1 **Chronic stimulation induces compensatory potassium channel activity that restores**
2 **calcium oscillations in pancreatic islets in vitro**

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Abstract

Insulin pulsatility is important to hepatic response in regulating blood glucose. Growing evidence suggests that insulin-secreting pancreatic β cells can compensate for chronic disruptions of pulsatility to rescue this physiologically important behavior. We determined the time scale for compensation and examined potential ion channels underlying compensation. We induced the compensation 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 compensation 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 compensation 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 [40]. The periodic insulin pulses produced at stimulatory glucose levels are due to bursting electrical activity; Ca^{2+} entry during each burst evokes insulin exocytosis [31]. ATP-sensitive K^+ (K(ATP)) channels largely dictate the pattern of activity in β cells [38], and may contribute to packaging electrical impulses into bursts [27]. 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 [15, 36]. 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 [6, 18]. Again, a mutation in the K(ATP) channel is a common cause of the disorder [20]. 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.

72   In light of these negative consequences of K(ATP) channel mutations in humans it is

73   surprising that, in mouse islets, mutations that eliminate functional K(ATP) channels do not

74   typically interfere with bursting electrical activity and the associated Ca^{2+} oscillations (11, 37,

75   41). This is unexpected, since pharmacological manipulations that acutely block K(ATP)

76   channels convert the bursting to tonic spiking with an elevated Ca^{2+} level (Fig. 1, and (22)). It is

77   indicative of a compensation mechanism that acts to restore oscillatory islet activity. Such

78   activity results in the secretion of insulin pulses, and it has been demonstrated that the liver

79   responds better to pulsatile insulin than to insulin maintained at a constant level (26). Bursting

80   electrical activity, and accompanying Ca^{2+} oscillations, are therefore beneficial from a

81   physiological perspective (17, 19, 40).

82   In this study, we examined how islets respond in vitro to persistent blockade of K(ATP)

83   channel activity by tolbutamide for up to 24h. In contrast to acute exposure to this β cell

84   stimulator, we demonstrate that islets chronically exposed to tolbutamide behave very differently

85   during subsequent acute exposure than do those islets without the prior chronic exposure to the

86   stimulator. With prior chronic tolbutamide treatment (12 to 24 h), many islets exhibit Ca^{2+}

87   oscillations, rather than an elevated Ca^{2+} level upon subsequent acute tolbutamide exposure.

88   Thus, the β cells compensate during the chronic tolbutamide exposure so as to rescue bursting,

89   and this rescue becomes evident with 12 h of tolbutamide treatment.

90   A previous study showed that overnight exposure of islets to high glucose (11 mM),

91   rather than low glucose (2.8 mM), caused a left shift in the glucose dose-response curve, and this

92   was attributed to decreased trafficking of K(ATP) channels to the plasma membrane (14). We

93   observed the opposite, a right-shifted glucose dose-response curve with overnight exposure to

94   tolbutamide. Using mathematical modeling, we demonstrate that an increase in the K(ATP)

95   current conductance can account for our data on chronic tolbutamide exposure. The model also

96   predicts that this mechanism can be evoked by chronic exposure of the islets to KCl, which

97   directly depolarizes the β cells. This prediction was tested and, indeed, chronic exposure to KCl

98   evoked the compensation mechanism so that islets exhibited Ca^{2+} oscillations during subsequent

99   acute exposure to tolbutamide. Once again, the islets adapted to chronic stimulation so as to

100 facilitate oscillatory activity.

101   While mathematical modeling suggests that increased K(ATP) conductance can explain

102 the compensation data, it is likely that additional ion channels are affected by the chronic

103 tolbutamide or KCl treatment. We tested the effects of several K^+ channel blockers on islets with

104 and without prior chronic tolbutamide treatment and found that the responses to the Kir2.1

105 channel antagonist ML133 and the general voltage-dependent K^+-channel blocker

106 tetraethylammonium (TEA) were different in treated versus untreated islets. This suggests that

107 both Kir2.1 and TEA-sensitive currents are affected by the tolbutamide treatment, and thus

108 contribute to the compensation.

109   Overall, our study reveals that islet β cells, when chronically stimulated, compensate so

110 as to restore oscillatory activity. Using tolbutamide as the stimulating agent, we found that this

111 compensation process first becomes evident with 12 h of exposure, but is of greater magnitude

112 with longer exposure times (exposures of up to 24 h were investigated). The evidence suggests

113 that changes in K^+ conductances are the ionic mechanisms for the compensation. The findings

114 demonstrate that β cells have the adaptive capacity to restore oscillatory activity in the face of

115 chronic stimulation, which would be valuable for the maintenance of glucose homeostasis under

116 the challenge of chronic overstimulation.

117

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 (8). 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 °C and 5% CO_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+})

140   was measured using the ratiometric Ca^{2+} indicator fura-2 AM as described previously in greater

141   detail (13). The use of Cell Tracker Red to record control vs. treated groups of islets

142   simultaneously is described in (9).

143

144   *Data analysis*

145   A Fisher Exact test (2x2 contingency) was used to compare the percentages of oscillating

146   vs. non-oscillating islets either treated with a stimulator or untreated. For glucose dose-response

147   curves, a two-point moving average was used to smooth inter-recording Ca^{2+} differences between

148   the two separate 3-glucose-step recordings used to form the 6-point curve. A two-tailed

149   Student's t-test was used for all other comparisons unless stated otherwise.

150

151   *Mathematical model*

152   A mathematical model was used to test whether a small compensating increase in K(ATP)

153   channel conductance could account for the data on chronic tolbutamide exposure and to predict

154   the compensating effects of chronic KCl exposure. The mathematical model is based on the Dual

155   Oscillator Model described in (7). This includes a first module describing the cellular electrical

156   activity, a second module for glycolysis, and a third one for mitochondrial metabolism. Since we

157   have recently found evidence supporting a key role for Ca^{2+} activation of the enzyme pyruvate

158   dehydrogenase (28, 29), we add this effect to the model. All equations and parameter values are

159   described in the computer code that can be downloaded from

160   http://www.math.fsu.edu/~bertram/software/islet.

161

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

163
$$I_{K(ATP)} = g_{K(ATP)} o_\infty (V - V_K),$$

164   where $g_{K(ATP)}$ is the maximum conductance, V is the plasma membrane potential, V_K is the K$^+$

165   Nernst potential, and o_∞ is the fraction of activated channels that depends on the ADP and ATP

166   concentrations and is given by

167
$$o_\infty(ADP, ATP) = \frac{0.08\left(1+2\frac{MgADP^-}{k_{dd}}\right)+0.89\left(\frac{MgADp^-}{k_{dd}}\right)^2}{\left(1+\frac{MgADP^-}{k_{dd}}\right)^2\left(1+\frac{ADP^{3-}}{k_{td}}+\frac{ATP^{4-}}{k_{tt}}\right)}.$$

168   Here, $MgADP^- = 0.165ADP$, $ADP^{3-} = 0.135ADP$, and $ATP^{4-} = 0.05ATP$, while the parameters

169   k_{dd}, k_{tt}, and k_{td} represent the dissociation constants, which describe the binding equilibrium of

170   the various nucleotide forms. In this paper, acute tolbutamide treatment is simulated by

171   decreasing the constant k_{tt} from 1 µM to 0.91 µM to mimic an increase in the ATP affinity

172   caused by the addition of the drug, leading to channel closure. Chronic tolbutamide

173   compensation is simulated by increasing the maximum conductance $g_{K(ATP)}$ from 19,700 pS to

174   21,500 pS, reflecting either increased trafficking of K(ATP) channels to the membrane or

175   increased gene expression of the channels (or a combination of both). Application of 30 mM KCl

176   is simulated by increasing the K$^+$ Nernst potential from $V_K = -75$ mV to -72 mV.

177

Results

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

180   Islets were acutely treated with a 30-min exposure to tolbutamide, and islet activity was

181   measured using fura-2AM fluorescence to measure intracellular Ca^{2+} ratiometrically (Fig. 1).

182   The recording began in 11 mM glucose (abbreviated 11G) to establish a baseline for normal islet

183 activity. In this condition, the 12 islets were all oscillating, as is typical for islets in 11G. Upon

184 the addition of tolbutamide at 20 min, Ca^{2+} levels rose, and oscillations ceased. This is the result

185 of K(ATP) channel blockage leading to membrane depolarization. After 30 min in the

186 tolbutamide-treated condition, tolbutamide was washed out with 11G, Ca^{2+} levels dropped, and

187 the islets resumed oscillatory activity in most cases. The effects on mean Ca^{2+} levels during each

188 treatment phase are quantified in Fig. 1B. Both the peak response to tolbutamide and the mean

189 response in the final 20 min of treatment were significantly elevated over control and washout

190 ($p<0.001$ for each). The mean Ca^{2+} level during the washout phase (calculated for the last 20

191 min) did not differ significantly from that of the 20 min control phase. Thus, our data show that

192 tolbutamide terminates Ca^{2+} oscillations and is reversible on the short term.

193

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

195   While the acute effect of tolbutamide application on islet activity has been demonstrated

196 previously (22), chronic effect of the drug on islet oscillations has not been systematically

197 studied. We approached this by incubating normal healthy islets in 11G plus tolbutamide (250

198 µM) for a range of durations (which we refer to as *chronic tolbutamide treatment*), then testing

199 the effects of subsequent 20 min re-exposure to 11G plus tolbutamide (*acute tolbutamide*

200 *treatment*). After 4 hours of chronic tolbutamide treatment (Fig. 2A), islets exhibited a steady

201 elevated Ca^{2+} level during acute tolbutamide treatment. There are no oscillations while

202 tolbutamide is present, but oscillations soon recover in most islets upon washout with 11G alone,

203 as in Fig. 1. In the 20 h tolbutamide treatment group (Fig. 2B), however, the islets oscillate

204 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 compensation that

206 occurred during the longer treatment.

207   To determine the compensation time scale, a total of 200 islets were separated into

208 groups with different incubation durations: approximately half had chronic tolbutamide treatment

209 and 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 compensation 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 compensation to chronic*

220 *tolbutamide treatment*

221   The compensation 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 compensation must also be such that in 11G

225 the islet still bursts, as in Fig. 2B during washout. If compensation increases K$^+$ conductance too

226 much, then the cell would be silent in the absence of tolbutamide (i.e., in 11G alone).

227   One possibility is that compensation 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 (7).

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 compensation 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

249   to KCl, which directly depolarizes the islet by reducing the gradient of K^+ across the plasma

250   membrane. This treatment excludes the possibility of tolbutamide desensitization, since

251   tolbutamide would only be applied acutely. But if compensation occurs so that bursting is

252   produced both during acute tolbutamide application and with 11G alone, what behavior should

253   one expect to see if the treated islet is acutely exposed to KCl?

254   To answer this question, we again employed the DOM. As before, the compensation to

255   chronic stimulation (here due to KCl) was simulated by increasing the K(ATP) channel

256   conductance (to the same level as in Fig. 3). In both treated and untreated model islets, acute

257   application of KCl (simulated by decreasing the K^+ Nernst potential) converted bursting to tonic

258   spiking, so that the Ca^{2+} concentration was pinned to an elevated level (Fig. 4). However, when

259   the model islets were subsequently exposed to tolbutamide (and not KCl), the untreated islet still

260   showed a plateau Ca^{2+} level, while the treated islet exhibited oscillations both during acute

261   tolbutamide treatment and after washout in 11G. Thus, the model illustrates that if stimulation

262   through chronic KCl exposure induces the same compensatory effects as stimulation through

263   chronic tolbutamide exposure, then the compensation would be sufficient to rescue bursting

264   during acute tolbutamide stimulation, but insufficient to rescue bursting during KCl stimulation,

265   which affects the current through all K^+ channels.

266

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

268   We tested the model prediction of Fig. 4 by incubating a population of islets in 11G plus

269   30 mM KCl for 24 h. We then followed the stimulus protocol described in Fig. 4. In a

270   representative example of an untreated islet (Fig. 5A), the Ca^{2+} concentration is elevated

271 throughout the acute KCl exposure. When exposed to tolbutamide in 11G, the Ca^{2+} level

272 remained elevated, and oscillations occurred only once the tolbutamide was removed. A

273 representative islet that was pretreated for 24 h with KCl (Fig. 5B) produced similar Ca^{2+}

274 responses during the acute KCl application. When switched to tolbutamide, however, the treated

275 islet produced a substantial drop in Ca^{2+} that rebounded into oscillations throughout the acute

276 tolbutamide treatment. The islet continued to show Ca^{2+} oscillations during the washout in 11G.

277   Responses to the stimulus protocol for the population of untreated (n=15) and 24 h KCl-

278 treated (n=12) islets are quantified in Fig. 5C, D. During acute KCl stimulation, the mean Ca^{2+}

279 level was elevated in both treated and untreated islets (Fig. 5C) and there were no Ca^{2+}

280 oscillations. With acute tolbutamide stimulation, however, the mean Ca^{2+} level was significantly

281 higher in the untreated than in the treated islets. This difference was due to the activity patterns

282 in the two populations. Fewer than 25% of the islets in the untreated population showed any

283 evidence of oscillations in tolbutamide. In contrast, the vast majority of the treated islets

284 exhibited oscillations during acute tolbutamide exposure (Fig. 5D). When switched to 11G alone,

285 there was no significant difference in the percent of islets exhibiting oscillations. Thus, the

286 experiments support the model prediction that chronic treatment with KCl could lead to a

287 compensating increase in K(ATP) conductance that rescues oscillations when the treated islet is

288 acutely stimulated by tolbutamide, but this is masked when the greater acute KCl stimulus is

289 applied. Importantly, they also demonstrate that the rescue of Ca^{2+} oscillations during acute

290 tolbutamide application is not due to tolbutamide desensitization.

291

292 *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 compensation*

313   Modeling has provided evidence that compensation to chronic stimulation could be

314   through increased K(ATP) conductance in the β cell membrane. However, changes in the

315   conductance level of other K^+ channel types could also be involved in the compensation process.

316   To determine if voltage-dependent K^+ channels are involved, we employed the non-specific

317   voltage-dependent channel blocker tetraethylammonium (TEA). If the conductance level of a

318   voltage-dependent K^+ channel is increased as a result of chronic tolbutamide treatment, then the

319   effect of blocking this channel should be greater in the treated islet than in the untreated islet.

320   We tested this by 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 compensation to chronic tolbutamide exposure. We tested a blocker of the A-type K^+

329   channel, 4-aminopyridine, but found no statistical difference between tolbutamide-treated and

330   untreated islets (data not shown).

331   Next, we employed an antagonist for inward-rectifying Kir2.1 channels, which have been

332   implicated in compensation in islets with functional K(ATP) channels genetically knocked out

333   (45). This channel blocker, ML133, was applied to both untreated and treated islets, as in the

334   TEA study. In both sets of islets, there was an increase in the Ca^{2+} oscillation amplitude when

335   ML133 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 compensation to chronic tolbutamide exposure.

339

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 (32, 40). There is evidence that this pulsatility

345 serves a physiological role (12, 24, 25, 34, 40), and is often lost in disease or during aging (21,

346 33, 35). It is perhaps more surprising that islet β cells are so good at compensating for

347 manipulations that interfere with the oscillatory activity that normally occurs at stimulatory

348 glucose levels.

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

350   The ion channels responsible for glucose sensing, K(ATP) channels, are composed of

351 four sulfonylurea-sensitive (SUR1) subunits and four pore-forming Kir6.2 subunits, and

352 functional channels require both sets of subunits (30). In SUR1 homozygous knockout mice,

353 islets typically exhibit bursting activity (11, 41) which result in Ca^{2+} oscillations (45). This is in

354 spite of the depolarizing effect that removal of these channels should have, which would lead to

355 tonic spiking in the absence of compensation, as in the case of acute sulfonylurea application

356 (16, 22). Similarly, Ca^{2+} oscillations have been reported from islets of Kir6.2 knockout mice

357 (37).

358   Our study was performed to determine whether oscillations could be restored following

359   chronic exposure to the stimulator tolbutamide, a drug used in the management of type 2 diabetes

360   (23). A previous study from Annello et al. showed that while overnight treatment with

361   tolbutamide reduced the insulin content of mouse islets, it had a mild attenuation of the mean

362   Ca^{2+} response to a glucose step from 3 mM to 15 mM, which did not reach significance (2). We

363   observed a similar attenuation of the Ca^{2+} response to glucose stimulation in the 12-20 mM range

364   following chronic tolbutamide exposure. Any Ca^{2+} oscillations in the Annello study (if they

365   occurred), were obscured by averaging over several islets. We found that oscillations during

366   acute tolbutamide exposure were often recovered following 12 h or more of chronic tolbutamide

367   exposure (Fig. 2). The extent of recovery was greater with longer exposure, saturating at 20 h

368   exposure, where ~80% of the treated islets exhibited Ca^{2+} oscillations. When the acute

369   tolbutamide challenge was removed, oscillations persisted (in 11G). Thus, the mechanism that

370   rescues oscillations in tolbutamide-stimulated islets also allows for oscillations in 11G alone.

371

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

373   One possibility is that the chronically treated islets become desensitized to tolbutamide.

374   To check this, we used KCl instead of tolbutamide to chronically stimulate islets, and only then

375   applied tolbutamide acutely. Once again, we observed a preponderance of oscillatory activity in

376   the treated islets during acute tolbutamide exposure, in contrast to the control islets incubated in

377   11G alone (Fig. 5). This supports the conclusion that it is the chronic stimulation, and not

378   tolbutamide desensitization, that accounts for the rescue of oscillations during acute tolbutamide

379   exposure. This conclusion is supported by a prior study that found that while chronic tolbutamide

380   exposure greatly reduced insulin secretion during a later acute tolbutamide challenge, the

381   membrane potential of single β cells was still responsive to the acute tolbutamide, arguing that

382   any desensitization occurs downstream of the cell's electrical activity (39).

383

384   *Multiple mechanisms to maintain pulsatility*

385   Our mathematical modeling suggests that the compensation mechanism could be through

386   increased K(ATP) channel conductance (Figs. 3, 4). However, this increase would be quite

387   small, since if the increase is too large, then the cell would fail to exhibit electrical activity once

388   the tolbutamide is removed and only glucose remains. A prior study confirmed that the

389   expression level of K(ATP) channel subunits is not adversely affected by chronic tolbutamide

390   treatment in clonal insulin-secreting BRIN-BD11 cells (5), though the resolution of such

391   measurements is not fine enough to pick up the small change in expression levels that would be

392   predicted if in fact the compensation involved increased K(ATP) channel subunit expression.

393   Rather than increased channel expression, it is also possible that compensation could be due to

394   increased K(ATP) channel trafficking to the membrane.

395   There are other potential mechanisms of compensation to restore pulsatility under chronic

396   stimulation. Genetic manipulation of K(ATP)-channels to eliminate K(ATP)-channel

397   conductance precludes the possibility of increased channel expression or increased numbers of

398   these channels inserted in to the plasma membrane; nevertheless, islets are oscillatory in these

399   models (11, 37, 41). In SUR1 knockout mice, it has been demonstrated that there is increased

400   inward rectifying K^+ current, and mathematical modeling was used to show that an inward

401   rectifying Kir2.1 current would be capable of recovering bursting oscillations (45). The

402   upregulation of such a current could be achieved through Ca^{2+}-sensitive gene transcription (42-

403   44). Consistent with this view, our findings with the Kir2.1-specific inhibitor ML133 suggest

404   that Kir2.1 activity is also increased following ~24 h of chronic stimulation. An additional, as of

405   yet unidentified, TEA-sensitive K^+ channel may also play a role. It is also possible that

406   compensation for chronic stimulation can be achieved by a mechanism other than gene

407   transcription. It was shown that islets exposed overnight to stimulatory glucose levels have

408   decreased K(ATP) conductance relative to those maintained in low glucose overnight, and that

409   this is due to decreased trafficking of K(ATP) channels to the plasma membrane rather than

410   decreased gene transcription (14).

411

412   *Possible feedback effects of compensation on metabolism*

413   A result of the compensation is a change in the glucose dose-response curve, such that

414   islets treated overnight with tolbutamide have a smaller response to glucose, as determined by

415   the mean Ca^{2+} level, at glucose levels >8 mM (Fig. 6). This reduced response is protective

416   against overstimulation, and is the opposite of what was seen when islets were treated overnight

417   with 11 mM glucose, versus 2.8 mM glucose (14). With tolbutamide exposure, the direct

418   influence is on the cell's electrical activity and Ca^{2+} dynamics, and the elevated Ca^{2+} level

419   associated with tolbutamide exposure likely leads to a reduction in the ATP/ADP ratio due to

420   ATP hydrolysis by Ca^{2+} pumps in the plasma and endoplasmic reticulum membranes (10).

421   Indeed, data show an increase in ATP/ADP when the islet is hyperpolarized and Ca^{2+} level

422   reduced with application of the K(ATP) channel activator diazoxide (29). In contrast, incubating

423   an islet in a solution with higher glucose content will lead to an increase in the ATP/ADP ratio

424   due to increased metabolism. It might be expected that chronic treatment with tolbutamide has

425 the opposite effect on the glucose dose-response curve than chronic treatment with a higher

426 glucose level.

427

428 *Conclusions*

429   We show that chronic stimulation of pancreatic islets triggers a compensatory

430 hyperpolarizing mechanism to restore normal endogenous oscillatory activity in the presence of

431 overstimulation. This study suggests that maintaining the ability to secrete insulin in a pulsatile

432 manner appears to be very important to overall function as evidenced by the drive to maintain

433 this function despite strong changes in physiological input. We identified several compensatory

434 changes, involving K(ATP) channels and additional K^+ channels, and a decrease in glucose

435 sensitivity. The net result is rescue of pulsatility, and its regulation by glucose. Further

436 identification and characterization of these mechanisms may aid in developing appropriate

437 therapeutic interventions for congenital metabolic diseases related to K(ATP) channels, such as

438 neonatal diabetes and congenital hyperinsulinism.

439

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558

559   **Figure Legend.**

560

561   **Figure 1:** Acute tolbutamide application terminates Ca^{2+} oscillations, leading to a sustained

562   elevated level. (A) Example of fura-2 traces of the intracellular Ca^{2+} level from n=12 islets

563   starting in 11 mM glucose (11G) for 20 min and then exposed to 250 uM tolbutamide for 30 min

564   (plus 11G), followed by a washout back to 11G for 40 min. (B) Mean Ca^{2+} values (340/380 nm

565   ratio) for 11G (control, first 20 min), peak value measured during tolbutamide treatment (peak

566   tolb), the mean Ca^{2+} level during the last 20-min of tolbutamide exposure (tolb), and the last 20

567   min of washout (wash). ***=$p<0.001$ and N.S (not significant) are compared to control (11G).

568

569   **Figure 2:** Islets show a duration-dependent response to tolbutamide that restores oscillations. (A-

570   B) Multiple traces are shown for islets incubated in 250 μM tolbutamide and 11G for 4 h (A,

571   n=6) or 20 hours (B, n=8). These islets were maintained in 11G plus tolbutamide for the first 20

572   minutes followed by a washout into 11G. (C) The fraction of oscillating islets for multiple

573   tolbutamide treatment durations of 4, 8, 12, 16, and 20 h ($\pm$ one hour). Untreated islets were

574   maintained in 11G media for the same durations and were recorded simultaneously with the

575   tolbutamide-treated islets. A two-tailed Chi square test was used for statistics: *=$p<0.05$,

576   ***=$p<0.001$.

577

578   **Figure 3:** Mathematical modeling demonstrates the feasibility of increased K(ATP) conductance

579   as a compensation mechanism. (A) Acute treatment with tolbutamide converts a bursting islet,

580   with accompanying Ca^{2+} oscillations, into tonic spiking, in which the Ca^{2+} concentration is

581   pinned to an elevated level. (B) In a model "treated islet" (an islet treated chronically with

582   tolbutamide), Ca^{2+} oscillations persist during an acute tolbutamide application.

583

584   **Figure 4:** Model simulation predicting how an islet exhibiting compensation through an increase

585   in K(ATP) conductance at the same level as that of Fig. 3 would respond to KCl, tolbutamide,

586   and 11G alone. (A) In an untreated islet, the Ca^{2+} level is at an elevated plateau (reflecting tonic

587   spiking) when stimulated by either KCl or tolbutamide. (B) In a treated islet, the Ca^{2+} level

588   plateaus during stimulation with KCl, but oscillates during acute tolbutamide stimulation.

589

590   **Figure 5:** Experimental data support model predictions. In all cases, 11G is present. (A)

591   Representative Ca^{2+} trace for an untreated islet, exhibiting elevated Ca^{2+} plateaus when

592   stimulated by either KCl or tolbutamide, but oscillating in 11G alone. (B) Representative Ca^{2+}

593   trace for an islet with 24-hour treatment with 30 mM KCl. As predicted, the islet exhibits an

594   elevated Ca^{2+} plateau when stimulated with KCl but oscillates when stimulated with tolbutamide

595   or with 11G alone. (C) Quantification of mean Ca^{2+} levels for untreated and KCl-treated islets

596   for each acute treatment phase. (D) Percent of islets displaying oscillations during each treatment

597   phase. A two-tailed t-test was used for statistic in (C) and chi square in (D): ***=p<0.05,**

598   *****=p<0.001,** N.S. = not significant.

599

600   **Figure 6:** Islets incubated for 24 hours in 250 μM tolbutamide plus 11G display a glucose-

601   dependent reduction in Ca^{2+} levels versus those incubated in 11G alone. (A) Averaged Ca^{2+}

602   traces during three consecutive glucose exposures of 0, 8, and 16 mM for N=13 untreated (blue)

603   and N=9 treated islets (orange). Averaged Ca^{2+} traces during three consecutive glucose

604   exposures of 4, 12, and 20 mM for N=12 untreated (blue) and N=9 treated islets (orange). Data

605   in (A-B) represent one of four trials. (C) Glucose dose-response curves computed using mean

606   Ca^{2+} levels over a range of glucose values for all four trials. Data were combined and two-tailed

607   t-tests were used at each glucose concentration: *=$p<0.05$, **=$p<0.01$. (D) EC50s for four trials

608   show a right shift in EC50 among tolbutamide-treated islets versus those incubated in 11G alone.

609   (E) Mean of the EC50s from the four individual trials *=$p<0.05$ determined by paired t-test.

610

611   **Figure 7:** Voltage-dependent K^+ channels and Kir2.1 channels participate in compensation to

612   chronic (~24 h) tolbutamide treatment. (A) In an untreated islet, the voltage-dependent K^+

613   channel blocker TEA does not affect the amplitude of Ca^{2+} oscillations. (B) In a treated islet,

614   TEA increases the oscillation amplitude. (C) Mean Ca^{2+} response to TEA between the untreated

615   and treated islets (n=24 for control, n=21 for tolbutamide treated). (D-E) In both untreated and

616   treated islets, the Kir2.1 channel blocker ML133 increases the amplitude of Ca^{2+} oscillations. (F)

617   The mean Ca^{2+} response to ML133 is larger in treated islets compared to untreated islets (n=18

618   for control and tolbutamide treated). A two-tailed t-test was used for statistics: *=$p<0.05$.

619   ***=$p<0.001$.

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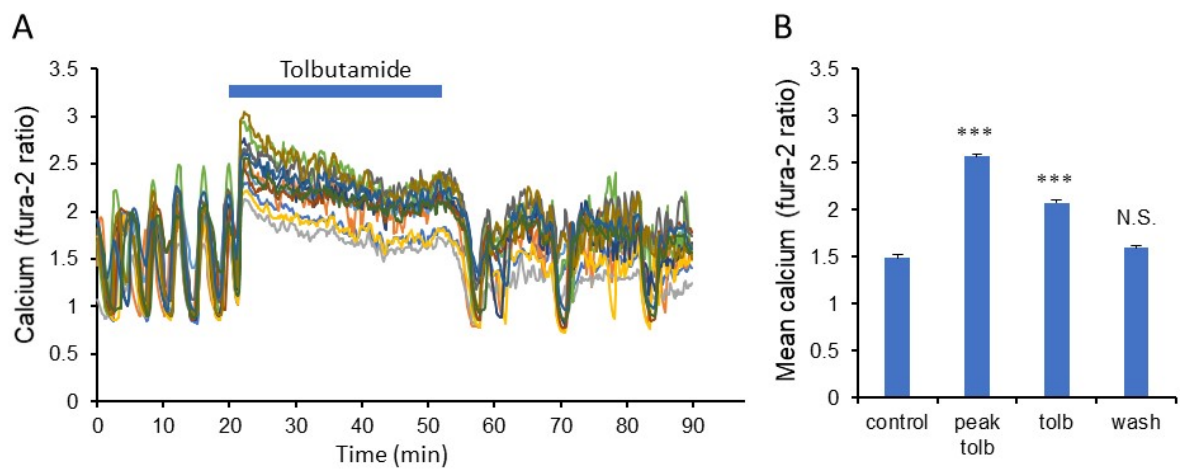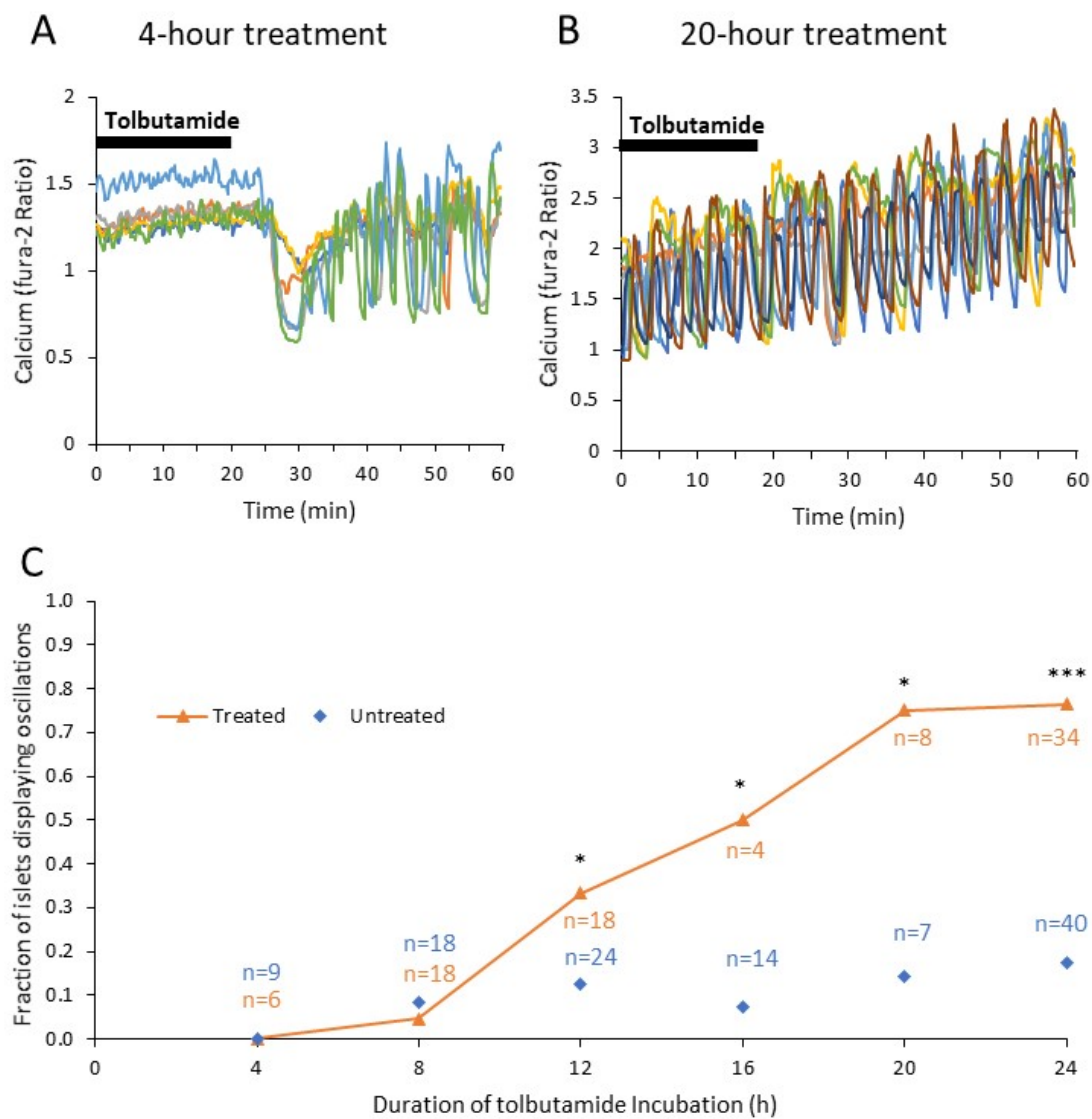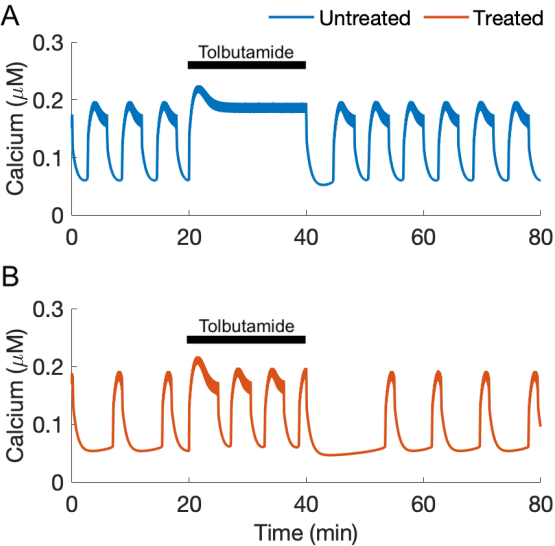
621   **Figure 1.**

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624   **Figure 2.**

627   **Figure 3.**
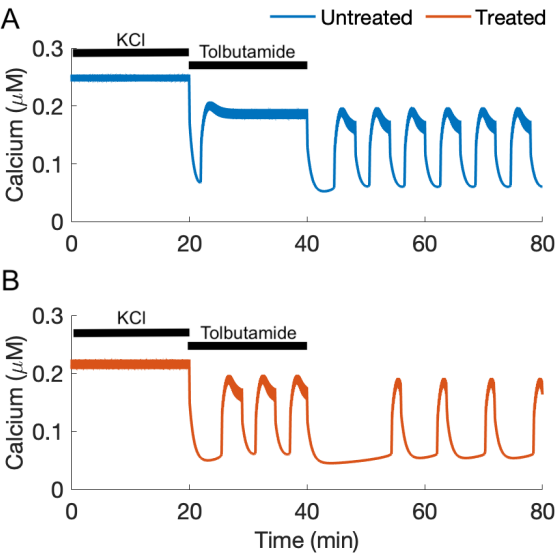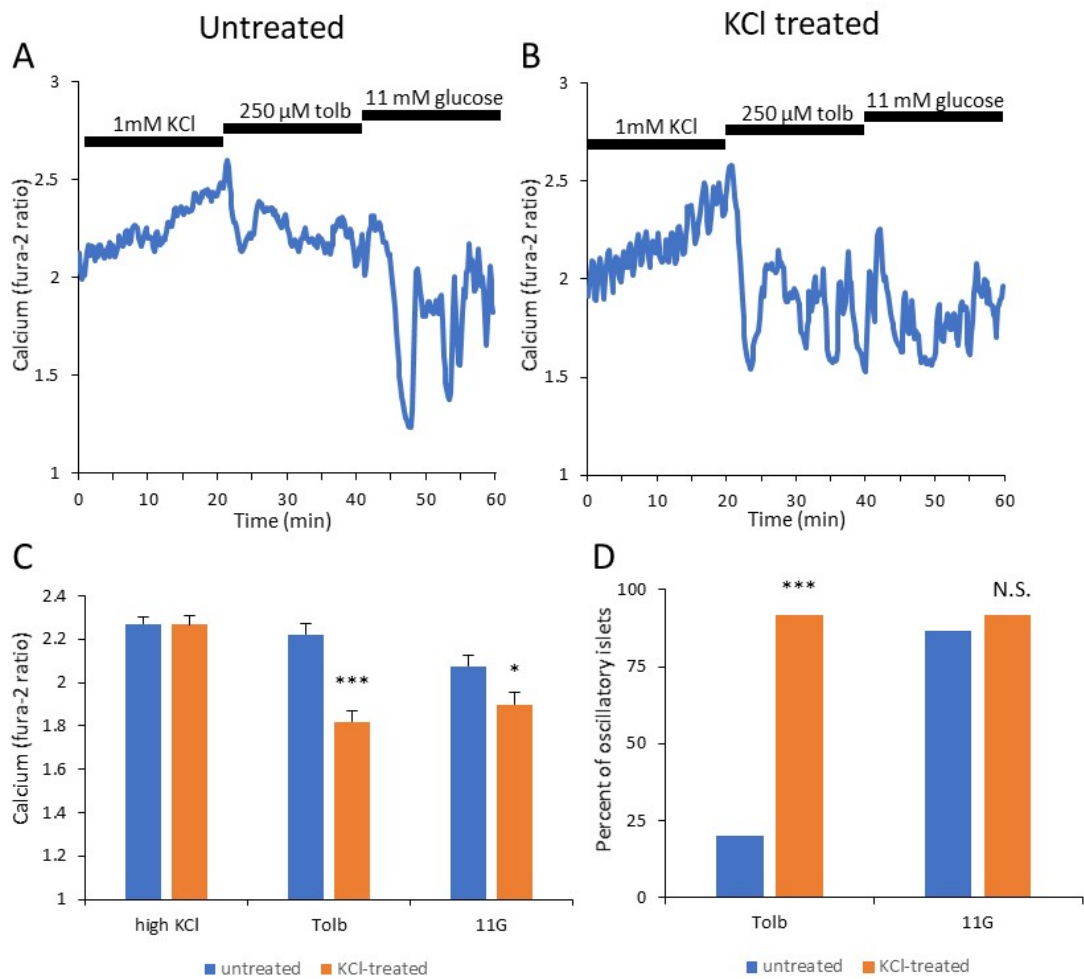
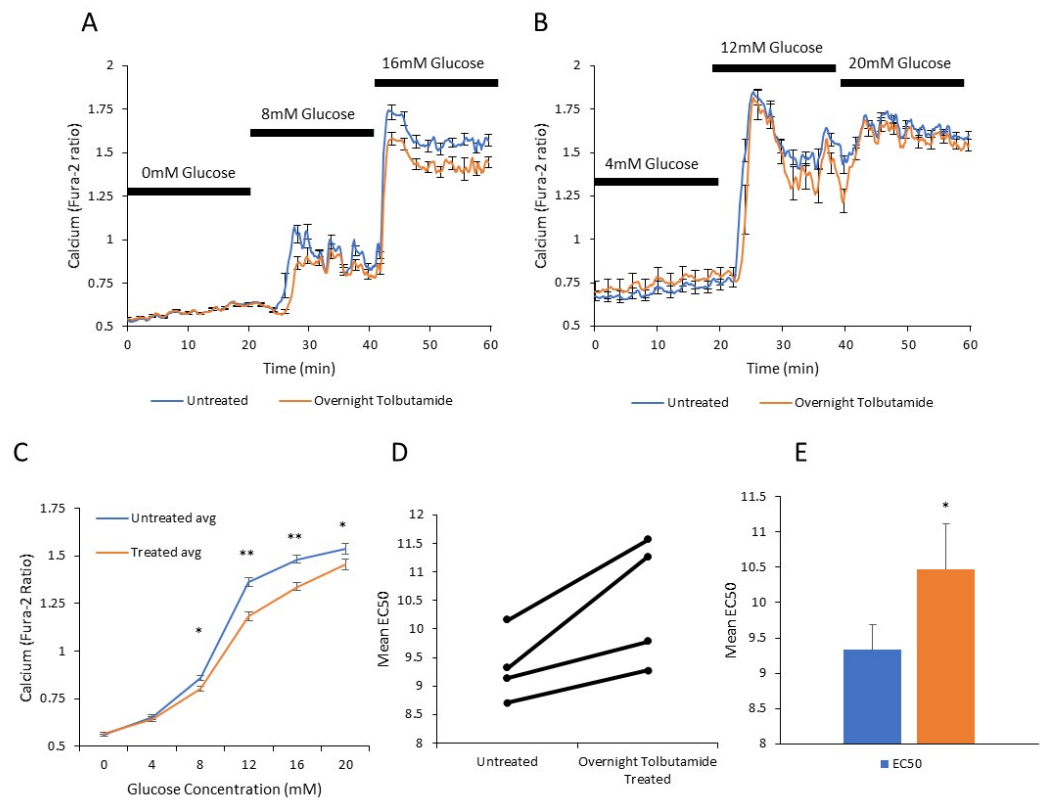

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630 **Figure 4.**

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636 **Figure 5.**

639   **Figure 6.**

641   **Figure 7.**

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