulses of insulin from pancreatic β cells when glucose is high following a meal and small pulses when glucose is low during sleep or during a period of fasting (46). The periodic insulin pulses produced at stimulatory glucose levels are due to bursting electrical activity; Ca^{2+} entry during each burst evokes insulin exocytosis (36). ATP-sensitive K^+ (K(ATP)) channels largely dictate the pattern of activity in β cells (43), and may contribute to packaging electrical impulses into bursts (32). These channels are regulated by glucose via metabolism (4), and thus provide a unique energy-sensing mechanism that serves as a key component in the insulin secretory pathway.

As important as K(ATP) channels are to insulin secretion, it is not surprising that defects in K(ATP) channels are associated with metabolic disorders. A mutation in the genes encoding the K(ATP) channel is responsible for approximately half of all cases of diabetes developing within the first six months of life (18, 41). Specifically, mutations in the KCNJ11 gene, which codes for the pore-forming subunit of the K(ATP) channel (Kir6.2), or mutations in the ABCC8 gene, which codes for the sulfonylurea receptor subunits, can result in neonatal diabetes (1). Another disorder, persistent hypoglycemic hyperinsulinemia (PHHI, also sometimes called congenital hyperinsulinism) is due to constitutive insulin secretion (8, 23). Again, a mutation in the K(ATP) channel is a common cause of the disorder (25). Even mutations with minor effects on K(ATP) channel function can result in diabetes in adulthood (3). These disorders thus highlight the importance of understanding how K(ATP) channels work in health and disease.

In light of these negative consequences of K(ATP) channel mutations in humans it is surprising that, in mouse islets, mutations that eliminate functional K(ATP) channels do not typically interfere with bursting electrical activity and the associated Ca^{2+} oscillations (14, 42, 48). This is unexpected, since pharmacological manipulations that acutely block K(ATP) channels convert the bursting to tonic spiking with an elevated Ca^{2+} level (Fig. 1, and (27)). It is indicative of a adaptation mechanism that acts to restore oscillatory islet activity. Such activity results in the secretion of insulin pulses, and it has been demonstrated that the liver responds better to pulsatile insulin than to insulin maintained at a constant level (31). Bursting electrical activity, and accompanying Ca^{2+} oscillations, are therefore beneficial from a physiological perspective (22, 24, 46).

In this study, we examined how islets respond in vitro to persistent blockade of K(ATP) channel activity by tolbutamide for up to 24h. In contrast to acute exposure to this β cell stimulator, we demonstrate that islets chronically exposed to tolbutamide behave very differently during subsequent acute exposure than do those islets without the prior chronic exposure to the stimulator. With prior chronic tolbutamide treatment (12 to 24 h), many islets exhibit Ca^{2+} oscillations, rather than an elevated Ca^{2+} level upon subsequent acute tolbutamide exposure. Thus, the β cells adapt during the chronic tolbutamide exposure so as to rescue bursting, and this rescue becomes evident with 12 h of tolbutamide treatment.

A previous study showed that overnight exposure of islets to high glucose (11 mM), rather than low glucose (2.8 mM), caused a left shift in the glucose dose-response curve, and this was attributed to decreased trafficking of K(ATP) channels to the plasma membrane (17). We observed the opposite, a right-shifted glucose dose-response curve with overnight exposure to tolbutamide. Using mathematical modeling, we demonstrate that an increase in the K(ATP)

current conductance can account for our data on chronic tolbutamide exposure. The model also predicts that this mechanism can be evoked by chronic exposure of the islets to KCl, which directly depolarizes the β cells. This prediction was tested and, indeed, chronic exposure to KCl evoked the adaptation mechanism so that islets exhibited Ca^{2+} oscillations during subsequent acute exposure to tolbutamide. Once again, the islets adapted to chronic stimulation so as to facilitate oscillatory activity.

While mathematical modeling suggests that increased K(ATP) conductance can explain the adaptation data, it is likely that additional ion channels are affected by the chronic tolbutamide or KCl treatment. We tested the effects of several K^+ channel blockers on islets with and without prior chronic tolbutamide treatment and found that the responses to the Kir2.1 channel antagonist ML133 and the general voltage-dependent K^+-channel blocker tetraethylammonium (TEA) were different in treated versus untreated islets. This suggests that both Kir2.1 and TEA-sensitive currents are affected by the tolbutamide treatment, and thus contribute to the adaptation.

Overall, our study reveals that islet β cells, when chronically stimulated, adapt so as to restore oscillatory activity. Using tolbutamide as the stimulating agent, we found that this adaptation process first becomes evident with 12 h of exposure, but is of greater magnitude with longer exposure times (exposures of up to 24 h were investigated). The evidence suggests that changes in K^+ conductances are the ionic mechanisms for the adaptation. The findings demonstrate that β cells have the adaptive capacity to restore oscillatory activity in the face of chronic stimulation, which would be valuable for the maintenance of glucose homeostasis under the challenge of chronic overstimulation.

Materials and Methods.

Mice.

Adult male CD1 mice at 8-20 weeks of age were used for all studies. Mice were purchased from Envigo (Indianapolis, IN) and housed at Ohio University until needed for experiments. All protocols used in these studies were approved by the Ohio University Institutional Animal Care and Use Committee.

Islet isolation

Pancreatic islets were isolated and cultured as described previously (11). Following isolation, islets were cultured overnight in RPMI-1640 media (Invitrogen) with 11mM glucose, 10% fetal bovine serum, and 1% penicillin/streptomycin. All drug treatments or supplements used for chronic conditions were made up in RPMI-1640, and all experiments were conducted within 1-2 days of islet isolation.

Measurements of intracellular calcium

Islets were loaded with 1μM fura-2AM in a mixture of two modified KRB solutions: low glucose solution = 3 mM glucose, 134.5 mM NaCl, 3 mM $CaCl_2$, 5 mM KCl, 2 mM $MgCl_2$, and 10 mM HEPES (pH 7.4); high glucose solution = 28 mM glucose 122 mM NaCl, 3 mM $CaCl_2$, 5 mM KCl, 2 mM $MgCl_2$, and 10 mM HEPES (pH 7.4). These solutions were mixed in proportional volumes to produce various glucose concentrations as needed. Islets were loaded for 30 min at 37 ^{o}C and 5% $C0_2$ in solutions identical to the starting solution for each recording and then transferred to the recording chamber for an additional 10 min. Intracellular calcium (Ca^{2+})

was measured using the ratiometric Ca^{2+} indicator fura-2 AM as described previously in greater detail (16). The use of Cell Tracker Red to record control vs. treated groups of islets simultaneously is described in (12).

Data analysis

A Fisher Exact test (2x2 contingency) was used to compare the percentages of oscillating vs. non-oscillating islets either treated with a stimulator or untreated. For glucose dose-response curves, a two-point moving average was used to smooth inter-recording Ca^{2+} differences between the two separate 3-glucose-step recordings used to form the 6-point curve. A two-tailed Student's t-test was used for all other comparisons unless stated otherwise.

Mathematical model

A mathematical model was used to test whether a small compensating increase in K(ATP) channel conductance could account for the data on chronic tolbutamide exposure and to predict the compensating effects of chronic KCl exposure. The mathematical model is based on the Dual Oscillator Model described in (9). This includes a first module describing the cellular electrical activity, a second module for glycolysis, and a third one for mitochondrial metabolism. Since we have recently found evidence supporting a key role for Ca^{2+} activation of the enzyme pyruvate dehydrogenase (33, 34), we add this effect to the model. All equations and parameter values are described in the computer code that can be downloaded from http://www.math.fsu.edu/~bertram/software/islet.

In the model, the current through K(ATP) channels is described by:

$$I_{K(ATP)} = g_{K(ATP)} o_\infty (V - V_K),$$

where $g_{K(ATP)}$is the maximum conductance, V is the plasma membrane potential, V_K is the K^+ Nernst potential, and o_∞ is the fraction of activated channels that depends on the ADP and ATP concentrations and is given by

$$o_\infty(ADP, ATP) = \frac{0.08\left(1+2\frac{MgADP^-}{k_{dd}}\right)+0.89\left(\frac{MgADp^-}{k_{dd}}\right)^2}{\left(1+\frac{MgADP^-}{k_{dd}}\right)^2\left(1+\frac{ADP^{3-}}{k_{td}}+\frac{ATP^{4-}}{k_{tt}}\right)} \quad .$$

Here, $MgADP^- = 0.165ADP$, $ADP^{3-} = 0.135ADP,$ and $ATP^{4-} = 0.05ATP$, while the parameters k_{dd}, k_{tt}, and k_{td}represent the dissociation constants, which describe the binding equilibrium of the various nucleotide forms. In this paper, acute tolbutamide treatment is simulated by decreasing the constant k_{tt} from 1 μM to 0.91 μM to mimic an increase in the ATP affinity caused by the addition of the drug, leading to channel closure. Chronic tolbutamide adaptation is simulated by increasing the maximum conductance $g_{K(ATP)}$ from 19,700 pS to 21,500 pS, reflecting either increased trafficking of K(ATP) channels to the membrane or increased gene expression of the channels (or a combination of both). Application of 30 mM KCl is simulated by increasing the K^+ Nernst potential from $V_K = -75$ mV to -72 mV.

Results

Short-term exposure to tolbutamide terminates islet oscillations and is immediately reversible

Islets were acutely treated with a 30-min exposure to tolbutamide, and islet activity was measured using fura-2AM fluorescence to measure intracellular Ca^{2+} ratiometrically (Fig. 1). The recording began in 11 mM glucose (abbreviated 11G) to establish a baseline for normal islet

activity. In this condition, the 12 islets were all oscillating, as is typical for islets in 11G. Upon the addition of tolbutamide at 20 min, Ca^{2+} levels rose, and oscillations ceased. This is the result of K(ATP) channel blockage leading to membrane depolarization. After 30 min in the tolbutamide-treated condition, tolbutamide was washed out with 11G, Ca^{2+} levels dropped, and the islets resumed oscillatory activity in most cases. The effects on mean Ca^{2+} levels during each treatment phase are quantified in Fig. 1B. Both the peak response to tolbutamide and the mean response in the final 20 min of treatment were significantly elevated over control and washout ($p<0.001$ for each). The mean Ca^{2+} level during the washout phase (calculated for the last 20 min) did not differ significantly from that of the 20 min control phase. Thus, our data show that tolbutamide terminates Ca^{2+} oscillations and is reversible on the short term.

Ca^{2+} oscillations are restored following long-term exposure to tolbutamide

While the acute effect of tolbutamide application on islet activity has been demonstrated previously (27), chronic effect of the drug on islet oscillations has not been systematically studied. We approached this by incubating normal healthy islets in 11G plus tolbutamide (250 μM) for a range of durations (which we refer to as *chronic tolbutamide treatment*), then testing the effects of subsequent 20 min re-exposure to 11G plus tolbutamide (*acute tolbutamide treatment*). After 4 hours of chronic tolbutamide treatment (Fig. 2A), islets exhibited a steady elevated Ca^{2+} level during acute tolbutamide treatment. There are no oscillations while tolbutamide is present, but oscillations soon recover in most islets upon washout with 11G alone, as in Fig. 1. In the 20 h tolbutamide treatment group (Fig. 2B), however, the islets oscillate despite the presence of tolbutamide and do not show the same overstimulation and heightened

205 Ca^{2+} levels that were observed in the 4-h treatment group. This demonstrates a adaptation that
206 occurred during the longer treatment.

207 To determine the adaptation time scale, a total of 200 islets were separated into groups
208 with different incubation durations: approximately half had chronic tolbutamide treatment and
209 the remaining islets were in a control group incubated with 11G alone. The fraction of islets
210 exhibiting Ca^{2+} oscillations is plotted for each incubation duration in Fig. 2C. At 4 and 8 hours of
211 tolbutamide exposure, there is no difference between treated and untreated groups in the fraction
212 of islets displaying oscillations in the presence of 250 μM tolbutamide plus 11G. At 12 hours, a
213 separation between treated and untreated groups starts to emerge, with 33% of tolbutamide-
214 treated islets oscillating versus only 13% of untreated islets ($p=0.04$). The difference between
215 treated and untreated groups grows with the exposure time to chronic tolbutamide. By 20 hours,
216 the adaptation appears to reach its maximum with ~75% of chronic tolbutamide-treated islets
217 oscillating compared to 18% of untreated controls.

218

219 *Modeling shows that increased K(ATP) conductance may mediate adaptation to chronic*
220 *tolbutamide treatment*

221 The adaptation to chronic tolbutamide exposure shown in Fig. 2 could, in principle, be
222 due to increased K^+ conductance or decreased Ca^{2+} conductance in the β cell membrane. Such
223 changes would decrease cell activity so that electrical bursting occurs in the presence of
224 tolbutamide, rather than tonic spiking. However, the adaptation must also be such that in 11G the
225 islet still bursts, as in Fig. 2B during washout. If adaptation increases K^+ conductance too much,
226 then the cell would be silent in the absence of tolbutamide (i.e., in 11G alone).

227 One possibility is that adaptation is achieved by increasing the total K(ATP) channel
228 conductance in the cell membrane, either through increased gene expression of the K(ATP)
229 channel subunits or through increased K(ATP) channel trafficking. To test whether increased
230 K(ATP) channel conductance can both rescue bursting during the acute tolbutamide application
231 and allow bursting to occur in the absence of tolbutamide, we used mathematical modeling. As
232 described in Methods, a modification of the Dual Oscillator Model (DOM) was employed (9).

233 Figure 3A shows a model simulation of an acute application of tolbutamide to an
234 "untreated islet". In 11G alone the model islet exhibits bursting, and the accompanying Ca^{2+}
235 oscillations are shown. When tolbutamide is added (simulated by decreasing the dissociation
236 constant k_{tt}), the model islet exhibits tonic spiking, and the Ca^{2+} concentration is pinned at an
237 elevated level, as in Fig. 1A. When tolbutamide is removed (parameter k_{tt} returned to its baseline
238 value), the model islet returns to a bursting state. In the model "treated islet", with increased
239 K(ATP) channel conductance, the islet is again bursting prior to acute application of
240 tolbutamide, and it continues to burst when the tolbutamide is applied (Fig. 3B). When
241 tolbutamide is removed the model islet again bursts. Therefore, increasing the total K(ATP)
242 conductance is a viable adaptation mechanism that is compatible with the data from Fig. 2.

243

244 *Modeling suggests an experiment that excludes the possibility of tolbutamide desensitization*

245 Another possible explanation for the data of Fig. 2 is that the islet becomes desensitized
246 to tolbutamide with chronic exposure. Our hypothesis, in contrast, is that the sustained activity
247 induced by chronic tolbutamide exposure leads to a compensatory increase in K(ATP)
248 conductance. If so, then this should also be the case in response to chronic exposure of the islet

to KCl, which directly depolarizes the islet by reducing the gradient of K^+ across the plasma membrane. This treatment excludes the possibility of tolbutamide desensitization, since tolbutamide would only be applied acutely. But if adaptation occurs so that bursting is produced both during acute tolbutamide application and with 11G alone, what behavior should one expect to see if the treated islet is acutely exposed to KCl?

To answer this question, we again employed the DOM. As before, the adaptation to chronic stimulation (here due to KCl) was simulated by increasing the K(ATP) channel conductance (to the same level as in Fig. 3). In both treated and untreated model islets, acute application of KCl (simulated by decreasing the K^+ Nernst potential) converted bursting to tonic spiking, so that the Ca^{2+} concentration was pinned to an elevated level (Fig. 4). However, when the model islets were subsequently exposed to tolbutamide (and not KCl), the untreated islet still showed a plateau Ca^{2+} level, while the treated islet exhibited oscillations both during acute tolbutamide treatment and after washout in 11G. Thus, the model illustrates that if stimulation through chronic KCl exposure induces the same compensatory effects as stimulation through chronic tolbutamide exposure, then the adaptation would be sufficient to rescue bursting during acute tolbutamide stimulation, but insufficient to rescue bursting during KCl stimulation, which affects the current through all K^+ channels.

Chronic KCl exposure rescues Ca^{2+} oscillations during acute tolbutamide exposure

We tested the model prediction of Fig. 4 by incubating a population of islets in 11G plus 30 mM KCl for 24 h. We then followed the stimulus protocol described in Fig. 4. In a representative example of an untreated islet (Fig. 5A), the Ca^{2+} concentration is elevated

throughout the acute KCl exposure. When exposed to tolbutamide in 11G, the Ca^{2+} level remained elevated, and oscillations occurred only once the tolbutamide was removed. A representative islet that was pretreated for 24 h with KCl (Fig. 5B) produced similar Ca^{2+} responses during the acute KCl application. When switched to tolbutamide, however, the treated islet produced a substantial drop in Ca^{2+} that rebounded into oscillations throughout the acute tolbutamide treatment. The islet continued to show Ca^{2+} oscillations during the washout in 11G.

Responses to the stimulus protocol for the population of untreated (n=15) and 24 h KCl-treated (n=12) islets are quantified in Fig. 5C, D. During acute KCl stimulation, the mean Ca^{2+} level was elevated in both treated and untreated islets (Fig. 5C) and there were no Ca^{2+} oscillations. With acute tolbutamide stimulation, however, the mean Ca^{2+} level was significantly higher in the untreated than in the treated islets. This difference was due to the activity patterns in the two populations. Fewer than 25% of the islets in the untreated population showed any evidence of oscillations in tolbutamide. In contrast, the vast majority of the treated islets exhibited oscillations during acute tolbutamide exposure (Fig. 5D). When switched to 11G alone, there was no significant difference in the percent of islets exhibiting oscillations. Thus, the experiments support the model prediction that chronic treatment with KCl could lead to a compensating increase in K(ATP) conductance that rescues oscillations when the treated islet is acutely stimulated by tolbutamide, but this is masked when the greater acute KCl stimulus is applied. Importantly, they also demonstrate that the rescue of Ca^{2+} oscillations during acute tolbutamide application is not due to tolbutamide desensitization.

Compensatory effects of chronic tolbutamide exposure reduces Ca^{2+} responses to glucose

293 Since pancreatic islets must respond to a wide range of glucose concentrations, it is
294 important to understand the effects that chronic tolbutamide treatment have on the response to
295 glucose throughout this range. We examined Ca^{2+} responses to glucose stimulation at 4 mM
296 intervals from 0 to 20 mM glucose following 24 h exposure to tolbutamide. The mean Ca^{2+}
297 traces for both tolbutamide-treated (orange, averaged over n=13 islets) and untreated (blue,
298 averaged over n=9 islets) islets are shown for 0, 8, and 16 mM glucose in Fig. 6A and for 4, 12,
299 and 20 mM glucose in Fig. 6B (n=12 untreated and n=9 treated islets). The treated groups show
300 substantially lower mean Ca^{2+} levels at glucose concentrations of 12 and 16 mM. Three
301 additional pairs of glucose trials were conducted and combined to form the glucose dose curve
302 shown in Fig. 6C. The most substantial difference between untreated and chronic tolbutamide-
303 treated islets is again at 12 mM glucose and 16 mM glucose.

304 To further characterize this change in glucose sensitivity, we examined the effective
305 concentration for half maximal stimulation (EC50) for each set of untreated and tolbutamide-
306 treated islets. Figure 6D shows that there was a shift of 0.5-2 mM glucose in the EC50 between
307 untreated to tolbutamide-treated islets across four separate trials. Figure 6E shows the mean
308 EC50 over the population of untreated islets or tolbutamide-treated islets. Chronic tolbutamide
309 treatment resulted in a statistically significant increase of 1.14 ± 0.38 mM in the EC50 for
310 glucose; a right shift of the glucose dose-response curve.

311

312 *Several K^+ channel types may be involved in adaptation*

313 Modeling has provided evidence that adaptation to chronic stimulation could be through
314 increased K(ATP) conductance in the β cell membrane. However, changes in the conductance

315 level of other K^+ channel types could also be involved in the adaptation process. To determine if
316 voltage-dependent K^+ channels are involved, we employed the non-specific voltage-dependent
317 channel blocker tetraethylammonium (TEA). If the conductance level of a voltage-dependent K^+
318 channel is increased as a result of chronic tolbutamide treatment, then the effect of blocking this
319 channel should be greater in the treated islet than in the untreated islet. We tested this by
320 evaluating the effect of TEA on Ca^{2+} oscillations in 11G.

321 In an untreated islet (Fig. 7A), TEA has minimal effect on the peak, baseline, or
322 amplitude of the Ca^{2+} oscillations. In a tolbutamide-treated islet (Fig. 7B), TEA has a more
323 substantial effect on oscillations, noticeably increasing their amplitude. TEA was applied to sets
324 of untreated and sets of treated islets in two separate trials. The mean Ca^{2+} level was computed
325 before and after TEA application for each set. The difference (after TEA – before TEA) is
326 quantified in Fig. 7C. As in the traces of Fig. 7A-B, the effect of TEA application is larger in
327 treated versus the untreated islets, suggesting that voltage-sensitive K^+ channels are involved in
328 the adaptation to chronic tolbutamide exposure. We tested a blocker of the A-type K^+ channel, 4-
329 aminopyridine, but found no statistical difference between tolbutamide-treated and untreated
330 islets (data not shown).

331 Next, we employed an antagonist for inward-rectifying Kir2.1 channels, which have been
332 implicated in adaptation in islets with functional K(ATP) channels genetically knocked out (54).
333 This channel blocker, ML133, was applied to both untreated and treated islets, as in the TEA
334 study. In both sets of islets, there was an increase in the Ca^{2+} oscillation amplitude when ML133
335 was applied, which is evident in the representative examples (Fig. 7D, E) and in the
336 quantification of the net change in Ca^{2+} (Fig. 7F). However, the effect was larger in the

337 tolbutamide treated islets than in the untreated islets (Fig. 7F). This suggests that increased
338 Kir2.1 conductance may play a role in the adaptation to chronic tolbutamide exposure.

339

340 **Discussion**

341 Hormone secretion is often pulsatile. For example, luteinizing hormone, follicle
342 stimulating hormone, testosterone, glucagon, growth hormone, cortisol levels all exhibit
343 oscillations on some time scale. It is therefore of little surprise that insulin levels are also
344 oscillatory in humans and many other species (37, 46). There is evidence that this pulsatility
345 serves a physiological role (15, 29, 30, 39, 46), and is often lost in disease or during aging (26,
346 38, 40). It is perhaps more surprising that islet β cells are so good at adapting to manipulations
347 that interfere with the oscillatory activity that normally occurs at stimulatory glucose levels.

348 *K(ATP) channels and the restoration of oscillatory Ca^{2+}*

349 The ion channels responsible for glucose sensing, K(ATP) channels, are composed of
350 four sulfonylurea-sensitive (SUR1) subunits and four pore-forming Kir6.2 subunits, and
351 functional channels require both sets of subunits (35). In SUR1 homozygous knockout mice,
352 islets typically exhibit bursting activity (14, 48) which result in Ca^{2+} oscillations (54). This is in
353 spite of the depolarizing effect that removal of these channels should have, which would lead to
354 tonic spiking in the absence of adaptation, as in the case of acute sulfonylurea application (19,
355 27). Similarly, Ca^{2+} oscillations have been reported from islets of Kir6.2 knockout mice (42).

356 Our study was performed to determine whether oscillations could be restored following
357 chronic exposure to the stimulator tolbutamide, a drug used in the management of type 2 diabetes
358 (28). Studies using clonal BRIN-BD11 cells found that 18 h tolbutamide exposure reduced the

359 insulin release in response to secretagogues, including tolbutamide (5), and that chronic exposure
360 reduced K(ATP)-channel-independent insulinotropic actions of sulfonylureas including
361 tolbutamide (7). Another study showed that while overnight treatment with tolbutamide reduced
362 the insulin content of mouse islets, it had a mild attenuation of the mean Ca^{2+} response to a
363 glucose step from 3 mM to 15 mM, which did not reach significance (2). We observed a similar
364 attenuation of the Ca^{2+} response to glucose stimulation in the 12-20 mM range following chronic
365 tolbutamide exposure. Any Ca^{2+} oscillations in the Annello study (if they occurred), were
366 obscured by averaging over several islets. We found that oscillations during acute tolbutamide
367 exposure were often recovered following 12 h or more of chronic tolbutamide exposure (Fig. 2).
368 The extent of recovery was greater with longer exposure, saturating at 20 h exposure, where
369 ~80% of the treated islets exhibited Ca^{2+} oscillations. When the acute tolbutamide challenge was
370 removed, oscillations persisted (in 11G). Thus, the mechanism that rescues oscillations in
371 tolbutamide-stimulated islets also allows for oscillations in 11G alone.

372 While it is well established that tolbutamide is a potent blocker of K(ATP) channels (43),
373 it has other indirect effects on β cells. For example, the elevated intracellular Ca^{2+} level that
374 results from acute tolbutamide exposure regulates signaling through phospholipase C, which in
375 turn affects insulin secretion (47). Tolbutamide has also been shown to affect Cl^- channels.
376 Whole cell patch clamp (50) and patch-clamp from excised membrane patches (51) have shown
377 that tolbutamide reduces the Cl^- current through cystic fibrosis transmembrane conductance
378 regulator (CFTR) channels, which are regulated by ATP. Another study showed that tolbutamide
379 potentiates another Cl^- channel, a volume-regulated ion channel (VRAC) in rat β cells (10).
380 While such Cl^- channel modulation may supplement the blockage of K(ATP) channels, the net
381 effect is a conversion of bursting to tonic spiking with acute tolbutamide exposure and a return to

382 bursting with chronic exposure. We believe that it is the resulting elevated Ca^{2+} and loss of Ca^{2+}
383 oscillations that drive the adaptation during chronic tolbutamide exposure, rather than the
384 specific channels that are affected by tolbutamide. Evidence for this is the adaptation that occurs
385 with depolarization through chronic elevated KCl exposure (Fig. 5).

386

387 *Tolbutamide desensitization does not appear to be a factor in restoring oscillations*

388 One possibility is that the chronically treated islets become desensitized to tolbutamide.
389 To check this, we used KCl instead of tolbutamide to chronically stimulate islets, and only then
390 applied tolbutamide acutely. Once again, we observed a preponderance of oscillatory activity in
391 the treated islets during acute tolbutamide exposure, in contrast to the control islets incubated in
392 11G alone (Fig. 5). This supports the conclusion that it is the chronic stimulation, and not
393 tolbutamide desensitization, that accounts for the rescue of oscillations during acute tolbutamide
394 exposure. This conclusion is supported by a prior study that found that while chronic tolbutamide
395 exposure greatly reduced insulin secretion during a later acute tolbutamide challenge, the
396 membrane potential of single β cells was still responsive to the acute tolbutamide, arguing that
397 any desensitization occurs downstream of the cell's electrical activity (44).

398

399 *Multiple mechanisms to maintain pulsatility*

400 Our mathematical modeling suggests that the adaptation mechanism could be through
401 increased K(ATP) channel conductance (Figs. 3, 4). This increased conductance is the reason for
402 a transient quiescence following acute tolbutamide exposure in the model simulations (Figs. 3,
403 4). This transient quiescence is sometimes seen in experimental traces, though not always. For

example, it is present in islets with 4 h tolbutamide treatment (Fig. 2A), but not in islets with 20 h treatment (Fig. 2B). This variability likely reflects the fact that the increased K(ATP) conductance caused by chronic stimulation would be quite small, since if the increase is too large the cell would fail to exhibit electrical activity once the tolbutamide is removed and only glucose remains. A prior study confirmed that the expression level of K(ATP) channel subunits is not adversely affected by chronic tolbutamide treatment in clonal insulin-secreting BRIN-BD11 cells (6), though the resolution of such measurements is not fine enough to pick up the small change in expression levels that would be predicted if in fact the adaptation involved increased K(ATP) channel subunit expression. Rather than increased channel expression, it is also possible that adaptation could be due to increased K(ATP) channel trafficking to the membrane.

There are other potential mechanisms of adaptation to restore pulsatility under chronic stimulation. Genetic manipulation of K(ATP)-channels to eliminate K(ATP)-channel conductance precludes the possibility of increased channel expression or increased numbers of these channels inserted in to the plasma membrane; nevertheless, islets are oscillatory in these models (14, 42, 48). In SUR1 knockout mice, it has been demonstrated that there is increased inward rectifying K^+ current, and mathematical modeling was used to show that an inward rectifying Kir2.1 current would be capable of recovering bursting oscillations (54). The upregulation of such a current could be achieved through Ca^{2+}-sensitive gene transcription (49, 52, 53). Consistent with this view, our findings with the Kir2.1-specific inhibitor ML133 suggest that Kir2.1 activity is also increased following ~24 h of chronic stimulation. An additional, as of yet unidentified, TEA-sensitive K^+ channel may also play a role. It is also possible that adaptation for chronic stimulation can be achieved by a mechanism other than gene transcription. It was shown that islets exposed overnight to stimulatory glucose levels have decreased K(ATP)

conductance relative to those maintained in low glucose overnight, and that this is due to decreased trafficking of K(ATP) channels to the plasma membrane rather than decreased gene transcription (17).

Possible feedback effects of adaptation on metabolism

A result of the adaptation is a change in the glucose dose-response curve, such that islets treated overnight with tolbutamide have a smaller response to glucose, as determined by the mean Ca^{2+} level, at glucose levels >8 mM (Fig. 6). Prior studies have shown that chronic exposure of islets to sulfonylureas result in a large reduction in the glucose-stimulated insulin release as well as release evoked by other stimulators (21, 45). This reduction is more dramatic than the reduction in the mean Ca^{2+} level shown in Fig. 6, and likely reflects additional negative effects of tolbutamide downstream of Ca^{2+} (7, 44). This reduced response is protective against overstimulation, and is the opposite of what was seen when islets were treated overnight with 11 mM glucose, versus 2.8 mM glucose (17). With tolbutamide exposure, the direct influence is on the cell's electrical activity and Ca^{2+} dynamics, and the elevated Ca^{2+} level associated with tolbutamide exposure likely leads to a reduction in the ATP/ADP ratio due to ATP hydrolysis by Ca^{2+} pumps in the plasma and endoplasmic reticulum membranes (13). Indeed, data show an increase in ATP/ADP when the islet is hyperpolarized and Ca^{2+} level reduced with application of the K(ATP) channel activator diazoxide (34). In contrast, incubating an islet in a solution with higher glucose content will lead to an increase in the ATP/ADP ratio due to increased metabolism. It might be expected that chronic treatment with tolbutamide has the opposite effect on the glucose dose-response curve than chronic treatment with a higher glucose level.

Conclusions

We show that chronic stimulation of pancreatic islets triggers a compensatory hyperpolarizing mechanism to restore normal endogenous oscillatory activity in the presence of overstimulation. This study suggests that maintaining the ability to secrete insulin in a pulsatile manner appears to be very important to overall function as evidenced by the drive to maintain this function despite strong changes in physiological input. We identified several adaptive changes, involving K(ATP) channels and additional K^+ channels, and a decrease in glucose sensitivity. The net result is rescue of pulsatility, and its regulation by glucose. This rescue of pulsatility, which facilitates the actions of the liver in reducing glucose (31), is countered by the dramatic reduction in glucose-induced insulin secretion that has been demonstrated in previous studies (44, 45). The net effect is a time-dependent decline in the efficacy of treatment with antidiabetic agents (20).

Acknowledgements

Isabella Marinelli was supported by the Basque government through BERC 2018-2021, the Spanish State Research Agency through BCAM Severo Ochoa excellence accreditation SEV-2017-0718 and AEI/FEDER-UE project MULTIQUANT RT12018-093416-B-100. Richard Bertram was supported by grant number DMS-1853342 from the National Science Foundation. Craig Nunemaker was supported by grant number R15 DK121247 from the National Institutes of Health, the Osteopathic Heritage Foundation, and the Heritage College of Osteopathic Medicine.

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606 **Figure Legend.**

607

608 **Figure 1:** Acute tolbutamide application terminates Ca^{2+} oscillations, leading to a sustained
609 elevated level. (A) Example of fura-2 traces of the intracellular Ca^{2+} level from n=12 islets
610 starting in 11 mM glucose (11G) for 20 min and then exposed to 250 uM tolbutamide for 30 min
611 (plus 11G), followed by a washout back to 11G for 40 min. (B) Mean Ca^{2+} values (340/380 nm
612 ratio) for 11G (control, first 20 min), peak value measured during tolbutamide treatment (peak
613 tolb), the mean Ca^{2+} level during the last 20-min of tolbutamide exposure (tolb), and the last 20
614 min of washout (wash). ***=$p<0.001$ and N.S (not significant) are compared to control (11G).

615

616 **Figure 2:** Islets show a duration-dependent response to tolbutamide that restores oscillations. (A-
617 B) Multiple traces are shown for islets incubated in 250 μM tolbutamide and 11G for 4 h (A,
618 n=6) or 20 hours (B, n=8). These islets were maintained in 11G plus tolbutamide for the first 20
619 minutes followed by a washout into 11G. (C) The fraction of oscillating islets for multiple
620 tolbutamide treatment durations of 4, 8, 12, 16, and 20 h (± one hour). Untreated islets were
621 maintained in 11G media for the same durations and were recorded simultaneously with the
622 tolbutamide-treated islets. A two-tailed Chi square test was used for statistics: *=$p<0.05$,
623 ***=$p<0.001$.

624

625 **Figure 3:** Mathematical modeling demonstrates the feasibility of increased K(ATP) conductance
626 as an adaptation mechanism. (A) Acute treatment with tolbutamide converts a bursting islet, with
627 accompanying Ca^{2+} oscillations, into tonic spiking, in which the Ca^{2+} concentration is pinned to

an elevated level. (B) In a model "treated islet" (an islet treated chronically with tolbutamide), Ca^{2+} oscillations persist during an acute tolbutamide application.

Figure 4: Model simulation predicting how an islet exhibiting adaptation through an increase in K(ATP) conductance at the same level as that of Fig. 3 would respond to KCl, tolbutamide, and 11G alone. (A) In an untreated islet, the Ca^{2+} level is at an elevated plateau (reflecting tonic spiking) when stimulated by either KCl or tolbutamide. (B) In a treated islet, the Ca^{2+} level plateaus during stimulation with KCl, but oscillates during acute tolbutamide stimulation.

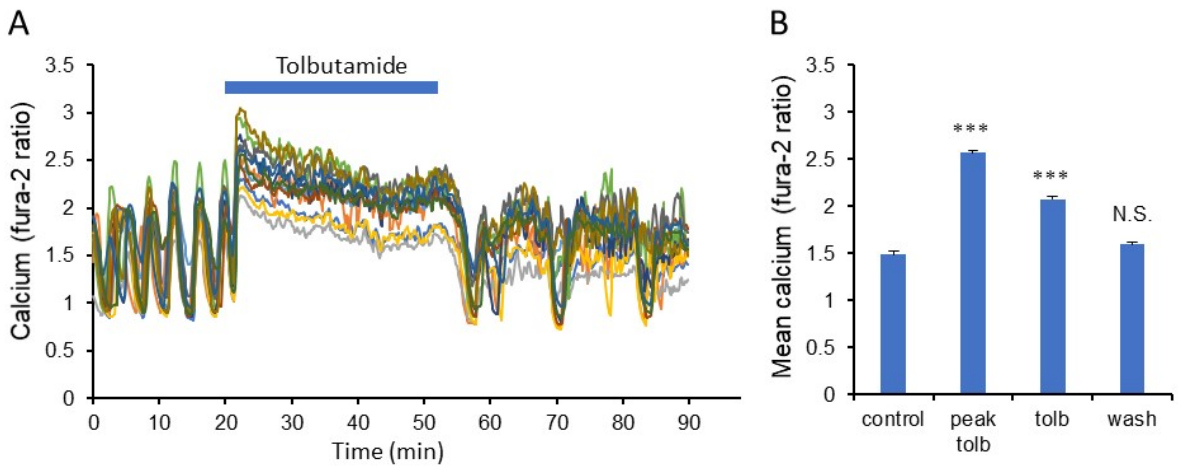
Figure 5: Experimental data support model predictions. In all cases, 11G is present. (A) Representative Ca^{2+} trace for an untreated islet, exhibiting elevated Ca^{2+} plateaus when stimulated by either KCl or tolbutamide, but oscillating in 11G alone. (B) Representative Ca^{2+} trace for an islet with 24-hour treatment with 30 mM KCl. As predicted, the islet exhibits an elevated Ca^{2+} plateau when stimulated with KCl but oscillates when stimulated with tolbutamide or with 11G alone. (C) Quantification of mean Ca^{2+} levels for untreated and KCl-treated islets for each acute treatment phase. (D) Percent of islets displaying oscillations during each treatment phase. A two-tailed t-test was used for statistic in (C) and chi square in (D): *=$p<0.05$, ***=$p<0.001$, N.S. = not significant.

Figure 6: Islets incubated for 24 hours in 250 μM tolbutamide plus 11G display a glucose-dependent reduction in Ca^{2+} levels versus those incubated in 11G alone. (A) Averaged Ca^{2+} traces during three consecutive glucose exposures of 0, 8, and 16 mM for N=13 untreated (blue)

and N=9 treated islets (orange). Averaged Ca^{2+} traces during three consecutive glucose exposures of 4, 12, and 20 mM for N=12 untreated (blue) and N=9 treated islets (orange). Data in (A-B) represent one of four trials. (C) Glucose dose-response curves computed using mean Ca^{2+} levels over a range of glucose values for all four trials. Data were combined and two-tailed t-tests were used at each glucose concentration: *=$p<0.05$, **=$p<0.01$. (D) EC50s for four trials show a right shift in EC50 among tolbutamide-treated islets versus those incubated in 11G alone. (E) Mean of the EC50s from the four individual trials *=$p<0.05$ determined by paired t-test.

Figure 7: Voltage-dependent K^+ channels and Kir2.1 channels participate in adaptation to chronic (~24 h) tolbutamide treatment. (A) In an untreated islet, the voltage-dependent K^+ channel blocker TEA does not affect the amplitude of Ca^{2+} oscillations. (B) In a treated islet, TEA increases the oscillation amplitude. (C) Mean Ca^{2+} response to TEA between the untreated and treated islets (n=24 for control, n=21 for tolbutamide treated). (D-E) In both untreated and treated islets, the Kir2.1 channel blocker ML133 increases the amplitude of Ca^{2+} oscillations. (F) The mean Ca^{2+} response to ML133 is larger in treated islets compared to untreated islets (n=18 for control and tolbutamide treated). A two-tailed t-test was used for statistics: *=$p<0.05$. ***=$p<0.001$.

668 **Figure 1.**



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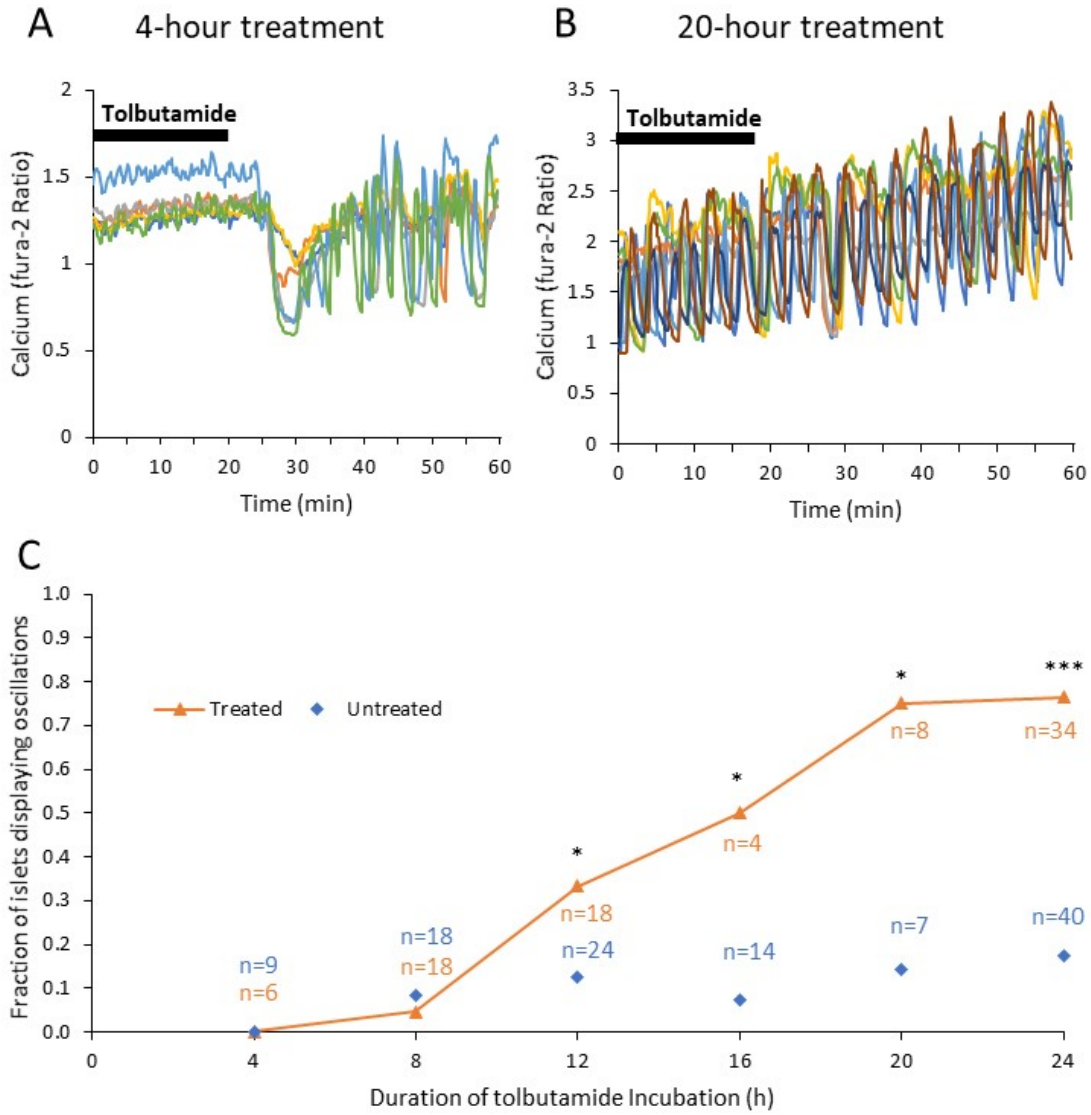
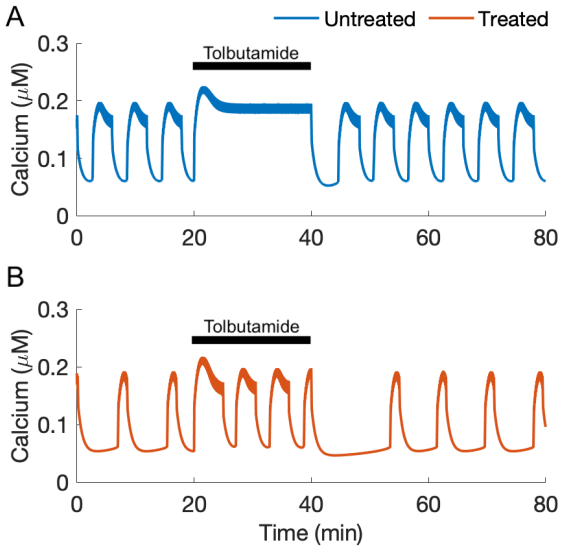
670

Figure 2.


A
4-hour treatment
Tolbutamide
Calcium (fura-2 Ratio)
Time (min)
B
20-hour treatment
Tolbutamide
Calcium (fura-2 Ratio)
Time (min)
C
Fraction of islets displaying oscillations
Treated
Untreated
n=9
n=6
n=18
n=18
n=18
n=24
n=4
n=14
n=8
n=7
n=34
n=40
*
*
*
***
Duration of tolbutamide Incubation (h)

674 **Figure 3.**



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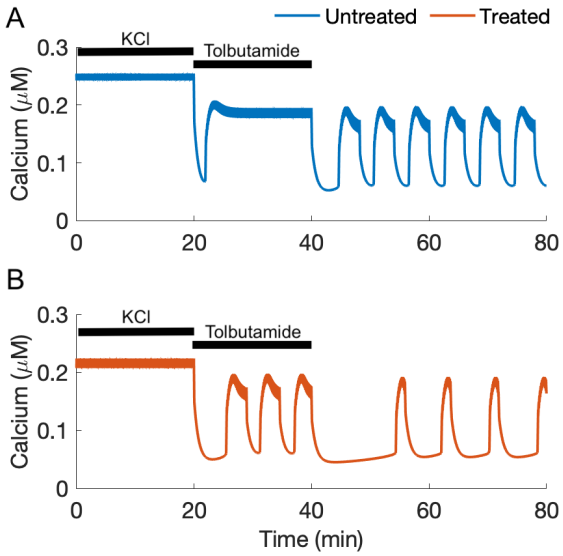
677 **Figure 4.**



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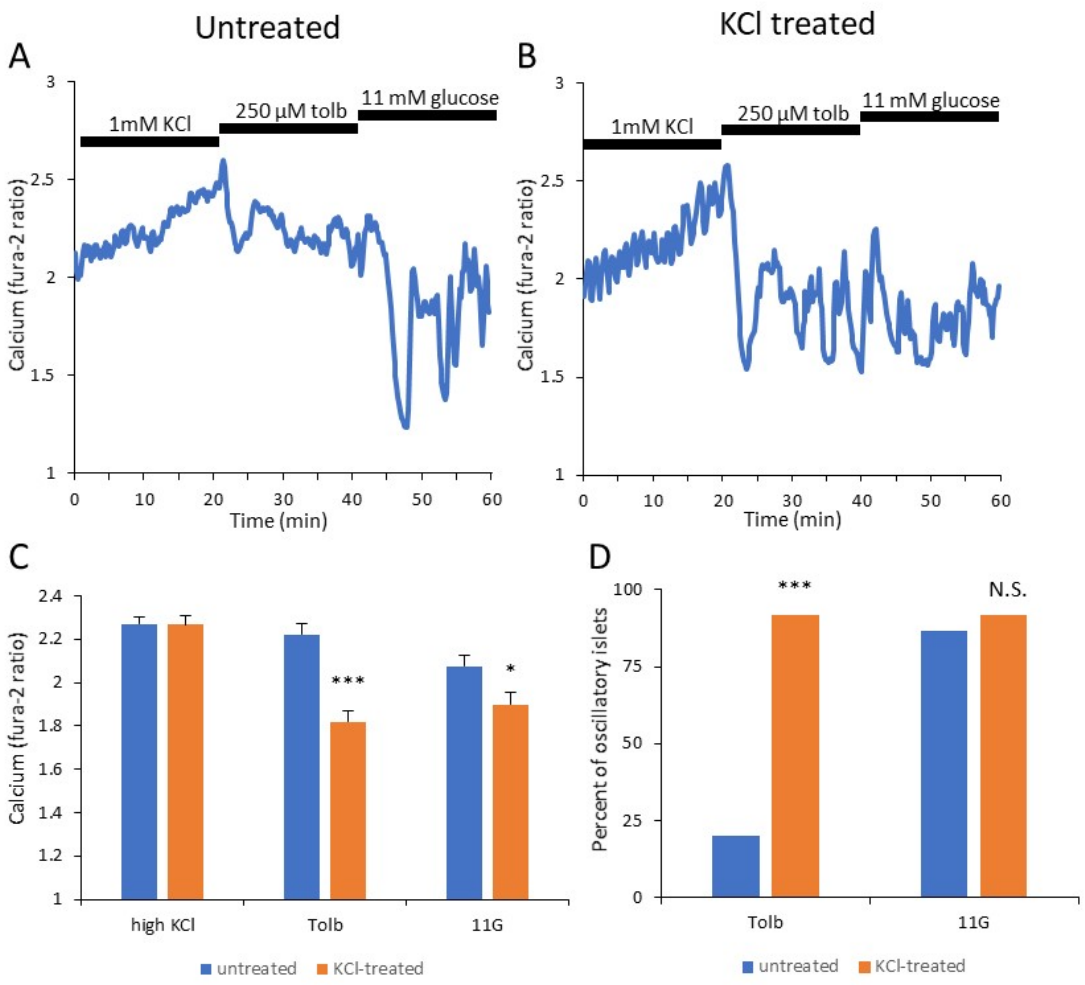
683 **Figure 5.**



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686 **Figure 6.**

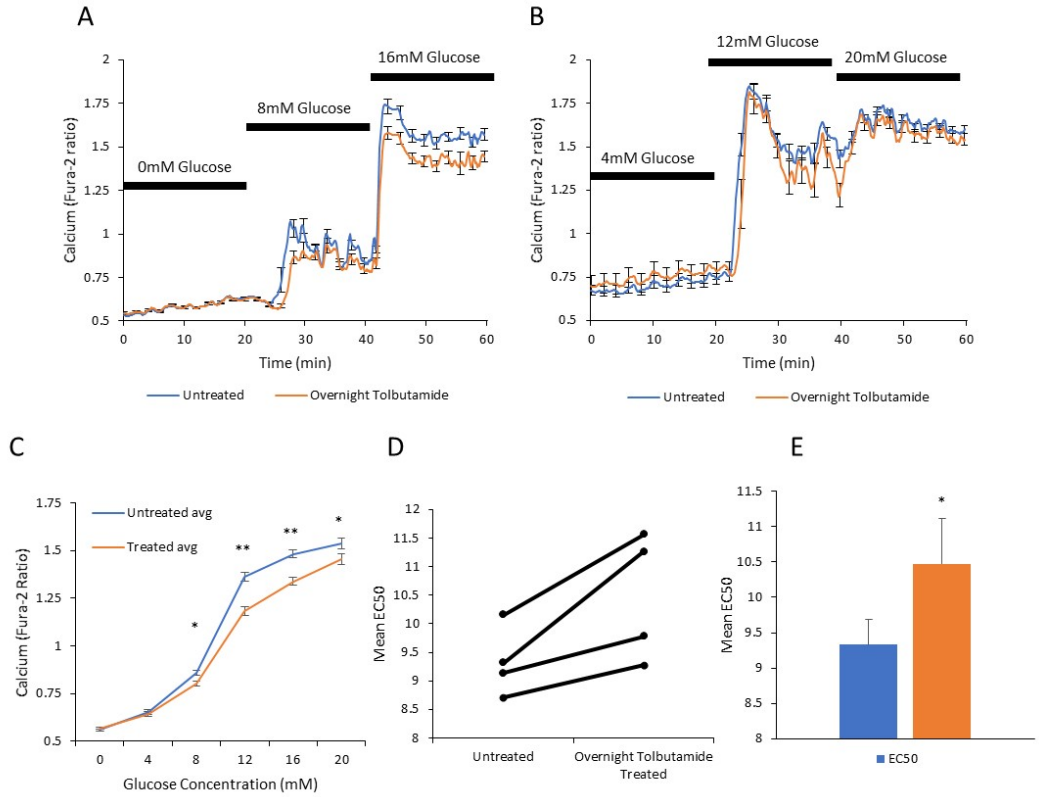

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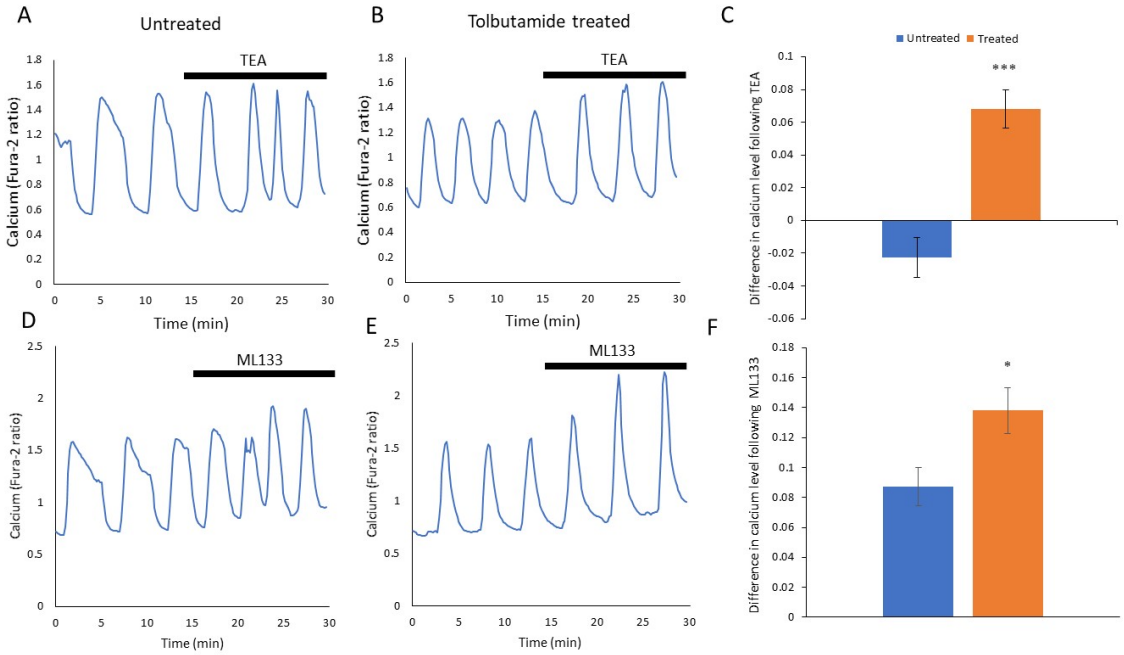
Figure 7.


A
Untreated
TEA
Calcium (Fura-2 ratio)
Time (min)
B
Tolbutamide treated
TEA
Calcium (Fura-2 ratio)
Time (min)
C
Untreated
Treated
***
Difference in calcium level following TEA
D
ML133
Calcium (Fura-2 ratio)
Time (min)
E
ML133
Calcium (Fura-2 ratio)
Time (min)
F
*
Difference in calcium level following ML133