

cAMP-Activated Protein Kinase–Independent Potentiation of Insulin Secretion by cAMP Is Impaired in SUR1 Null Islets

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Whereas the loss of ATP-sensitive K⁺ channel (K_{ATP} channel) activity in human pancreatic β-cells causes severe hypoglycemia in certain forms of hyperinsulinemic hypoglycemia, similar channel loss in sulfonylurea receptor-1 (SUR1) and Kir6.2 null mice yields a milder phenotype that is characterized by normoglycemia, unless the animals are stressed. While investigating potential compensatory mechanisms, we found that incretins, specifically glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic peptide (GIP), can increase the cAMP content of Sur1KO islets but do not potentiate glucose-stimulated insulin release. This impairment is secondary to a restriction in the ability of Sur1KO β-cells to sense cAMP correctly. Potentiation does not appear to require cAMP-activated protein kinase (PKA) because H-89 (*N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide) and KT5720, inhibitors of PKA, do not affect stimulation by GLP-1, GIP, or exendin-4 in wild-type islets, although they block phosphorylation of cAMP-response element-binding protein. The impaired incretin response in Sur1KO islets is specific; the stimulation of insulin release by other modulators, including mastoparan and activators of protein kinase C, is conserved. The results suggest that the defect responsible for the loss of cAMP-induced potentiation of insulin secretion is PKA independent. We hypothesize that a reduced release of insulin in response to incretins may contribute to the unexpected normoglycemic phenotype of Sur1KO mice versus the pronounced hypoglycemia seen in neonates with loss of K_{ATP} channel activity. *Diabetes* 51:3440–3449, 2002

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[Ca²⁺]_i, cytosolic Ca²⁺ concentration; [cAMP]_i, intracellular content of cAMP; CREB, cAMP-response element-binding protein; Epac, exchange protein directly activated by cAMP; GEF, guanine-nucleotide exchange factor; GHS, glucose-induced insulin secretion; GIP, glucose-dependent insulinotropic peptide; GLP-1, glucagon-like peptide-1; H-89, *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide; HI, hyperinsulinemic hypoglycemia; IBMX, 3-isobutyl-1-methylxanthine; K_{ATP} channel, ATP-sensitive K⁺ channel; K_d, estimated dissociation constant; K_i, constant for inhibition; KRBB, Krebs-Ringer bicarbonate buffer; PKA, cAMP-activated protein kinase; PKC, protein kinase C; PKI, protein kinase inhibitor; Rp-cAMPS, (Rp)-Adenosine 3',5'-monophosphorothioate; SUR1, sulfonylurea receptor-1; TPA, 12-O-tetradecanoylphorbol 13-acetate.

Insulin secretion is a unique example of exocytosis controlled by metabolic, ionic, and hormonal pathways. An imbalance in insulin release due to disruption of these pathways can produce the profound changes in glucose homeostasis associated with either hyperglycemia (diabetes) or hypoglycemia (e.g., hyperinsulinemic hypoglycemia [HI]). In pancreatic β-cells, ATP-sensitive K⁺ channels (K_{ATP} channels), composed of Kir6.2 and the sulfonylurea receptor-1 (SUR1), and voltage-gated Ca²⁺ channels are key players linking increased glucose metabolism to elevation of cytosolic Ca²⁺ concentration ([Ca²⁺]_i). Membrane depolarization induced by closure of K_{ATP} channels, secondary to changes in ADP/ATP resulting from glucose metabolism, leads to the generation of Ca²⁺-dependent action potentials and [Ca²⁺]_i oscillations, which are considered to be a major mediator of insulin secretion. Sulfonylureas that block β-cell K_{ATP} channels are commonly used to restore insulin secretion in type 2 diabetes. The loss or constitutive closure of β-cell K_{ATP} channels, as a result of mutations in either subunit, is a cause of both dominant and recessive forms of HI (HI-SUR1 or HI-Kir6.2), characterized by elevated plasma insulin values inconsistent with the observed hypoglycemia (reviewed in 1,2). Studies on islets isolated from patients diagnosed with HI are consistent with hypersecretion of insulin (3,4). By comparison, the clinical phenotype of mice lacking β-cell/neuronal K_{ATP} channels is strikingly normal. Kir6.2 null (Kir6.2KO) (5) and Sur1KO (6) mice are normoglycemic when fed, displaying only mild glucose intolerance, consistent with their loss of first phase and attenuated second phase of insulin release. Sur1KO mice exhibit greater hypoglycemia upon fasting, consistent with their inability to rapidly repolarize their β-cells and reduce insulin release (6). No compensating ionic mechanisms have been identified, and the electrophysiological phenotype of isolated K_{ATP}KO mouse β-cells, i.e., constant membrane depolarization, presence of Ca²⁺-dependent action potentials, and elevated oscillating [Ca²⁺]_i in low glucose, is quite similar to that of β-cells from HI neonates (compare 5–7); therefore, it is unclear why K_{ATP}KO islets lack the elevated basal insulin release observed in HI islets (3,4).

In a search for differences in the regulation of insulin secretion, we discovered that the incretins (8) glucagon-like peptide-1 (GLP-1) and glucose-dependent insulin-

tropic peptide (GIP) will increase cAMP content in both Sur1KO and wild-type islets. However, the increased cAMP potentiates glucose-induced insulin secretion only from wild-type islets, not from Sur1KO islets. We hypothesize that the impaired response of Sur1KO β -cells to elevated cAMP will reduce insulin secretion in response to incretins, and possibly other hormones, and thus provide one compensatory mechanism contributing to the normoglycemia of Sur1KO mice. Although the molecular nature of the defect remains to be described, we show that it does not impair the potentiation of insulin release by carbachol and phorbol esters and is upstream of the GTP-dependent step activated by mastoparan.

RESEARCH DESIGN AND METHODS

Animals. The generation of Sur1KO mice has been described previously (6).

Peptides. Human GIP, human GLP-1-(7-36)amide, and exendin-4 were purchased from Sigma Chemical (St. Louis, MO).

Islet isolation and insulin measurements. The methods of islet isolation and culture have been described elsewhere (6). Briefly, animals were anesthetized using sodium pentobarbital (65 mg/kg). Islets were isolated after digestion of the pancreas by intraductal injection of 1 mg/ml collagenase P (Boehringer-Mannheim, Indianapolis, IN) dissolved in Krebs-Ringer bicarbonate buffer (KRB) solution as described elsewhere (9,10). For batch experiments, the islets were incubated overnight in RPMI 1640 medium supplemented with 10% fetal bovine serum and test concentrations of glucose equilibrated with 5% CO₂/95% air, pH 7.4, at 37°C. Islets were preincubated in media containing 2.8 mmol/l glucose before incubation in 0.75 ml of medium containing test concentrations of glucose. Perfusion was done following Komatsu et al. (11) with modifications. One-hundred islets in a column of Bio-Gel P-10 (Bio-Rad, Richmond, CA) were continuously perfused at 37°C with Hepes-NaHCO₃ KRB (equilibrated with 5% CO₂/95% air, pH 7.4) at a flow rate of 0.75 ml/min. After preincubation for 30 min in 2.8 mmol/l glucose, solutions were changed as indicated in the figures. Test substances were added to the basal media without adjustment of the final osmolarity and were present from 10 through 90 min. Insulin assays were done using radioimmunoassay kits from Linco Research (St. Charles, MO) with rat insulin as a standard. cAMP was measured by radioimmunoassay using kits from Amersham Pharmacia Biotech (Piscataway, NJ).

Assay of PKA in permeabilized islets. cAMP-activated protein kinase (PKA) activity was assayed in permeabilized islets following Maller et al. (12). Briefly, islets were permeabilized at 4°C with digitonin (25 mg/l) in (mmol/l) 20 HEPES, 137 NaCl, 5.4 KCl, 0.3 NaH₂PO₄, 0.4 KH₂PO₄, 10 MgCl₂, 25 Na β -glycerol phosphate, 5 EGTA, 5.6 mmol/l glucose, and 20 μ mol/l Na₃VO₄, pH 7.2, and incubated with 10 μ mol/l ATP plus 2.5 μ Ci [γ -³²P]ATP and 0.1 μ mol/l "Kemptide" (Leu-Arg-Arg-Ala-Ser-Leu-Gly) in the absence or presence of 5 μ mol/l cAMP, 5 μ mol/l cAMP plus 10 nmol/l protein kinase inhibitor (PKI), the classic Walsh heat-stable PKI (13), or 5 μ mol/l cAMP plus H-89 (*N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide), a synthetic PKA inhibitor (14). After a 10-min incubation at 30°C, the phosphorylated peptide was isolated on DEAE-cellulose, washed three times with ice-cold 75 mmol/l H₃PO₄, and counted in a scintillation counter.

Assay of phosphorylated and total cAMP-response element-binding protein (CREB) in wild-type islets. Islets were isolated into RPMI medium supplemented with 1% BSA and 25 mmol/l HEPES, pH 7.4, and then cultured overnight in RPMI supplemented with 10% fetal bovine serum, 10 mmol/l HEPES, pH 7.4, 2 mmol/l glutamine, 1 mmol/l pyruvate, and 50 μ mol/l β -mercaptoethanol. Islets were washed once with RPMI containing 2.8 mmol/l glucose and 1% BSA and then washed three times with a KRB solution buffered with 25 mmol/l HEPES, pH 7.4, supplemented with 1% BSA and 2.8 mmol/l glucose (10-min incubation per wash). The washed islets were preincubated for 40 min with 3-isobutyl-1-methylxanthine (IBMX; 500 μ mol/l) or IBMX plus 5 or 12.5 μ mol/l H-89 or KT5720 before being stimulated for 20 min by addition of glucose to 16.7 mmol/l. The islets were solubilized with boiling SDS sample buffer, subject to electrophoresis, and transferred to nitrocellulose. Antiphosphorylated CREB and anti-CREB antibodies (Cell Signaling Technology, Beverly, MA) were used following the manufacturers recommendations, detected using an horseradish peroxidase-conjugated second-step anti-mouse antibody (Sigma Chemical, St. Louis, MO), and visualized by chemiluminescence.

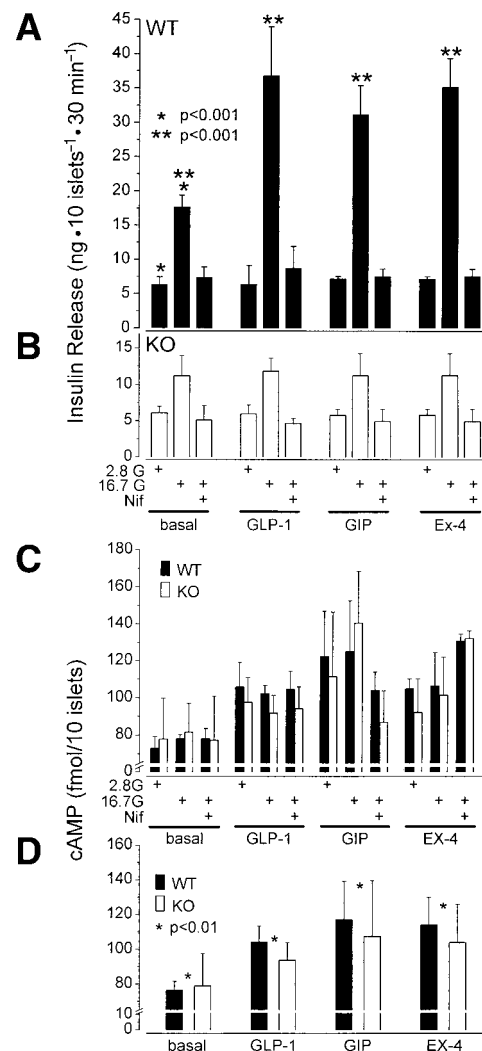


FIG. 1. Incretins increase [cAMP]_i but fail to potentiate glucose-induced insulin secretion from Sur1KO islets. Islets were incubated overnight in RPMI 1640 medium plus 11.1 mmol/l glucose (G) and 10% fetal bovine serum. Insulin secretion (A and B) or cAMP content (C and D) was measured after 30 min in KRB solution (6) containing 2.8 mmol/l glucose, 16.7 mmol/l glucose, or 16.7 mmol/l glucose plus 1 μ mol/l nifedipine in the presence of GLP-1 (10 nmol/l), GIP (10 nmol/l), or exendin-4 (Ex-4; 10 nmol/l) as indicated. D: Averaged cAMP contents for wild-type and Sur1KO islets for the four treatment groups in C. The values for islets treated with incretins are significantly different from the basal level ($P < 0.01$). The means \pm SD from four independent experiments are shown for all data.

RESULTS

Glucose-induced insulin release from Sur1KO islets is not potentiated by incretins. Based on the estimated dissociation constant (K_D) for GLP-1 (~200 pmol/l) (15) and plasma levels of GLP-1-(7-3)amide in mice after challenge with gastric glucose (~15 pmol/l) (16), we used saturating concentrations (10 nmol/l) of GLP-1-(7-36)amide, GIP, and exendin-4, a GLP-1 receptor agonist (17), to show that these peptides stimulate insulin secretion from wild-type but not Sur1KO islets (Fig. 1A and B). Stimulation of wild-type islets requires elevated glucose and is abolished by the L-type Ca²⁺ channel blocker, nifedipine. The incretins comparably increase the steady-state islet cAMP content, indicating that the lack of response of Sur1KO islets is not due to altered coupling of incretin receptors with adenylyl cyclases (Fig. 1C). One-

way ANOVA indicated that there were no significant differences between the wild-type and Sur1KO values within a group or with glucose or nifedipine concentration; therefore, we averaged the wild-type and Sur1KO values within a group (Fig. 1D).

The potentiation of insulin release by cAMP is impaired in Sur1KO islets. To manipulate the cAMP content further, we inhibited phosphodiesterases with IBMX and stimulated adenylyl cyclases with forskolin. The intracellular content of cAMP ($[cAMP]_i$) is elevated similarly in both wild-type and Sur1KO islets after incubation (30 min) with forskolin, IBMX, or both agents (Fig. 2A). One-way ANOVA indicates there are no significant differences between the conditions within the four treatment groups, i.e., no treatment, plus IBMX, plus forskolin, or plus both agents; therefore, we averaged the values for the four islet groups. The grouped data are 86.79 ± 33.02 , 254.87 ± 116.25 , $2,471.9 \pm 948.87$, and $10,905 \pm 3,167.3$ fmol of cAMP/10 islets \pm SD for no treatment versus treatments with IBMX, forskolin, or IBMX plus forskolin, respectively. ANOVA on this grouped data indicates that the approximate threefold difference with and without the addition of IBMX does not reach statistical significance ($P > 0.05$), whereas the differences between the control (no treatment) and other treatments are highly significant ($P < 0.001$).

Figure 2B shows the result of elevating $[cAMP]_i$ on glucose-dependent insulin release from wild-type versus Sur1KO islets. The data in Fig. 2A and B were obtained from the same islets and are replotted in Fig. 2C, giving insulin release as a function of cAMP content. Increased $[cAMP]_i$ has little effect on insulin release from wild-type islets in low glucose (2.8 mmol/l), whereas in high glucose (16.7 mmol/l), even the threefold average increase in $[cAMP]_i$ after treatment with IBMX potentiates insulin secretion ~ 15 -fold above basal. In depolarized Sur1KO islets (6) with elevated $[Ca^{2+}]_i$ (18,19), superphysiologic concentrations of cAMP, >100 -fold above basal values, significantly increased insulin release even in low glucose. Insulin release from Sur1KO islets in low glucose was about six times the basal rate of release or nearly twice as great as that obtained from wild-type islets in high glucose alone. In high glucose, Sur1KO islets exhibit a restricted stimulatory response at all $[cAMP]_i$ values relative to that observed from wild-type islets (Fig. 2B and C). The effect is most evident at low $[cAMP]_i$ values where wild-type islets display their full response. The difference in insulin release from Sur1KO islets in low and high glucose, both with elevated $[Ca^{2+}]_i$, is consistent with the known Ca^{2+} -dependent stimulation of insulin secretion by products of glucose metabolism (20–23, reviewed in 24) and long-chain acyl CoA (25, reviewed in 26). Consistent with earlier reports (27,28), increasing glucose metabolism did not raise $[cAMP]_i$ in wild-type or Sur1KO islets significantly. The data show that there is no significant difference in the ability of wild-type versus Sur1KO islets to generate cAMP and suggest that the impairment in Sur1KO animals is in a cAMP-effector pathway.

Perfusion experiments were done at different $[cAMP]_i$ to examine the kinetics of glucose-induced insulin secretion and confirm the loss of cAMP responsiveness in Sur1KO islets. To compare the responses of wild-type

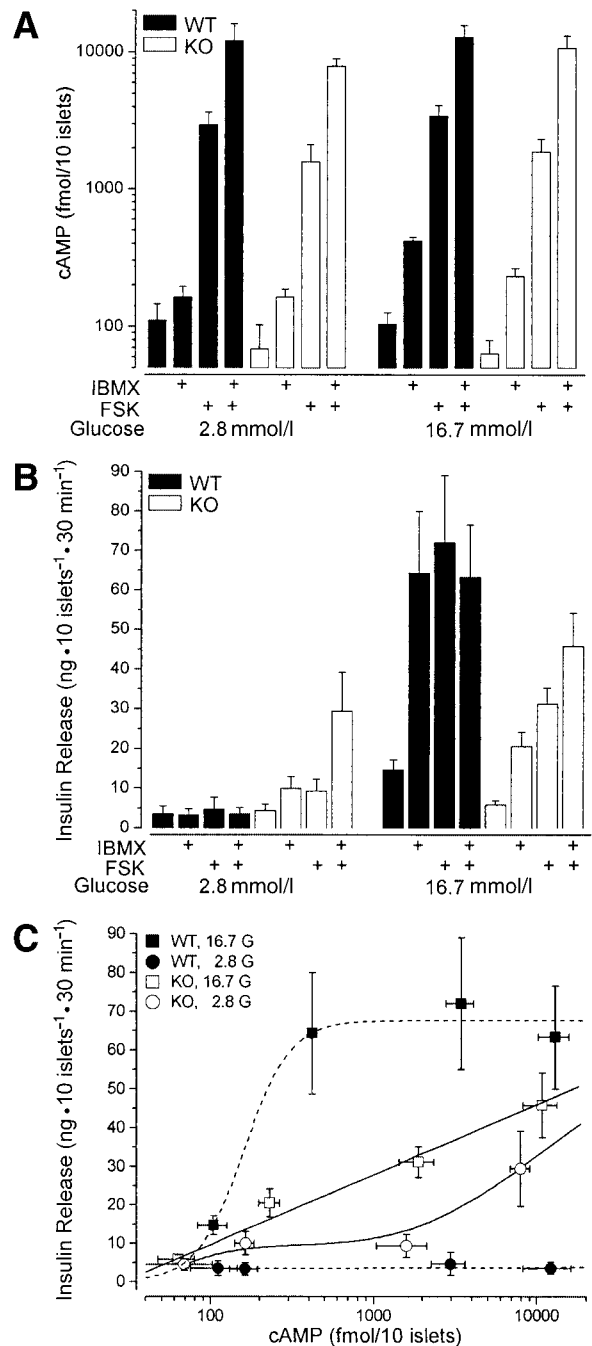


FIG. 2. Dependence of insulin release on the cAMP content of wild-type and Sur1KO islets. To determine the effect of cAMP content on insulin release, freshly isolated islets were incubated in KRB solution supplemented with IBMX (500 μ mol/l), forskolin (10 μ mol/l), or both agents for 30 min with either 2.8 or 16.7 mmol/l glucose as indicated. **A:** Islets were collected and extracted by boiling in Na-acetate buffer (0.2 mmol/l, pH 6.2) for determination of cAMP. **B:** Collected media were assayed for insulin. **C:** Insulin secretion data plotted versus the islet cAMP content. The solid lines identify the Sur1KO data, and dashed lines are used for the wild-type data. The data are the means \pm SD from four or five independent experiments.

versus Sur1KO islets directly, we calculated the stimulated glucose-induced insulin secretion (GIIS) by subtracting the secretory values induced by high glucose alone from the values obtained when the stimulating agent was present. The procedure is illustrated in Fig. 3A using IBMX-potentiated glucose-induced insulin secretion as an

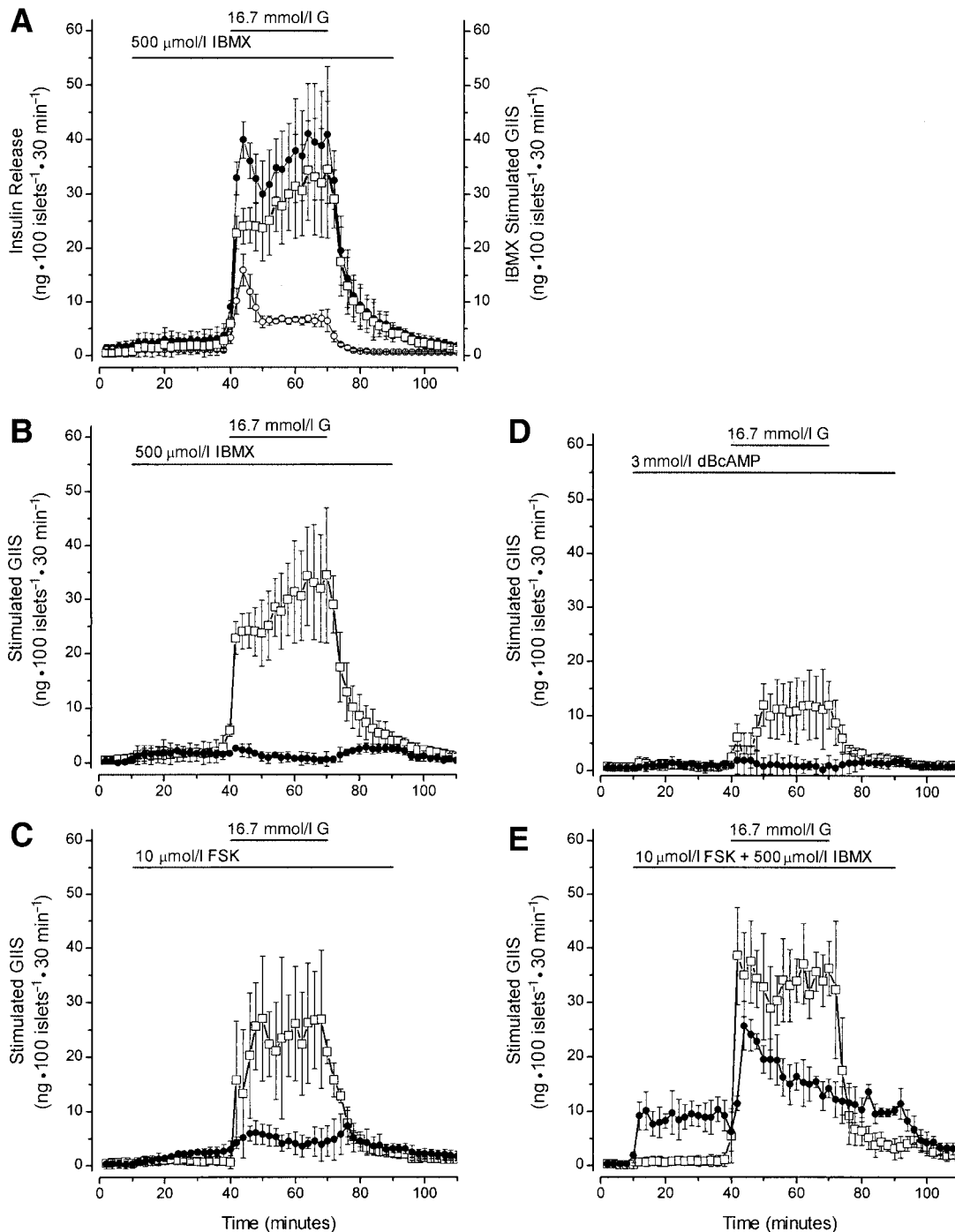


FIG. 3. Kinetics of glucose-induced insulin release from wild-type versus Sur1KO islets at different $[cAMP]_i$. **A:** Potentiation of insulin release from wild-type islets by 500 $\mu\text{mol/l}$ IBMX, which elevates islet cAMP content approximately threefold. To facilitate comparison of the data from wild-type and Sur1KO islets under the different conditions, we have defined the difference between the mean insulin values from treated and untreated islets to equal the stimulated glucose-induced insulin secretion or "stimulated GIIS." Insulin values are given on the left axis, and the Stimulated GIIS values are given on the right axis. IBMX (500 $\mu\text{mol/l}$) was present from 10 to 90 min. **B–E:** Comparison of the stimulated GIIS response (values on left axes) from wild-type and Sur1KO islets challenged with IBMX (**B**), forskolin (FSK) (**C**), dibutyryl-cAMP (dBcAMP) (**D**), or IBMX plus forskolin (**E**). The concentrations of each agent are given over the bars indicating time of application. The glucose (G) concentration was elevated from 2.8 to 16.7 mmol/l, as indicated by the bars. The data are the means \pm SD from three experiments.

example. Fig. 3B–E compare stimulated GIIS from Sur1KO with wild-type islets in which $[cAMP]_i$ was varied with IBMX, dibutyryl cAMP, forskolin, or forskolin plus IBMX, respectively. At all $[cAMP]_i$ values, the response of Sur1KO islets is impaired versus wild-type islets. The differential response of Sur1KO versus wild-type islets in high versus low glucose is most evident in Fig. 3E, where $[cAMP]_i$ is

elevated ~ 100 -fold (Fig. 2) and significantly stimulates insulin release in 2.8 mmol/l glucose. The results show that superphysiologic $[cAMP]_i$ together with elevated $[Ca^{2+}]_i$ can drive insulin release, even when the rate of glucose metabolism is low.

Insulin release from Sur1KO islets is potentiated by activators of protein kinase C pathways. Activation of

protein kinase C (PKC) pathways by 500 nmol/l 12-O-tetradecanoylphorbol 13-acetate (TPA) stimulates insulin release from both wild-type and Sur1KO islets. In high glucose, the rates of release from wild-type versus Sur1KO islets were indistinguishable (Fig. 4A), suggesting saturation of the stimulatory effect. In low glucose, TPA significantly potentiates secretion from Sur1KO versus wild-type islets. Carbamylcholine (100 μ mol/l) potentiates insulin release from wild-type islets (Fig. 4B); stimulation is greatest in high glucose but is significant even in low glucose. Insulin secretion from Sur1KO islets is markedly stimulated by carbamylcholine in both low and high glucose (Fig. 4C). Comparison of the release stimulated by glucose alone versus glucose plus carbamylcholine shows that there is a quantitatively greater effect on insulin release from Sur1KO islets, $\sim 3.7 \pm 0.2$ -fold more than the control versus $\sim 2.0 \pm 0.1$ -fold more for wild type, due mainly to the lower rate of secretion from the knockout islets. The results are consistent with the idea that the pathways by which PKC stimulates insulin secretion remain functional in Sur1KO islets.

The potentiation of glucose-induced insulin release from wild-type islets is not sensitive to PKA inhibitors. It is not well understood where cAMP acts in the exocytotic pathway. Some have suggested that phosphorylation via PKA is critical for the action of cAMP in β -cells (29–32), but an early study (28) and recent reports indicate PKA-independent pathways are important in islets (33–36) and in the regulation of other endocrine systems (37). We tested whether inhibitors of PKA would block phosphorylation in wild-type islets and thus mimic the incretin refractory phenotype of Sur1KO islets. Three PKA inhibitors were used: H-89 (14), the reportedly more specific KT5720 (38), and (Rp)-Adenosine 3',5'-monophosphothioate (Rp-cAMPS) (39,40), which inhibits PKA and is resistant to phosphodiesterase but is expected to compete with cAMP for binding sites on Epac (exchange protein directly activated by cAMP)/cAMP-GEF (guanine-nucleotide exchange factor) proteins (41,42) without their activation (43). Neither H-89 or KT5720 markedly altered the potentiating effect of GLP-1 (Fig. 5) at 5 μ mol/l, a concentration ~ 100 -fold greater than the constants for inhibition (K_i s) of PKA (48 and 60 nmol/l for H-89 [14] and KT5720 [38]), respectively. Rp-cAMPS (100 μ mol/l; K_i for PKA ~ 11 μ mol/l) did significantly inhibit potentiation of insulin release by elevated $[cAMP]_i$, reducing secretion to $\sim 30\%$ of the control.

Figure 6A and B summarize dose-response curves for H-89 and KT5720 on insulin release from wild-type islets. Neither H-89 (25 μ mol/l) nor KT5720 (25 μ mol/l) produced a statistically significant effect when all the data were analyzed by ANOVA. Preincubation with H-89 did not significantly affect the potentiation of glucose-induced insulin release by different concentrations of IBMX (Fig. 6C); the estimated half-maximal activation values for IBMX were 9.5 ± 3.3 vs. 13.8 ± 1.2 μ mol/l in the presence and absence of 5 μ mol/l H-89, respectively. Perfusion experiments (Fig. 6D) show preincubation of islets with H-89 (5 μ mol/l) does not markedly affect the kinetics of insulin release.

Wild-type and Sur1KO islets have equivalent levels of PKA that are inhibited by H-89 and KT5720. Digitonin

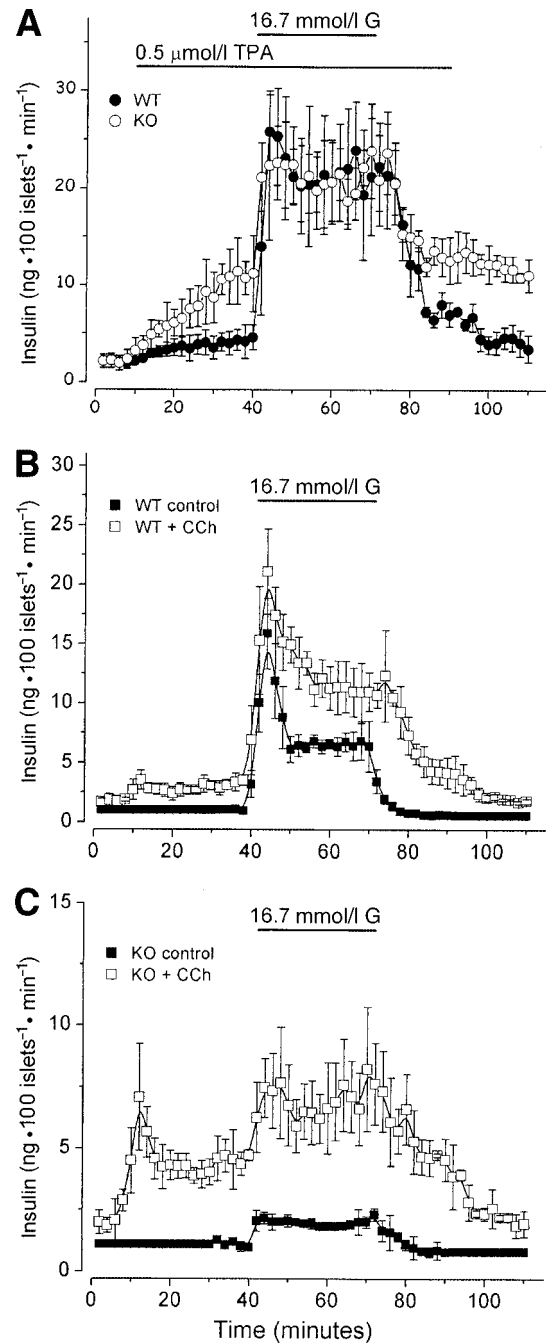


FIG. 4. Activators of PKC potentiate glucose-induced insulin secretion from both wild-type and Sur1KO islets. **A:** TPA (500 nmol/l) potently stimulates glucose-induced insulin release from both wild-type and Sur1KO islets, and the rates of release in 16.7 mmol/l glucose (G) are the same. Note that TPA also stimulates secretion from Sur1KO islets, with elevated $[Ca^{2+}]_i$, even in 2.8 mmol/l glucose. For comparison, the control secretion data, in the absence of TPA, for wild-type and Sur1KO islets are given in **B** and **C**, respectively. **B** and **C:** Wild-type (**B**) and Sur1KO islets (**C**) were assayed in the presence or absence of 100 μ mol/l carbamylcholine (CCh). CCh was present from 10 through 90 min in **B** and **C**. The stimulatory effect in the presence of elevated glucose is quantitatively greater for Sur1KO islets (**C**), increasing secretion $\sim 3.7 \pm 0.2$ -fold above the control vs. $\sim 2.0 \pm 0.1$ -fold for wild type (**B**), based on total insulin released between 40 and 80 min. The glucose concentration was elevated from 2.8 to 16.7 mmol/l, as indicated by the bars. The data are the means \pm SD from three experiments.

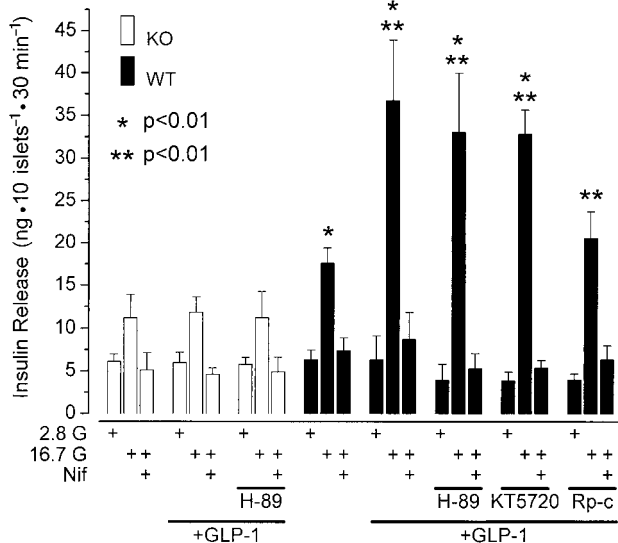


FIG. 5. PKA inhibitors do not markedly affect the potentiation of glucose-induced insulin release by GLP-1. Insulin secretion from isolated islets was determined as described in Fig. 1. Islets were incubated with or without 10 nmol/l GLP-1. H-89 and KT5720 were used at 5 μ mol/l, Rp-cAMP (Rp-c) was used at 100 μ mol/l, and nifedipine (Nif) was used at 1 μ mol/l. The data are the means \pm SD from four independent experiments.

(25 mg/l)-permeabilized islets incubated with γ [³²P] ATP and a PKA substrate, Leu-Arg-Arg-Ala-Ser-Leu-Gly (12), were used to estimate PKA activity. The results show that both wild-type and Sur1KO islets have comparable levels of kinase activity, which is stimulated 8- to 10-fold by 5 μ mol/l cAMP, is inhibited by nanomolar concentrations of PKI (13) and by H-89 (Fig. 7A).

To show that H-89 and KT5720 were able to inhibit PKA in intact islet preparations, we assessed their ability to block cAMP-stimulated phosphorylation of CREB in intact wild-type islets under the same conditions used to test their effect on insulin release. Although influenced by a variety of signals, including Ca²⁺, growth factors, and cellular stress (see 37,44 for reviews), phosphorylation of CREB is used as a reporter of cAMP-dependent kinase activity. H-89 (Fig. 7A) and KT5720 inhibited the increase in islet CREB phosphorylation observed when [cAMP]_i was increased by IBMX treatment. The results are expressed as the ratio of phosphorylated CREB to total CREB, estimated using specific antibodies (Fig. 7C). Basal phosphorylation of CREB due to the activity of other kinases is approximately one-third the level stimulated by addition of IBMX. H-89 and KT5720 (5 μ mol/l) completely blocked the stimulatory effect of IBMX on CREB phosphorylation consistent with their hydrophobic nature and estimated K_D values in the nanomolar range (14,38). The results indicate that both compounds can enter islets and inhibit PKA(s) and confirm a report in insulinoma cells and rat islets (45).

The insulinotropic peptide mastoparan stimulates insulin release from Sur1KO islets. Mastoparan is a cationic, amphiphilic, 14 amino acid peptide that stimulates insulin release (46) reportedly at a late stage of exocytosis (47,48), an effect that is attenuated by pertussis toxin (46), independent of increased [Ca²⁺]_i (47,49), augmented by nutrients, and dependent on GTP (48). Mastoparan prompts insulin release from Sur1KO islets in the

absence of external Ca²⁺, and this effect is augmented by glucose, 3.5 \pm 0.8-fold for wild-type vs. 1.6 \pm 0.9-fold for Sur1KO islets (Fig. 8). The apparent lower rates of insulin release from Sur1KO islets are consistent with their lower insulin content. The insulin contents (ng/50 islets; mean \pm SD) for males are 5,192.2 \pm 344.3 vs. 2,904.2 \pm 556.1 and for females are 4,774.5 \pm 572.7 vs. 2,174.4 \pm 498.3, for wild-type versus Sur1KO islets, respectively. The values for fractional secretion for the three mastoparan challenges show that the normalized rates of secretion are essentially the same. The fractional values for each challenge are 1) 0.6 \pm 0.1 vs. 1.4 \pm 0.6, 2) 2.0 \pm 0.3 vs. 2.3 \pm 1.0, 3) 2.0 \pm 0.5 vs. 2.4 \pm 1.0, given as percentage of total insulin content \pm SD for wild-type versus Sur1KO islets, respectively. The augmentation by glucose appears to be abolished by prior treatment with mastoparan, because a third exposure to mastoparan in the absence of glucose gives an equivalent bolus of insulin to that obtained with 16.7 mmol/l glucose. The results suggest there is no general secretory defect in Sur1KO β -cells and are consistent with the idea that the cAMP-dependent step is upstream of the GTP-dependent Ca²⁺-independent step targeted by mastoparan (Fig. 8).

DISCUSSION

We have shown that isolated islets from Sur1KO mice, lacking β -cell/neuronal-type K_{ATP} channels, have an impaired insulin secretory response to cAMP. One physiologic consequence of this biochemical defect is that although the cAMP content of Sur1KO islets increases upon stimulation with incretins, the rate of insulin release is not affected, consistent with a defect in cAMP sensing. Our data suggest the cAMP response in wild-type islets is nonlinear; a small increase in [cAMP]_i (<50% above basal values) is sufficient to increase insulin release two- to threefold when the glucose concentration is high. Blocking phosphodiesterase activity with IBMX elevates [cAMP]_i about threefold above basal values, which is sufficient to saturate the potentiating effect of cAMP on glucose-induced insulin release from wild-type islets. Superphysiologic [cAMP]_i, \sim 100-fold higher than basal levels, obtained with IBMX plus forskolin, has no stimulatory effect on insulin release from wild-type islets in low glucose, but does strongly potentiate insulin release from Sur1KO islets, which have elevated [Ca²⁺]_i, even in low glucose. The impaired response to incretins does not appear to result from a generalized defect in exocytosis, since insulin release is comparably stimulated from wild-type and Sur1KO islets by activators of PKC pathways and by mastoparan, which is reported to activate exocytosis at a late, GTP-dependent step (47,48).

What biochemical mechanisms are impaired when SUR1 is missing is not known. SUR1 is the regulatory subunit of SUR1/Kir6.2, β -cell/neuronal-type K_{ATP} channels, but has not generally been considered to interact with other proteins or known to serve a function in exocytosis beyond modulating Ca²⁺ entry via regulation of membrane potential. K_{ATP} channels are reported to be inhibited by GLP-1 (50). Various mechanisms have been put forward to account for this inhibition, including phosphorylation of SUR1/Kir6.2 subunits by PKA (although phosphorylation by PKA [51] or by PKC [52] is also reported to increase the

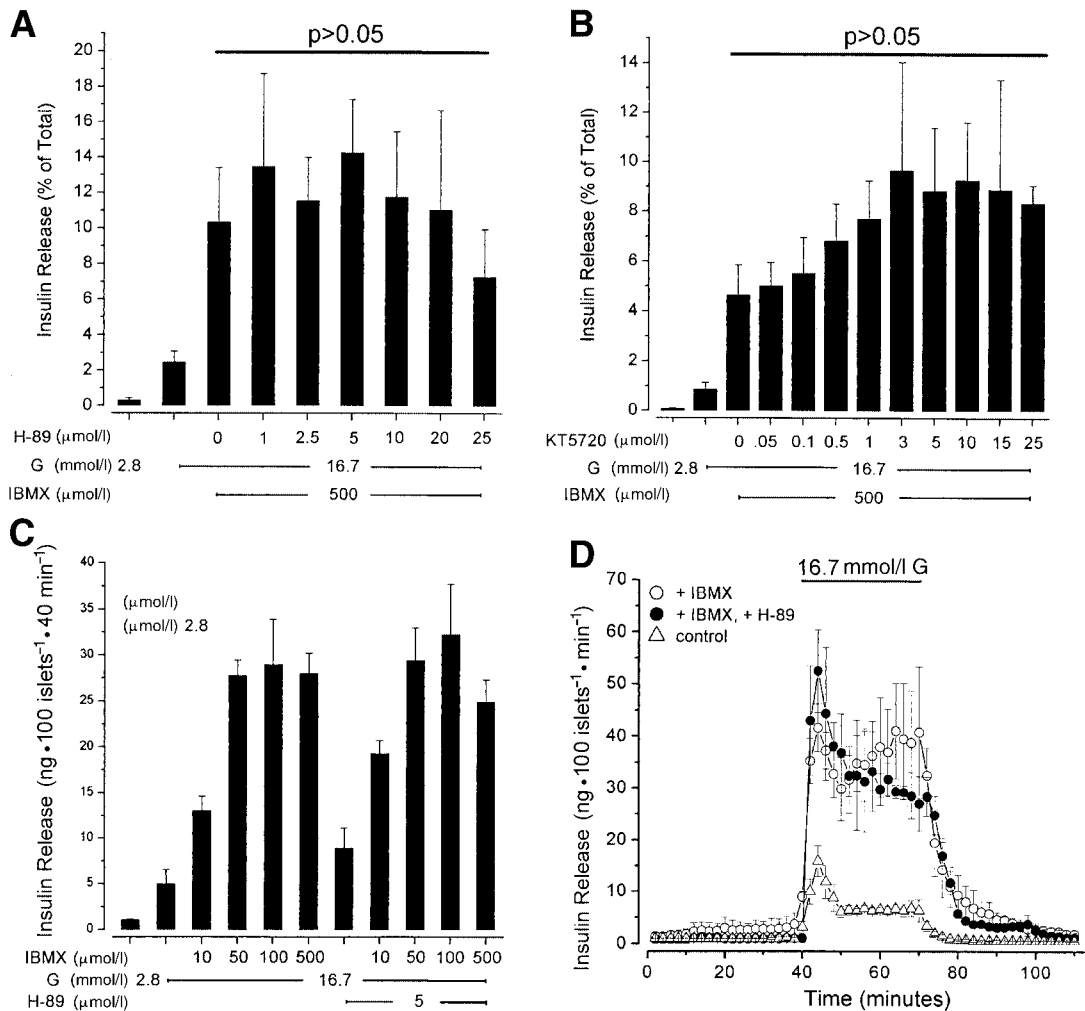


FIG. 6. Summary of the effects of H-89 and KT5720 on glucose-induced insulin release from wild-type islets. Effect of increasing concentrations of H-89 (A) and KT5720 (B) on the potentiation of insulin release by elevated [cAMP]_i. C: H-89 (5 μmol/l) does not affect the potentiation of insulin release by different concentrations of IBMX. D: Islets were perfused with H-89 for 40 min before switching to media containing IBMX, which was present from 10 through 90 min. The inhibitor does not significantly affect the kinetics of glucose-induced insulin release potentiated by elevated [cAMP]_i. Insulin secretion from isolated islets was determined as described in Fig. 1. The values are the means ± SD from three or more experiments.

open probability of K_{ATP} channels), by direct binding of G-protein $\beta\gamma$ subunits to SUR1 (53), by stimulation of glucose transport or metabolism (50), and by increasing the sensitivity of K_{ATP} channels to inhibitory ATP (54). We think the simple explanation that loss of a potential GLP-1 target, i.e., K_{ATP} channels, can account for the observed loss of incretin sensitivity is unlikely. If GLP-1 stimulates secretion by inhibiting K_{ATP} channel activity, then insulin secretion from Sur1KO islets, a model system for constitutively closed K_{ATP} channels, should exhibit sustained oversecretion equivalent to that stimulated by GLP-1, rather than the moderate, regulated secretion we observed.

How increased cAMP potentiates insulin secretion is not well understood. A number of reports have stressed the importance of PKA-dependent phosphorylation (29–32), but some early reports argued that basal and glucose-induced insulin secretion are PKA independent (28,55). Exocytosis has been reported to be activated directly by cAMP (33,56), and two recent reports (34,36) have described interactions between SUR1, Epac2/cAMP-GEF II (41,42), Rab3A, and Rim, proteins involved in vesicle

trafficking and fusion (57). Kashima et al. (36) report both PKA-dependent and -independent effects of increased [cAMP]_i on glucose-induced secretion from islets treated with antisense oligonucleotides. We observed that two PKA inhibitors, H-89 and KT5720, had no statistically significant effect on stimulation of wild-type insulin secretion by GLP-1 or by IBMX and forskolin. The reason(s) for the different responses in these studies is not clear. A third PKA inhibitor, Rp-cAMPS, did significantly inhibit glucose-induced insulin secretion. Our results are consistent with the possibility that SUR1, or SUR1 assembled with Kir6.2, can interact with a cAMP sensing pathway in β -cells, possibly Epac2/cAMP-GEFII, as suggested by Ozaki et al. (34), that does not use PKA as an effector. The results suggest the impaired reaction is upstream of the step(s) activated by the Ca^{2+} -independent GTP-dependent reaction(s) activated by mastoparan.

What contribution the impaired secretory response to cAMP of Sur1KO islets makes to the normoglycemia of the knockout mice versus the profound hypoglycemia of HI neonates lacking functional K_{ATP} channels is not known. We considered several possibilities. First, that loss of the

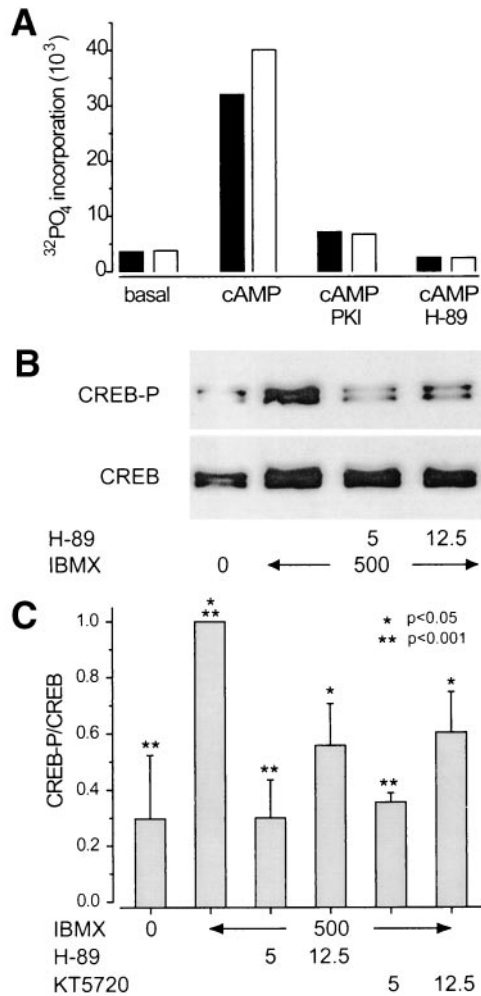


FIG. 7. H-89 and KT5720 inhibit β -cell PKA. **A:** Wild-type (■) and Sur1KO (□) islets were permeabilized with digitonin and then incubated with Kemptide, a synthetic peptide PKA substrate, and [γ -³²P]ATP to assay PKA activity stimulated by cAMP (5 μ M). The estimated PKA activities were comparable. PKI (10 nmol/l) and H-89 (1 μ M) inhibited transfer of ³²P-ATP. **B:** The level of phosphorylation of CREB was estimated using antiphosphorylated CREB and anti-CREB antibodies. IBMX was used at 500 μ M to elevate cAMP; H-89 and KT5720 were used at 5 and 12.5 μ M to inhibit PKA. **C:** The ratio of phosphorylated CREB to total CREB was estimated by densitometry of bands on X-ray films. Data from three experiments were normalized by setting the stimulated values to 1. The results are the means \pm SD.

incretin response in Sur1KO islets disrupts the enteroinsular axis in the animals, reducing insulin secretion and glucose disposal, thus contributing to their normoglycemia. There is little insulin release after intraperitoneal injection of glucose into Kir6.2KO (5) and Sur1KO animals (6), and we anticipate that postprandial insulin levels in Sur1KO mice should be reduced versus wild type. However, although the data are controversial, GLP-1 has been reported to increase glucose uptake in peripheral tissues (compare 58,59 with 60–62) and thus may contribute to glucose disposal in Sur1KO mice. We are not aware of any data on the incretin responsiveness of hyperinsulinemic-hypoglycemic neonates. A second possibility is that there is a basic difference in cAMP sensing in mouse versus human islets, as has been suggested to explain the difference in kinetics of second-phase secretion of mouse versus rat and human islets (63). The available comparative data for knockout mouse versus HI islets are insuffi-

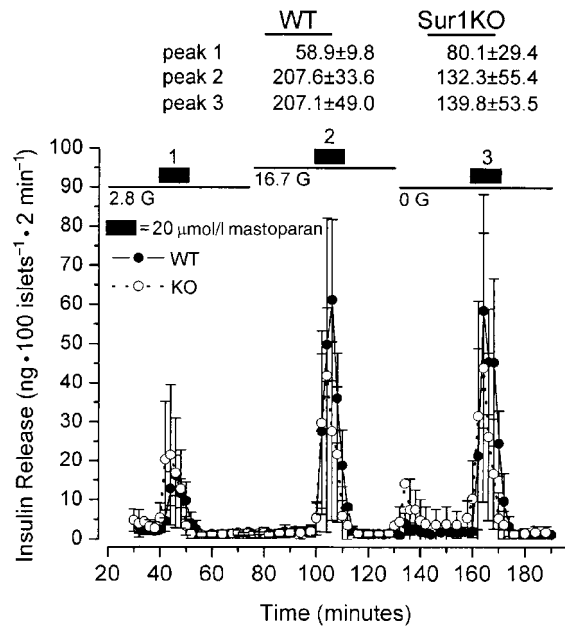


FIG. 8. Mastoparan stimulates insulin release from WT and Sur1KO islets. Freshly isolated islets were stimulated with 20 μ M mastoparan (■) in KRB medium (6), supplemented with 1 mmol/l EGTA, at the indicated concentrations of glucose (G). The total insulin (ng/100 islets) secreted with each mastoparan challenge is summarized at the top. The values are the means \pm SD for $n = 4$ (WT) or $n = 3$ (Sur1KO) experiments. The values for total insulin (\pm SD) were obtained by summing the values under the peaks and applying standard error propagation analysis.

cient to address this question. Rates of insulin turnover have not been compared directly, but the insulin content of Sur1KO islets reported here is \sim 60% of wild-type values versus \sim 2.5% reported for HI islets (4), suggesting a higher turnover rate for the latter. Kaiser et al. (3) have also reported that cells derived from HI islets exhibit a high rate of glucose-independent insulin secretion, which is stimulated further by IBMX and forskolin. Similarly, Straub et al. (4) have shown that secretion from HI β -cells, determined by capacitance measurements, is potentiated by forskolin. However, in these studies, as in our experiments with IBMX and forskolin, [cAMP]_i is increased to superphysiologic levels where insulin secretion is significantly increased in the Sur1KO islets, and it is difficult to extrapolate to a physiologic condition. Finally, it is important to consider that SUR1-regulated K⁺ channels are present in nonpancreatic tissues, where their loss could contribute to glycemic control, for example, through counterregulatory responses that may well differ between rodents and humans.

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