1 Regulation of cAMP accumulation and activity by distinct phosphodiesterase subtypes in INS-1
2 cells and human pancreatic β-cells

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21   **Abstract**

22   Pancreatic β-cells express multiple phosphodiesterase (PDE) subtypes, but the specific roles for

23   each in β-cell function, particularly in humans, is not clear. We evaluated the cellular role of

24   PDE1, PDE3, and PDE4 activity in the rat insulinoma cell line INS-1 and in primary human β-

25   cells using subtype-selective PDE inhibitors. Using a genetically encoded, FRET-based cAMP

26   sensor, we found that the PDE1 inhibitor 8MM-IBMX and the PDE4 inhibitor rolipram elevated

27   cAMP levels above baseline in the absence and presence of 18 mM glucose in INS-1 cells.

28   Inhibition of PDE1 or PDE4 potentiated glucose-stimulated insulin secretion in INS-1 cells. In

29   contrast, the inhibition of PDE3 with cilostamide had little effect on cAMP levels or glucose-

30   stimulated insulin secretion. PDE1 inhibition, but not PDE3 of PDE4 inhibition, reduced

31   palmitate-induced caspase-3/7 activation, and enhanced CREB phosphorylation in INS-1 cells.

32   In human β-cells, only PDE3 or PDE4 inhibition increased cAMP levels in 1.7 mM glucose, but

33   PDE1, PDE3, or PDE4 inhibition potentiated cAMP levels in 16.7 mM glucose. Inhibition of

34   PDE1 or PDE4 increased cAMP levels to a greater extent in 16.7 mM glucose than in 1.7 mM

35   glucose in human β-cells. In contrast, elevation of cAMP levels by PDE3 inhibition was not

36   different at these glucose concentrations. PDE1 inhibition also potentiated insulin secretion

37   from human islets, suggesting that the role of PDE1 may be conserved between INS-1 cells and

38   human pancreatic β-cells. Our results suggest that inhibition of PDE1 may be a useful strategy

39   to potentiate glucose-stimulated insulin secretion, and to protect β-cells from the toxic effects of

40   excess fatty acids.

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43   **Introduction**

44   Pancreatic β-cells secrete the blood glucose-lowering hormone insulin to maintain glucose

45   homeostasis in the body (1). Pancreatic β-cell dysfunction and cell death underlies the

46   development of type 2 diabetes (2). At the cellular level, glucose-stimulated insulin secretion

47   (GSIS) is driven by Ca^{2+} influx through the L-type voltage-gated Ca^{2+} channels (L-VGCC) $Ca_v1.2$

48   and $Ca_v1.3$ (3), and release of Ca^{2+} from the endoplasmic reticulum (ER) (4). GSIS is further

49   regulated by the second messenger 3',5'-cyclic adenosine monophosphate (cAMP), which is

50   generated by the enzyme adenylyl cyclase (AC) (5). In addition to enhancing GSIS, cAMP

51   promotes pancreatic β-cell mass through increased replication (6) and decreased apoptosis (7).

52   Both glucose (8-10) and incretin hormones (11), such as glucagon-like peptide-1 (GLP-1), are

53   capable of stimulating cAMP production and subsequent activation of the cAMP effector

54   proteins Protein Kinase A (PKA) and Exchange Protein Directly Activated by cAMP (Epac) (12).

55   PKA and Epac regulate insulin secretion through proximal effects on the machinery involved in

56   exocytosis at the plasma membrane (13-15) and distal effects on ER Ca^{2+} release channels (16,

57   17). cAMP signaling is compartmentalized to microdomains within the cell, including near sites

58   of ER Ca^{2+} release, by phosphodiesterase enzymes (PDE), which degrade cAMP to 5'-AMP.

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60   PDE1, PDE3, PDE4, and PDE8 are widely-regarded as the primary PDE subtypes

61   responsible for regulating cytosolic cAMP levels and GSIS in rodent β-cell lines, and rodent and

62   human islets (18). PDE1 is the only subtype that is regulated by Ca^{2+}/Calmodulin (19, 20) and is

63   predicted to serve a critical role in pancreatic β-cells where Ca^{2+} dynamics and signaling are

64   prominent (21). Indeed, the subtype-selective PDE1 inhibitor 8-methoxymethyl-3-isobutyl-1-

65   methylxanthine (8MM-IBMX) elevated resting and glucose-stimulated cAMP levels and

66   enhanced GSIS in βTC3 cells (21), MIN6 cells, primary mouse β-cells (22) and intact islets from

67   rat and human (23). Yet, PDE3 appears to be the primary PDE in mouse, rat, and human islets,

68   and the pancreatic β-cell lines INS-1 and MIN6. PDE3 overexpression led to reduced glucose-

69   stimulated cAMP production, impaired GSIS (24, 25) and glucose intolerance (26), whereas

70   PDE3 inhibition (22, 23, 27) or PDE3 knockdown (28, 29) elevated resting and glucose-

71   stimulated cAMP levels and GSIS (30). It has also been suggested that leptin and insulin-like

72   growth factor 1 attenuate insulin secretion through PDE3B activation (31, 32). Subtype-selective

73   inhibitors and knockdown of PDE4 or PDE8 also elevated resting cAMP levels in INS-1 cells

74   (28, 30), βTC3 cells (21), and intact rat and human islets (23, 27), suggesting that PDE4 and

75   PDE8 may too be required. PDE8 knockdown enhanced GSIS in INS-1 cells (28, 30) and MIN6

76   cells (22), which directly correlates with PDE8-mediated regulation of resting cAMP levels.

77   PDE4 inhibition, on the other hand, enhanced GSIS in clonal pancreatic β-cell lines (28, 30) but

78   not in primary islets (23, 27), revealing a disconnect between clonal and primary β-cells.

79   Furthermore, PDE4 activity was elevated in MIN6 cells and primary mouse β-cells following

80   stimulation with glucose, as compared with resting conditions (22). The paucity of information

81   regarding PDE activity in human β-cells and the disparate results in various rodent models have

82   hampered the development of selective PDE inhibition as a strategy for treatment of type 2

83   diabetes.

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85   Using subtype-selective inhibitors of PDE1, PDE3, PDE4, and PDE8, we performed a

86   side-by-side comparison of the role of each subtype in INS-1 cells and for the first time, in single

87   human pancreatic β-cells. We used a state-of-the-art fluorescent sensor of cAMP to evaluate

88   PDE-mediated regulation of cAMP under resting conditions and during glucose stimulation. The

89   latter allowed us to directly compare our findings with the ability of each PDE inhibitor to

90   potentiate GSIS. Furthermore, we revealed a novel Ca^{2+}-dependent mechanism by which PDE

91   inhibition can potentiate GSIS in INS-1 cells. Finally, we show that PDE inhibition not only

92   enhanced GSIS but also promoted INS-1 cell survival in the face of stress.

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95   **Materials and Methods**

96   *Chemicals and Reagents*- D-glucose was purchased from Mallinckrodt Chemicals (Dublin,

97   Ireland). IBMX (3-Isobutyl-8-(methoxymethyl)-1-methyl-1H-purine-2,6(3H,7H)-dione) was

98   purchased from Sigma-Aldrich (St. Louis, MO). 8MM-IBMX (3-Isobutyl-8-(methoxymethyl)-1-

99   methyl-1H-purine-2,6(3H,7H)-dione) was purchased from Santa Cruz Biotechnology (Dallas,

100 TX). Cilostamide (N-cyclohexyl-N-methyl-4-(1,2- dihydro-2-oxo-6-quinolyloxy) butyramide),

101 rolipram (4-[3-(cyclopentyloxy)-4-methoxyphenyl]-2-pyrrolidinone), PF-04671536 (5-Methyl-3-

102 [[(2R)-4 (thiazolylmethyl)-2-morpholino]methyl]-3H-1,2,3-trazolol[4,5]pyrimidin-7-amine), and

103 forskolin were purchased from Tocris Bioscience (Bristol, UK). PF-04957325 (3-[[(2R)-4-(1,3-

104 thiazol-2-ylmethyl)morpholin-2-yl]methyl]-5-(trifluoromethyl)triazolo[4,5-d]pyrimidin-7-amine) was

105 purchased from MedChemExpress (Monmouth Junction, NJ). Unless otherwise indicated, all

106 other reagents were purchased from Sigma-Aldrich.

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108 *INS-1 Cell Culture*- INS-1 cells were cultured in complete RPMI-1640 media containing 11 mM

109 glucose, 10 mM HEPES, 10% fetal bovine serum (FBS), 11 mg/mL sodium pyruvate, 10,000

110 units/mL penicillin, 10,000 μg/mL streptomycin, and 50 μM β-mercaptoethanol at 37°C and 5%

111 CO_2 (33). In some experiments, INS-1 cells were serum-deprived overnight using minimal

112 RPMI-1640 media containing 2.5 mM glucose, 10 mM HEPES, 11 mg/mL sodium pyruvate,

113 10,000 units/mL penicillin, 10,000 μg/mL streptomycin and 50 μM β-mercaptoethanol

114 supplemented with 0.1% fatty acid-free bovine serum albumin (BSA).

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116 *Intracellular Ca^{2+} Assay*- INS-1 cells were plated in 96-well black-walled plates (Corning Life

117 Sciences, Corning, NY) and incubated for at least 24h at 37°C and 5% CO_2. Cells were washed

118 once with 200 μL Phosphate Buffered Saline (PBS) and incubated in 100 μL Krebs-Ringer

119 HEPES Buffer (KRBH) containing 5 μM Fura2-AM (Molecular Probes, Eugene, OR) for 1h at

120 room temperature. KRBH contained 134 mM NaCl, 3.5 mM KCl, 1.2 mM KH_2PO_4, 0.5 mM

121      $MgSO_4$, 1.5 mM $CaCl_2$, 5 mM $NaHCO_3$ and 10 mM HEPES and was supplemented with 0.05%

122   fatty-acid free BSA (pH 7.4). Following this 1h incubation, KRBH containing Fura2-AM was

123   removed, and the cells were washed once with 200 µL KRBH. Cells were pretreated for 30 min

124   with 100 µL KRBH containing the working concentration of inhibitors or KRBH alone.

125   Intracellular Ca^{2+} assays were performed using a Synergy 4 Multi-Mode Plate Reader (BioTek

126   Instruments, Winooski, VT). Fura2 fluorescence was measured using a 508/20 nm band-pass

127   emission filter and 340/11 nm or 380/20 nm band-pass excitation filters. For acute application

128   of caffeine or PDE inhibitors, a 15 sec baseline was recorded prior to injection of 100 µL of a 2X

129   concentration of stimulant, and fluorescence was recorded for an additional 2 min.

130   Fluorescence was collected from both channels every 0.7 sec. For long-term stimulation with

131   PDE Inhibitors in combination with glucose or glucose + forskolin, a 5 min baseline was

132   recorded prior to injection of 100 µL of a 2X concentration of stimulant, and fluorescence was

133   recorded for an additional 1h. Fluorescence was collected from both channels every 0.7 sec.

134   To assess changes in cytosolic Ca^{2+}, the fluorescent signal collected using the 340 nm

135   excitation filter was divided by the fluorescent signal collected using the 380 nm excitation filter

136   (340/380 nm) for each well over the entire time-course. The average ratio during the first min

137   was subtracted from the entire time-lapse to yield a baseline of zero. Each treatment was

138   performed in quadruplicate, and the average 340/380 nm ± SE was determined for the time-

139   course. At least three independent experiments were performed for each treatment, and the

140   average AUC ± SE was determined.

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142   *Insulin Secretion-* INS-1 cells were plated in 24-well plates (Corning Life Sciences, Corning,

143   NY) and incubated for at least 24h at 37°C and 5% CO_2. Cells were washed once with PBS and

144   incubated with 500 µL KRBH alone or containing the working concentration of inhibitors for 30

145   min at 37°C and 5% CO_2. Following pretreatment, 500 µL KRBH containing a 2X concentration

146   of glucose or glucose + PDE inhibitors along with the working concentration of the same

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147   inhibitors used in the pretreatment was added to each well and incubated for an additional 1h at

148   37°C and 5% CO_2. Following this incubation, the supernatant (1 mL) was transferred from each

149   well to 1.5 mL Eppendorf tubes and stored at 20°C until assayed. Secreted insulin was

150   measured using the High-Range Insulin ELISA kit (ALPCO, Salem, NH), according to the

151   manufacturer's instructions. After supernatant removal, cells from each well were immediately

152   lysed in 200 µL Cell lysis buffer (20 mM Na_2HPO_4 + 150 mM NaCl + 0.1% Triton X-100, pH 7.4)

153   + freshly added protease inhibitors (800 nM aprotinin, 50 µM leupeptin, 1 µg/mL pepstatin, 1

154   mM benzamidine, 1 mM 4-(2-Aminoethyl) benzenesulfonylfluoride,10 µg/mL calpain inhibitor I

155   and 10 µg/mL calpain inhibitor II). Cell lysis buffer was transferred from each well to 1.5 mL

156   tubes, placed on ice for 20 min and centrifuged at 14,000 x g for 10 min. The supernatant was

157   transferred to fresh 1.5 mL tubes and stored at -20°C until assayed. Cellular protein was

158   measured using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA),

159   according to the manufacturer's instructions. Each sample was assayed in duplicate. Secreted

160   insulin was normalized to protein content, both of which were measured using the PowerWave

161   Plate Reader (BioTek Instruments, Winooski, VT). A calibration curve was generated using

162   standards provided in each kit, and the amount of secreted insulin and protein content were

163   determined. The amount of insulin secreted (ng) was divided by protein content (mg) to yield an

164   insulin/protein ratio for each well. Each treatment was performed in triplicate, and the average

165   insulin/protein ratio was calculated among like wells. The average insulin/protein ratio of each

166   treatment was normalized to the untreated condition to yield percent basal. This value was

167   again normalized to the glucose treatment to yield percent glucose. At least three independent

168   experiments were performed for each treatment, and the average percent glucose ± SE was

169   determined.

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171   *CREB Phosphorylation Assay-* INS-1 cells were plated in 96-well black-walled plates and

172   incubated for at least 24h at 37°C and 5% CO_2. Cells were incubated for an additional 24h in

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173   minimal RPMI media. Cells were washed twice with PBS and incubated for 2h in KRBH at 37°C

174   and 5% CO_2. The preincubation buffer was removed and replaced with KRBH alone or

175   containing the working concentration of inhibitors for 30 min at 37°C and 5% CO_2. Cells were

176   treated for 10 min with KRBH alone or KRBH containing the working concentration of PDE

177   inhibitors. Following this incubation, cells were fixed with 4% formaldehyde and stored at 4°C

178   until assayed. Total CREB and pCREB were measured using the Human/Mouse/Rat Phospho-

179   CREB (S133) Cell-based ELISA (R&D Systems, Minneapolis, MN), according to the

180   manufacturer's instructions. Using a Synergy 4 Multi-Mode Plate Reader (BioTek Instruments,

181   Winooski, VT), total CREB was measured at 450 nm with excitation at 360 nm, and pCREB was

182   measured at 600 nm with excitation at 540 nm. The fluorescent signal of pCREB was divided

183   by the fluorescent signal of total CREB to yield a pCREB/total CREB ratio for each well. Each

184   treatment was performed in duplicate, and the average pCREB/total CREB was calculated

185   between these two wells. The average pCREB/total CREB among background wells (no

186   primary antibody) was subtracted from the values obtained from other wells. The average

187   pCREB/total CREB ratio of each treatment was normalized to the untreated condition to yield

188   percent basal. In some cases, the basal value was subtracted and the resulting values were

189   normalized to the glucose treatment to yield percent glucose. At least three independent

190   experiments were performed for each treatment, and the average percent basal or percent

191   glucose ± SE was calculated.

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193   *Caspase-3/7 Glo Assay-* INS-1 cells were plated in 96-well white-walled plates (Corning Life

194   Sciences, Corning, NY) and incubated for at least 24h at 37°C and 5% CO_2. INS-1 cells were

195   treated with a 3:1 molar ratio of palmitate to BSA, with and without the working concentration of

196   PDE inhibitors, or BSA alone (untreated control). To prepare the stock solution of palmitate,

197   sodium palmitate (100 mM) (Santa Cruz Biotechnology, Dallas, TX) was dissolved in 50%

198   ethanol by incubating at 55°C for 10-15 min with frequent vortexing. As an untreated control,

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199   50% ethanol that did not contain palmitate was also prepared. A 1:200 dilution (50 µL) of

200   palmitate (500 µM final) or control solution (0.25% ethanol final) was added to 10 mL minimal

201   RPMI media containing 1% fatty acid-free BSA. Tubes containing palmitate + BSA or BSA

202   alone were incubated in a 37˚C water bath for 30 min to promote conjugation. INS-1 cells were

203   treated for 12h at 37ºC and 5% CO_2. Following incubation, treatments were removed and

204   replaced with 50 µL of fresh media. 50 µL of Caspase-3/7 Glo Reagent (ProMega, Madison, WI)

205   was added to each well to yield a 100 µL total volume. As a background control, 50 µL RPMI-

206   1640 + 50 µL Caspase-3/7 Glo Reagent were added to several wells that did not contain INS-1

207   cells. The plate was shaken at 500 rpm for 30 sec and incubated in the dark at room

208   temperature for 2h. Luminescence was recorded using a Synergy 4 Multi-Mode Plate Reader

209   (BioTek Instruments, Winooski, VT). A 1s integration time was used in all experiments;

210   however, the sensitivity was changed to achieve an average luminescence recording of 500 in

211   the background wells. Each treatment was performed in triplicate, and the average

212   luminescence signal was calculated among these wells. The average luminescent signal of

213   each treatment was normalized to the untreated condition to yield percent basal. This value

214   was again normalized to palmitate to yield percent palmitate. At least three independent

215   experiments were performed for each treatment, and the average percent palmitate ± SE was

216   determined.

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218   *Human pancreatic islets*- Human islets were acquired from the Integrated Islet Distribution

219   Program (City of Hope National Medical Center, Duarte, CA). The average purity and viability of

220   the human islet preparations used in these studies was 92% and 94%, respectively. All the

221   donors were classified as nondiabetics, and the cause of death was reported as stroke (4

222   donors), head trauma (4 donors), anoxia (2 donors), or CNS tumor (1 donor). The average age

223   was 40 years old (range 28-62), the average weight was 225.3 pounds (range 170-296) and the

224   average body mass index was 33.0 (range 23.7-49.9). Upon receipt, human islets were

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225   centrifuged at 200 x g for 5 min and re-suspended in RPMI-1640 media containing 11 mM

226   glucose, 10 mM HEPES, 10% FBS, 10,000 units/mL penicillin and 10,000 µg/mL streptomycin

227   (human islet media). Islets were plated in 24-well plates (30-40 islets/well) for FRET-based

228   imaging experiments or ultra-low attachment 60 mm culture dishes (Corning Life Sciences,

229   Corning, NY) for single islet insulin secretion assay. Islets were incubated for at least 24h at

230    $37^{\circ}C$ and 5% CO_2 for recovery.

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232   *Dissociation and transfection of human pancreatic islets-* Human islets were dissociated from

233   one well of a 24-well plate (30-40 islets). Islets were transferred to a 1.5 mL Eppendorf tube

234   and centrifuged at 200 x g for 5 min. The media was carefully removed and replaced with 1 mL

235   warm Versene (Thermo Fisher Scientific, Waltham, MA). The islets were re-suspended by

236   pipetting up and down 5X, and centrifuged at 200 x g for 5 min. The Versene was carefully

237   removed and replaced with 1 mL warm Accutase (Thermo Fisher Scientific, Waltham, MA). The

238   islets were re-suspended by pipetting up and down 5X and placed on a rocker at room

239   temperature for 5 min for constant agitation. Following agitation, the islets were briefly placed in

240   a 37°C water bath and re-suspended by pipetting up and down 5X. The 1.5 mL tube was

241   placed on the rocker at room temperature for an additional 5 min. Following agitation, the islets

242   were briefly warmed in a 37°C water bath and re-suspended for a final time by pipetting up and

243   down 5X. The dissociated islets were pelleted at 200 x g for 5 min, and the Accutase solution

244   was carefully removed and replaced with 100 µL human islet media. The islets were

245   resuspended and transferred to the center of a 40 mm coverslip coated with poly-D-lysine

246   placed in a 60 mm dish. The dissociated islet cells were allowed to settle for 10-15 min prior to

247   transfection. 500 ng Epac-S^{H187} + 1 µL P3000 + 1 µL Lipofectamine 3000 (Life Technologies,

248   Grand Island, NY) was used for transfection. The DNA + P3000 and lipofectamine 3000 were

249   added to separate 1.5 mL tubes, each containing 10 µL OPTI-MEM. The contents of both tubes

250   were combined to yield a total volume of 20 µL, and the DNA and lipofectamine were allowed to

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251   complex at room temperature for 5 min. Following 4-6h incubation at 37°C and 5% CO_2, fresh

252   human islet media was carefully added to the 60 mm dish as not to disturb the dissociated

253   islets. Dissociated cells were imaged at 48h post-transfection.

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255   *Single human islet insulin secretion assay-* Using a dissection microscope, single human islets

256   (50-100 μm in diameter) were transferred from an ultra-low attachment 60 mm dish to 96-well V-

257   bottom clear-walled plate (Corning Life Sciences, Corning, NY) containing 100 μL human islet

258   media and incubated for 48h at 37°C and 5% CO_2. Following this incubation, the cells were

259   washed once with 50 μL warm KRBH + 1.7 mM glucose and incubated with 100 μL warm KRBH

260   + 1.7 mM glucose for 30 min at 37°C and 5% CO_2. The pretreatment was removed and

261   replaced with 100 μL warm KRBH + 1.7 mM glucose (1.7G) or KRBH + 16.7 mM glucose

262   (16.7G) with and without the working concentration of PDE inhibitors, and incubated for an

263   additional 1h at 37°C and 5% CO_2. The supernatants containing secreted insulin were

264   transferred to fresh wells on the same 96-well V-bottom plate. Single human islets were lysed

265   using 100 μL RIPA Buffer (50 mM Tris + 150 mM NaCl + 1% Triton X-100 + 0.5% Sodium

266   Deoxycholate + 0.1% SDS) + freshly added protease inhibitors. The 96-well V-bottom plate

267   containing cell lysate and secreted insulin was wrapped in parafilm and stored at 20˚C until

268   assayed. Secreted insulin and insulin content were diluted 1:10 and 1:100, respectively, to fall

269   in the dynamic range of the kit, and each sample was assayed in duplicate using the Human

270   Insulin Chemiluminescence ELISA (ALPCO, Salem, NH), according to the manufacturer's

271   instructions. Within 5-15 min of adding the chemiluminescent substrate, luminescence was

272   detected using the Synergy 4 Multi-Mode Plate Reader using a 1s integration time and detector

273   sensitivity of 150. A calibration curve was generated using standards provided in the kit, and

274   the amount of secreted insulin and insulin content were determined. The resulting values were

275   corrected for dilution, and the amount of secreted insulin was divided by total insulin (secreted

276   insulin + insulin content) to yield a secreted insulin/total insulin percentage for each well. Each

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277 treatment was performed in triplicate, and the average % secreted/total insulin ± SE was

278 calculated.

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280 *FRET-based cAMP imaging-* To measure cAMP accumulation in the presence of PDE

281 inhibitors, INS-1 cells were transfected with 1 μg Epac-S^{H187} DNA using 2.5 μL Lipofectamine

282 2000 (Life Technologies, Grand Island, NY), according to the manufacturer's instructions.

283 Following 4-6h incubation at 37°C and 5% CO_2, the cells were split into 60 mm dishes

284 containing 40 mm glass coverslips coated with poly-D-lysine and imaged at 48h post-

285 transfection. INS-1 cells and dissociated human islet cells were imaged using a Nikon A1

286 Confocal and Perfect Focus Ti-E Inverted Microscope equipped with an Apo TIRF 60x Oil DIC

287 N2 (NA 1.49) objective lens. 40 mm coverslips were assembled into a RC-31 imaging chamber

288 (Warner instruments, Hamden, CT) and attached to a six-channel perfusion mini-valve system

289 (VC-6M). The donor signal of Epac-S^{H187} was collected using the following confocal

290 parameters: 457 nm laser line of a Multi-Argon laser (457/476/488/514 nm), a 400-457/514 nm

291 primary dichroic mirror, a 520 nm long-pass dichroic mirror and a 485/35 nm band-pass

292 emission filter. The FRET signal of Epac-S^{H187} was collected using the following confocal

293 parameters: 457 nm laser line of a Multi-Argon laser (457/476/488/514 nm), a 400-457/514 nm

294 primary dichroic mirror, a 565 nm long-pass dichroic mirror and a 538/33 nm band-pass

295 emission filter. Confocal images of donor and FRET signal were collected sequentially every 2

296 sec using the following parameters: 512 x 512 total pixels, 4.8 μs pixel dwell, pinhole size of 1.2

297 AU and varied zoom factor, laser power and detector gain. A baseline signal was collected for 1

298 min prior to stimulation. KRBH containing the working concentration of IBMX or subtype-

299 selective PDE inhibitors were perfused over the cells, and once a new baseline was achieved,

300 KRBH was used as a wash. For experiments with INS-1 cells, KRBH in the basal or

301 "unstimulated" condition contained no glucose. For experiments with human β-cells, basal

302 KRBH contained 1.7 mM glucose. Time-lapse recordings of donor and FRET signal were

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303   analyzed using NIS Elements 4.0 software. To assess changes in cAMP accumulation, the

304   ratio of donor to FRET signal of Epac-S^{H187} was determined for each cell over the time-course,

305   to yield several single cell traces. Each trace was normalized to the average ratio during the

306   first min, to yield a baseline of one. At least three independent experiments were performed for

307   each treatment. The percent change from baseline was determined for each treatment and the

308   average ± SE was determined.

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310   *Data Analysis-* Data were analyzed using GraphPad Prism 7. Data are represented as the

311   average ± SE. Statistical analysis was performed using one-way or two-way ANOVA with the

312   Tukey post-hoc test. Alternatively, the student's unpaired t-test was used. $P < 0.05$ was

313   considered significant.

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Results

PDE-mediated regulation of resting and glucose-stimulated cAMP levels in INS-1 cells

The role of PDE1, PDE3, PDE4 and PDE8 in regulating both resting and glucose-stimulated

cAMP levels in pancreatic β-cells is poorly understood. We used the Epac1-based, high-affinity,

high-dynamic range FRET sensor Epac-S^{H187} to evaluate the effect of subtype-selective PDE

inhibitors on cAMP levels in INS-1 cells with real-time resolution (34). We transiently transfected

INS-1 cells with Epac-S^{H187} and imaged cells by confocal microscopy at 48 hours post-

transfection in KRBH without glucose. Application of the pan PDE inhibitor IBMX (100 µM)

resulted in a robust increase in cAMP levels (34.99 ± 1.94%) that recovered to baseline upon

drug removal (Fig 1A). We sequentially applied the subtype-selective inhibitors 8MM-IBMX,

cilostamide, and rolipram to test the role of the IBMX-sensitive PDE1, PDE3, and PDE4,

respectively (Fig 1A). The concentrations of PDE inhibitors used in this study were 5-20 times

the reported IC_{50} values (35-37) and were comparable with those used in a similar study

performed in MIN6 cells (22). Application of 100 µM 8MM-IBMX (17.87 ± 1.42%) and 10 µM

rolipram (10.6 ± 2.14%) significantly elevated cAMP levels above baseline, whereas 1 µM

cilostamide (5.04 ± 0.5%) had little effect (Fig 1B). Notably, 8MM-IBMX had a significantly

greater effect on resting cAMP levels than either cilostamide and rolipram. Furthermore, the

rolipram-induced increase in cAMP levels was significantly greater than cilostamide. Thus,

PDE1, PDE3, PDE4 regulate resting cAMP levels in INS-1 cells with a rank order of PDE1 >

PDE4 > PDE3. Summation of the percent increase in cAMP levels elicited by each subtype-

selective PDE inhibitor was 33.5%, which is comparable to the increase observed with IBMX.

Since PDE8 is not inhibited by IBMX (38), we examined the effect of the PDE8-selective

inhibitor PF-04957325 (39) on resting cAMP levels in INS-1 cells. We found that 100 nM PF-

04957325 (IC50 < 1 nM for both PDE8A and 8B) had no effect on cAMP levels under basal

conditions (S1 Fig). Additionally, the PDE8-selective inhibitor PF-04671536 (IC_{50} < 2 nM for

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354   both PDE8A and 8B)(42), didn't increase basal cAMP levels in INS-1 cells at concentrations up

355   to 5 µM (S1 Fig).

356   *Figure 1. PDE1 and PDE4 regulate basal cAMP levels and glucose-stimulated cAMP in*

357   *INS-1 cells* **A)** Subtype-selective PDE inhibitors and the pan PDE inhibitor IBMX raise basal

358   cAMP levels in INS-1 cells. The Epac-S^{H187} mTurquoise2/FRET ratio is directly proportional to

359   the cAMP level. Data shown is a representative experiment from a single cell. **B)** The percent

360   increase in cAMP levels above baseline elicited by PDE inhibitors in INS-1 cells under basal

361   conditions. IBMX (100 µM), 8MM-IBMX (100 µM) and rolipram (10 µM) significantly elevate

362   cAMP levels above baseline. Further, the percent increase elicited by each of the subtype-

363   selective inhibitors is significantly less than that of IBMX. Last, 8MM-IBMX caused a significantly

364   greater elevation in cAMP levels than either cilostamide (1 µM) or rolipram (***, $P < 0.001$

365   compared to baseline; ###, $P < 0.001$ compared to IBMX; †††, $P < 0.001$, ††, $P < 0.01$

366   compared to 8MM-IBMX; One-way ANOVA, Tukey post-hoc test). Data shown are mean ± SE

367   from 23 cells taken from four independent experiments. **C)** Subtype-selective PDE inhibitors and

368   the pan PDE inhibitor IBMX raise cAMP levels in the presence of 18 mM glucose in INS-1 cells.

369   Data shown is a representative experiment from a single cell. **D)** The percent increase in cAMP

370   levels above baseline elicited by PDE inhibitors in INS-1 cells in the presence of 18 mM

371   glucose. IBMX, 8MM-IBMX and rolipram significantly elevate cAMP levels above baseline,

372   whereas only IBMX and 8MM-IBMX are significantly greater than glucose. Last, 8MM-IBMX

373   caused a significantly greater elevation in cAMP levels than either cilostamide or rolipram. Data

374   shown are mean ± SE from 18 cells taken from four independent experiments. (***, $P < 0.001$, *,

375    $P < 0.05$ compared to baseline; ###, $P < 0.001$ compared to 18G; †††, $P < 0.001$, ††, $P < 0.01$

376   compared to 8MM-IBMX; One-way ANOVA, Tukey post-hoc test). **E)** Comparison of cAMP

377   accumulation stimulated by PDE inhibitors in the absence and presence of 18 mM glucose.

378   IBMX stimulated a greater increase in cAMP levels in the absence of glucose (basal), compared

379   to 18 mM glucose (***, $P < 0.001$; Student's unpaired t-test).

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380

381   PDE1 activity is upregulated by Ca^{2+}/Calmodulin (19, 20), while PDE3 and PDE4

382   activities are upregulated by PKA-mediated phosphorylation (40). Glucose elevates Ca^{2+} and

383   cAMP levels in pancreatic β-cells (8); therefore, it's plausible that the PDE profile is altered in

384   INS-1 cells following glucose stimulation. To test this, we stimulated INS-1 cells expressing

385   Epac-S^{H187} with 18 mM glucose and sequentially applied the PDE inhibitors. Fig 1C shows

386   initial application of glucose (18 mM) resulted in a small but detectable rise in cytosolic cAMP

387   levels (2.64 ± 0.83%). Similar to our findings under resting conditions, 100 µM IBMX (18 ±

388   1.93%), 100 µM 8MM-IBMX (24.45 ± 3.61%), and 10 µM rolipram (11.43 ± 3.66%) significantly

389   elevated cAMP levels in the presence of glucose, while 1 µM cilostamide (8.39 ± 1.92%) did not

390   (Fig 1D). Furthermore, 8MM-IBMX had a significantly greater effect on glucose-stimulated

391   cAMP levels compared with rolipram and cilostamide, suggesting that PDE1 is the primary

392   regulator of cAMP levels under resting and glucose-stimulated conditions. In addition, we

393   directly compared the effect of each PDE inhibitor under resting conditions with glucose

394   stimulation. We found that the PDE1-selective inhibitor 8MM-IBMX and PDE3-selective inhibitor

395   cilostamide trended toward increased cAMP levels in the presence of glucose, but this

396   difference did not reach statistical significance (Fig 1E). Interestingly, the IBMX-induced rise in

397   cAMP levels was significantly greater in the absence of glucose than in 18 mM glucose.

398

399   *PDE-mediated regulation of resting and glucose-stimulated cAMP levels in human β-cells*

400   Given that the rat-derived INS-1 cell line may not reflect human β-cell physiology, we examined

401   the roles of PDE1, PDE3, PDE4 and PDE8 in regulation of resting cAMP levels in isolated

402   pancreatic β-cells dissociated from human islets. Previous reports have demonstrated that

403   PDE1, PDE3 and PDE4 regulate cAMP levels in intact human islets; however, there is no

404   evidence which suggests that this is specific to β-cells (23, 41). Dissociated human islet cells

405   were transiently transfected with Epac-S^{H187} and imaged at 48 hours post-transfection by

406   confocal microscopy. To distinguish pancreatic β-cells from other islet cell types, we employed

407   a previously established pharmacological approach (22). Unlike other islet cell types,

408   pancreatic β-cells express both the GLP-1 receptor and α2-adrenergic receptor, which when

409   activated with their respective agonists, lead to AC activation and inhibition, respectively. At the

410   end of each imaging experiment, we applied the GLP-1R agonist GLP-1 (50 nM) followed by co-

411   administration of the α_2-adrenergic receptor agonist clonidine (1 µM). If a cell exhibited a GLP-

412   1-induced rise in cAMP levels that was dampened or abolished by application of clonidine, it

413   was classified as a pancreatic β-cell (Fig 2A-C). We used this approach to measure cAMP

414   levels in human pancreatic β-cells obtained from non-diabetic human donors (S1 Table) and

415   found that, as in INS-1 cells, application of 100 µM IBMX in low glucose (1.7 mM) resulted in a

416   robust increase in cAMP levels (29.45 ± 3.17%) (Fig 2). Further, 1 µM cilostamide (15.3 ±

417   2.94%) and 10 µM rolipram (12.53 ± 4.43%) significantly elevated resting cAMP levels, whereas

418   100 µM 8MM-IBMX (9.35 ± 1.53%) did not (Fig 2). This suggests that unlike INS-1 cells, PDE3

419   and PDE4, but not PDE1, regulates resting cAMP levels in human pancreatic β-cells. We also

420   examined the role of PDE8 in regulating cAMP levels in human pancreatic β-cells. We used the

421   PDE8 inhibitor PF-04671536 since it is reported to stimulate GSIS in human islets (42).

422   However, 5 µM PF-04671536 had no effect on basal cAMP levels in human pancreatic β-cells

423   (S2. Fig).

424

425   ***Figure 2. Regulation of basal cAMP levels by PDEs in human pancreatic β-cells* A-C)**

426   Subtype-selective PDE inhibitors and IBMX raise cAMP levels in pancreatic β-cells dissociated

427   from human islets under basal conditions (1.7 mM glucose). For each experiment, IBMX (100

428   µM) and either 8MM-IBMX (100 µM) (**A**), cilostamide (1 µM) (**B**) or rolipram (10 µM) (**C**) were

429   perfused onto the cell. Pancreatic β-cells were identified at the end of each experiment by

430   addition of GLP-1 (50 nM) with and without the α2-receptor agonist clonidine (1 µM). Data

431   shown are representative experiments from single human pancreatic β-cells. **D)** The percent

17

432   increase in cAMP levels above baseline elicited by PDE inhibitors in human pancreatic β-cells

433   under basal conditions. IBMX, cilostamide and rolipram significantly elevate cAMP levels above

434   baseline. The percent increase in cAMP levels stimulated by 8MM-IBMX and rolipram is

435   significantly less than that of IBMX (***, $P < 0.001$, *, $P < 0.05$ compared to baseline; ###, P <

436   0.001 compared to IBMX; One-way ANOVA, Tukey post-hoc test). Data shown are average ±

437   SE from 15 cells (8MM-IBMX), 7 cells (cilostamide), 12 cells (rolipram) and 34 cells (IBMX),

438   collected from four human islet preparations.

439   To compare regulation of resting and glucose-stimulated cAMP levels by PDEs in

440   human pancreatic β-cells, we examined the role of PDE1, PDE3, and PDE4 in regulation of

441   cAMP levels during 16.7 mM glucose stimulation in human pancreatic β-cells from non-diabetic

442   human donors (S1 Table). At the end of each experiment, pancreatic β-cells were again

443   identified by the characteristic increase in cAMP concentration stimulated by GLP-1 which is

444   rapidly reversed by clonidine (Fig 3A-C). We found that the pan PDE inhibitor IBMX (48.08 ±

445   3.25%), PDE1-selective inhibitor 8MM-IBMX (25.81 ± 2.75%), PDE3-selective inhibitor

446   cilostamide (15.99 ± 1.4%), and PDE4-selective inhibitor rolipram (27.58 ± 5.77%) each

447   significantly elevated cAMP levels in the presence of glucose (Fig 3D). A direct comparison of

448   PDE-mediated regulation of cAMP levels under resting (Fig 2) and stimulatory conditions (Fig 3)

449   revealed that the IBMX-induced rise was significantly elevated in the presence of glucose (Fig

450   3E). Consistent with this, 8MM-IBMX and rolipram had a significantly greater effect in the

451   presence of glucose compared with resting conditions, whereas cilostamide did not. Thus,

452   PDE1 and PDE4 are upregulated following glucose stimulation, suggesting that the activity of

453   these PDE subtypes is enhanced in the presence of glucose. In the case of PDE1, activity is

454   highly dependent on glucose stimulation in human pancreatic β-cells.

455   ***Figure 3. Regulation of glucose-stimulated cAMP by PDEs in human pancreatic β-cells* A-**

456   **C)** Subtype-selective PDE inhibitors and IBMX raise cAMP levels in pancreatic β-cells

457   dissociated from human islets under high glucose (16.7 mM) stimulation conditions. For each

18

458 experiment, IBMX (100 µM) and either 8MM-IBMX (100 µM) (**A**), cilostamide (1 µM) (**B**) or

459 rolipram (10 µM) (**C**) were perfused onto the cell. Pancreatic β-cells were identified using GLP-1

460 and clonidine as described for Figure 5. Data shown are representative experiments from single

461 human pancreatic β-cells. **D)** The percent increase in cAMP levels above baseline elicited by

462 PDE inhibitors in human pancreatic β-cells in the presence of 16.7 mM glucose. IBMX, 8MM-

463 IBMX, cilostamide, and rolipram all significantly elevate cAMP levels above baseline (***, $P <$

464 0.001; ###, $P < 0.001$ compared to IBMX; One-way ANOVA with Tukey post-hoc test). Data are

465 shown as mean ± SE from 27 cells (IBMX), 14 cells (8MM-IBMX), 17 cells (cilostamide), and 18

466 cells (rolipram). **E)** Comparison of cAMP accumulation stimulated by PDE inhibitors in 1.7 mM

467 (basal) or 18 Mm glucose.   IBMX, 8MM-IBMX, and rolipram stimulated a greater increase in

468 cAMP levels in 18 mM glucose, compared to 1.7 mM glucose (***, $P < 0.001$, *, $P < 0.01$;

469 Student's unpaired t-test).

470

471 *PDE-mediated regulation of GSIS in INS-1 cells and human islets*

472 Elevated cAMP concentrations can enhance GSIS (43); therefore, we tested whether PDE-

473 mediated regulation of glucose-stimulated cAMP levels in INS-1 cells (Fig 1) correlates with the

474 ability of subtype-selective PDE inhibitors to potentiate GSIS. Here, we measured insulin

475 secretion from INS-1 cells stimulated with 18 mM glucose in the absence or presence of PDE

476 inhibitors. 18 mM glucose significantly stimulated insulin secretion from INS-1 cells compared to

477 basal secretion (Fig 4A). Each experiment was normalized to insulin secretion in the presence

478 of 18 mM glucose alone. Treatment with 100 µM IBMX resulted in greater than a three-fold

479 increase (3.24 ± 0.17) in insulin secretion in INS-1 cells compared with glucose alone (Fig 4B).

480 Treatment of INS-1 cells with 18 mM glucose + 100 µM 8MM-IBMX (2.25 ± 0.15) or 10 µM

481 rolipram (1.79 ± 0.1) significantly potentiated insulin secretion compared with glucose alone,

482 whereas 1 µM cilostamide (1.12 ± 0.13) did not (Fig 4B). Moreover, potentiation of GSIS by

483 cilostamide was significantly less than that observed with IBMX, 8MM-IBMX, or rolipram. This

19

484   suggests that PDE1 and PDE4, but not PDE3, regulate GSIS in INS-1 cells, consistent with our

485   measurements of cAMP stimulation in Fig 1D, showing that PDE1 is the major subtype

486   degrading cAMP in the presence of 18 mM glucose, followed closely by PDE4. Lastly, we found

487   that PDE8-selective inhibitor PF-04957325 (100 nM) did not significantly potentiate GSIS in

488   INS-1 cells (S1 Fig). Thus, it appears that the major IBMX-insensitive PDE isoform found in

489   pancreatic β-cells does not play a significant role in either regulation of basal cAMP levels or

490   GSIS in INS-1 cells.

491   *Figure 4. Effect of PDE inhibitors on GSIS in INS-1 cells and human pancreatic islets* **A)**

492   Glucose (18 mM) significantly stimulates insulin secretion above basal levels in INS-1 cells (**,

493    $P < 0.01$, compared to 0 glucose; Student's unpaired t-test). Data shown are mean ± SE from

494   four independent experiments. **B)** Subtype-selective PDE inhibitors and pan the PDE inhibitor

495   IBMX potentiate insulin secretion stimulated with glucose (18 mM) in INS-1 cells. Each

496   experiment was normalized to the glucose response. IBMX (100 µM), 8MM-IBMX (100 µM) and

497   rolipram (10 µM) significantly stimulate insulin secretion, compared with glucose alone.

498   Potentiation of GSIS with cilostamide (1 µM) or rolipram is significantly less than IBMX. Further,

499   insulin secretion stimulated with cilostamide is significantly less than both 8MM-IBMX and

500   rolipram (**, $P < 0.01$, *, $P < 0.05$ compared to 18G; #, $P < 0.05$ compared to IBMX; †, $P < 0.05$

501   compared to 8MM-IBMX; ‡, $P < 0.05$ compared to rolipram; One-way ANOVA, Tukey post-hoc

502   test). Data shown are mean ± SE from four independent experiments. **C)** Subtype-selective

503   PDE inhibitors and the pan PDE inhibitor IBMX potentiate insulin secretion stimulated with

504   16.7G in intact human islets isolated from four separate donors. In donor 3, 8MM-IBMX was

505   significantly greater than 1.7G and 16.7G. In donor 4, IBMX was significantly greater than 1.7G

506   and 16.7G, whereas rolipram was significantly less than IBMX. There are no significant

507   differences among the treatment conditions for donor 5. Finally, in donor 6 we observed that

508   IBMX had a significant effect above 1.7G and 16.7G, and 8MM-IBMX was less than IBMX (**, P

509   < 0.01, *, $P < 0.05$ compared to 1.7G; ##, $P < 0.01$, #, $P < 0.05$ compared to 16.7G; †, $P < 0.05$

20

510   compared to IBMX; One-way ANOVA, Tukey post-hoc test). Data shown are mean ± SE from

511   four human islet donors, in which each treatment was performed in triplicate.

512   Given the distinct effects of PDE subtype inhibition on cAMP levels in human pancreatic

513   β-cells, we evaluated whether inhibitors of PDE1, PDE3, and PDE4 potentiate GSIS in isolated

514   human islets using a single-islet insulin secretion assay (44). In short, islets obtained from non-

515   diabetic human donors (S1 Table) were plated in a 96-well V-bottom plate, so that each well

516   held a single islet. Human islets were incubated with PDE inhibitors at low glucose (1.7 mM) for

517   30 min prior to a 1h incubation with high glucose (16.7 mM) with and without PDE inhibitors.

518   Insulin release was normalized to the insulin content of each islet. We found that 16.7 mM

519   glucose stimulated a subtle increase in insulin secretion compared with 1.7 mM glucose in all

520   four donors (Fig 4C), ranging from a 1.11-fold increase to 2.03-fold (S1 Table). However, in

521   none of the donors did this apparent increase reach statistical significance. In contrast, the PDE

522   inhibitors had more robust effects on GSIS in some donors. The pan PDE inhibitor IBMX

523   trended toward potentiated GSIS in all four donors, and this reached statistical significance in

524   two of the four donors (donors 4 and 6). The PDE1-selective inhibitor 8MM-IBMX enhanced

525   GSIS in three donors (donors 3, 4 and 5), and this increase reached statistical significance in

526   donor 3. The PDE3-selective inhibitor cilostamide trended toward potentiation of GSIS in three

527   (donors 3, 5 and 6) and the PDE4-selective inhibitor rolipram trended toward significance in two

528   (donors 3 and 6), but none of these increases reached statistical significance. Taken together,

529   PDE1 appears to play a role in regulating GSIS in intact human islets, though there is clearly

530   heterogeneity amongst individuals.

531

532   *PDE-mediated regulation of Ca^{2+} dynamics in INS-1 cells*

533   8MM-IBMX and rolipram potentiated GSIS in INS-1 cells (Fig 4B), suggesting that PDE1 and

534   PDE4 are the major PDE subtypes that regulate GSIS in INS-1 cells. PDE-mediated regulation

535   of GSIS presumably occurs following the rise in cytosolic cAMP levels (Fig 1) and subsequent

21

536   activation of the cAMP effector proteins, PKA (45) and Epac (46). However, both IBMX and

537   8MM-IBMX are derivatives of xanthine, as is caffeine, a potent activator of the ryanodine

538   receptor (RyR) (47). Therefore, we examined whether or not, like caffeine, IBMX and 8MM-

539   IBMX, and potentially cilostamide and rolipram, enhanced GSIS by stimulating RyR-mediated

540   Ca^{2+} release. Using the fluorescent Ca^{2+} sensor Fura2-acetoxymethyl (AM), we found that

541   acute application of caffeine (5 mM) stimulated a rapid and robust increase in cytosolic Ca^{2+} (Fig

542   5A&B). In contrast, application of IBMX and each of the subtype-selective PDE inhibitors, at the

543   concentrations used to stimulate cAMP accumulation, did not markedly elevate cytosolic Ca^{2+}

544   levels in INS-1 cells. Quantification of the area under the curve (AUC) showed that all of the of

545   the PDE inhibitors stimulated a significantly smaller increase in intracellular Ca^{2+} concentration

546   compared to caffeine: 100 µM IBMX (14.86 ± 4.9%), 100 µM 8MM-IBMX (23.9 ± 2.23%), 1 µM

547   cilostamide (22.67 ± 1.92%) or 10 µM rolipram (15.22 ± 2.41%). Thus, potentiation of GSIS by

548   IBMX, 8MM-IBMX and rolipram in INS-1 cells is likely not due to direct activation of RyR.

549   Alternatively, it's possible that PDE-mediated enhancement of GSIS occurs via activation of

550   PKA and Epac, which can enhance ER Ca^{2+} release (16, 17). As expected, 18 mM glucose

551   stimulated a rise in cytosolic Ca^{2+} that peaked approximately 10 min post-stimulation (Fig 5C).

552   However, co-application of IBMX or any of the subtype-selective PDE inhibitors did not further

553   increase intracellular Ca^{2+} levels in comparison with glucose alone (Fig 5D). Since the increase

554   in cAMP levels stimulated by glucose alone is relatively modest, it's possible that a more intense

555   stimulation of cAMP levels might potentiate the Ca^{2+} response to glucose. Therefore, in an

556   attempt to amplify the changes in cytosolic Ca^{2+}, we stimulated INS-1 cells with glucose (18

557   mM) + the AC activator forskolin (25 µM). Glucose + forskolin stimulated a biphasic Ca^{2+}

558   transient in INS-1 cells characterized by a rapid early peak followed by a prolonged plateau

559   phase (Fig 5E). Co-application of the pan PDE inhibitor IBMX (150 ± 27.5%) and PDE1-

560   selective inhibitor 8MM-IBMX (162 ± 15.48%) selectively enhanced the first phase of the Ca^{2+}

561   transient stimulated with glucose + forskolin (Fig 5E&F). Notably, we've previously

22

562   demonstrated the first phase of the tolbutamide-stimulated Ca^{2+} transient is due to ER Ca^{2+}

563   release (48). In contrast, neither the PDE3-selective inhibitor cilostamide (110 ± 6.99%) nor the

564   PDE4-selective inhibitor rolipram (123 ± 10.2%) had any effect. Thus, under conditions that

565   strongly elevate Ca^{2+} and cAMP levels, PDE1 inhibition strongly enhances intracellular Ca^{2+}

566   levels, providing a potential Ca^{2+}-dependent mechanism by which PDE1 potentiates GSIS.

567

568   ***Figure 5. Effect of PDE inhibitors on intracellular Ca^{2+} dynamics in INS-1 cells* A)** Caffeine

569   robustly elevates intracellular Ca^{2+} in INS-1 cells when added acutely; however, the subtype-

570   selective PDE inhibitors and the pan PDE inhibitor IBMX have little effect. Data shown are the

571   mean ± SE from representative experiments performed in quadruplicate. **B)** AUC analysis of

572   Ca^{2+} dynamics stimulated with caffeine (5 mM), 8MM-IBMX (100 µM), cilostamide (1 µM),

573   rolipram (10 µM) or IBMX (100 µM). Each experiment was normalized to the response elicited

574   by caffeine. The Ca^{2+} response elicited by each of the subtype-selective PDE inhibitors as well

575   as IBMX was significantly less than that of caffeine (****, $P < 0.0001$ compared to caffeine; One-

576   way ANOVA, Tukey post-hoc test). Data shown are average ± SE from four independent

577   experiments. **C)** Glucose (18 mM) elevates intracellular Ca^{2+} levels in INS-1 cells but the

578   subtype-selective PDE inhibitors and the pan PDE inhibitor IBMX have no additional effect. Data

579   shown are the mean ± SE from representative experiments performed in quadruplicate. **D)** AUC

580   analysis of Ca^{2+} dynamics stimulated with glucose (18 mM) alone and in the presence of either

581   8MM-IBMX (100 µM), cilostamide (1 µM), rolipram (10 µM) or IBMX (100 µM). Each experiment

582   was normalized to the response elicited by 18G. There was no significant difference among the

583   treatment conditions. Data shown are average ± SE from four independent experiments. **E)**

584   Glucose (18 mM) + forskolin (25 µM) strongly elevates intracellular Ca^{2+} levels in INS-1 cells.

585   The PDE1 inhibitor 8MM-IBMX (100 µM) and IBMX (100 µM) potentiate the initial phase of the

586   response. Data shown are the mean ± SE from representative experiments performed in

587   quadruplicate. **F)** AUC analysis of Ca^{2+} dynamics stimulated with glucose (18 mM) + forskolin

23

588   (25 µM) alone and in the presence of either 8MM-IBMX (100 µM), cilostamide (1 µM), rolipram

589   (10 µM) or IBMX (100 µM). Each experiment was normalized to the response elicited by 18G +

590   forskolin. Addition of IBMX or 8MM-IBMX elicited a significantly greater elevation in intracellular

591   Ca^{2+} (AUC) than 18G + forskolin alone. Ca^{2+} AUC stimulated by 18G+forskolin in the presence

592   of cilostamide is significantly less than that in the presence of 8MM-IBMX (**, $P < 0.01$, *, $P <$

593   0.05 compared to 18G + forskolin; #, $P < 0.05$ compared to 8MM-IBMX; One-way ANOVA,

594   Tukey post-hoc test). Data shown are average ± SE from four independent experiments.

595

596   *PDE-mediated regulation of INS-1 cell survival*

597   cAMP not only enhances insulin secretion from pancreatic β-cells but also is an important

598   regulator of β-cell mass, enhancing proliferation and cell survival (49). Not surprisingly, cAMP

599   signaling is impaired in diabetic pancreatic β-cells (50). Given the robust effects of PDE

600   inhibitors on cAMP levels (Fig 1), we tested whether raising cAMP levels using subtype-

601   selective PDE inhibitors can rescue INS-1 cells from apoptosis caused by the saturated fatty

602   acid palmitate, which is a widely-used model of lipotoxicity associated with type 2 diabetes (51).

603   We exposed INS-1 cells to 500 µM palmitate at a 3:1 molar ratio with 1% BSA (52) in the

604   presence of PDE inhibitors and measured caspase-3/7 activation, an early marker of apoptosis,

605   at 12 hours post-treatment (53). Palmitate treatment significantly upregulated caspase-3/7

606   activity, compared with BSA alone (Fig 6A). Co-treatment of INS-1 cells with 100 µM IBMX

607   (61.35 ± 8.65%) significantly decreased palmitate-induced caspase-3/7 activation (Fig 6B).

608   Furthermore, we found that 100 µM 8MM-IBMX (64.81 ± 7.94%), but neither 1 µM cilostamide

609   (87.54 ± 3.31%) nor 10 µM rolipram (75.45 ± 9.84%), significantly decreased palmitate-induced

610   caspase 3/7 activation.

611   ***Figure 6. PDE1 inhibition reduces palmitate-induced apoptosis and stimulates CREB***

612   ***phosphorylation in INS-1 cells- A)*** Incubation of INS-1 cells with palmitate for 12h significantly

613   elevates caspase-3/7 activation (*, $P < 0.05$ compared to BSA; Student's unpaired t-test). Data

24

614   shown are average ± SE from six independent experiments. **B)** The PDE1 inhibitor 8MM-IBMX

615   (100 µM) and pan PDE inhibitor IBMX (100 µM) significantly decrease the level of caspase-3/7

616   activation induced by palmitate toxicity (**, $P < 0.01$, *, $P < 0.05$ compared to palmitate; One-

617   way ANOVA, Tukey post-hoc test). Each experiment was normalized to the level of palmitate

618   toxicity. Data shown are average ± SE from 4-6 independent experiments. **C)** The PDE1

619   inhibitor 8MM-IBMX (100 µM) and pan PDE inhibitor IBMX (100 µM) significantly stimulate

620   CREB phosphorylation above basal levels (****, $P < 0.0001$, *, $P < 0.05$ compared to basal; ##,

621    $P < 0.01$, #, $P < 0.05$ compared to IBMX; One-way ANOVA, Tukey post-hoc test). Data shown

622   are average ± SE from four independent experiments.

623

624   cAMP-response element binding protein (CREB) is a cAMP-dependent transcription

625   factor that regulates gene transcription and pancreatic β-cell survival following PKA-dependent

626   phosphorylation (7). Thus, a potential mechanism by which IBMX and 8MM-IBMX may activate

627   pro-survival pathways in INS-1 cells is through an elevation in cAMP levels (Fig 1) that

628   ultimately leads to PKA-mediated phosphorylation of CREB. To determine whether PDE

629   inhibition stimulates CREB phosphorylation, we treated INS-1 cells with IBMX and the subtype-

630   selective PDE inhibitors for 10 min, lysed the cells and measured CREB phosphorylation at

631   S133 (pCREB) and total CREB expression using a commercially available ELISA kit. We

632   calculated the ratio of pCREB to total CREB and normalized each experiment to basal

633   pCREB/total CREB. Using this approach, we found that 100 µM IBMX (171 ± 14.32%) and 100

634   µM 8MM-IBMX (137 ± 8.05%) significantly elevated pCREB/total CREB, compared with basal

635   conditions (Fig 6C). In contrast, neither 1 µM cilostamide (125 ± 5.94% basal) nor 10 µM

636   rolipram (120 ± 2.27% basal) had a significant effect. Taken together, the pro-survival effect of

637   the PDE-selective inhibitor 8MM-IBMX could be the result of elevated cAMP levels (Fig 1) and

638   subsequent activation of the cAMP-responsive transcription factor CREB.

639

Discussion

In this study, we examined the role of PDE1, PDE3, PDE4, and PDE8 in regulating resting and glucose-stimulated cAMP levels and downstream signaling in INS-1 cells and primary human pancreatic β-cells. We found that PDE1 and PDE4, but not PDE3 or PDE8, regulate resting and glucose-stimulated cAMP levels in INS-1 cells (Fig 1, S1 Fig), and this correlates with the ability of PDE1- and PDE4-selective inhibitors to enhance GSIS in these cells (Fig 4B). However, 8MM-IBMX was the only subtype-selective inhibitor to enhance glucose + forskolin-stimulated Ca^{2+} transients (Fig 5E&F), suggesting that PDE1 may regulate GSIS by modulating Ca^{2+} dynamics. In contrast to INS-1 cells, we found that PDE3 and PDE4, but not PDE1 or PDE8, regulate resting cAMP levels in primary human β-cells (Fig 2, S2 Fig). Strikingly, inhibition of PDE1 and PDE4 activity significantly increased cAMP accumulation following stimulation with 16.7 mM glucose in human β-cells (Fig 3). Consistent with this, the PDE1-selective inhibitor 8MM-IBMX had the greatest effect on GSIS in human islets (Fig 4C). Finally, we found that PDE1 inhibition reduced palmitate-induced caspase-3/7 activation (Fig 6B) and induced CREB phosphorylation (Fig 6C) in INS-1 cells. Taken together, our results suggest that PDE1-mediated regulation of cAMP levels is important for regulation of GSIS and pancreatic β-cell survival.

PDE-mediated regulation of resting and glucose-stimulated cAMP levels in INS-1 cells and human pancreatic β-cells

We elected to study the role of PDE1, PDE3, PDE4, and PDE8 in PDE-mediated regulation of cAMP levels because they are among the most highly-expressed PDEs in pancreatic β-cell lines and islets from rat, mouse, and human (21, 28, 30, 41). Subtype-selective inhibitors and siRNA-mediated knockdown have revealed an important role for PDE1, PDE3, PDE4, and PDE8 in regulation of resting cAMP levels and GSIS (21-23). However, individual studies have focused on one or two PDE subtypes and have not performed a direct comparison of PDE1,

26

666   PDE3, PDE4, and PDE8. The few studies that have examined PDE1, PDE3, PDE4, and PDE8

667   differed substantially in approach from the current study. For example, subtype-selective

668   inhibitors of PDE1 and PDE4, but not PDE3, elevated cAMP content in homogenates of the

669   mouse insulinoma β-cell line βTC3, suggesting that PDE1 and PDE4 are required (21).

670   However, studying the effect of PDE inhibitors on cAMP dynamics in living pancreatic β-cells in

671   real-time, as we performed in this study, is more likely to reflect cAMP dynamics in vivo. A

672   systematic comparison of PDE1, PDE3, PDE4, and PDE8 was performed in the mouse

673   insulinoma cell line MIN6 and primary mouse pancreatic β-cells using a plasma membrane-

674   localized fluorescent cAMP sensor and TIRF microscopy (22). The authors concluded that

675   PDE3 is the primary regulator of resting cAMP levels. However, PDE3 is commonly associated

676   with cellular membranes, especially the plasma membrane (54, 55). PDE1 and PDE4, on the

677   other hand, are associated with cellular sub-compartments, including the ER (56). Therefore,

678   an approach that detects mainly sub-plasmalemmal cAMP may underestimate the

679   contributions of PDE1 and PDE4. In the current study, we used a robust fluorescent cAMP

680   sensor localized to the cytosol, Epac-S^{H187} (34), and found that under resting conditions, PDEs

681   regulate resting cAMP levels in INS-1 cells with a rank order of PDE1 > PDE4 > PDE3 = PDE8

682   (Fig 1A&B, S1 Fig). In contrast, we found that in human pancreatic β-cells, PDE3 and PDE4,

683   but not PDE1 or PDE8, regulate resting cAMP levels (Fig 2, S2 Fig). Two earlier studies have

684   examined regulation of resting cAMP levels by PDE1, PDE3, and PDE4 in human islets (23,

685   41). However, the authors measured cAMP in cell lysates obtained from intact islets, which

686   included contributions from α- and δ-cells, in addition to β-cells (57). Therefore, the present

687   study, which specifically examines PDEs in isolated human pancreatic β-cells, is much less

688   likely to be influenced by contributions from other cell types resident within the pancreatic islet.

689   While PDE-mediated regulation of resting cAMP levels has been extensively studied,

690   regulation of cAMP levels by PDE1, PDE3, and PDE4 in the presence of glucose remains

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691   largely unexplored. This data is significant because it would be directly comparable with the

692   effect of subtype-selective PDE inhibitors on GSIS. In this study, we found that stimulatory

693   concentrations of glucose altered the PDE profile in INS-1 cells (Fig 1E) and human β-cells (Fig

694   3E). The IBMX-stimulated rise in cAMP levels was significantly greater in the absence of

695   glucose than in the presence of 18 mM glucose in INS-1 cells (Fig 1E); however, this was not

696   the case for the subtype-selective inhibitors. In fact, the PDE1-selective inhibitor 8MM-IBMX

697   and PDE3-selective inhibitor cilostamide trended toward a greater effect in the presence of

698   glucose. PDE activity is governed by the local cAMP concentration, which in turn is dependent

699   on the presence of cAMP-generating AC subtypes within cAMP microdomains or compartments

700   in the cell (58). PDE activity can also be regulated by protein kinase phosphorylation, such as

701   PKA-mediated phosphorylation and upregulation of PDE3 and PDE4 activities (40). In addition,

702   PDE1 activity is upregulated by Ca^{2+}/Calmodulin (19, 20). The observation that the IBMX-

703   stimulated rise in cAMP levels decreased in the presence of glucose, while the effect of

704   subtype-selective inhibitors did not change, suggests that PDE1, PDE3 and PDE4 transition

705   (Fig 1D) from regulating different cAMP compartments under resting conditions (subtype-

706   selective inhibitors added up to IBMX) to regulating a similar cAMP microdomain during glucose

707   stimulation (subtype-selective inhibitors are roughly equivalent to IBMX). Indeed, stimulus-

708   induced changes in the components of cAMP microdomains, including PDEs, have been

709   reported previously (56, 59). Glucose elevates Ca^{2+} and cAMP levels and stimulates PKA

710   activity (8, 10, 12); therefore, glucose stimulation of PDE1 and PDE3 activities may be due to

711   regulation by Ca^{2+}/Calmodulin and PKA, respectively. In contrast with our findings in INS-1

712   cells, the IBMX-stimulated rise in cAMP levels was significantly elevated following glucose

713   stimulation of human β-cells (Fig 3E). This was accompanied by a significant increase in the

714   8MM-IBMX- and rolipram-induced elevations in cAMP levels. PDE4 activity is upregulated by

715   PKA- and CamKII-mediated phosphorylation (40, 60); therefore, it's possible that glucose

716   stimulates PKA- or CamKII-dependent phosphorylation of PDE4 in human β-cells. Our finding

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717   that PDE1 and PDE4, but not PDE3, activities are upregulated by glucose in human β-cells may

718   also be explained in the context of the affinity of the different PDEs for cAMP. The resting

719   cAMP concentration in mammalian cells is estimated to be near 1 μM (61, 62), close to the K_m

720   for cAMP of PDE1C and PDE4 isoforms, below the K_m for cAMP of PDE1A and 1B, but much

721   higher than that of all PDE3 isoforms, which have K_m values for cAMP of between 20 and 150

722   nM (63). Therefore, PDE3 activity is likely maximal under resting conditions, whereas the

723   activities of PDE1 and PDE4 would be expected to increase in response to a glucose-stimulated

724   elevation in cAMP levels.

725

726   *PDE-mediated regulation of Ca^{2+} dynamics and GSIS in INS-1 cells and human islets*

727   We found that inhibition of PDE1 and PDE4 potentiated GSIS INS-1 cells (Fig 4B), while

728   inhibition of PDE3 (Fig 4B) and PDE8 (S1 Fig) did not. Unfortunately, none of our experiments

729   with human islets resulted in significant GSIS over basal secretion. However, in two donors

730   (donors 4&6), IBMX increased insulin secretion over glucose alone, and in another donor (donor

731   3), inhibition of PDE1 significantly increased insulin secretion over glucose alone (Fig 4C).

732   Inhibition of PDE1 trended toward potentiation of GSIS in two additional donors (donors 4&5).

733   Our result in INS-1 cells is consistent with our finding that inhibition of PDE1 or PDE4

734   potentiates glucose-stimulated cAMP levels (Fig 1C&D). Other studies on PDE-regulation of

735   GSIS demonstrate that PDE3 is involved in regulating GSIS (24, 26-28, 64). One exception to

736   this is that the PDE1-selective inhibitor 8MM-IBMX was shown to potentiate GSIS in βTC3 cells

737   (21), in agreement with our study. A direct comparison of GSIS regulation by PDE1, PDE3,

738   PDE4, and PDE8 showed that knockdown of each subtype enhanced GSIS in INS-1 cells;

739   however, there was no difference among them (30). Another study found that PDE1 is an

740   important regulator of GSIS in MIN6 cells, but also reported that PDE3 and PDE8 regulate GSIS

741   as well (22). In human islets PDE1, PDE3, and PDE4, together comprised the vast majority of

29

742   total PDE activity, although other PDEs were detected by western blot (41). This is consistent

743   with our conclusion that PDE1 inhibition can potentiate GSIS in human islets (Fig 4C).

744

745   The rise in cAMP levels stimulated by the pan PDE inhibitor IBMX, PDE1-selective

746   inhibitor 8MM-IBMX and PDE4-selective inhibitor rolipram in INS-1 cells (Fig 1) and human

747   pancreatic β-cells (Fig 2&3) likely resulted in activation of the cAMP effectors PKA and Epac

748   (12); however, it is only in the presence of glucose that PDE inhibition has the ability to stimulate

749   insulin secretion (65). PKA and Epac enhance insulin secretion at the site of exocytosis through

750   phosphorylation of exocytotic substrates (13) and direct interactions with scaffolding proteins

751   (66), SNARE proteins (67) and ion channels (15), respectively. PKA and Epac can also

752   enhance insulin secretion through more distal actions on ER Ca^{2+} channels IP_3R and RyR. PKA

753   directly phosphorylates the IP_3R to stimulate ER Ca^{2+} release (68-70), whereas Epac is reported

754   to regulate RyR-mediated Ca^{2+} release through a Rap1/PLCε/CamKII signaling pathway (16,

755   71). Thus, we tested the possibility that PDE inhibition regulates $[Ca^{2+}]_{in}$ under various

756   conditions.   While we could not detect any change in cytosolic Ca^{2+} levels with the subtype-

757   selective PDE inhibitors or IBMX in the absence or presence of 18 mM glucose, we found that

758   the PDE1-selective inhibitor 8MM-IBMX potentiated the Ca^{2+} rise stimulated with 18mM glucose

759   + the AC activator forskolin to a level comparable with IBMX (Fig 5E&F). We've previously

760   reported that 25 μM forskolin, in the presence of IBMX, stimulates a robust increase in [cAMP]

761   as assessed with the Epac-S^{H187} sensor in INS-1 cells (72). Thus, while the effect of IBMX and

762   8MM-IBMX on cAMP levels in INS-1 cells is readily detectable even under resting conditions

763   (Fig 1A&B), they only significantly increase global Ca^{2+} levels over that stimulated by 18 mM

764   glucose when transmembrane ACs are strongly stimulated. Interestingly, inhibition of neither

765   PDE3 nor PDE4 potentiated the Ca^{2+} transient stimulated by 18 mM glucose + forskolin (Fig

766   5F). Together, our results suggest that inhibition of PDE1 could potentially enhance the efficacy

767   of incretin hormones, such as GLP-1 or gastrointestinal inhibitory peptide, that activate

768   transmembrane ACs through G-protein coupled receptors (73). In cardiomyocytes, cAMP

769   microdomains containing PDEs, ACs, the cAMP effectors PKA and Epac, are anchored near

770   target substrates, including ER Ca^{2+} release channels, by A-Kinase Anchoring Protein (AKAP)

771   (74-76). Indeed, evidence of a cAMP microdomain containing AKAP150, PKA and AC8 has

772   been demonstrated in pancreatic β-cells (77-79). Whether PDE1 is present, and the precise

773   sub-cellular localization of this cAMP microdomain, remains to be determined.

774

775   *PDE inhibitors as a potential treatment for type 2 diabetes*

776   As demonstrated by GLP-1 receptor stimulation, elevated cAMP levels not only enhance GSIS

777   but can stimulate pancreatic β-cell proliferation (80) and cell survival (81). Indeed, the pan PDE

778   inhibitor IBMX has the ability to rescue pancreatic β-cells from cell death (Fig 6B) induced by the

779   unsaturated fatty acid palmitate (82). Palmitate exposure recapitulates key aspects of

780   pancreatic β-cell dysfunction observed in diabetes, including disruption of glucose-stimulated

781   cAMP oscillations (50, 51). We also showed that the PDE1-selective inhibitor 8MM-IBMX

782   significantly reduces palmitate-induced caspase-3/7 activation in INS-1 cells, while the PDE3-

783   selective inhibitor cilostamide and PDE4-selective inhibitor rolipram do not (Fig 6B). Consistent

784   with our observation, PDE1A is strongly upregulated in INS-1 832/13 cells in response to 48 hr

785   palmitate exposure (83).

786

787   Activation of the cAMP-response element binding protein (CREB) is associated with β-

788   cell survival (7, 84). Furthermore, IBMX has been shown to induce CREB phosphorylation via a

789   PKA-dependent mechanism in other cell types (85). In our study, we found that IBMX and

790   8MM-IBMX significantly stimulated CREB phosphorylation above resting levels, while

791   cilostamide and rolipram did not (Fig 6C), suggesting that PDE1 inhibition may suppress

792   pancreatic β-cell apoptosis via a CREB-dependent mechanism. Since GSIS and pancreatic β-

793   cell mass are compromised in type 2 diabetics (2), it's possible that PDE1 inhibitors could have

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794   clinical applications for treating this disease (86). A recent study demonstrated that IBMX, the

795   PDE3 inhibitors cilostamide and milrinone, the PDE4 inhibitor rolipram, and the PDE4/10

796   inhibitor dipyridamole stimulated rat pancreatic β-cell replication, and that the latter reduces

797   serum glucose levels in humans (6). However, the PDE1 inhibitor 8MM-IBMX did not

798   significantly stimulate β-cell replication in this study, suggesting that PDE1 may regulate GSIS

799   and pancreatic β-cell survival but not proliferation. It will be of interest to determine if specific

800   PDE1 isoforms play distinct roles in the regulation of pancreatic β-cell function and survival.

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823

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References

1. Rorsman P, Eliasson L, Renstrom E, Gromada J, Barg S, Gopel S. The Cell Physiology of Biphasic Insulin Secretion. News Physiol Sci. 2000;15:72-7.

2. Weir GC, Bonner-Weir S. Five stages of evolving beta-cell dysfunction during progression to diabetes. Diabetes. 2004;53 Suppl 3:S16-21.

3. Ohta M, Nelson J, Nelson D, Meglasson MD, Erecinska M. Effect of Ca++ channel blockers on energy level and stimulated insulin secretion in isolated rat islets of Langerhans. J Pharmacol Exp Ther. 1993;264:35-40.

4. Johnson JD, Kuang S, Misler S, Polonsky KS. Ryanodine receptors in human pancreatic beta cells: localization and effects on insulin secretion. FASEB J. 2004;18:878-80.

5. Ramos LS, Zippin JH, Kamenetsky M, Buck J, Levin LR. Glucose and GLP-1 stimulate cAMP production via distinct adenylyl cyclases in INS-1E insulinoma cells. J Gen Physiol. 2008;132:329-38.

6. Zhao Z, Low YS, Armstrong NA, Ryu JH, Sun SA, Arvanites AC, et al. Repurposing cAMP-modulating medications to promote beta-cell replication. Mol Endocrinol. 2014;28:1682-97.

7. Jhala US, Canettieri G, Screaton RA, Kulkarni RN, Krajewski S, Reed J, et al. cAMP promotes pancreatic beta-cell survival via CREB-mediated induction of IRS2. Genes Dev. 2003;17:1575-80.

8. Dyachok O, Idevall-Hagren O, Sagetorp J, Tian G, Wuttke A, Arrieumerlou C, et al. Glucose-induced cyclic AMP oscillations regulate pulsatile insulin secretion. Cell Metabol. 2008;8:26-37.

9. Kitaguchi T, Oya M, Wada Y, Tsuboi T, Miyawaki A. Extracellular calcium influx activates adenylate cyclase 1 and potentiates insulin secretion in MIN6 cells. Biochem J. 2013;450:365-73.

858   10.   Landa LR, Jr., Harbeck M, Kaihara K, Chepurny O, Kitiphongspattana K, Graf O, et al.

859   Interplay of Ca2+ and cAMP signaling in the insulin-secreting MIN6 beta-cell line. J Biol Chem.

860   2005;280:31294-302.

861   11.   Dyachok O, Isakov Y, Sagetorp J, Tengholm A. Oscillations of cyclic AMP in hormone-

862   stimulated insulin-secreting beta-cells. Nature. 2006;439:349-52.

863   12.   Idevall-Hagren O, Barg S, Gylfe E, Tengholm A. cAMP mediators of pulsatile insulin

864   secretion from glucose-stimulated single beta-cells. J Biol Chem. 2010;285:23007-18.

865   13.   Song WJ, Seshadri M, Ashraf U, Mdluli T, Mondal P, Keil M, et al. Snapin mediates

866   incretin action and augments glucose-dependent insulin secretion. Cell Metab. 2011;13:308-19.

867   14.   Skelin M, Rupnik M. cAMP increases the sensitivity of exocytosis to Ca(2)+ primarily

868   through protein kinase A in mouse pancreatic beta cells. Cell Calcium. 2011;49:89-99.

869   15.   Kang G, Chepurny OG, Malester B, Rindler MJ, Rehmann H, Bos JL, et al. cAMP sensor

870   Epac as a determinant of ATP-sensitive potassium channel activity in human pancreatic beta

871   cells and rat INS-1 cells. J Physiol. 2006;573:595-609.

872   16.   Dzhura I, Chepurny OG, Kelley GG, Leech CA, Roe MW, Dzhura E, et al. Epac2-

873   dependent mobilization of intracellular Ca(2)+ by glucagon-like peptide-1 receptor agonist

874   exendin-4 is disrupted in beta-cells of phospholipase C-epsilon knockout mice. J Physiol.

875   2010;588:4871-89.

876   17.   Kang G, Joseph JW, Chepurny OG, Monaco M, Wheeler MB, Bos JL, et al. Epac-

877   selective cAMP analog 8-pCPT-2'-O-Me-cAMP as a stimulus for Ca2+-induced Ca2+ release

878   and exocytosis in pancreatic beta-cells. J Biol Chem. 2003;278:8279-85.

879   18.   Tengholm A. Cyclic AMP dynamics in the pancreatic beta-cell. Ups J Med Sci.

880   2012;117:355-69.

881   19.   Goraya TA, Cooper DM. Ca2+-calmodulin-dependent phosphodiesterase (PDE1):

882   current perspectives. Cell Signal. 2005;17:789-97.

883   20.   Goraya TA, Masada N, Ciruela A, Cooper DM. Sustained entry of Ca2+ is required to

884   activate Ca^{2+}-calmodulin-dependent phosphodiesterase 1A. J Biol Chem. 2004;279:40494-504.

885   21.   Han P, Werber J, Surana M, Fleischer N, Michaeli T. The calcium/calmodulin-dependent

886   phosphodiesterase PDE1C down-regulates glucose-induced insulin secretion. J Biol Chem

887   1999;274:22337-44.

888   22.   Tian G, Sagetorp J, Xu Y, Shuai H, Degerman E, Tengholm A. Role of

889   phosphodiesterases in the shaping of sub-plasma-membrane cAMP oscillations and pulsatile

890   insulin secretion. J Cell Sci. 2012;125:5084-95.

891   23.   Parker JC, VanVolkenburg MA, Ketchum RJ, Brayman KL, Andrews KM. Cyclic AMP

892   phosphodiesterases of human and rat islets of Langerhans: contributions of types III and IV to

893   the modulation of insulin secretion. Biochem Biophys Res Commun. 1995;217:916-23.

894   24.   Harndahl L, Jing XJ, Ivarsson R, Degerman E, Ahren B, Manganiello VC, et al. Important

895   role of phosphodiesterase 3B for the stimulatory action of cAMP on pancreatic beta-cell

896   exocytosis and release of insulin. J Biol Chem. 2002;277:37446-55.

897   25.   Walz HA, Wierup N, Vikman J, Manganiello VC, Degerman E, Eliasson L, et al. Beta-cell

898   PDE3B regulates Ca2+-stimulated exocytosis of insulin. Cell Signal. 2007;19:1505-13.

899   26.   Harndahl L, Wierup N, Enerback S, Mulder H, Manganiello VC, Sundler F, et al. Beta-

900   cell-targeted overexpression of phosphodiesterase 3B in mice causes impaired insulin

901   secretion, glucose intolerance, and deranged islet morphology. J Biol Chem. 2004;279:15214-

902   22.

903   27.   Shafiee-Nick R, Pyne NJ, Furman BL. Effects of type-selective phosphodiesterase

904   inhibitors on glucose-induced insulin secretion and islet phosphodiesterase activity. Brit J

905   pharmacol. 1995;115:1486-92.

906   28.   Dov A, Abramovitch E, Warwar N, Nesher R. Diminished phosphodiesterase-8B

907   potentiates biphasic insulin response to glucose. Endocrinol. 2008;149:741-8.

36

908   29.   Choi YH, Park S, Hockman S, Zmuda-Trzebiatowska E, Svennelid F, Haluzik M, et al.

909   Alterations in regulation of energy homeostasis in cyclic nucleotide phosphodiesterase 3B-null

910   mice. J Clin Invest. 2006;116:3240-51.

911   30.   Waddleton D, Wu W, Feng Y, Thompson C, Wu M, Zhou YP, et al. Phosphodiesterase 3

912   and 4 comprise the major cAMP metabolizing enzymes responsible for insulin secretion in INS-

913   1 (832/13) cells and rat islets. Biochem Pharmacol. 2008;76:884-93.

914   31.   Zhao AZ, Bornfeldt KE, Beavo JA. Leptin inhibits insulin secretion by activation of

915   phosphodiesterase 3B. J Clin Invest. 1998;102:869-73.

916   32.   Zhao AZ, Zhao H, Teague J, Fujimoto W, Beavo JA. Attenuation of insulin secretion by

917   insulin-like growth factor 1 is mediated through activation of phosphodiesterase 3B. Proc Natl

918   Acad Sci U S A. 1997;94:3223-8.

919   33.   Asfari M, Janjic D, Meda P, Li G, Halban PA, Wollheim CB. Establishment of 2-

920   mercaptoethanol-dependent differentiated insulin-secreting cell lines. Endocrinol. 1992;130:167-

921   78.

922   34.   Klarenbeek J, Goedhart J, van Batenburg A, Groenewald D, Jalink K. Fourth-Generation

923   Epac-Based FRET Sensors for cAMP Feature Exceptional Brightness, Photostability and

924   Dynamic Range: Characterization of Dedicated Sensors for FLIM, for Ratiometry and with High

925   Affinity. PloS one. 2015;10:e0122513.

926   35.   Hidaka H, Hayashi H, Kohri H, Kimura Y, Hosokawa T, Igawa T, et al. Selective inhibitor

927   of platelet cyclic adenosine monophosphate phosphodiesterase, cilostamide, inhibits platelet

928   aggregation. J Pharmacol Exp Ther. 1979;211:26-30.

929   36.   Schwabe U, Miyake M, Ohga Y, Daly JW. 4-(3-Cyclopentyloxy-4-methoxyphenyl)-2-

930   pyrrolidone (ZK 62711): a potent inhibitor of adenosine cyclic 3',5'-monophosphate

931   phosphodiesterases in homogenates and tissue slices from rat brain. Mol Pharmacol.

932   1976;12:900-10.

933 37.   Ahn HS, Bercovici A, Boykow G, Bronnenkant A, Chackalamannil S, Chow J, et al.

934 Potent tetracyclic guanine inhibitors of PDE1 and PDE5 cyclic guanosine monophosphate

935 phosphodiesterases with oral antihypertensive activity. J Med Chem. 1997;40:2196-210.

936 38.   Soderling SH, Beavo JA. Regulation of cAMP and cGMP signaling: new

937 phosphodiesterases and new functions. Current opinion in cell biology. 2000;12:174-9.

938 39.   Vang AG, Ben-Sasson SZ, Dong H, Kream B, DeNinno MP, Claffey MM, et al. PDE8

939 regulates rapid Teff cell adhesion and proliferation independent of ICER. PloS one.

940 2010;5(8):e12011.

941 40.   Murthy KS, Zhou H, Makhlouf GM. PKA-dependent activation of PDE3A and PDE4 and

942 inhibition of adenylyl cyclase V/VI in smooth muscle. Am J Physiol Cell Physiol. 2002;282:C508-

943 17.

944 41.   Heimann E, Jones HA, Resjo S, Manganiello VC, Stenson L, Degerman E. Expression

945 and regulation of cyclic nucleotide phosphodiesterases in human and rat pancreatic islets. PloS

946 one. 2010;5:e14191.

947 42.   DeNinno MP, Wright SW, Etienne JB, Olson TV, Rocke BN, Corbett JW, et al. Discovery

948 of triazolopyrimidine-based PDE8B inhibitors: exceptionally ligand-efficient and lipophilic ligand-

949 efficient compounds for the treatment of diabetes. Bioorg Med Chem Lett. 2012;22:5721-6.

950 43.   Liu G, Jacobo SM, Hilliard N, Hockerman GH. Differential modulation of Cav1.2 and

951 Cav1.3-mediated glucose-stimulated insulin secretion by cAMP in INS-1 cells: distinct roles for

952 exchange protein directly activated by cAMP 2 (Epac2) and protein kinase A. J Pharmacol Exp

953 Ther. 2006;318:152-60.

954 44.   Truchan NA, Brar HK, Gallagher SJ, Neuman JC, Kimple ME. A single-islet microplate

955 assay to measure mouse and human islet insulin secretion. Islets. 2015;7:e1076607.

956 45.   Gromada J, Brock B, Schmitz O, Rorsman P. Glucagon-like peptide-1: regulation of

957 insulin secretion and therapeutic potential. Basic Clin Pharmacol Toxicol. 2004;95:252-62.

958   46.   Leech CA, Chepurny OG, Holz GG. Epac2-dependent rap1 activation and the control of

959   islet insulin secretion by glucagon-like peptide-1. Vitam Horm. 2010;84:279-302.

960   47.   Kong H, Jones PP, Koop A, Zhang L, Duff HJ, Chen SR. Caffeine induces Ca2+ release

961   by reducing the threshold for luminal Ca2+ activation of the ryanodine receptor. Biochem J.

962   2008;414:441-52.

963   48.   Wang Y, Jarrard RE, Pratt EP, Guerra ML, Salyer AE, Lange AM, et al. Uncoupling of

964   Cav1.2 from Ca(2+)-induced Ca(2+) release and SK channel regulation in pancreatic beta-cells.

965   Mol Endocrinol. 2014;28:458-76.

966   49.   Garber AJ. Incretin effects on beta-cell function, replication, and mass: the human

967   perspective. Diabetes Care. 2011;34 Suppl 2:S258-63.

968   50.   Tian G, Sol ER, Xu Y, Shuai H, Tengholm A. Impaired cAMP Generation Contributes to

969   Defective Glucose-Stimulated Insulin Secretion After Long-term Exposure to Palmitate.

970   Diabetes. 2015;64:904-15.

971   51.   Biden TJ, Boslem E, Chu KY, Sue N. Lipotoxic endoplasmic reticulum stress, beta cell

972   failure, and type 2 diabetes mellitus. Trends Endocrinol Metab. 2014;25:389-98.

973   52.   Hoppa MB, Collins S, Ramracheya R, Hodson L, Amisten S, Zhang Q, et al. Chronic

974   palmitate exposure inhibits insulin secretion by dissociation of Ca(2+) channels from secretory

975   granules. Cell Metab. 2009;10:455-65.

976   53.   Brentnall M, Rodriguez-Menocal L, De Guevara RL, Cepero E, Boise LH. Caspase-9,

977   caspase-3 and caspase-7 have distinct roles during intrinsic apoptosis. BMC Cell Biol.

978   2013;14:32.

979   54.   Penmatsa H, Zhang W, Yarlagadda S, Li C, Conoley VG, Yue J, et al.

980   Compartmentalized cyclic adenosine 3',5'-monophosphate at the plasma membrane clusters

981   PDE3A and cystic fibrosis transmembrane conductance regulator into microdomains. Mol Biol

982   Cell. 2010;21:1097-110.

983   55.   Shakur Y, Takeda K, Kenan Y, Yu ZX, Rena G, Brandt D, et al. Membrane localization

984   of cyclic nucleotide phosphodiesterase 3 (PDE3). Two N-terminal domains are required for the

985   efficient targeting to, and association of, PDE3 with endoplasmic reticulum. J Biol Chem.

986   2000;275:38749-61.

987   56.   Carlisle Michel JJ, Dodge KL, Wong W, Mayer NC, Langeberg LK, Scott JD. PKA-

988   phosphorylation of PDE4D3 facilitates recruitment of the mAKAP signalling complex. Biochem

989   J. 2004;381:587-92.

990   57.   Brissova M, Fowler MJ, Nicholson WE, Chu A, Hirshberg B, Harlan DM, et al.

991   Assessment of human pancreatic islet architecture and composition by laser scanning confocal

992   microscopy. J Histochem Cytochem. 2005;53:1087-97.

993   58.   Baillie GS. Compartmentalized signalling: spatial regulation of cAMP by the action of

994   compartmentalized phosphodiesterases. FEBS J. 2009;276:1790-9.

995   59.   Richter W, Day P, Agrawal R, Bruss MD, Granier S, Wang YL, et al. Signaling from

996   beta1- and beta2-adrenergic receptors is defined by differential interactions with PDE4. EMBO

997   J. 2008;27:384-93.

998   60.   Mika D, Richter W, Conti M. A CaMKII/PDE4D negative feedback regulates cAMP

999   signaling. Proc Natl Acad Sci U S A. 2015;112:2023-8.

1000   61.   Borner S, Schwede F, Schlipp A, Berisha F, Calebiro D, Lohse MJ, et al. FRET

1001   measurements of intracellular cAMP concentrations and cAMP analog permeability in intact

1002   cells. Nat Protoc. 2011;6:427-38.

1003   62.   Koschinski A, Zaccolo M. Activation of PKA in cell requires higher concentration of

1004   cAMP than in vitro: implications for compartmentalization of cAMP signalling. Sci

1005   Rep. 2017;7:14090.

1006   63.   Omori K, Kotera J. Overview of PDEs and their regulation. Circ Res. 2007;100:309-27.

1007   64.   Walz HA, Harndahl L, Wierup N, Zmuda-Trzebiatowska E, Svennelid F, Manganiello VC,

1008   et al. Early and rapid development of insulin resistance, islet dysfunction and glucose

1009   intolerance after high-fat feeding in mice overexpressing phosphodiesterase 3B. J Endocrinol.

1010   2006;189:629-41.

1011   65.   Komatsu M, Sato Y, Yamada S, Yamauchi K, Hashizume K, Aizawa T. Triggering of

1012   insulin release by a combination of cAMP signal and nutrients: an ATP-sensitive K+ channel-

1013   independent phenomenon. Diabetes. 2002;51 Suppl 1:S29-32.

1014   66.   Shibasaki T, Sunaga Y, Fujimoto K, Kashima Y, Seino S. Interaction of ATP sensor,

1015   cAMP sensor, Ca^{2+} sensor, and voltage-dependent Ca^{2+} channel in insulin granule exocytosis. J

1016   Biol Chem. 2004;279:7956-61.

1017   67.   Vikman J, Svensson H, Huang YC, Kang Y, Andersson SA, Gaisano HY, et al.

1018   Truncation of SNAP-25 reduces the stimulatory action of cAMP on rapid exocytosis in insulin-

1019   secreting cells. Am J Physiol Endocrinol Metab. 2009;297:E452-61.

1020   68.   Dyachok O, Tufveson G, Gylfe E. Ca^{2+}-induced Ca^{2+} release by activation of inositol

1021   1,4,5-trisphosphate receptors in primary pancreatic beta-cells. Cell Calcium. 2004;36:1-9.

1022   69.   Holz GG, Leech CA, Heller RS, Castonguay M, Habener JF. cAMP-dependent

1023   mobilization of intracellular Ca^{2+} stores by activation of ryanodine receptors in pancreatic beta-

1024   cells. A Ca^{2+} signaling system stimulated by the insulinotropic hormone glucagon-like peptide-1-

1025   (7-37). J Biol Chem. 1999;274:14147-56.

1026   70.   Soulsby MD, Wojcikiewicz RJ. The type III inositol 1,4,5-trisphosphate receptor is

1027   phosphorylated by cAMP-dependent protein kinase at three sites. Biochem J. 2005;392:493-7.

1028   71.   Dzhura I, Chepurny OG, Leech CA, Roe MW, Dzhura E, Xu X, et al. Phospholipase C-

1029   epsilon links Epac2 activation to the potentiation of glucose-stimulated insulin secretion from

1030   mouse islets of Langerhans. Islets. 2011;3:121-8.

1031   72.   Pratt EP, Salyer AE, Guerra ML, Hockerman GH. Ca^{2+} influx through L-type Ca^{2+}

1032   channels and Ca^{2+}-induced Ca^{2+} release regulate cAMP accumulation and Epac1-dependent

1033   ERK 1/2 activation in INS-1 cells. Mol Cell Endocrinol. 2016;419:60-71.

1034   73.   Velasco M, Diaz-Garcia CM, Larque C, Hiriart M. Modulation of Ionic Channels and

1035   Insulin Secretion by Drugs and Hormones in Pancreatic Beta Cells. Mol Pharmacol.

1036   2016;90:341-57.

1037   74.   Dodge KL, Khouangsathiene S, Kapiloff MS, Mouton R, Hill EV, Houslay MD, et al.

1038   mAKAP assembles a protein kinase A/PDE4 phosphodiesterase cAMP signaling module.

1039   EMBO J. 2001;20:1921-30.

1040   75.   Dodge-Kafka KL, Soughayer J, Pare GC, Carlisle Michel JJ, Langeberg LK, Kapiloff MS,

1041   et al. The protein kinase A anchoring protein mAKAP coordinates two integrated cAMP effector

1042   pathways. Nature. 2005;437:574-8.

1043   76.   Nijholt IM, Dolga AM, Ostroveanu A, Luiten PG, Schmidt M, Eisel UL. Neuronal

1044   AKAP150 coordinates PKA and Epac-mediated PKB/Akt phosphorylation. Cell Signal.

1045   2008;20:1715-24.

1046   77.   Hinke SA, Navedo MF, Ulman A, Whiting JL, Nygren PJ, Tian G, et al. Anchored

1047   phosphatases modulate glucose homeostasis. EMBO J. 2012;31:3991-4004.

1048   78.   Lester LB, Langeberg LK, Scott JD. Anchoring of protein kinase A facilitates hormone-

1049   mediated insulin secretion. Proc Natl Acad Sci U S A. 1997;94:14942-7.

1050   79.   Willoughby D, Masada N, Wachten S, Pagano M, Halls ML, Everett KL, et al.

1051   AKAP79/150 interacts with AC8 and regulates Ca^{2+}-dependent cAMP synthesis in pancreatic

1052   and neuronal systems. J Biol Chem. 2010;285:20328-42.

1053   80.   Rabinovitch A, Blondel B, Murray T, Mintz DH. Cyclic adenosine-3',5'-monophosphate

1054   stimulates islet B cell replication in neonatal rat pancreatic monolayer cultures. J Clin Invest.

1055   1980;66:1065-71.

1056   81.   Cunha DA, Ladriere L, Ortis F, Igoillo-Esteve M, Gurzov EN, Lupi R, et al. Glucagon-like

1057   peptide-1 agonists protect pancreatic beta-cells from lipotoxic endoplasmic reticulum stress

1058   through upregulation of BiP and JunB. Diabetes. 2009;58:2851-62.

1059   82.   Kwon G, Pappan KL, Marshall CA, Schaffer JE, McDaniel ML. cAMP Dose-dependently

1060   prevents palmitate-induced apoptosis by both protein kinase A- and cAMP-guanine nucleotide

1061   exchange factor-dependent pathways in beta-cells. J Biol Chem. 2004;279:8938-45.

1062   83.   Malmgren S, Spegel P, Danielsson AP, Nagorny CL, Andersson LE, Nitert MD, et al.

1063   Coordinate changes in histone modifications, mRNA levels, and metabolite profiles in clonal

1064   INS-1 832/13 beta-cells accompany functional adaptations to lipotoxicity. J Biol Chem.

1065   2013;288:11973-87.

1066   84.   Costes S, Broca C, Bertrand G, Lajoix AD, Bataille D, Bockaert J, et al. ERK1/2 control

1067   phosphorylation and protein level of cAMP-responsive element-binding protein: a key role in

1068   glucose-mediated pancreatic beta-cell survival. Diabetes. 2006;55:2220-30.

1069   85.   Liu H, Tang JR, Choi YH, Napolitano M, Hockman S, Taira M, et al. Importance of

1070   cAMP-response element-binding protein in regulation of expression of the murine cyclic

1071   nucleotide phosphodiesterase 3B (Pde3b) gene in differentiating 3T3-L1 preadipocytes. J Biol

1072   Chem. 2006;281:21096-113.

1073   86.   Butler AE, Janson J, Bonner-Weir S, Ritzel R, Rizza RA, Butler PC. Beta-cell deficit and

1074   increased beta-cell apoptosis in humans with type 2 diabetes. Diabetes. 2003;52:102-10.

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1076   **Supporting Information Captions**

1077   **S1 Table- Human Islet Donor Characteristics**

1078   **S1 Fig- PDE 8-selective inhibitors don't potentiate cAMP levels or insulin secretion in**

1079   **INS-1 cells-** (A) Application of 100 nM PF4957325 on INS-1 resulted in no increase in cAMP

1080   over baseline. Subsequent application of 100 µM IBMX resulted in increased cAMP ranging

1081   from 7-49% over baseline (n= 14 cells) (B) PF4957325 does not potentiate glucose-stimulated

1082   insulin secretion in INS-1 cells. Stimulation of INS-1 cells with 18 mM glucose resulted in a
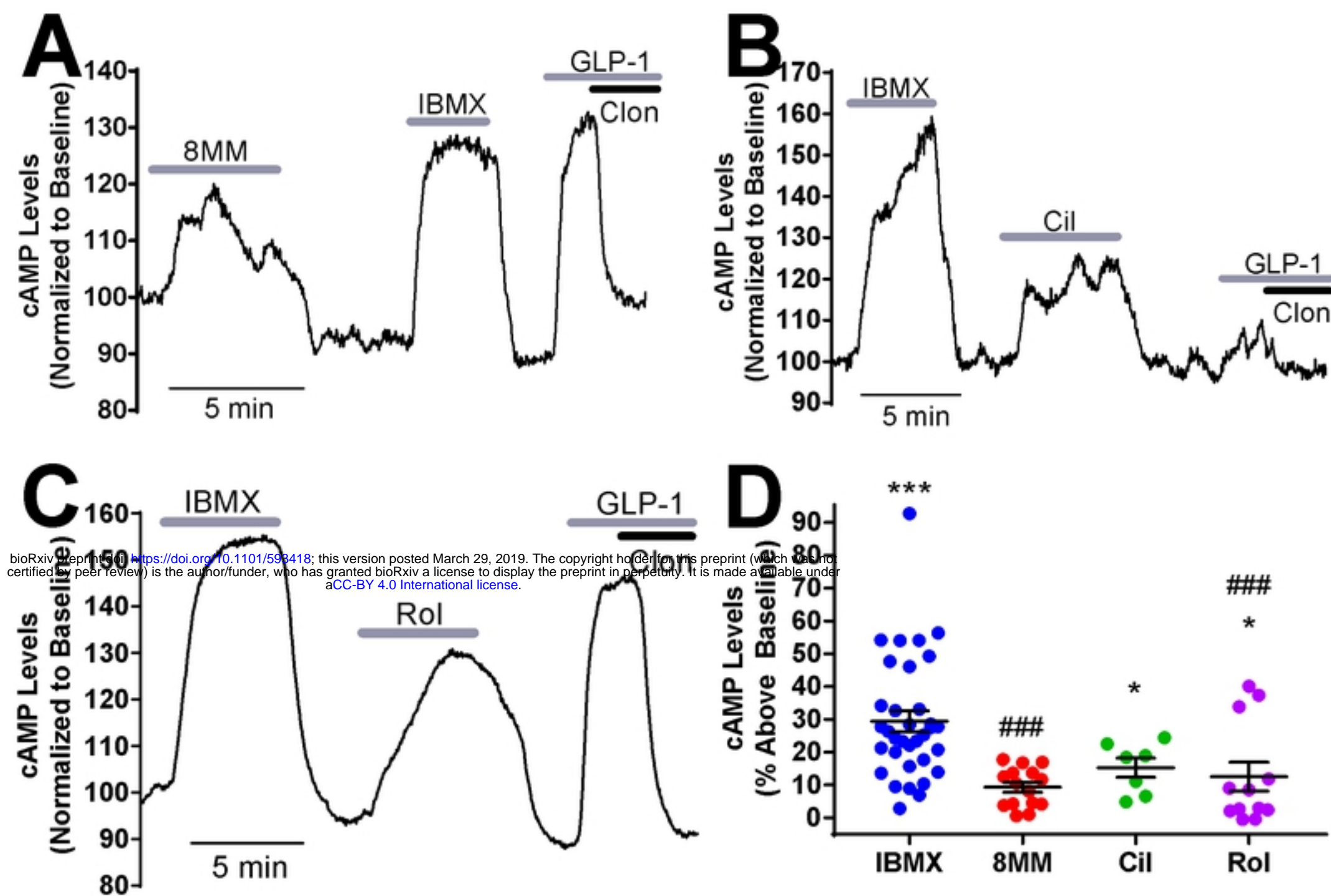
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1083   significant increase in insulin secretion over basal secretion. Co-stimulation with 100 nM

1084   PF4957325 failed to result in a significant increase in secretion compared to 18 mM glucose

1085   alone(n=3). (C) PF04671536 does not increase intracellular cAMP in INS-1 cells. Application of

1086   increasing concentrations of PF04671536 failed to increase in cAMP over baseline. Application

1087   of 100 µM IBMX resulted in increases in cAMP ranging from 23-113% over baseline (n=11

1088   cells).

1089   **S2 Fig- The PDE8-selective inhibitor PF04671536 does not increase basal cAMP levels in**

1090   **human pancreatic β-cells.** Application of 100 nM PF04671536 does not result in any increase

1091   in cAMP over baseline. Application of 100 µM IBMX increased cAMP ranging from 40-101%.

1092   GLP-1 and clonidine were used to identify β-cells (n=4).

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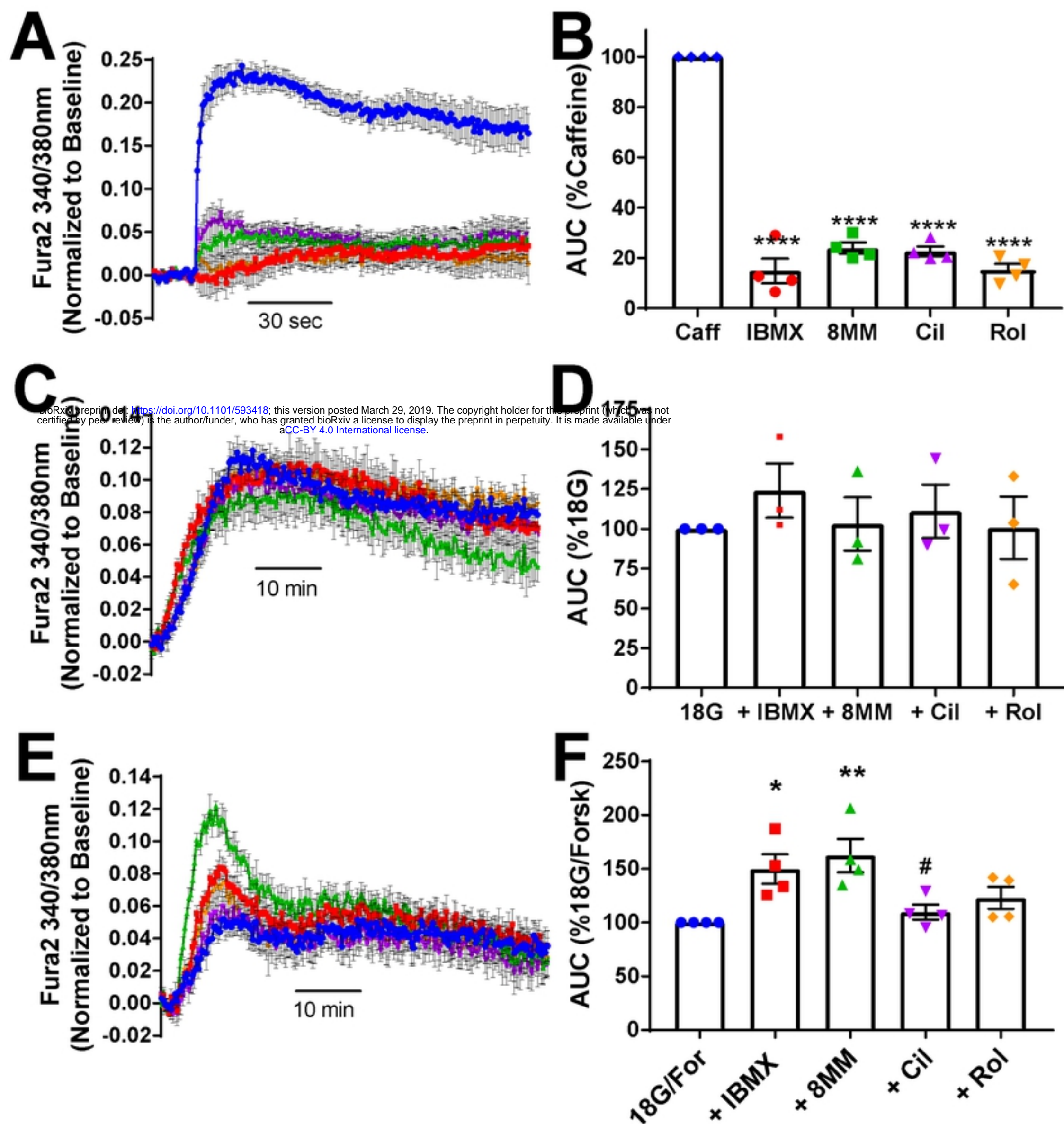
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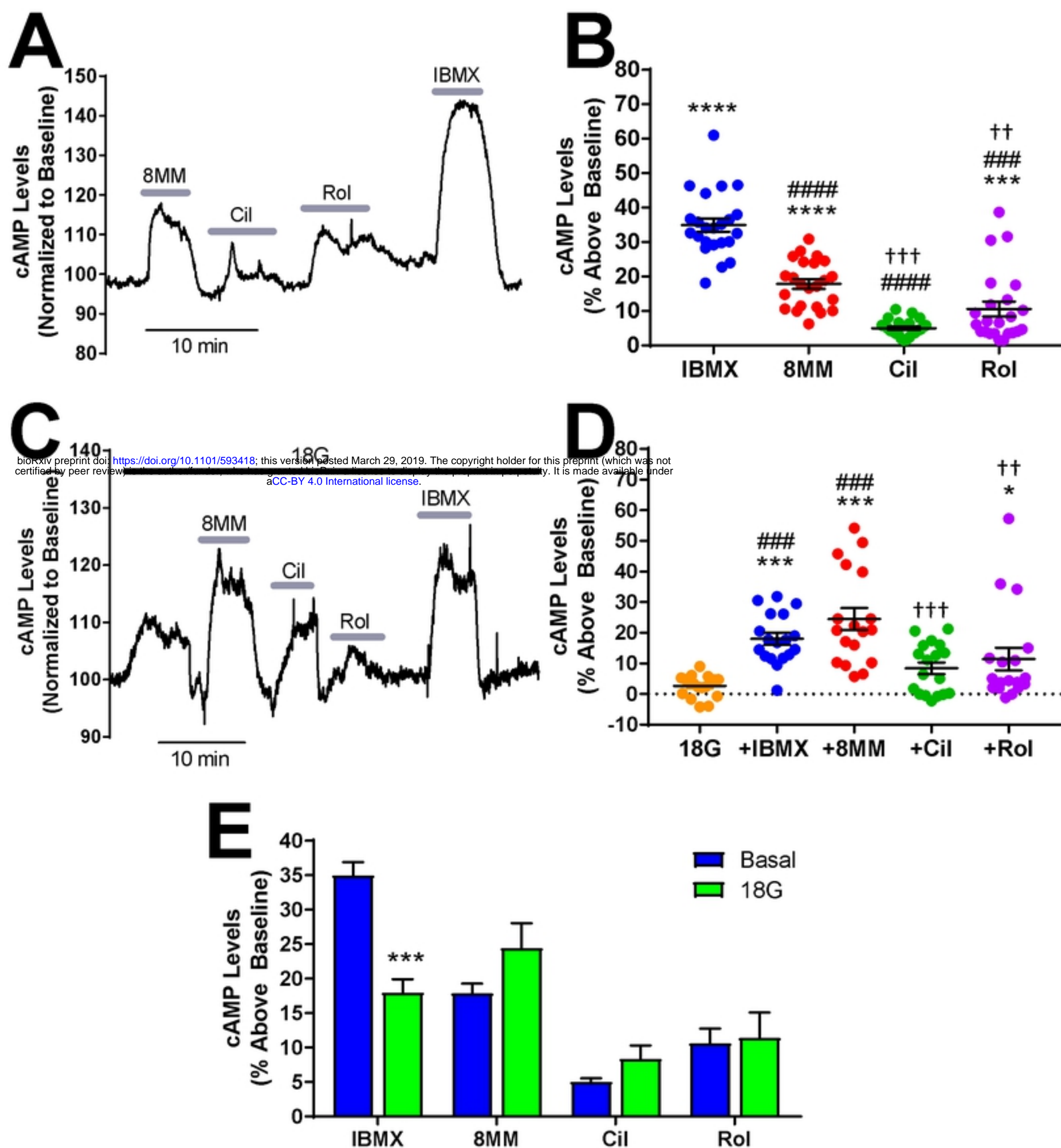
Pratt et al., Figure 1

Figure 1

Pratt et al. Figure 2

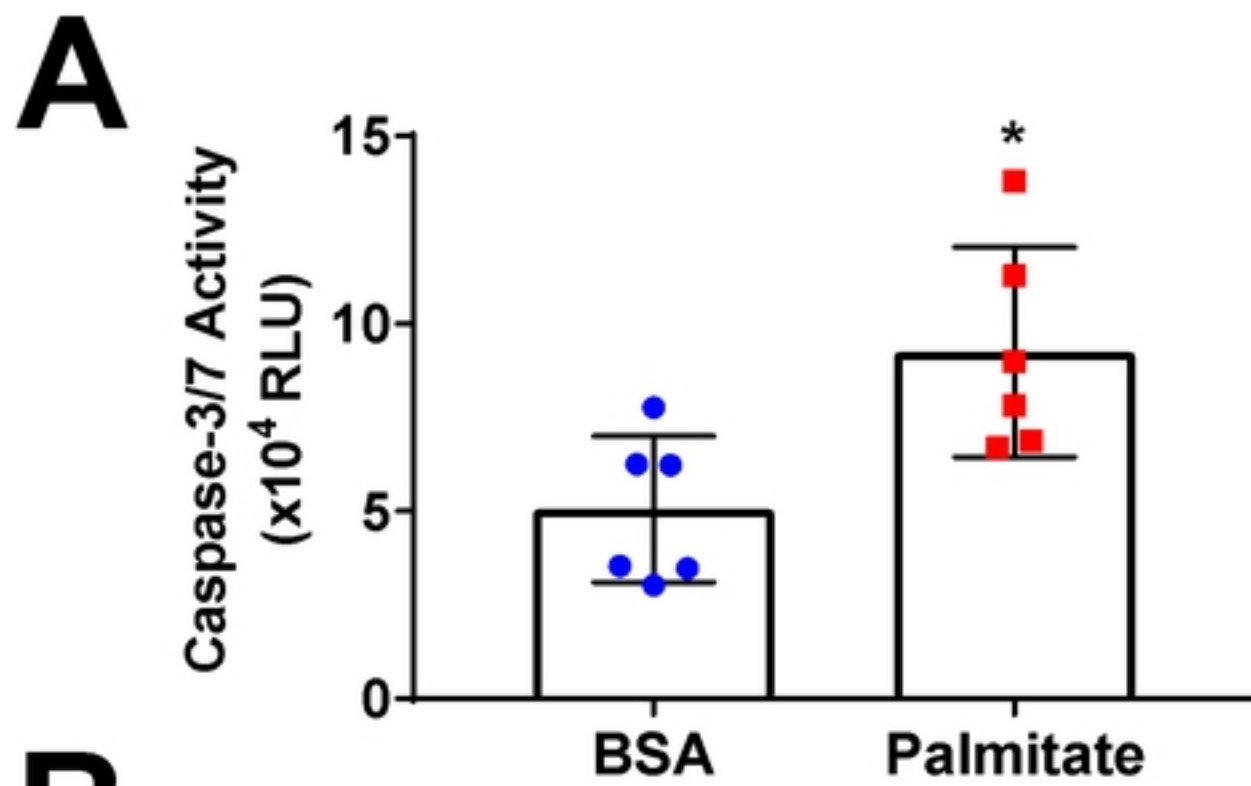Figure 2

Pratt et al. Figure 3

Figure 3

INS-1

A

B

Human Islets

Legend:
- 1.7G
- 16.7G
- + IBMX
- + 8MM-IBMX
- + Cilostamide
- + Rolipram
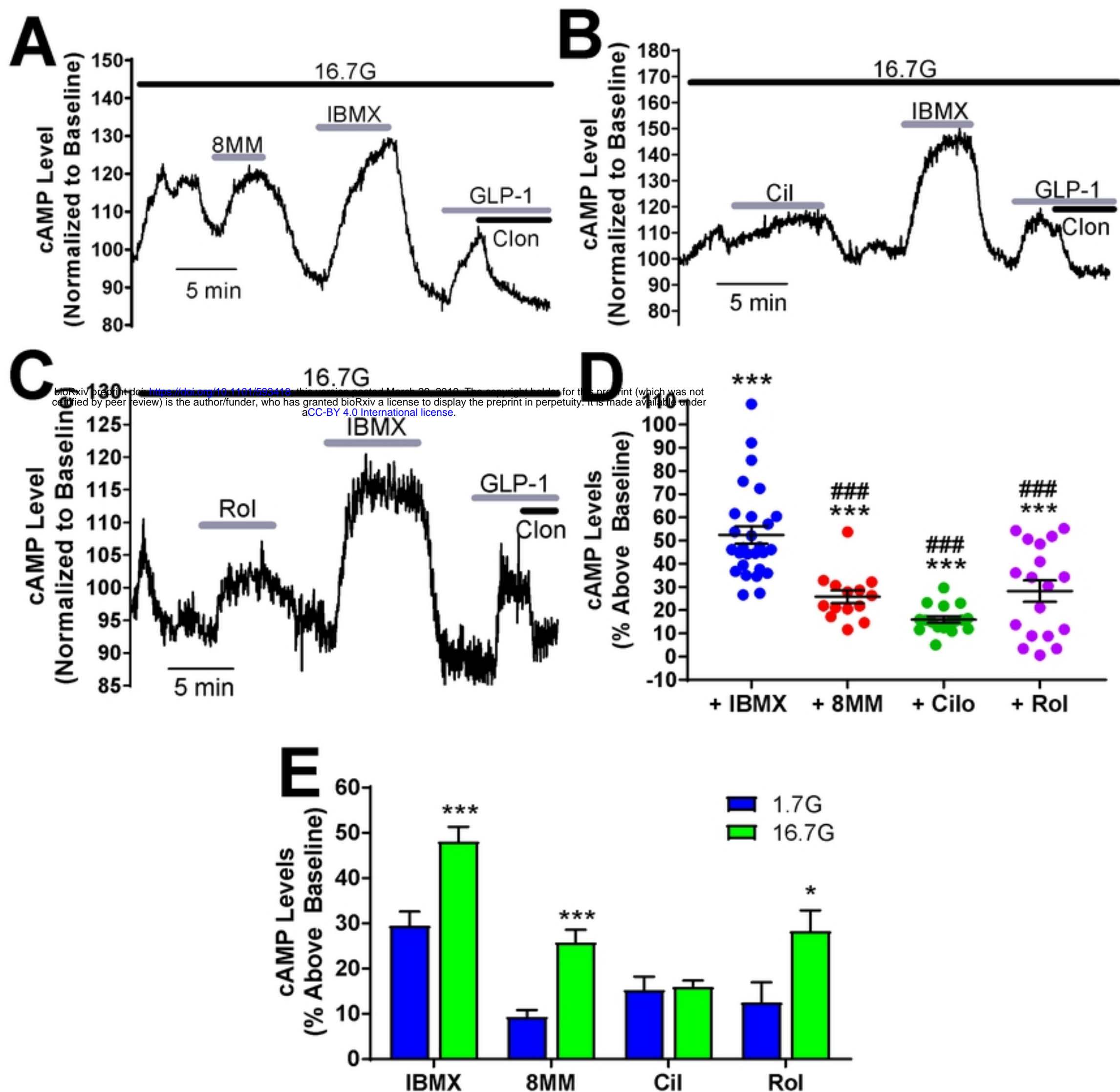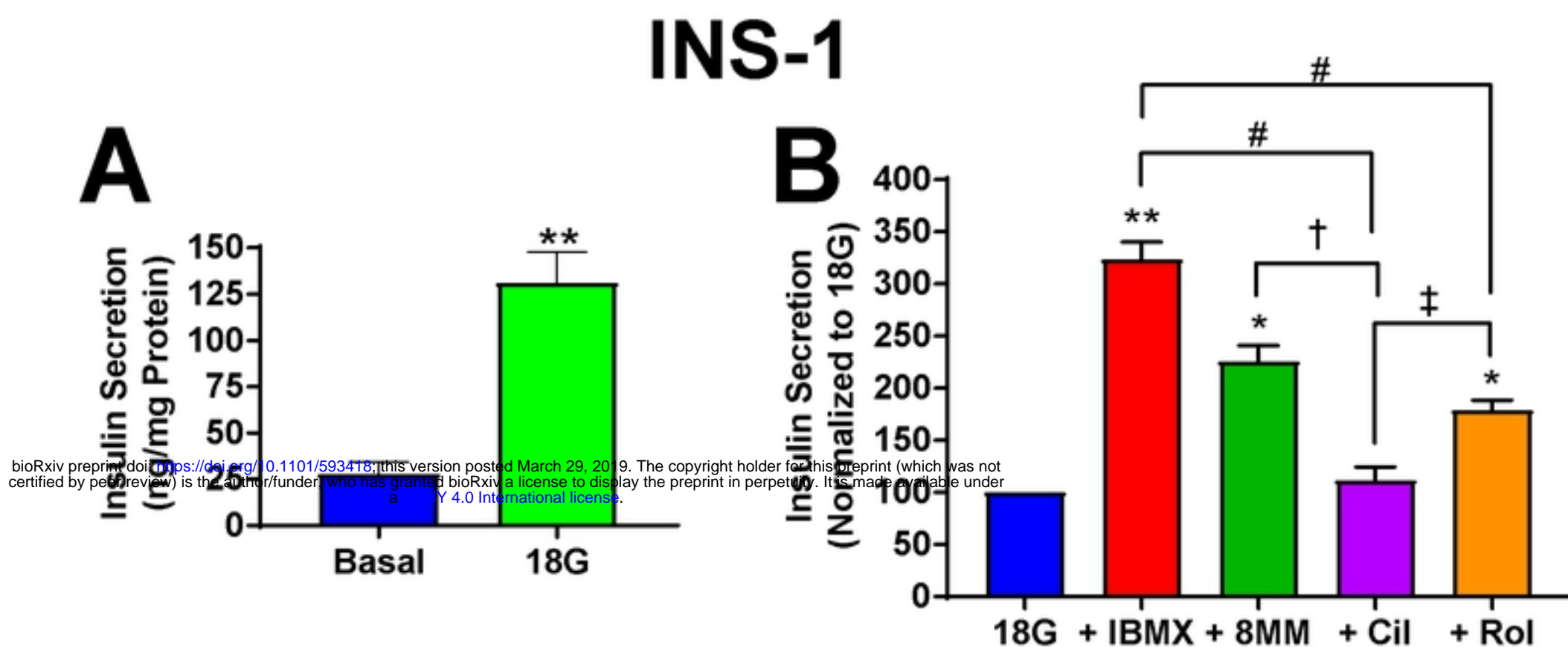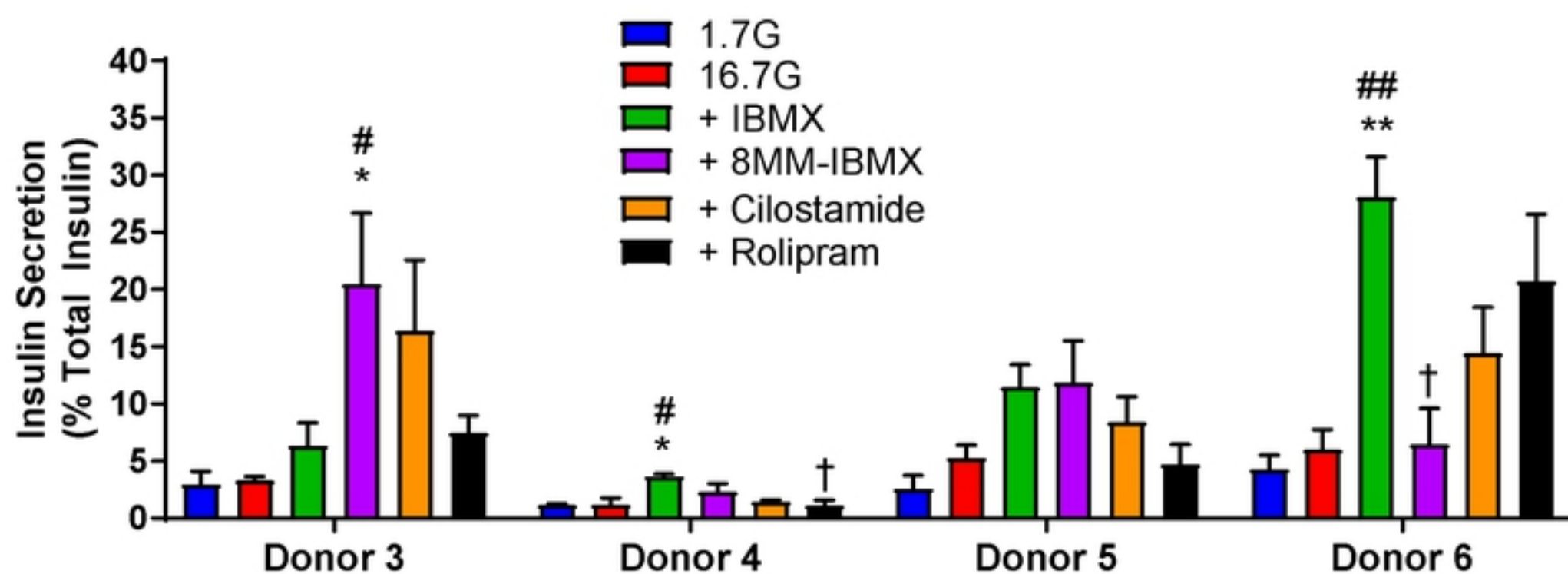
Pratt et al. Figure 4

Figure 4

Pratt et al. Figure 5

Figure 5

Pratt et al. Figure 6

Figure 6