Massive Augmentation of Stimulated Insulin Secretion Induced by Fatty Acid–Free BSA in Rat Pancreatic Islets

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Incubation of rat pancreatic islets for 4–6 h with 100 μmol/l fatty acid–free BSA induced a 3- to 10-fold enhancement of insulin release to a subsequent challenge with 16.7 mmol/l glucose, without changing the typical biphasic pattern of the response. A similar enhancement was observed with other stimuli, such as leucine, depolarizing concentrations of KCl and tolbutamide, pointing to a general phenomenon and common mechanism for the augmentation. Norepinephrine completely blocked the stimulated response. The protein kinase C (PKC) inhibitor Ro 31-8220, which acts at the ATP-binding site and inhibits all PKC isoforms, strongly inhibited the enhancement of a subsequent glucose challenge when present during the BSA pretreatment period. In contrast, Go 6976, an inhibitor of conventional PKC isoforms, was without effect, even at the high concentration of 1 μmol/l. Preincubation with calphostin C, which competes for the diacylglycerol (DAG)-binding site, therefore inhibiting conventional, novel, and PKC isoforms of the PKD type, completely abolished the enhancing effect of the BSA but did not affect secretion in islets treated with 10 μmol/l fatty acid–free BSA. We conclude that the remarkable enhancement of insulin release is due to a change in glucose signaling and activation of a novel PKC isoform or a DAG-binding protein. *Diabetes* 53:3152–3158, 2004

Fatty acids play complex roles in the control of insulin secretion and stimulate or inhibit secretion, depending upon the conditions (1–5). The acute insulinotropic action depends on chain length and degree of saturation and entails conversion of long-chain fatty acids to their respective CoA esters that are thought to be active signaling molecules (6–11). Long-term exposure is associated with a reduction in glucose-stimulated secretion. These "lipotoxic" phenomena are thought to be due to several effects including decreased glucose metabolism (12), effects on phosphofructokinase and hexokinase (13), and inhibition of proinsulin biosynthesis (14).

In light of these deleterious effects on the stimulation of insulin release by chronic exposure of the β-cell to fatty acids, a reverse approach to deplete the β-cells of fatty acids was used. It is known that pancreatic islets export substantial amounts of fatty acids to the medium (15). Therefore, we incubated rat pancreatic islets for several hours in the presence of fatty acid–free BSA to trap fatty acids as they exit and thus deplete the islets. This treatment resulted in an extraordinary 3- to 10-fold increase in secretion rates compared with those of controls. The data indicate that under normal conditions the β-cell operates under a strong inhibitory tone.

RESEARCH DESIGN AND METHODS

Protein kinase C (PKC) inhibitors were from Calbiochem (La Jolla, CA), as was arachidonyltrifluoromethyl ketone (AACOCF3). BSA (Fraction V) was from Sigma (order no.: A-7888 [non–fatty acid depleted] and A-7033 (fatty acid depleted; lot nos.: 082K0768, 101K1437, 092K0858, 082K1150, 092K0857, and 102K1264) and one fatty acid–depleted BSA from Roche (lot no: 93166322; Basel, Switzerland). For some experiments the fatty acid–free BSA was purified by charcoal treatment as follows. A 10% solution of fatty acid–free BSA in double distilled water was mixed with 5% Norit A charcoal. The pH was reduced to 3.0 by the addition of 0.2 N HCl and the solution stirred for 1 h at 4°C. After centrifugation for 20 min at 20,000*g*, the supernatant was removed and the pH adjusted to 7.0 with 0.2 N NaOH. After protein determination, the purified BSA was diluted out into RPMI medium at the appropriate concentrations.

Islet isolation. Male Sprague Dawley rats (250–350 g) and C57Bl/6 mice (25–30 g) (Charles River, Wilmington, MA) had food and water ad libitum except for the fasting experiments (food was withheld 24 h before experiment). Islets were isolated in a Krebs-Ringer bicarbonate HEPES buffer (KRBH) of the following composition (in mmol/l): 129 NaCl, 5 $NaHCO_3$, 4.8 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 10 HEPES, 2.5 $CaCl_2$, 0.1% BSA, and 2.8 mmol/l glucose at pH 7.4 using a collagenase digestion technique (16).

Islet perifusion. Islets were incubated for up to 6 h in RPMI medium containing 5.6 mmol/l glucose and the indicated fatty acid–free BSA concentrations before perifusion. During both preperifusion and perifusion, 10 μmol/l fatty acid–free BSA was used in the KRBH buffer. After loading 12 islets into 70-μl chambers they were perifused at 1 ml/min. After a 20-min equilibration period at 2.8 mmol/l glucose, sample collection started. The samples were subjected to radioimmunoassay (17).

Cell culture and static incubations. INS-1 and INS 832/13 cell lines were a gift from Dr. C.B. Newgard. Passages 61–70 were used. They were cultured in RPMI containing 11.1 mmol/l glucose with 10% FBS, 10 mmol/l HEPES, 2 mmol/l glutamine, 1 mmol/l sodium pyruvate, 50 μmol/l β-mercaptoethanol, 100 units/ml penicillin, and 100 μg/ml streptomycin. INS 832/13 cells were cultured in 5 mmol/l glucose in serum-deprived medium 24 h before the experiments. The cells were exposed for 4 h to RPMI supplemented with 5 mmol/l glucose containing either 10 or 100 μmol/l fatty acid–free BSA. After one wash, cells were incubated in KRBH with either 2.8 or 16.7 mmol/l glucose. Aliquots of the supernatants were assayed for the insulin released.

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DAG, diacylglycerol; α-KIC, α-ketoisocaproic acid; KRBH, Krebs-Ringer bicarbonate HEPES buffer; PKC, protein kinase C; PLD, phospholipase D; TDP, time-dependent potentiation.

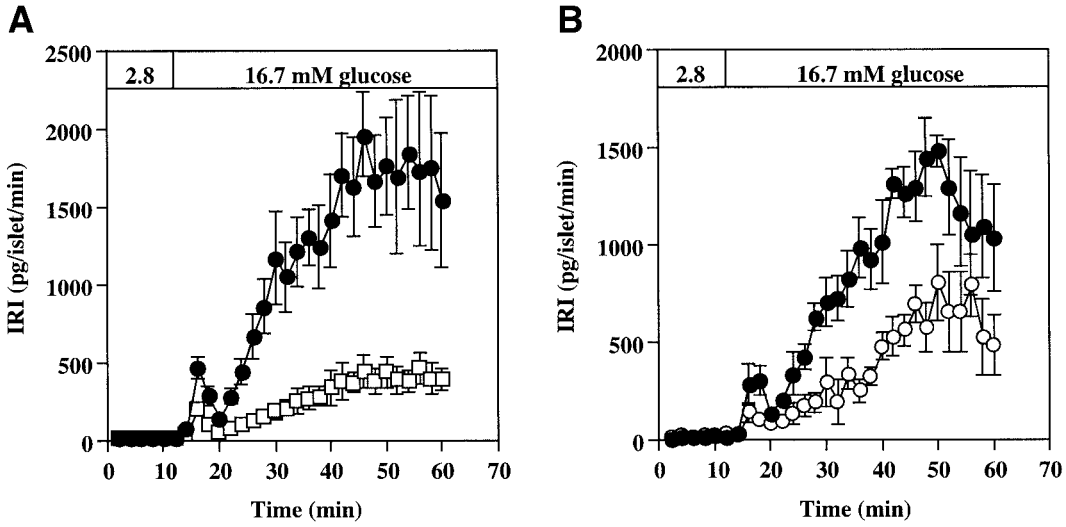

FIG. 1. *A*: Insulin secretory response to 16.7 mmol/l glucose by isolated rat islets that had been preincubated for 4 h in RPMI medium containing 5.6 mmol/l glucose and either 10 μmol/l fatty acid–free BSA (□) or 100 μmol/l fatty acid–free BSA (●). Following the preincubation, the islets were perifused with KRBH buffer containing 10 μmol/l fatty acid–free BSA and 2.8 mmol/l glucose for 20 min (the equilibration period). Perifusate samples were taken for insulin radioimmunoassay from 0 to 60 min. At 10 min the perifusates were changed to KRBH buffer containing 10 μmol/l fatty acid–free BSA and 16.7 mmol/l glucose. Results are means ± SEM, *n* = 6. *B*: Insulin secretory response to 16.7 mmol/l glucose by isolated rat islets that had been preincubated for 4 h in RPMI medium containing 5.6 mmol/l glucose and either 100 μmol/l BSA that was not fatty acid free (○) or 100 μmol/l fatty acid–free BSA (●). Following the preincubation, the islets were perifused with KRBH buffer containing 10 μmol/l fatty acid–free BSA and 2.8 mmol/l glucose for 20 min (the equilibration period). Perifusate samples were taken for insulin radioimmunoassay from 0 to 60 min. At 10 min the perifusates were changed to KRBH buffer containing 10 μmol/l fatty acid–free BSA and 16.7 mmol/l glucose. Results are means ± SEM, *n* = 4. IRI, immunoreactive insulin.

RESULTS

No significant difference was detected in the response to 16.7 mmol/l glucose between islets that had been preincubated for 4 h with 10 μmol/l fatty acid–free BSA μmol/l or BSA that was not fatty acid free (data not shown). Consequently, we used 10 μmol/l fatty acid–free BSA in all control experiments. The effect of incubating rat pancreatic islets for 4 h in RPMI containing 100 μmol/l fatty acid–free BSA compared with control islets with 10 μmol/l fatty acid–free BSA is shown in Fig. 1*A*. Under control conditions, the basal release rate was 18 ± 2 pg · islet^{-1} · min^{-1}. Stimulation with 16.7 mmol/l glucose resulted in a biphasic response with a first-phase peak of 210 ± 28 pg · islet^{-1} · min^{-1} after 6 min, a nadir after 10 min of 88 ± 30 pg · islet^{-1} · min^{-1}, and a rising second phase that reached a plateau of 381 ± 116 pg · islet^{-1} · min^{-1} at 46 min. Under test conditions (preincubation with 100 μmol/l BSA), the basal release rate was 17 ± 2 pg · islet^{-1} · min^{-1}. Stimulation with 16.7 mmol/l glucose resulted in a first-phase peak of 469 ± 66 pg · islet^{-1} · min^{-1}, a nadir of 130 ± 15 pg · islet^{-1} · min^{-1}, and a rising second phase that reached a plateau of 1,958 ± 350 pg · islet^{-1} · min^{-1}. The maximum rates for the first and second phases were significantly different. Thus, both phases of release were augmented by preincubation with 100 μmol/l fatty acid–free BSA, while the basal release rate and the rate at the nadir were unaffected. At the peak of the first phase the secretion rate was 135% higher, and at the plateau of the second phase the secretion rate was 435% higher. Notably, the temporal profiles of the biphasic responses under the two conditions were identical, with the same time for the first-phase peak, the nadir, and the second-phase plateau. In separate experiments, the response to 11.1 mmol/l glucose following incubation with 100 μmol/l fatty acid–free BSA was augmented by 437% (integrated insulin release over 50 min) relative to islets incubated with 10 μmol/l fatty acid–free BSA (n = 4). When islets manifesting the augmented response were exposed to 10 μmol/l norepinephrine, insulin release rates returned to basal values (data not shown). Thus, insulin is being released by the normal mechanisms of exocytosis.

In view of this remarkable phenomenon, two possibilities were examined further: *1*) that the augmentation was due to an impurity in the batch of fatty acid–free BSA used and *2*) that the effect was indeed due to extraction of fatty acids from the islets. On the premise that an impurity would not likely be present in all lots of BSA, we tested an additional five lots of fatty acid–free BSA from Sigma. All lots induced augmentation (data not shown). Additionally, a lot from Roche (Basel, Switzerland) also induced a 580% augmentation of the response to 16.7 mmol/l glucose, n = 4) (data not shown). Additionally, BSA was treated with charcoal (18) and compared with non–charcoal-treated BSA. Enhancement of glucose-stimulated secretion was similar in both cases (data not shown). Taken together, these data suggest that the effect of the fatty acid–free BSA is not due to an impurity.

To seek evidence that augmentation is indeed due to extraction of fatty acids, the following experiments were performed. In the first, the effect of preincubation with 100 μmol/l BSA (not fatty acid free) was compared with 100 μmol/l fatty acid–free BSA. While there may have been some enhancement of release in the islets pretreated with BSA (Fig. 1*B*), the paired islets treated with fatty acid–free BSA released insulin at much higher rates. This suggests that the lack of fatty acids in the BSA, and likely extraction of fatty acids from the islets, is the cause of the augmentation.

In the second series of experiments, fatty acids were added to the RPMI medium used in the incubations. RPMI

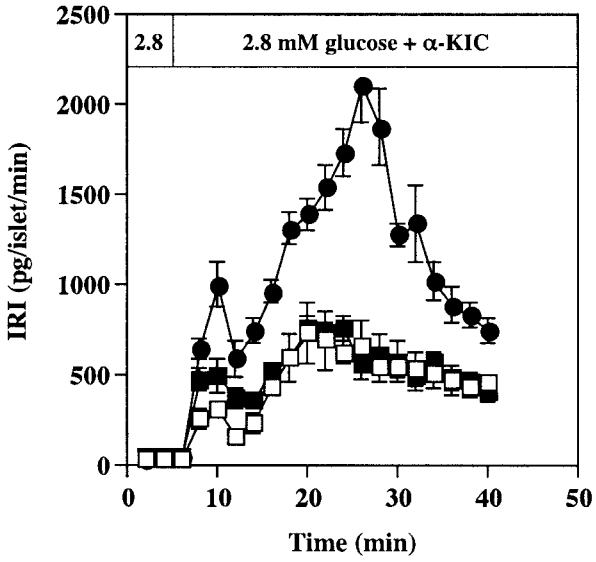

FIG. 2. Insulin secretory response to 20 mmol/l α-KIC by isolated rat islets that had been preincubated for 4 h in RPMI medium containing 5.6 mmol/l glucose and either 10 μmol/l fatty acid–free BSA (□), 100 μmol/l fatty acid–free BSA (●), or 100 μmol/l fatty acid–free BSA plus 20 μmol/l free palmitate (■). Following the preincubation, the islets were perifused with KRBH buffer containing 10 μmol/l fatty acid–free BSA and 2.8 mmol/l glucose for 20 min (the equilibration period). Perifusate samples were taken for insulin radioimmunoassay from 0 to 40 min. At 4 min the perifusates were changed to KRBH buffer containing 10 μmol/l fatty acid–free BSA and 20 mmol/l α-KIC. Results are means ± SEM, $n = 4$. IRI, immunoreactive insulin.

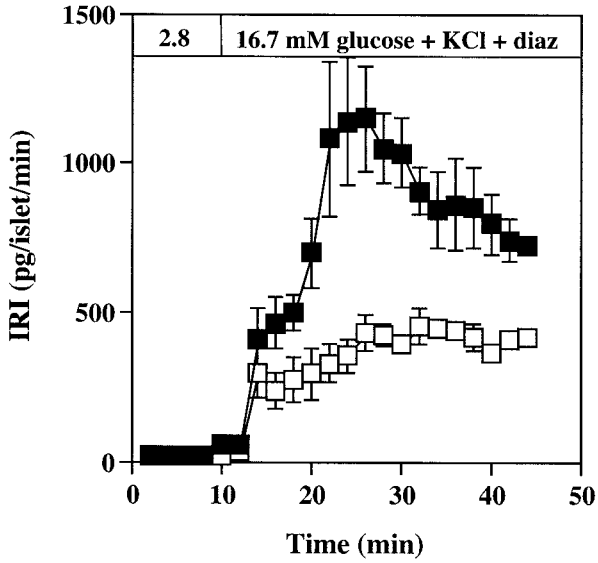

FIG. 3. Insulin secretory response to 40 mmol/l KCl, 250 μmol/l diazoxide, and 16.7 mmol/l glucose by isolated rat islets that had been preincubated for 4 h in RPMI medium containing 5.6 mmol/l glucose and either 10 μmol/l fatty acid–free BSA (□) or 100 μmol/l fatty acid–free BSA (■). Following the preincubation, the islets were perifused with KRBH buffer containing 10 μmol/l fatty acid–free BSA and 2.8 mmol/l glucose for 20 min (the equilibration period). Perifusate samples were taken for insulin radioimmunoassay from 0 to 44 min. At 10 min the perifusates were changed to KRBH buffer containing 10 μmol/l fatty acid–free BSA and 16.7 mmol/l glucose, KCl, and diazoxide. Results are means ± SEM, $n = 4$. IRI, immunoreactive insulin.

containing 340 μmol/l palmitate with 100 μmol/l fatty acid–free BSA (free palmitate ~1 μmol/l) and RPMI containing 600 μmol/l caproate and 100 μmol/l fatty acid–free BSA (free caproate ~10 μmol/l) failed to affect the augmentation. However, buffer containing 600 μmol/l palmitate with 100 μmol/l fatty acid–free BSA (~10 μmol/l free palmitate) reduced the response (integrated over 50 min) by 30% (data not shown). Free fatty acid estimates are according to Spector et al. (19). Next, we increased the free fatty acid concentration to 20 μmol/l during the preincubation period. In addition, since prolonged fatty acid treatment can inhibit subsequent glucose-induced secretion (14), we took advantage of the fact that α-ketoisocaproic acid (α-KIC)-induced stimulation is immune to prior fatty acid treatment (14). In islets exposed for 4 h to 100 μmol/l fatty acid–depleted BSA (vs. 10 μmol/l), we observed augmentation of the α-KIC–induced secretion (255% higher than the low BSA-treated islets). However, pretreatment of paired islets with a free palmitate concentration of 20 μmol/l (7.1 mol/mol) essentially obliterated the augmentation (>95% inhibition) (Fig. 2), supporting the idea that augmentation is due to extraction of fatty acids.

Preincubation for 4 h with 100 μmol/l fatty acid–free BSA also augmented the responses to 10 mmol/l leucine (500%), 40 mmol/l KCl (270%), and 250 μmol/l tolbutamide (450%) over a 20-min exposure. Thus augmentation is not limited to glucose or nutrient secretagogues. Similarly, the response of the ATP-sensitive K^+ channel–independent pathway tested by stimulating the islets with 16.7 mmol/l glucose in the presence of KCl and diazoxide showed marked augmentation (Fig. 3).

The secretion rate of islets from 24-h–fasted rats was augmented by 450% (data not shown), similar to that seen in islets from fed rats (435%). When mouse islets were preincubated for 4 h in 100 μmol/l fatty acid–free BSA, no significant enhancement of glucose-stimulated insulin secretion was observed. During the first phase (0–14 min), control islets (10 μmol/l BSA) released 931 ± 106 vs. 1,129 ± 93 pg insulin per islet for the test islets ($P > 0.17$, $n = 4$). During the second phase (14–40 min), control islets released 2,148 ± 289 vs. 2,265 ± 249 pg insulin per islet for the test islets ($P > 0.37$, $n = 4$). Additionally, preincubation with 100 μmol/l fatty acid–free BSA for 4 h failed to affect the responsiveness of two clonal β-cell lines: the INS-1 cell, which does not manifest the ATP-sensitive K^+ channel–independent pathway of glucose signaling (20), and the INS 832/13 cell, which does (20,21). INS-1 control cells in the presence of 16.7 mmol/l glucose released 59 ± 3 ng · well^{-1} · 30 min^{-1}; the test cells also released 59 ± 3 ng · well^{-1} · 30 min^{-1}. INS 832/13 control cells in the presence of 16.7 mmol/l glucose released 71 ± 5 ng · well^{-1} · 30 min^{-1} and the test cells 73 ± 3 ng · well^{-1} · 30 min^{-1}.

In time course studies, preincubation with 100 μmol/l BSA for 1 h had no effect. An enhanced secretory response was detectable after 2 h and was maximal between 4 and 6 h (data not shown). In view of this, it seemed possible that de novo protein synthesis could be involved. Therefore, experiments were performed in which 10 μmol/l cycloheximide—chosen because it inhibits protein synthesis in pancreatic islets by >90% (22)—was included during the 4-h preincubation period with 100 μmol/l BSA. Cycloheximide reduced the first phase of glucose-induced secretion by 50%, as reported previously for freshly isolated islets (22), and slightly delayed the onset of the second

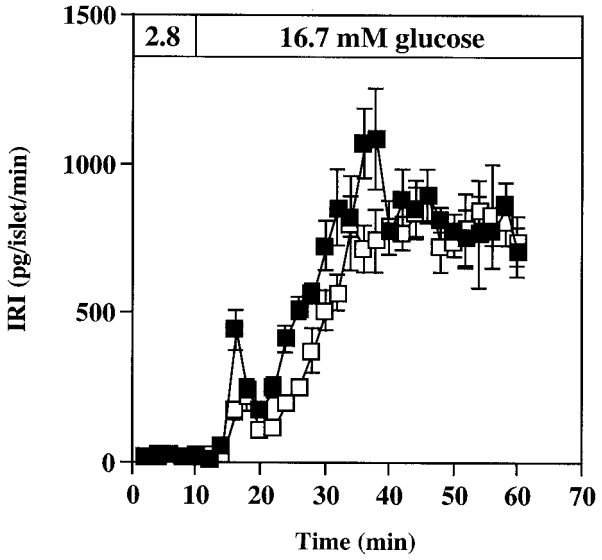

FIG. 4. Insulin secretory response to 16.7 mmol/l glucose by isolated rat islets that had been preincubated for 4 h in RPMI medium containing 100 μmol/l fatty acid–free BSA and 5.6 mmol/l glucose in the presence (■) or absence (□) of 10 μmol/l cycloheximide. Following the preincubation, the islets were perifused with KRBH buffer containing 10 μmol/l fatty acid–free BSA for 20 min (the equilibration period). Perifusate samples were taken for insulin radioimmunoassay from 0 to 60 min. At 10 min the perifusates were changed to KRBH buffer containing 10 μmol/l fatty acid–free BSA and 16.7 mmol/l glucose. Results are means ± SEM, n = 4. IRI, immunoreactive insulin.

phase. However, peak rates at 50–60 min were similar to those of controls (Fig. 4). Therefore, augmentation is not due to de novo protein synthesis.

Because of the potential involvement of lipids in the augmentation, inhibitors of different pathways activated by lipids were studied. When islets were incubated for 4 h in 100 μmol/l fatty acid–free BSA in the presence and absence of 50 μmol/l AACOCF3, a specific inhibitor of phospholipase A_2 (23), no inhibition of the biphasic response to 16.7 mmol/l glucose was seen. When 100 nmol/l wortmannin was used there was no inhibition of glucose-stimulated insulin secretion under control (10 μmol/l fatty acid–free BSA) or augmented (100 μmol/l fatty acid–free BSA) conditions. This rules out the involvement of PI 3-kinase. Raloxifene (50 μmol/l), which inhibits phospholipase D (PLD)1 an PLD2 with a half-maximal effective concentration of 4 μmol/l (24), did not affect the augmentation of insulin release (data not shown). This would argue against a role for PLD.

The next experiments explored the potential involvement of PKC and used inhibitors targeting different subclasses of PKC. To differentiate between effects of these inhibitors on glucose stimulus-secretion coupling and their effects on BSA-induced augmentation, two conditions were studied: *1*) effects on acute glucose-induced secretion with freshly isolated islets and *2*) inclusion of the inhibitors only during the 4-h preincubation period. The concentrations used were chosen according to their previous use and effectiveness in pancreatic islets (25–27).

Ro 31-8220 binds to the ATP-binding site, thereby inhibiting the phosphorylating activity of all PKC isoforms (25). Ro 31-8220 (10 μmol/l), added 20 min before the stimulus of 16.7 mmol/l glucose to freshly isolated islets and present throughout the experiment, inhibited both phases

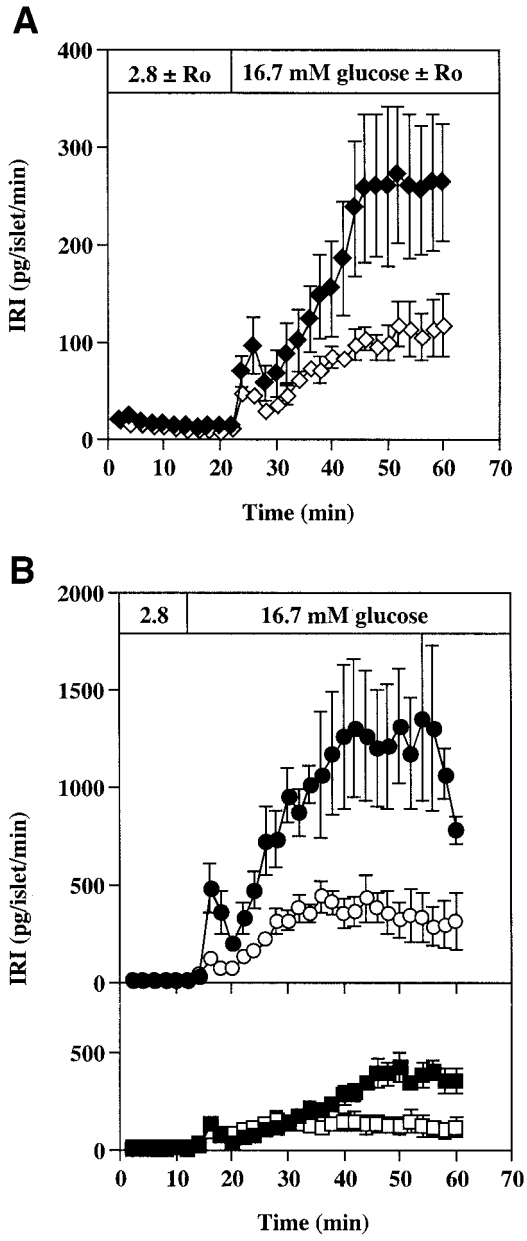

FIG. 5. *A*: The effect of Ro 31-8220 on the insulin secretory response to 16.7 mmol/l glucose by freshly isolated rat islets. The islets were preperifused with KRBH buffer containing 2.8 mmol/l glucose for 20 min before one set of islets (◇) was exposed to 10 μmol/l Ro 31-8220 at 0 min. At 20 min the perifusates were changed to KRBH buffer containing 16.7 mmol/l glucose with Ro 31-8220 (◇) or without Ro 31-8220 (◆). Results are means ± SEM, n = 4. *B*: Shown in top panel is the effect of Ro 31-8220 on the insulin secretory response to 16.7 mmol/l glucose by isolated rat islets that had been preincubated for 4 h in RPMI medium containing 100 μmol/l fatty acid–free BSA and 5.6 mmol/l glucose in the absence (●) or presence (○) of 10 μmol/l Ro 31-8220. Shown in the bottom panel is the effect of 4 h preincubation with 10 μmol/l fatty acid–free BSA in the absence (■) or presence (□) of 10 μmol/l Ro 31-8220. Following the preincubations, the islets were perifused with KRBH buffer containing 10 μmol/l fatty acid–free BSA and 2.8 mmol/l glucose for 20 min (the equilibration period). At 10 min the perifusates were changed to KRBH buffer containing 10 μmol/l fatty acid–free BSA and 16.7 mmol/l glucose. Ro 31-8220 was not present at any time during the perifusions. Results are means ± SEM, n = 4. IRI, immunoreactive insulin.

of glucose-stimulated insulin secretion by >50% (Fig. 5*A*). When Ro 31-8220 was present only during the 4 h preincubation period with 100 μmol/l fatty acid–free BSA, it decreased both phases of the augmented glucose-stimu-

lated insulin secretion but to a larger extent than seen under acute conditions (Fig. 5*B*). Similar results to those in freshly isolated islets were obtained with Ro 31-8220 when islets were preincubated for 4 h with low (10 μmol/l) fatty acid–free BSA (lower Fig. 5*B*). These results suggest the involvement of a PKC in glucose-stimulated insulin secretion and, more importantly for this work, in the mechanism by which augmentation is induced. Therefore, additional PKC inhibitor studies were performed.

Go 6976 (1 μmol/l), a selective inhibitor of conventional PKC isoforms, had no effect on glucose-stimulated insulin secretion when added acutely or on the subsequent secretory response when present during the 4-h preincubation period (data not shown). Thus, the conventional PKCs, α, βI, βII, and γ, appear not to be involved in glucose-stimulated insulin secretion and also not involved in the induction of augmentation.

Calphostin C acts at the DAG-binding site and inhibits conventional and novel PKC isoforms and DAG-binding proteins. Calphostin C (1 μmol/l) had no effect on insulin release stimulated acutely by glucose in freshly isolated islets (Fig. 6*A*), as reported previously (26). However, when freshly isolated islets were preincubated for 30 min with 1 μmol/l calphostin C, a substantial inhibition of the glucose response was observed (Fig. 6*B*). This occurred despite the calphostin C not being present during the perifusion. Thus the effect of calphostin C to inhibit glucose-stimulated insulin secretion is either time dependent or the compound must be present in the cell before the stimulation with glucose begins, i.e., it is possible that stimulation with glucose blocks the effect of the calphostin C. When the drug was present during the 4-h incubation of islets with 10 μmol/l fatty acid–free BSA, no inhibition was observed (Fig. 6*C*). This is remarkable because a 30-min preincubation with the drug inhibits glucose-stimulated release (Fig. 6*B*). Even more remarkable, when the drug was present during the 4-h incubation of islets with 100 μmol/l fatty acid–free BSA, the response to glucose stimulation was abolished (Fig. 6*C*). Thus the augmented insulin secretion of islets treated with 100 μmol/l fatty acid–free BSA is completely dependent on a signaling pathway that is inhibited by calphostin C. In summary, glucose-stimulated insulin secretion in freshly isolated islets is not inhibited by acute administration of calphostin C. However, it can be inhibited if the islets are preincubated with calphostin C for 30 min before the glucose challenge. A 4-h incubation with 10 μmol/l fatty acid–free BSA changes β-cell signaling so that the β-cell is no longer inhibited by calphostin C. A 4-h incubation with 100 μmol/l fatty acid–free BSA switches the β-cell from its normal glucose signaling to an alternate pathway that results in massive augmentation and is totally blocked by calphostin C. This pathway is presumed to involve a novel PKC or a DAG-binding protein.

DISCUSSION

Rat pancreatic islets preincubated for 4 h with a high concentration of fatty acid–free BSA to deplete them of endogenous fatty acids responded to glucose and other secretagogues with extraordinarily high rates of secretion. These high rates were not seen when the islets were preincubated with fatty acid–containing BSA. Importantly, basal and nadir secretion were unchanged. The results suggest that the fatty acid content of the islet, or fatty acid derivatives, exert an inhibitory tone on the rate of secretion. Augmentation of the biphasic response to glucose is interesting in several respects. While both phases of release were augmented, they were not augmented to the same extent. The second phase was augmented to a greater extent than the first. As the first phase is thought to be due to the release of an "immediately releasable" pool of granules docked at the β-cell plasma membrane (20,28), augmentation of this phase implies an increase in the size of the immediately releasable pool. The fact that the rate of insulin secretion at the nadir was not augmented ($P = 0.13$, $n = 6$, Fig. 1*A*) suggests that the immediately releasable pools in both test and control islets have been depleted at this time and that the second phase must develop independently. That the second phase is augmented to a greater extent than the first phase (435 vs. 135%) suggests that the size of the immediately releasable pool of docked granules is limited.

It is not clear how fatty acids exert an inhibitory tone on the β-cell or how depletion of fatty acids leads to a massive augmentation of insulin release. Trying to narrow the field of possible mediators, we focused on signaling pathways linked to secretion and affected by lipids. We conclude from the lack of effect of wortmannin, raloxifene, and AACOCF3 (inhibitors of PI 3-kinase, PLD, and cytosolic phospholipase A_2, respectively) that their involvement in the augmentation is unlikely. Another class of kinases regulated by lipids is the large family of PKCs. These can be divided into three subclasses, as follows. *1*) The conventional (classical) PKC, consisting of α, β_I, and β_{II} isoforms (γ is not expressed in the β-cell). This group is activated by Ca^{2+}, DAG, and phosphatidylserine. *2*) The novel PKC isoforms δ, ε, η, and θ, which respond to DAG and PS but are unresponsive to Ca^{2+}. *3*) The atypical PKC isoforms, comprised of ζ and ι/λ, which are Ca^{2+} and DAG insensitive and mainly regulated by phosphatidylinositol 4-phosphate (PIP_3) and other lipid mediators (29). To differentiate between these different subclasses, we used three inhibitors: Go 6976, which inhibits conventional PKC isoforms, Ro 31-8220, which acts at the ATP-binding site and inhibits all PKCs, and calphostin C, which interferes with DAG binding and inhibits both conventional and novel isoforms. On freshly isolated islets, we confirmed previous reports of ~50% inhibition of glucose-stimulated secretion by Ro 31-8220 and no effect of Go 6976. However, whereas acute addition of calphostin C did not affect glucose-induced secretion, a 30-min preincubation with the compound inhibited secretion by 50% (to the same extent as Ro 31-8220). This points to a delayed effect of calphostin C accompanied by an irreversible inhibition of the target, at least for the time studied. It also indicates that acute glucose-stimulated exocytosis likely involves the activation of a novel PKC isoform because we can exclude conventional isoforms (no inhibition by Go 6976) and atypical PKC isoforms, which would not be affected by calphostin C. Inclusion of these inhibitors during the preincubation period with 100 μmol/l BSA produced essentially the same results but with one very important difference. Pretreatment with calphostin C in the high-BSA–pretreated islets almost obliterated the subsequent

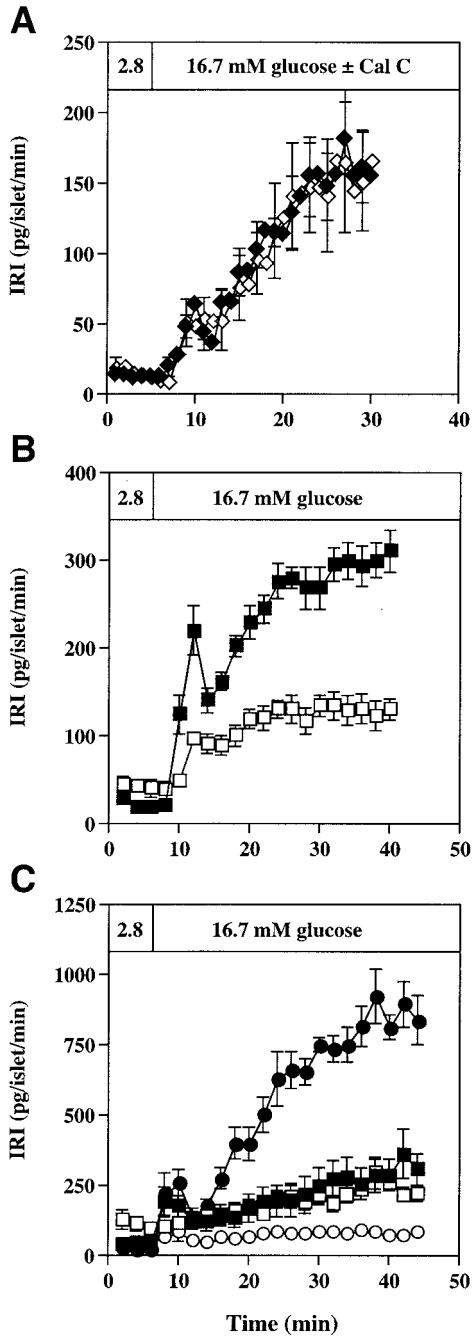

FIG. 6. ***A*****: The effect of 1 μmol/l calphostin C on the insulin secretory response of freshly isolated rat islets to 16.7 mmol/l glucose. The islets were perifused with KRBH buffer containing 2.8 mmol/l glucose for 40 min before sampling was started. At 5 min the perifusate was changed to KRBH buffer containing 16.7 mmol/l glucose with or without calphostin C. ♦, control islets; ◇, calphostin C–treated islets. Results are means ± SEM, $n = 4$. *B*: The effect of preincubation with calphostin C on the insulin secretory response of freshly isolated rat islets to 16.7 mmol/l glucose. The islets were preincubated with KRBH buffer containing 2.8 mmol/l glucose with or without 1 μmol/l calphostin C for 30 min before transfer to the perifusion chambers. After a 20-min preperifusion at 2.8 mmol/l glucose, sampling was started. At 6 min the perifusate was changed to KRBH buffer containing 16.7 mmol/l glucose. ■, control islets; □, calphostin C–treated islets. Results are means ± SEM, $n = 4$. *C*: The effect of calphostin C on the insulin secretory response to 16.7 mmol/l glucose by isolated rat islets that had been preincubated for 4 h in RPMI medium containing 10 μmol/l fatty acid–free BSA and 5.6 mmol/l glucose in the absence (■) or presence (□) of 1 μmol/l calphostin C and for 4 h in RPMI medium containing 100 μmol/l fatty acid–free BSA and 5.6 mmol/l glucose in the absence (●) or presence (○) of 1 μmol/l calphostin C. Following the preincubation, the islets were perifused with KRBH buffer containing 10 μmol/l fatty acid–free BSA and 2.8 mmol/l glucose for 20 min (the equilibration period). At 6 min the perifusate was changed to KRBH buffer containing 16.7 mmol/l glucose and 10 μmol/l fatty acid–free BSA. Results are means ± SEM, $n = 4$. IRI, immunoreactive insulin.**

glucose-stimulated secretion (~95% inhibition), while hardly affecting secretion in the low-BSA–pretreated control group. This is in contrast to Ro 31-8220, which reduced secretion in low- and high-BSA–pretreated islets, albeit its efficiency was increased in the latter. There are two possible explanations: *1*) pretreatment with high BSA changes the β-cell signaling to increase the input of a novel PKC isoform to more efficiently stimulate subsequent secretion and *2*) the greater inhibition of secretion in the presence of calphostin C compared with Ro 31-8220 is due to a change in β-cell signaling but now to the activation of an additional signaling molecule. A likely candidate would be a DAG-binding protein, such as Munc13, that is not subject to inhibition by Ro 31-8220. In chromaffin cells, overexpression of Munc13 enhances both the rapid and sustained phases of Ca^{2+}-stimulated exocytosis (30). Furthermore, in the β-cell, overexpression of Munc13 also amplifies insulin secretion (31).

Supportive evidence for the involvement of a PKC or DAG-binding protein lies in the fact that the augmentation induced by high BSA pretreatment is a memory effect that persists for at least 90 min after reversal to low BSA concentrations. This is reminiscent of time-dependent potentiation (TDP), which, in addition to being induced by glucose, can be induced by phorbol-12-myristate-13-acetate (PMA) (32), a compound that activates PKC isoforms and DAG-binding proteins (33). The fact that the augmentation induced by treatment with fatty acid–free BSA occurs in rat islets and not in mouse islets is also reminiscent of the finding that TDP can be induced in rat but not in mouse islets (34,35). Thus some part of the augmentation may rely on mechanisms similar to those underlying TDP.

It remains to be determined how depletion of fatty acids in the β-cell leads to increased activity of these isoforms or of DAG-binding proteins and the augmentation of secretion. One possibility is that depletion of fatty acids leads to an increased breakdown of triglycerides in the cell in an attempt to replace the fatty acids lost and that this results in an increased flux of potential messenger molecules. It might also be that the loss of fatty acid(s) from the cell (palmitate being the major form in the β-cell) would result in lipid remodeling and the appearance of novel fatty acid derivatives with increased activity relative to the derivatives of palmitate. This, for example, might lead to novel forms of DAG with increased potency on PKC or DAG-binding proteins.

Despite our lack of knowledge of the biochemical mechanisms involved, the augmentation of secretion by 100 μmol/l fatty acid–free BSA can be ascribed to an effect on the docked granules. The prompt increase and augmentation of the rate of insulin secretion during the first phase in response to glucose indicates that the size of the immediately releasable pool has been increased. The size of this pool and of the first phase of release are thought to be due to a rate-limiting step that converts granules in a readily releasable pool to an immediately releasable pool, a pool that has a limit to the number of granules contained

within it (28). Also, the magnitude of the second phase of release is thought to be due to the rate at which this conversion between the pools takes place (28). After 40 min of stimulation by glucose at the very high rates of secretion that are induced by a 4-h exposure to 100 μmol/l fatty acid–free BSA, the number of docked granules in the rat β-cell is reduced by two-thirds (S.G.S., G. Shanmugam, and G.W.G.S., unpublished observation). Nevertheless, there is still a surplus of granules docked at the plasma membrane that is available for release. The fact that the number of docked granules has decreased during stimulation by glucose under these conditions means that granule translocation and docking failed to keep up with the very high rate of secretion. Despite this, docked granules are still present when insulin secretion increased to very high rates. Thus, pretreatment of islets with fatty acid–free BSA acts on the docked granule pool and accelerates the rate of conversion of docked readily releasable granules to the immediately releasable state.

Regardless of the mechanism of action, these findings open up the possibility that manipulation of the fatty acid/lipid composition of the islet could radically increase insulin secretion. This could be of use in diabetic patients, where insulin secretion is insufficient, and/or in those who suffer from lipotoxicity. It also raises the possibility of engineering cells with a greater capacity for insulin secretion than normal β-cells, while still responding to the same range of glucose concentrations.

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