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Exendin-(9–39) Corrects Fasting Hypoglycemia in *SUR-1*$^{-/-}$ Mice by Lowering cAMP in Pancreatic β-Cells and Inhibiting Insulin Secretion*

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Congenital hyperinsulinism is a disorder of pancreatic β-cell function characterized by failure to suppress insulin secretion in the setting of hypoglycemia, resulting in brain damage or death if untreated. Loss-of-function mutations in the K_{ATP} channel (composed of two subunits: Kir6.2 and SUR-1) are responsible for the most common and severe form of congenital hyperinsulinism. Most patients are unresponsive to available medical therapy and require palliative pancreatectomy. Similar to the human condition, the *SUR-1*$^{-/-}$ mouse is hypoglycemic when fasted and hyperglycemic when glucose-loaded. We have previously reported that the glucagon-like peptide-1 receptor antagonist exendin-(9–39) raises fasting blood glucose in normal mice. Here we examine the effect of exendin-(9–39) on fasting blood glucose in *SUR-1*$^{-/-}$ mice. Mice were randomized to receive exendin-(9–39) or vehicle. Fasting blood glucose levels in *SUR-1*$^{-/-}$ mice treated with exendin-(9–39) were significantly higher than in vehicle-treated mice and not different from wild-type littermates. Exendin-(9–39) did not further worsen glucose tolerance and had no effect on body weight and insulin sensitivity. Isolated islet perifusion studies demonstrated that exendin-(9–39) blocked amino acid-stimulated insulin secretion, which is abnormally increased in *SUR-1*$^{-/-}$ islets. Furthermore, cAMP content in *SUR-1*$^{-/-}$ islets was reduced by exendin-(9–39) both basally and when stimulated by amino acids, whereas cytosolic calcium levels were not affected. These findings suggest that cAMP plays a key role in K_{ATP}-independent insulin secretion and that the GLP-1 receptor is constitutively active in *SUR-1*$^{-/-}$ β-cells. Our findings indicate that exendin-(9–39) normalizes fasting hypoglycemia in *SUR-1*$^{-/-}$ mice via a direct effect on insulin secretion, thereby raising exendin-(9–39) as a potential therapeutic agent for K_{ATP} hyperinsulinism.

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Congenital hyperinsulinism is a genetic disorder of pancreatic β-cell function characterized by failure to suppress insulin secretion in the setting of hypoglycemia, resulting in brain damage or death if inadequately treated. Loss-of-function mutations in the K_{ATP}-sensitive channel (composed of two subunits: Kir6.2 and SUR-1) are responsible for the most common and severe form of hyperinsulinism (K_{ATP}HI). K_{ATP}-sensitive channels couple the metabolic state of the β-cell to membrane potential by sensing changes in intracellular ATP concentration. In pancreatic β-cells, closure of the channel in response to elevation of the ATP/ADP ratio following stimulation with glucose leads to depolarization of the membrane and activation of voltage-dependent calcium channels with resultant exocytosis of insulin-containing granules (1). Thus, K_{ATP}-sensitive channels play a critical role in the triggering pathway of glucose-stimulated insulin secretion (2). In contrast, the amplifying pathway of insulin release operates independently of K_{ATP}-sensitive channels and functions to augment fuel-stimulated insulin secretion (3). Electrophysiological studies of islets from infants with K_{ATP}HI show reduction of K_{ATP} channel activity and spontaneously active voltage-dependent Ca^{2+} channels (reviewed in Ref. 4). Diazoxide, the mainstay of medical therapy for hyperinsulinism, suppresses insulin by promoting the opening of the β-cell K_{ATP} channel and is ineffective in patients with K_{ATP}HI. Thus, most of these patients require surgical palliation by near-total pancreatectomy, which is not curative but carries a high risk of either persistent hypoglycemia or insulin-requiring diabetes (5).

An animal model harboring a targeted inactivation of the SUR-1 gene (*SUR-1*$^{-/-}$ mouse) reproduces the key pathophysiological features of K_{ATP}HI. *SUR-1*$^{-/-}$ mice are both significantly more hypoglycemic when fasted and significantly more hyperglycemic when glucose-loaded compared with control animals (6). Interestingly, *SUR-1*$^{-/-}$ mice secrete a nearly normal amount of insulin in response to a meal despite markedly impaired glucose-stimulated insulin secretion (7). Isolated

SUR-1$^{-/-}$ islets exhibit all of the features expected to result from nonfunctional K_{ATP} channels, including β-cell depolarization (6) and elevation of intracellular calcium (7–9). Furthermore, base-line insulin release in cultured *SUR-1*$^{-/-}$ islets is higher compared with control islets (8, 9). Seghers *et al.* (6) demonstrated a lack of first phase insulin secretion in response to glucose and attenuated second phase insulin release in *SUR-1*$^{-/-}$ islets. The authors concluded that in the absence of K_{ATP} channels, insulin release is regulated by a slow, K_{ATP}-independent glucose stimulated mechanism. In contrast to the defect in glucose-stimulated insulin release, isolated *SUR-1*$^{-/-}$ islets are hypersensitive to amino acids. After stimulation with an amino acid mixture, insulin release increases by 3-fold in *SUR-1*$^{-/-}$ islets, whereas normal islets do not respond to amino acids in the absence of a concomitant glucose stimulus. Glutamine plays a prominent role in mediating amino acid stimulation of insulin release in *SUR-1*$^{-/-}$ islets, and this effect requires functional calcium channels as well as β-cell depolarization (8).

GLP-1 (glucagon-like peptide-1) is an incretin hormone secreted by the intestinal L-cells in response to ingested nutrients that acts on β-cells to augment insulin release in response to glucose (10). GLP-1 stimulates insulin secretion by binding to a guanine nucleotide binding protein-coupled receptor (11), resulting in the activation of adenylate cyclase and generation of cAMP (12). GLP-1 and agents that increase cAMP levels stimulate insulin secretion by both PKA-dependent and -independent mechanisms (13). Studies of the GLP-1 response in *SUR-1*$^{-/-}$ islets have provided mixed results. Nakazaki *et al.* (14) reported that despite a normal cAMP elevation to GLP-1, stimulation with GLP-1 did not affect insulin release in *SUR-1*$^{-/-}$ islets. In contrast, Eliasson *et al.* (15) demonstrated that GLP-1 stimulates insulin secretion in *SUR-1*$^{-/-}$ islets, although the magnitude of the response was reduced to ~50% of wild-type islets. The latter study also found an impairment of the PKA-independent component of exocytosis in *SUR-1*$^{-/-}$ islets (15). Further, Doliba *et al.* (16) demonstrated that GLP-1 (as well as acetylcholine) restores glucose-responsive insulin secretion in *SUR-1*$^{-/-}$ islets, again supporting the concept that elevation of intracellular cAMP levels further augments insulin secretion in the face of persistently high intracellular calcium levels.

Exendin-(9–39), a derivative of the nonmammalian peptide exendin-4, acts as a specific and competitive antagonist of the GLP-1 receptor and impairs glucose tolerance in humans (17) as well as in a variety of animal models (18–20). Studies in murine β-cells have shown that exendin-(9–39) decreases basal cAMP levels, acting as an inverse agonist of the GLP-1 receptor (21). We have previously shown that exendin-(9–39) results in a persistent elevation of fasting blood glucose levels in wild-type BALB/c mice (22). Based on this observation and on the evidence for GLP-1 responsiveness of *SUR-1*$^{-/-}$ islets, we hypothesized that exendin-(9–39) would increase fasting blood glucose in *SUR-1*$^{-/-}$ mice. Here we show that antagonism of the GLP-1 receptor by exendin-(9–39) corrects fasting hypoglycemia in *SUR-1*$^{-/-}$ mice via a direct effect on insulin secretion. Further, exendin-(9–39) decreases basal and stimulated cAMP levels significantly in isolated *SUR-1*$^{-/-}$ islets, mirroring its impact on the insulin secretory response of these islets. Taken together, these data identify exendin-(9–39) as a potential therapeutic agent in human patients with congenital hyperinsulinism.

MATERIALS AND METHODS

Animals—SUR-1$^{-/-}$ mice were kindly provided by Dr. Mark A. Magnuson. The generation and genotyping of *SUR-1*$^{-/-}$ mice were previously described (7). Mice are maintained in a C57Bl/6 genetic background. 12–18-week *SUR-1*$^{-/-}$ and wild-type littermate control mice were used in all experiments. Mice were maintained on a 12/12-h light/dark cycle and were fed a standard rodent chow diet. All procedures were approved and carried out according to the University of Pennsylvania Institutional Animal Care and Use Committee guidelines.

Exendin-(9–39) Administration—Alzet miniosmotic pumps (model 2002; Alza, Palo Alto, CA) were implanted subcutaneously to deliver exendin-(9–39) (Bachem Bioscience, King of Prussia, PA) at a rate of 150 pmol/kg/min or vehicle (0.9% NaCl, 1% bovine serum albumin) for 2 weeks.

Glucose Homeostasis—For determination of fasting blood glucose levels, mice were fasted for 12–16 h. Oral glucose tolerance testing was carried after a 12–16-h fast by administering 2 g/kg of dextrose by oral gavage (feeding needles; Popper and Sons, Inc., Hyde Park, NY). For insulin tolerance testing, mice received 0.5 units/kg of insulin intraperitoneally after a 4-h fast. Blood glucose levels were measured using a hand-held glucose meter (FreeStyle; TheraSense, Alameda, CA). Insulin and glucagon were measured by ELISA (Mouse Endocrine Immunoassay Panel; Linco Research, Inc., St. Charles, MO).

Islet Studies—Islets were isolated by collagenase digestion and cultured for 3 days in RPMI 1640 medium containing 10 mM glucose. The culture medium was supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, and 50 μg/ml streptomycin. Islets were incubated at 37 °C in a 5% CO_2, 95% air-humidified incubator. Batches of 100 cultured mouse islets were loaded onto a nylon filter in a chamber and perifused with Krebs-Ringer bicarbonate buffer (115 mM NaCl, 24 mM $NaHCO_3$, 5 mM KCl, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$, 10 mM HEPES, pH 7.4) with 0.25% bovine serum albumin at a flow rate of 2 ml/min. Perifusate solutions were gassed with 95% O_2, 5% CO_2 and maintained at 37 °C. Islets were stimulated with a ramp of amino acids. The mixture of 19 amino acids when used at a maximum concentration of 12 mM (about 3 times physiological concentration) had the following composition: 2 mM glutamine, 1.25 mM alanine, 0.53 mM arginine, 0.11 mM aspartate, 0.27 mM citrulline, 0.35 mM glutamate, 0.85 mM glycine, 0.22 mM histidine, 0.27 mM isoleucine, 0.46 mM leucine, 1.06 mM lysine, 0.14 mM methionine, 0.20 mM ornithine, 0.23 mM phenylalanine, 1 mM proline, 1.62 mM serine, 0.77 mM threonine, 0.21 mM tryptophan, 0.57 mM valine. Samples were collected every minute for insulin assays. Insulin was measured by radioimmunoassay (Linco Research Inc., St. Charles, MO).

cAMP Content Determination—Islets were isolated as above, hand-picked, and cultured for 3 days. Cultured islets were preincubated in glucose-free Krebs-Ringer bicarbonate buffer for 60 min, and 100 nM exendin-(9–39) was added 30 min into the preincubation period. Then, islets were exposed to different treatments for an additional 30 min in the presence of 0.1 mM

isobutylmethylzanthine. After incubation, islets were washed two times by cold glucose-free Hanks' buffer. cAMP was measured in islet lysates by an enzyme-linked immunosorbent assay (GE Healthcare).

Cytosolic Free Ca^{2+} Measurements—Mouse islets were isolated and cultured on poly-L-lysine-coated glass coverslips under the same conditions as described above. The perifusion procedure and cytosolic-free Ca^{2+} ($[Ca^{2+}]_i$) measurement were described previously (23). In brief, the coverslip with attached islets was incubated with 15 μM Fura-2 acetoxymethylester (Molecular Probes, Inc., Eugene, OR) in Krebs-Ringer bicarbonate buffer with 5 mM glucose for 35 min at 37 °C. Islets were then perifused with Krebs-Ringer bicarbonate buffer with 0.25% bovine serum albumin at 37 °C at a flow rate of 2 ml/min while various agents were applied. $[Ca^{2+}]_i$ was measured with a dual wavelength fluorescence microscope as previously described.

Statistical Evaluation—Data presented are mean ± S.E. and compared using Student's *t* test. For glucose and insulin tolerance testing, values were compared by repeated measures ANOVA.[3] Differences were considered significant at $p < 0.05$.

RESULTS

Twelve–18-week-old male *SUR-1*$^{-/-}$ and wild-type littermates underwent a base-line evaluation, including fasting blood glucose measurements and oral glucose tolerance testing, followed by randomization to treatment with exendin-(9–39) (150 pmol/kg/min) or vehicle (0.9% NaCl, 1% bovine serum albumin). Fasting blood glucose levels were determined after an overnight fast on days 3 and 7 of the infusion. In addition, oral glucose tolerance and insulin sensitivity were evaluated during treatment.

As previously reported (7), fasting blood glucose levels were significantly lower in *SUR-1*$^{-/-}$ mice compared with wild-type littermates (59.4 ± 1.5 mg/dl *versus* 75 ± 1.8 mg/dl; $p = 0.00000003$) (Fig. 1*A*), whereas body weight was not different (Fig. 1*B*). After an oral load of glucose, *SUR-1*$^{-/-}$ mice were glucose-intolerant when compared with littermate wild-type controls ($p < 0.0001$, repeated measures ANOVA) (Fig. 1*C*). The *SUR-1*$^{-/-}$ mice have a significant impairment of insulin secretion in response to an oral glucose load (wild-type *versus* *SUR-1*$^{-/-}$; $p = 0.02$; repeated measures ANOVA) (Fig. 1*D*).

Exendin-(9–39) was administered via an Azlet miniosmotic subcutaneous pump at a continuous infusion rate of 150 pmol/kg/min for 2 weeks. This dose was chosen based on results of a pilot study evaluating different doses previously shown to have an effect in normal humans and mice (17, 22, 24). On day 7, fasting blood glucose was significantly lower in vehicle-treated *SUR-1*$^{-/-}$ mice compared with vehicle-treated wild-type littermates ($p = 0.000002$) (Fig. 2). Treatment with exendin-(9–39) significantly raised fasting blood glucose levels in *SUR-1*$^{-/-}$ mice compared with vehicle-treated *SUR-1*$^{-/-}$ mice (82.2 ± 6.3 mg/dl *versus* 63.2 ± 4.9 mg/dl, $p = 0.03$, on day 3; 82 ± 4.7 mg/dl *versus* 56.4 ± 4.3 mg/dl, $p = 0.0006$, on day 7). Fasting blood glucose levels were not different in exendin-(9–39)-treated wild-type mice compared with vehicle-treated wild-type controls, in contrast to our previous observations in wild-type BALB/c mice. Exendin-(9–39) did not impact weight gain in *SUR-1*$^{-/-}$ nor wild-type littermate controls (data not shown).

During treatment, fasting insulin and glucagon levels were not significantly different among the treatment groups (Fig. 3*A*); however, in the setting of lower fasting blood glucose levels, insulin levels were inappropriately elevated in vehicle-treated *SUR-1*$^{-/-}$ mice and glucagon levels failed to rise as expected in response to the hypoglycemia. Insulin/glucose ratio is increased in *SUR-1*$^{-/-}$ mice compared with wild-type littermate control mice (Fig. 3*B*) (WT *versus* *SUR-1*$^{-/-}$; $p = 0.04$) and is normalized by exendin-(9–39) treatment (WT *versus* *SUR-1*$^{-/-}$Ex-(9–39); $p = 0.32$), suggestive of a direct islet effect of exendin-(9–39) on insulin secretion.

Despite the marked effect on fasting blood glucose levels, treatment with exendin-(9–39) did not significantly impair glucose tolerance in *SUR-1*$^{-/-}$ mice, except for a delay in the return to base-line blood glucose levels at the 120 min time point. Similarly, there was no effect on glucose tolerance in wild-type littermates during treatment with exendin-(9–39) (Fig. 4*A*). In agreement with these results, glucose-stimulated insulin secretion was not affected by exendin-(9–39) in wild-type mice and the already impaired insulin response to the oral glucose load characteristic of the *SUR-1*$^{-/-}$ mice was not further impaired by treatment (Fig. 4*B*).

To determine the mechanism of action for the effect of exendin-(9–39) on fasting blood glucose levels we assessed insulin sensitivity by an insulin tolerance test. Insulin sensitivity was not different between *SUR-1*$^{-/-}$ and wild-type littermates. Further, Exendin-(9–39) did not impact peripheral insulin sensitivity in any of the treatment groups (Fig. 4*C*).

Given the observed *in vivo* effects on insulin secretion but not insulin sensitivity, we next determined whether exendin-(9–39) exerts a direct effect on *SUR-1*$^{-/-}$ islet function. Since *SUR-1*$^{-/-}$ islets do not respond to glucose under our experimental conditions (8), we chose to examine the effect of exendin-(9–39) on the abnormal response of *SUR-1*$^{-/-}$ islets to fuel-induced insulin secretion (specifically, the hyperresponsiveness to amino acids). Isolated islets were perifused with a mixture of amino acids. As previously reported (8), *SUR-1*$^{-/-}$ islets abnormally released insulin in response to ramp stimulation by a physiologic mixture of 19 amino acids (using an increment of 0.04 mM/min for glutamine and 0.2 mM/min for the other amino acids). This response to amino acids was blocked by exendin-(9–39) (Fig. 5*A*). The insulin response to KCl was similar in the presence and absence of exendin-(9–39).

To examine the effect of exendin-(9–39) in wild-type islets, we incubated islets with 10 mM glucose and then introduced the ramp of amino acids. Li *et al.* (8) previously demonstrated that in the presence of 10 mM glucose, a ramp of amino acids stimulates insulin secretion in a concentration-dependent manner. Here we demonstrate that, as in *SUR-1*$^{-/-}$ islets, exendin-(9–39) specifically inhibits the response to amino acids in wild-type islets (Fig. 5*B*). In contrast, the acute response to glucose (10 mM) was not impaired by exendin-(9–39).

The effect of exendin-(9–39) on cAMP was determined in static incubations of isolated islets. In the absence of exogenous

[3] The abbreviation used is: ANOVA, analysis of variance.

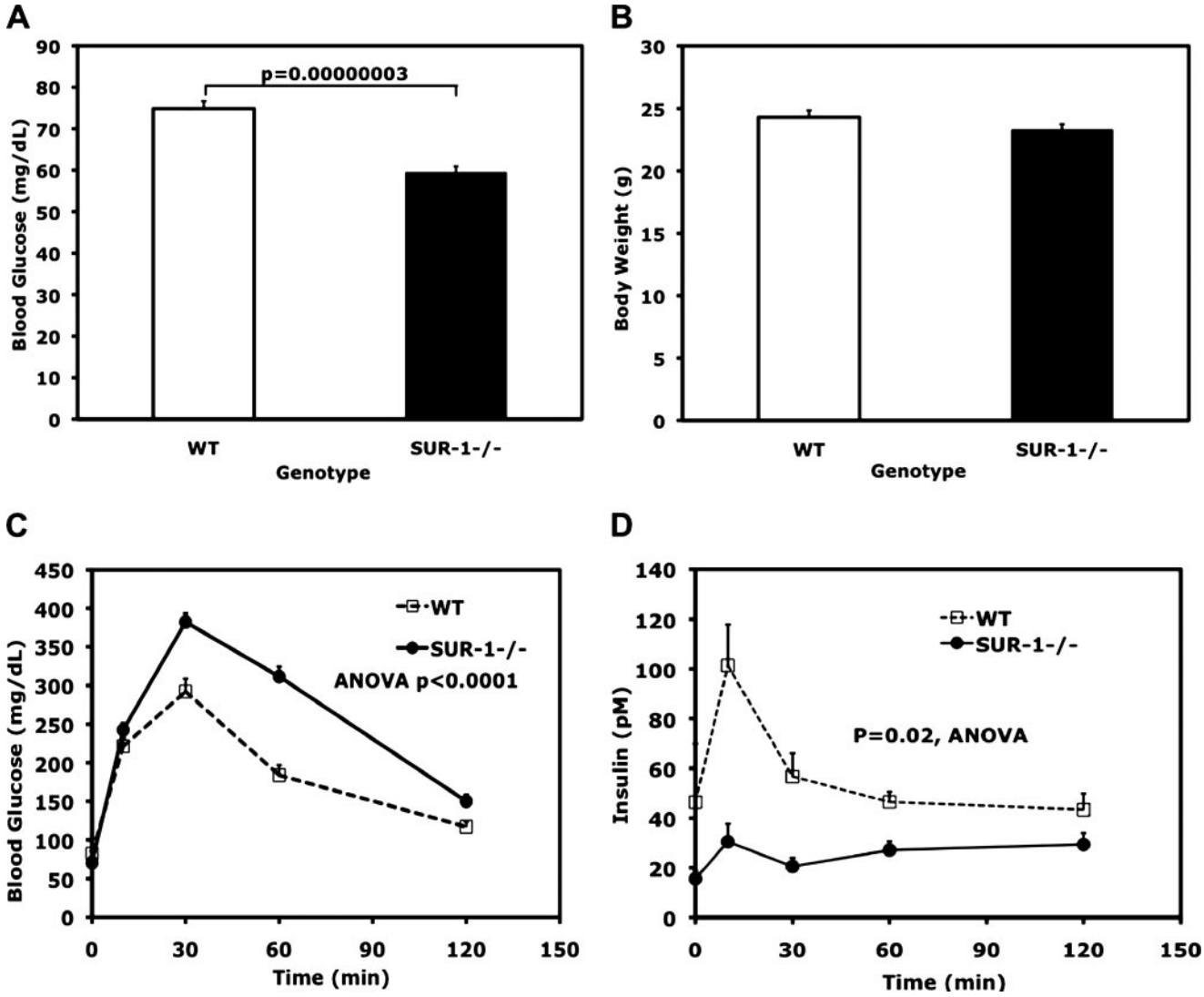

FIGURE 1. **Fasting hypoglycemia and impaired glucose tolerance in *SUR-1*$^{-/-}$ mice.** *A*, fasting blood glucose levels (in mg/dl) in *SUR-1*$^{-/-}$ mice ($n = 27$) and wild-type littermate controls ($n = 30$), $p = 0.00000003$. *B*, body weight (in g) in *SUR-1*$^{-/-}$ mice ($n = 27$) and wild-type littermate controls ($n = 30$). *C*, oral glucose tolerance (2 g/kg) in *SUR-1*$^{-/-}$ mice ($n = 23$) (*solid line* and *circles*) and wild-type littermate controls ($n = 25$) (*dashed line* and *open squares*), $p < 0.0001$, repeated measures ANOVA. *D*, insulin secretion in response to an oral glucose load (2 g/kg) in *SUR-1*$^{-/-}$ mice ($n = 8$) (*solid line* and *circles*) compared with wild-type littermate controls ($n = 9$) (*dashed line* and *open squares*), $p = 0.02$, repeated measures ANOVA.

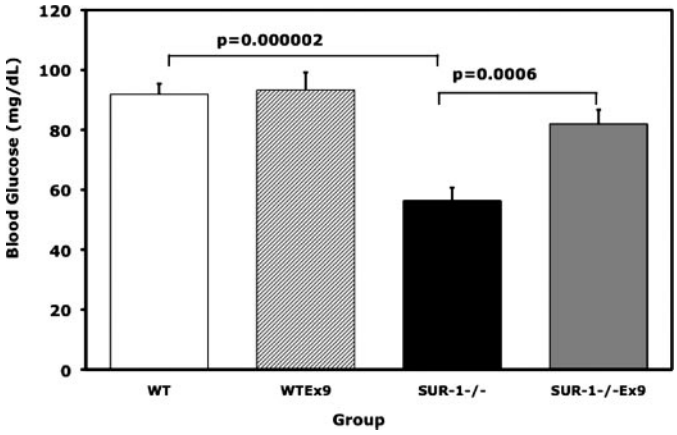

FIGURE 2. **Exendin-(9–39)-normalized fasting blood glucose levels in *SUR-1*$^{-/-}$ mice.** Blood glucose levels were determined after a 12–16-h fast on day 7. *White bar*, vehicle-treated wild-type littermates ($n = 13$); *hatched bar*, exendin-(9–39)-treated wild-type littermates ($n = 10$); *black bar*, vehicle-treated *SUR-1*$^{-/-}$ mice ($n = 11$); *gray bar*, exendin-(9–39)-treated *SUR-1*$^{-/-}$ mice ($n = 11$).

GLP-1, exendin-(9–39) significantly decreased basal intracellular cAMP in *SUR-1*$^{-/-}$ islets (40 ± 4 *versus* 21 ± 2 pmol/100 islets; $p < 0.05$) (Table 1). Amino acids significantly increased cAMP levels in *SUR-1*$^{-/-}$ islets compared with base line (73 ± 13 *versus* 40 ± 4 pmol/100 islets; $p < 0.05$). The amino acid-stimulated increase in cAMP was significantly reduced by exendin-(9–39) (73 ± 13 *versus* 24 ± 5 pmol/100 islets; $p < 0.05$). In these static incubations, the effect of exendin-(9–39) on cAMP levels mirrored the effect on insulin secretion, suggesting that exendin-(9–39) effects on insulin secretion in *SUR-1*$^{-/-}$ islets are mediated by changes in cAMP. Base-line insulin secretion was significantly reduced by exendin-(9–39) (221 ± 22 *versus* 126 ± 17 ng/100 islets/30 min; $p < 0.05$). As seen in the perifusion studies, amino acids significantly increased insulin secretion in *SUR-1*$^{-/-}$ islets (221 ± 22 *versus* 360 ± 32 ng/100 islets/30 min; $p < 0.01$), and exendin-(9–39) significantly reduced amino acid-stimulated insulin secretion (360 ± 32 *versus* 190 ± 35 ng/100 islets/30 min; $p < 0.01$).

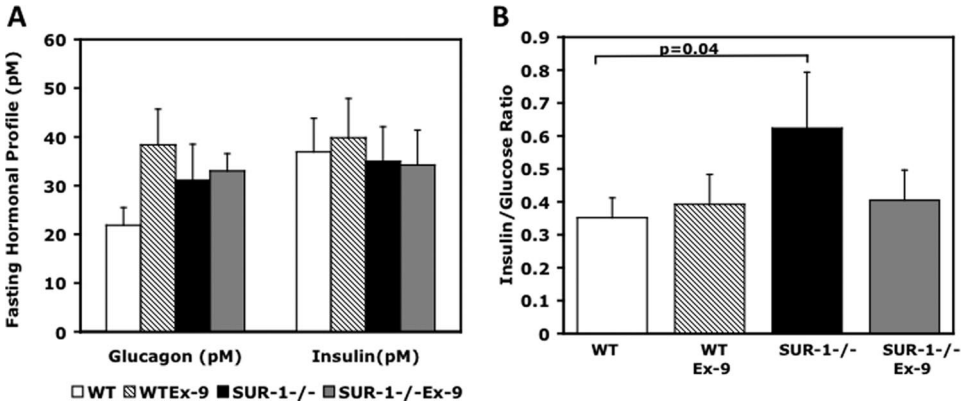

FIGURE 3. **Fasting hormonal profile.** *A*, fasting glucagon and insulin levels in vehicle-treated wild-type (n = 15) (*white bar*), exendin-(9–39)-treated wild-type (n = 14) (*hatched bar*), vehicle-treated *SUR-1*$^{-/-}$ (n = 13) (*black bar*), and exendin-(9–39)-treated *SUR-1*$^{-/-}$ (n = 14) (*gray bar*). *B*, insulin/glucose ratio in vehicle-treated wild-type (n = 9) (*white bar*), exendin-(9–39)-treated wild type (n = 10) (*hatched bar*), vehicle-treated *SUR-1*$^{-/-}$ (n = 9) (*black bar*), and exendin-(9–39)-treated *SUR-1*$^{-/-}$ (n = 10) (*gray bar*).

Under glucose-free conditions, cAMP levels were higher in wild-type islets compared with *SUR-1*$^{-/-}$ islets (83 ± 3 *versus* 40 ± 4 pmol/100 islets; $p < 0.01$). This difference may be explained by the high insulin levels in *SUR-1*$^{-/-}$ islets. In support, previous reports have shown that insulin can reduce cellular cAMP by activating phosphodiesterase to induce cAMP degradation (25–28).

In wild-type islets, exendin-(9–39) significantly decreased basal intracellular cAMP in (83 ± 3 *versus* 47 ± 6 pmol/100 islets; $p < 0.01$) (Table 2). Cyclic AMP was not sig-

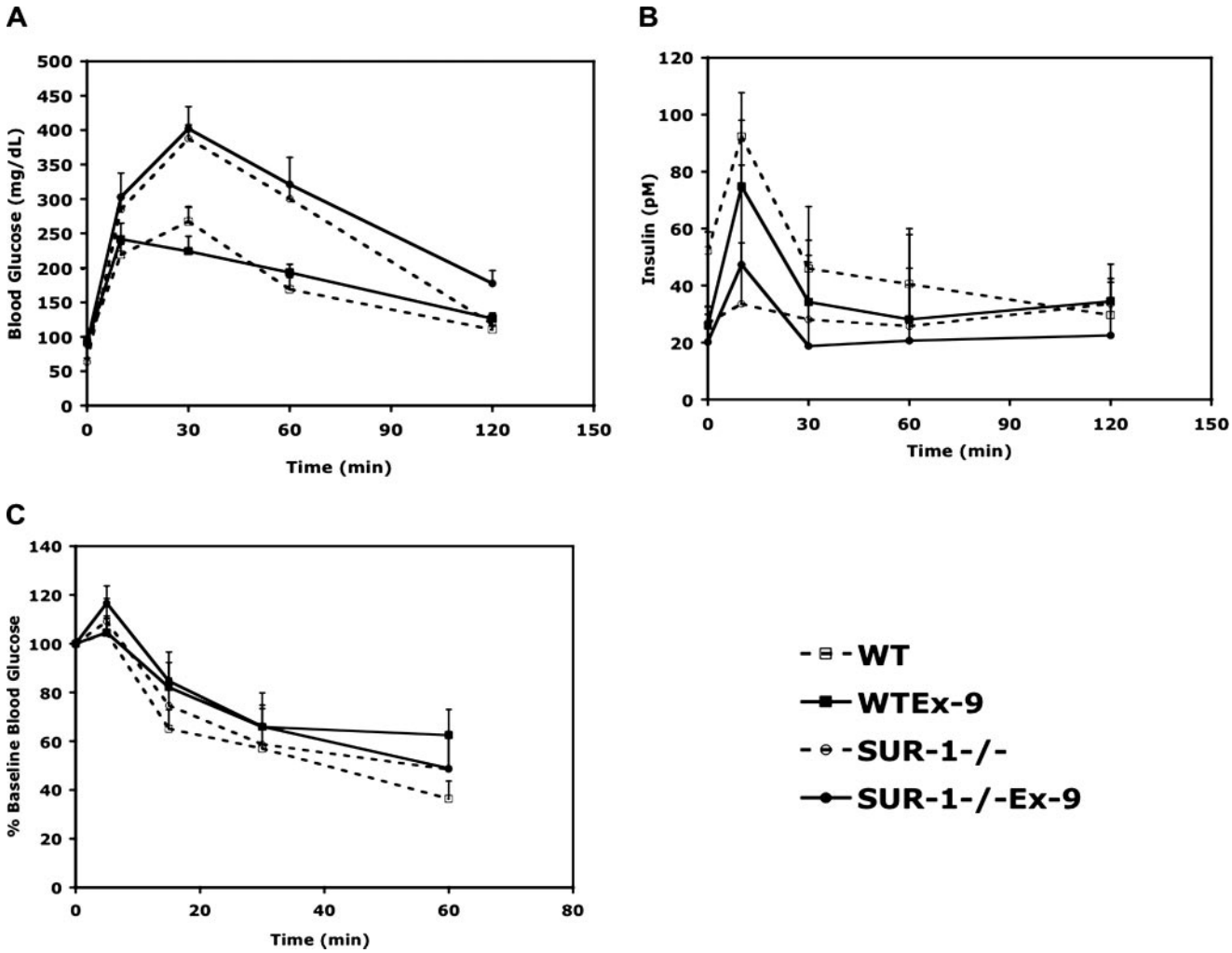

FIGURE 4. **Exendin-(9–39) did not influence glucose tolerance or insulin sensitivity.** *A*, blood glucose levels in response to an oral glucose load in vehicle-treated wild-type littermates (n = 10) (*dashed line/open squares*), exendin-(9–39)-treated wild-type (n = 8) (*solid line/solid squares*), vehicle-treated *SUR-1*$^{-/-}$ mice (n = 9) (*dashed line/open circles*), and exendin-(9–39)-treated *SUR-1*$^{-/-}$ (n = 9) (*solid line/solid circles*). Vehicle-treated wild type *versus* vehicle-treated *SUR-1*$^{-/-}$ (p = 0.001, repeated measures ANOVA) and vehicle-treated *SUR-1*$^{-/-}$ *versus* exendin-(9–39)-treated *SUR-1*$^{-/-}$ (p = 0.02 at time 120 min) are shown. *B*, insulin levels in response to an oral glucose load in vehicle-treated wild-type littermates (n = 10) (*dashed line/open squares*), exendin-(9–39)-treated wild-type (n = 8) (*solid line/solid squares*), vehicle-treated *SUR-1*$^{-/-}$ mice (n = 9) (*dashed line/open circles*), and exendin-(9–39)-treated *SUR-1*$^{-/-}$ (n = 9) (*solid line/solid circles*). *C*, blood glucose change (expressed as the percentage from base line) in response to an intraperitoneal injection of insulin in vehicle-treated wild-type mice (n = 15) (*dashed line/open squares*), exendin-(9–39)-treated wild-type mice (n = 14) (*solid line*/solid squares), vehicle-treated *SUR-1*$^{-/-}$ mice (n = 13) (*dashed line/open circles*), and exendin-(9–39)-treated *SUR-1*$^{-/-}$ mice (n = 14) (*solid line/solid circles*).

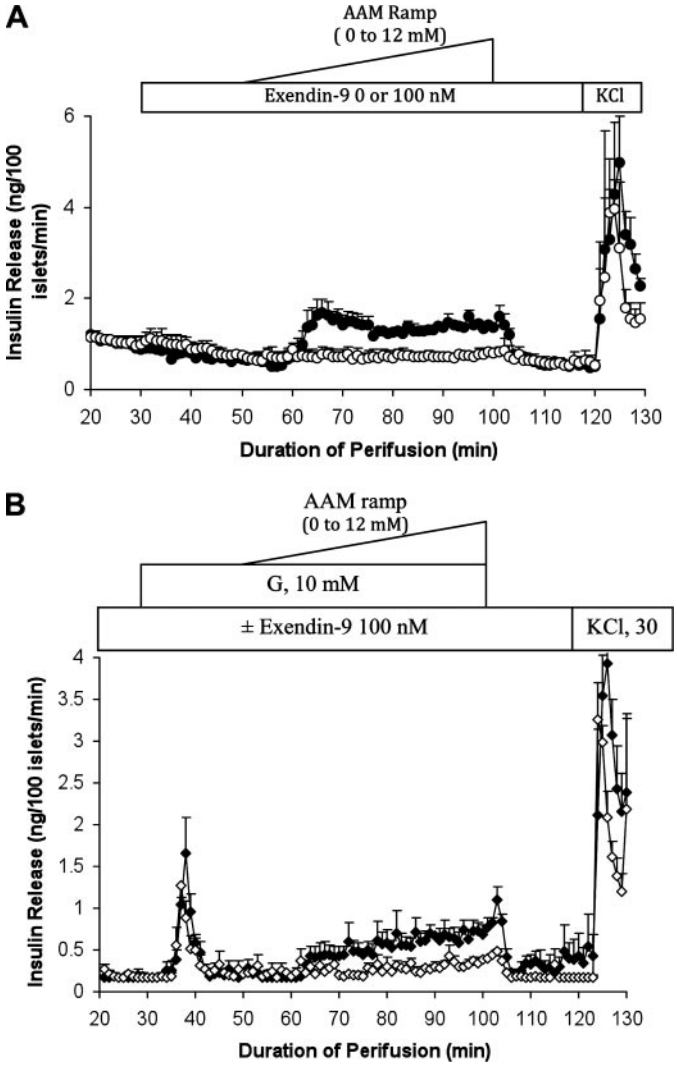

FIGURE 5. **Effect of exendin-(9–39) on fuel responsiveness of *SUR-1*$^{-/-}$ and wild-type islets.** Isolated islets from *SUR-1*$^{-/-}$ mice and wild-type littermates were cultured for 3 days in RPMI 1640 medium containing 10 mM glucose. *A*, *SUR-1*$^{-/-}$ islets were perifused with a ramp of a physiologic mixture of amino acids (0–12 mM) in the presence (*open circles*) or absence (*black circles*) of exendin-(9–39) at a concentration of 100 nM. Finally, 30 mM KCl was applied. *B*, wild-type islets were perifused with (*open diamonds*) or without (*black diamonds*) 100 nM exendin-(9–39). Perifused islets were then exposed to 10 mM glucose at 30 min, followed 20 min later by ramp of a physiologic mixture of amino acids (0–12 mM). Finally, 30 mM KCl were applied. Results are presented as means ± S.E. for 100 islets from three separate perifusions for each condition.

TABLE 1

Exendin-(9–39) reduces base-line and stimulated cytosolic cAMP content and insulin release in *SUR-1*$^{-/-}$ islets

Isolated *SUR-1*$^{-/-}$ mouse islets were cultured in 10 mM glucose for 3 days. Islets were preincubated in glucose-free KRBB for 60 min. 100 nM exendin-(9–39) was added after a 30-min preincubation. Then islets were exposed to different treatment conditions for an additional 30 min. All conditions contained 0.1 mM isobutylmethylzanthine. AAM, amino acid mixture.

Condition	cAMP content	Insulin secretion
	pmol/100 islets	*ng/100 islets/30 min*
Base line ($n = 3$)	40 ± 4	221 ± 22
100 nM exendin-(9–39) ($n = 3$)	21 ± 2[a]	126 ± 17[a]
4 mM AAM ($n = 8$)	73 ± 13[a,b]	360 ± 32[c,d]
100 nM exendin-(9–39)/4 mM AAM ($n = 4$)	24 ± 5[a]	190 ± 35

[a] $p < 0.05$, compared with base-line conditions.
[b] $p < 0.05$, compared with 100 nM exendin-9, 4 mM amino acid mixture.
[c] $p < 0.01$, compared with base-line conditions.
[d] $p < 0.01$, compared with 100 nM exendin-9, 4 mM amino acid mixture.

TABLE 2

Exendin-(9–39) reduces glucose and amino acid-stimulated cytosolic cAMP content and insulin release in wild-type islets

Isolated wild-type islets were cultured in 10 mM glucose for 3 days. Islets were preincubated in glucose-free KRBB for 60 min. 100 nM exendin-(9–39) was added after a 30-min preincubation. Then islets were exposed to different treatment conditions for an additional 30 min. All conditions contained 0.1 mM isobutylmethylzanthine. AAM, amino acid mixture.

Condition ($n = 3$)	cAMP content	Insulin secretion
	pmol/100 islets	*ng/100 islets/30 min*
Base line, 0 mM glucose	83 ± 3	17 ± 4
100 nM exendin-(9–39)	47 ± 6[a]	16 ± 6
10 mM glucose	62 ± 4[b]	153 ± 10[a]
10 mM glucose, 100 nM exendin-(9–39)	44 ± 13	72 ± 11[c]
10 mM glucose, 4 mM AAM	95 ± 6[d]	463 ± 27[c]
10 mM glucose, 4 mM AAM, 100 nM exendin-(9–39)	42 ± 2[e]	153 ± 18[e]

[a] $p < 0.01$, compared with 0 mM glucose.
[b] $p < 0.05$; compared with 0 mM glucose.
[c] $p < 0.01$, compared with 10 mM glucose.
[d] $p < 0.05$, compared with 10 mM glucose.
[e] $p < 0.01$, compared with 10 mM glucose, 4 mM amino acid mixture.

nificantly increased when glucose concentration was increased from 0 to 10 mM. Amino acids significantly increased cAMP levels in wild-type islets in the presence of 10 mM of glucose compared with base line (95 ± 6 *versus* 62 ± 4 pmol/100 islets; $p < 0.05$). The amino acid-stimulated increase in cAMP was significantly reduced by exendin-(9–39) (95 ± 6 *versus* 42 ± 2 pmol/100 islets; $p < 0.01$).

In wild-type islets, base-line insulin secretion (in the absence of any stimuli) was not affected by exendin-(9–39). Insulin secretion as expected was significantly stimulated by 10 mM of glucose (17 ± 4 *versus* 153 ± 10 ng/100 islets/30 min, $p < 0.01$). In contrast to the lack of effect on glucose-stimulated insulin secretion in the perifusion studies, exendin-(9–39) significantly reduced glucose-stimulated insulin secretion in these static incubations (153 ± 10 *versus* 72 ± 11 ng/100 islets/30 min; $p < 0.01$). Flamez *et al.* (29) have reported variable effects of exendin-(9–39), depending on the length of incubation; this may explain the different effect in our perifusion and static incubation experiments. As seen in the perifusion studies, amino acids significantly increased insulin secretion in wild-type islets in the presence of glucose (463 ± 27 *versus* 153 ± 10 ng/100 islets/30 min, $p < 0.01$), and exendin-(9–39) significantly reduced amino acid-stimulated insulin secretion (463 ± 27 *versus* 153 ± 18 ng/100 islets/30 min, $p < 0.01$).

Finally, we studied the effect of exendin-(9–39) on the characteristically elevated intracellular calcium concentration of *SUR-1*$^{-/-}$ islets (8). Exendin-(9–39) did not affect basal intracellular calcium (Fig. 6). As previously reported, amino acids caused a transient further rise in intracellular calcium. Exendin-(9–39) had no effect on the amino acid-stimulated rise in intracellular calcium, indicating that its effect on insulin secretion is distal to the effect of calcium.

DISCUSSION

Congenital hyperinsulinism due to mutations in the K_{ATP} channel is a devastating disease that is generally unresponsive to available medical therapies. We previously reported that a continuous infusion of exendin-(9–39) elevated fasting blood glucose levels in BALB/c mice (22), an effect that has also been

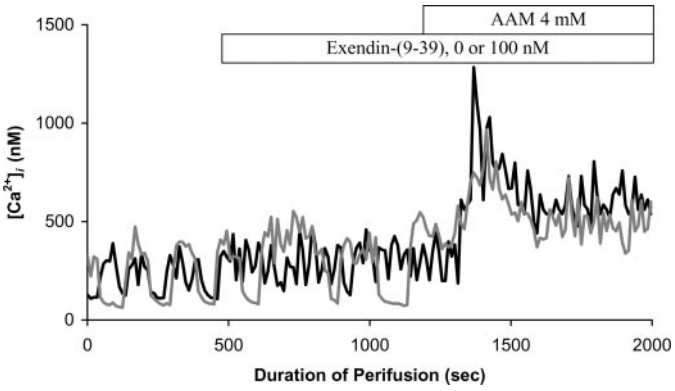

FIGURE 6. **Exendin-(9–39) did not impact $[Ca^{2+}]_i$ in *SUR-1*$^{-/-}$ islets.** Isolated *SUR-1*$^{-/-}$ mouse islets were cultured with 10 mM glucose for 3 days on coverslips. $[Ca^{2+}]_i$ was continuously measured by Fura-2 fluorescence in response to amino acids (4 mM) in the presence (*gray line*) or absence of exendin-(9–39) (*black line*). Representative experiments are shown. All studies were repeated at least three times and showed comparable results.

observed in baboons (19) and healthy human subjects (24). Although *SUR-1*$^{-/-}$ mice are relatively normoglycemic in the fed state (6, 7), they develop hypoglycemia with fasting, which allowed us to test the potential for exendin-(9–39) to normalize fasting blood glucose in the absence of functional K_{ATP} channels.

Our studies confirmed previous reports highlighting the phenotype of the *SUR-1*$^{-/-}$ mice (6, 7). Compared with wild-type littermates, *SUR-1*$^{-/-}$ mice are more hypoglycemic when fasted and more hyperglycemic when glucose-loaded, and the glucose intolerance is the result of impaired insulin secretion. The islet counterregulatory response to hypoglycemia is also impaired in the *SUR-1*$^{-/-}$ mice, since in the fasting state, when blood glucose is significantly lower than in wild-type littermates, insulin and glucagon levels are not different compared with normoglycemic wild-type littermates. These findings are in agreement with those of Shiota *et al.* (30), who reported an impaired glucagon secretory response in *SUR-1*$^{-/-}$ mice.

Administered as a continuous infusion, exendin-(9–39) significantly raised fasting blood glucose levels in *SUR-1*$^{-/-}$ mice without significantly impacting weight gain, glucose tolerance, or insulin sensitivity. However, we cannot entirely rule out a contribution by changes in hepatic insulin sensitivity, which could have been missed on the insulin tolerance test. The elevated fasting insulin/glucose ratio in *SUR-1*$^{-/-}$ mice was decreased by exendin-(9–39). We did not observe an effect of exendin-(9–39) on glucagon levels in the fasting state. These findings suggest that the effect of exendin-(9–39) on fasting blood glucose levels is mediated by the β-cell GLP-1 receptor with no significant impact on other peripheral or central GLP-1 receptor-mediated actions at the dose studied.

The striking *in vivo* impact of exendin-(9–39) on fasting blood glucose in the *SUR-1*$^{-/-}$ mice prompted us to explore its effects on the nutrient-induced insulin secretory response of *SUR-1*$^{-/-}$ islets. Because of the clinical observation that protein ingestion provokes hypoglycemia in patients with K_{ATP} hyperinsulinism (31) and our observation that *SUR-1*$^{-/-}$ islets hyperrespond to amino acids (8), we evaluated the impact of exendin-(9–39) on amino acid-stimulated insulin secretion. In islet perifusion studies, exendin-(9–39) suppressed amino acid-stimulated insulin secretion in both *SUR-1*$^{-/-}$ and wild-type islets. Although the mechanism by which amino acids, specifically glutamine, stimulates insulin secretion is not well understood (8), our findings suggest that cAMP plays a central role in amino acid-stimulated insulin secretion. Further, exendin-(9–39) decreased basal and amino acid-stimulated insulin secretion and intracellular cAMP accumulation in static incubation experiments. Thus, exendin-(9–39) corrects the abnormal pattern of insulin secretion responsible for hypoglycemia in the absence of K_{ATP} channels: elevated basal and amino acid-stimulated insulin secretion.

The results of isolated islet experiments, conducted in the absence of exogenous GLP-1 receptor ligand, suggest that the GLP-1 receptor is constitutively active in *SUR-1*$^{-/-}$ islets, and they support previous reports implicating exendin-(9–39) as an inverse agonist of the GLP-1 receptor (21, 29). Alternatively, this effect might be explained by antagonism of local intraislet GLP-1 (32) or by competition between exendin-(9–39) and glucagon for binding to the GLP-1 receptor on β-cells (33).

Nakazaki *et al.* (14) reported a loss of cAMP-induced potentiation of insulin secretion in *SUR-1*$^{-/-}$ islets. In their studies, GLP-1-induced elevation of cAMP was not mirrored by changes in insulin secretion in mutant islets. Interestingly and in contrast to these observations, the changes we observed in cAMP levels were mirrored by changes in insulin secretion. This discrepancy may be attributable to methodologic differences. In our studies, islets were cultured for 3 days prior to perifusion or static incubation experiments. We believe that this preculture recovery period is essential to restore a normal secretory response after the stress of islet isolation.

Cyclic AMP has been previously recognized as a critical physiological potentiator of insulin secretion (34). Our studies demonstrate the central role of cAMP in the K_{ATP} channel-independent pathway regulating insulin secretion. Cyclic AMP stimulates exocytosis by PKA-dependent pathways, through phosphorylation of downstream targets, including the K_{ATP} channel, and by PKA-independent mechanisms through the activation of guanine nucleotide exchange factors, such as cAMP-GEFII (also known as Epac2) (13, 35). The PKA-independent pathway is critical in the potentiation of insulin secretion by the incretin hormones GLP-1 and GIP (36). Furthermore, Epac2 plays an essential role in the first phase of insulin granule exocytosis potentiated by cAMP (37). Cyclic AMP promotes insulin granule exocytosis by increasing the size of the readily releasable pool and by accelerating the refilling of the readily releasable pool (12, 13, 15, 38). In pancreatic islets, the effect of Epac2 on insulin secretion depends on cytosolic calcium as well as cAMP (36), and it has been postulated that cAMP sensitizes the exocytotic machinery to calcium (39). Thus, we speculate that the inhibition of insulin secretion in *SUR-1*$^{-/-}$ islets by exendin-(9–39) is mediated by the effect of cAMP on a late calcium-dependent step in the exocytotic pathway (Fig. 7).

In summary, we have shown that exendin-(9–39) significantly raises fasting blood glucose levels in *SUR-1*$^{-/-}$ mice

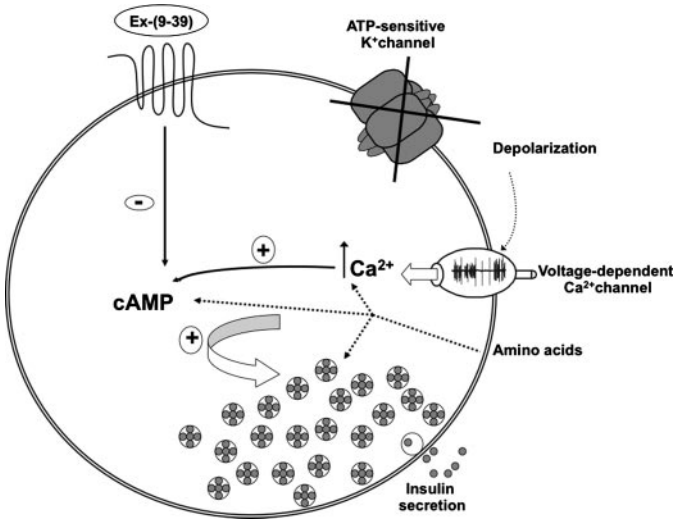

FIGURE 7. **Proposed mechanism of action of exendin-(9–39) in *SUR-1*$^{-/-}$ islets.** In *SUR-1*$^{-/-}$ mouse islets, plasma membrane depolarization results in elevated cytosolic Ca^{2+} and dysregulated insulin secretion. Exendin-(9–39) binds to the GLP-1 receptor and lowers base-line cAMP levels, resulting in decreased insulin secretion despite the elevated calcium levels. Similarly, by decreasing amino acid-stimulated cAMP accumulation, exendin-(9–39) inhibits amino acid-stimulated insulin secretion.

through a direct effect on insulin secretion that appears to be mediated at least in part by changes in intracellular cAMP accumulation. These findings have significant translational application, given the lack of effective medical therapies for children with congenital hyperinsulinism due to K_{ATP} mutations. The use of GLP-1 receptor antagonists to control hypoglycemia in congenital hyperinsulinism could have a beneficial effect on morbidity and long term outcome in this patient population.

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Exendin-(9–39) Corrects Fasting Hypoglycemia in *SUR-1*$^{-/-}$ Mice by Lowering cAMP in Pancreatic β-Cells and Inhibiting Insulin Secretion

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