Palmitate Potentiation of Glucose-Induced Insulin Release: A Study Using 2-Bromopalmitate

S.M. Parker, P.C. Moore, L.M. Johnson, and V. Poitout

The mechanisms whereby fatty acids (FA) potentiate glucose-induced insulin secretion from the pancreatic β cell are incompletely understood. In this study, the effects of palmitate on insulin secretion were investigated in isolated rat islets. Palmitate did not initiate insulin secretion at nonstimulatory glucose concentrations, but markedly stimulated insulin release at concentrations of glucose ≥ 5.6 mmol/L. At concentrations of palmitate ≥0.5 mmol/L, the important determinant of the potency of the FA was its unbound concentration. At total concentrations ≤ 0.5 mmol/L, both the total and unbound concentrations appeared important. Surprisingly, 2-bromopalmitate did not affect palmitate oxidation, but significantly diminished palmitate esterification into cellular lipids. Neither methyl palmitate, which is not activated into a long-chain acyl-CoA ester, nor 2-bromopalmitate affected glucose-stimulated insulin release. Further, 2-bromopalmitate partly inhibited the potentiating effect of palmitate. These results support the concept that FA potentiation of insulin release is mediated by FA-derived signals generated in the esterification pathway.

LONG-CHAIN FATTY acids (FA) are essential regulators of normal pancreatic β-cell function and are likely to play a role in the pathogenesis of β-cell dysfunction in type 2 diabetes (reviewed in McGarry and Dobbins[1] and Poitout).[2] In vivo, infusion of FA stimulates insulin secretion.[3,4] Physiologically, the ability of FA to potentiate glucose-stimulated insulin secretion (GSIS) is important after a period of fasting. McGarry et al[5-8] have demonstrated that GSIS in 18- to 24-hour fasted rats is dependent on circulating FA. Thus, infusion of the antilipolytic agent nicotinic acid in fasted rats completely inhibits the subsequent response to glucose, which is restored by simultaneous lipid infusion.[5] Similar observations were made after prolonged fasting in humans.[8] This group[1] has suggested that the FA dependency of glucose-induced insulin secretion might explain the early observation that islets isolated from fasted animals respond poorly to glucose,[9] although the essentiality of FA under these conditions is still debated.[10] In vitro, FA do not initiate insulin secretion in the absence of glucose, but strongly potentiate GSIS.[11-13] The ability of FA to potentiate GSIS in vitro is determined by their free (ie, unbound to proteins) concentration[14] and increases with their degree of saturation[14] and chain length.[11] FA potentiation of insulin secretion is dependent on the presence of guanosine 5′ triphosphate (GTP)[13] and extracellular Ca^{++}[12] and is prevented by blockade of FA metabolism.[13] However, whether the stimulatory effects of FA depend on their oxidation in the mitochondrion or the generation of lipid-derived signal in the cytosol is still unclear. Studies in insulin-secreting HIT-T15 cells have shown that the inhibitor of FA oxidation 2-bromopalmitate stimulates insulin release,[11] supporting the concept that FA-stimulation of insulin secretion was unrelated to their oxidation. In contrast, in a study in isolated mouse islets, 2-bromopalmitate did not affect insulin secretion and only partially inhibited the effect of palmitate on GSIS.[12] In this latter study, however, the effects of 2-bromopalmitate on FA metabolism were not measured. These discrepancies prompted us to ascertain the effects of 2-bromopalmitate on FA oxidation, FA esterification, and insulin secretion in isolated rat islets of Langerhans.

MATERIALS AND METHODS

Reagents

Palmitic acid (sodium salt), methyl palmitate, and FA-free bovine serum albumin (BSA) were from Sigma Aldrich (St Louis, MO). 2-bromopalmitate was from Aldrich (Milwaukee, WI). [1-^{14}C] palmitate was from NEN (Boston, MA).

FA Solutions

Stock solutions were prepared as follows: palmitic acid was dissolved in ethanol:H_20 (1:1, vol:vol) at 50°C at a final concentration of 150 mmol/L; 2-bromopalmitate and methyl palmitate were dissolved in ethanol at a final concentration of 300 mmol/L. Aliquots of stock solutions were complexed with FA-free BSA (10% solution in H_20) by stirring for 1 hour at 37°C and then diluted in culture media. The final molar ratio of FA:BSA was between 1:1 and 5:1. The final BSA concentration was ≤ 1.34% (vol:vol). The final ethanol concentration was ≤ 0.66% (vol:vol). All control conditions included a solution of vehicle (ethanol:H_20) mixed with FA-free BSA at the same concentration as the FA solution.

Animals

Six-week-old male Wistar rats were purchased from Harlan Sprague Dawley (Indianapolis, IN). Animals were housed on a 12-hour light/dark cycle with free access to water and standard laboratory chow. All procedures using animals were approved by the PNRI Institutional Animal Care and Use Committee.

Rat Islet Isolation and Culture

Rat islets were isolated by collagenase digestion as described[15] and cultured overnight in RPMI 1640 containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 μg/mL streptomycin, and 11.1 mmol/L glucose.

Insulin Secretion in Static Incubations

Batches of 10 islets each were washed twice in Krebs-Ringer Buffer (KRB) containing 2.8 mmol/L glucose for 20 minutes at 37°C, then

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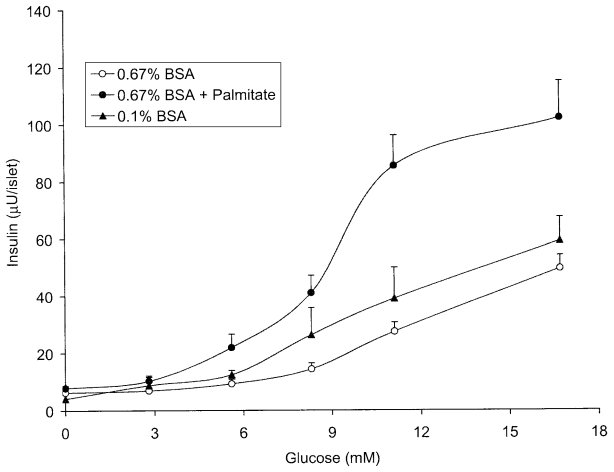

Fig 1. Effects of palmitate on glucose-stimulated insulin secretion. Isolated rat islets were incubated in the presence of increasing glucose concentrations with 0.5 mmol/L palmitate complexed to 0.67% BSA (n = 12), 0.67% BSA without palmitate (n = 12), or 0.1% BSA without palmitate (n = 4) for 1 hour at 37°C as described in Materials and Methods. Data are mean ± SE.

incubated for 60 minutes in the presence of various glucose and FA concentrations, as indicated in the figure legends. Each condition was run in duplicate. Insulin levels in samples collected from the static incubations were measured using the Sensitive Rat Insulin RIA kit (Linco Research, St Charles, MO).

Palmitate Oxidation and Esterification

Batches of 100 isolated islets each were prelabeled overnight in RPMI 1640 containing 11.1 mmol/L glucose, 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, 0.1% BSA, 0.5 μCi/μmol [1-^{14}C] palmitate, 0.1 mmol/L unlabeled palmitate, and 1 mmol/L carnitine at 37°C. Islets were then washed and incubated in KRB containing 0.1% BSA, 2.8 or 16.7 mmol/L glucose, 0.5 μCi/μmol [1-^{14}C] palmitate, 0.1 mmol/L unlabeled palmitate, and 1 mmol/L carnitine for 1 hour at 37°C. One milliliter incubation buffer was then transferred to an Erlenmeyer with a rubber cap and acidified with 100 μL 7% perchloric acid. Benzethonium hydroxide (400 μL) was then injected into small wells suspended to the rubber caps, and, after 16 hours at room temperature, trapped $^{14}CO_2$ was measured by liquid scintillation counting. Background counts from a control condition treated side by side with the samples, but without islets, were subtracted from the counts. Islets were harvested at the end of the final incubation for lipid extraction and thin-layer chromatography (TLC) analysis as described.[15]

Expression of Data and Statistics

Data are expressed as mean ± SE. Intergroup comparisons were performed by Student's paired *t* test or analysis of variance (ANOVA) with post hoc Bonferroni adjustment, where appropriate. $P < .05$ was considered significant.

RESULTS

Effects of Palmitate on Glucose-Induced Insulin Secretion

The effects of 0.5 mmol/L palmitate complexed to 0.1 mmol/L BSA were first studied in 1-hour static incubations in the presence of increasing glucose concentrations (Fig 1). Insulin secretion in the presence of palmitate was compared with that from islets in the absence of palmitate, but in the presence of the same amount of BSA, ie, 0.67% (= 0.1 mmol/L). In addition, to ascertain the influence of BSA itself on insulin secretion, we included an additional control condition containing 0.1% BSA (the usual BSA concentration in KRB). Glucose dose-dependently increased insulin release in all 3 conditions (ANOVA, $P \leq .001$). The presence of 0.67% BSA tended to decrease insulin secretion at glucose concentrations ≥ 8.3 mmol/L, but this difference did not reach statistical significance (not significant [NS], n = 4). Palmitate did not affect insulin secretion at concentrations of glucose ≤ 2.8 mmol/L, but significantly stimulated insulin secretion at glucose concentrations ≥ 5.6 mmol/L. At 16.7 mmol/L glucose, insulin secretion in the presence of palmitate was 2.1-fold ± 0.2-fold greater than in the absence of palmitate ($P \leq .01$, n = 12). To determine whether the effects of palmitate on insulin secretion were reversible, we performed an additional series of experiments in which islets were first incubated for 1 hour in the absence or presence of 0.5 mmol/L palmitate and 16.7 mmol/L glucose, then washed and incubated for an additional 1 hour with 16.7 mmol/L glucose in the absence of palmitate. Islets that had been first incubated with palmitate secreted 96.6% ± 17% of the amount of insulin secreted by the islets that had not previously been exposed to palmitate (n = 4, NS), indicating that the effects of palmitate on insulin secretion are fully reversible. We then investigated the effects of increasing concentrations of palmitate on insulin secretion at 11.1 mmol/L glucose (Fig 2). In the first series of experiments, the concentration of BSA was increased proportionally to that of palmitate, so that the molar ratio of palmitate:BSA remained constant at 5:1 (Fig 2A). Palmitate dose-dependently increased GSIS at 11.1 mmol/L glucose between 0 and 0.5 mmol/L (ANOVA, $P < .05$, n = 3), after which insulin secretion reached a plateau (Fig 2A). Interestingly, increasing concentrations of BSA in the controls apparently decreased GSIS at 11.1 mmol/L glucose, which is consistent with the tendency of BSA to lower insulin secretion observed in Fig 1 and, which could be attributed either to nonspecific binding of insulin to BSA or to depletion of FA from the cells in the presence of BSA. In the second series of experiments, the concentration of BSA was fixed at 0.1 mmol/L, and palmitate was increased so that the molar ratio of palmitate:BSA increased from 2:1 to 8:1 (Fig 2B). Under these conditions, palmitate dose-dependently increased insulin secretion (ANOVA, $P < .05$, n = 3).

Effects of 2-Bromopalmitate on Palmitate Metabolism and Glucose-Induced Insulin Secretion

Most of the studies using 2-bromopalmitate in islets have used the brominated derivative as an inhibitor of long-chain FA oxidation.[16] To verify 2-bromopalmitate effects on palmitate metabolism, we measured palmitate oxidation and esterification in isolated islets as described in Materials and Methods in the presence of 16.7 mmol/L glucose and 0.5 mmol/L palmitate with or without 0.5 mmol/L 2-bromopalmitate (Table 1). Surprisingly, we found that 2-bromopalmitate did not affect palmitate oxidation, but significantly reduced palmitate esterification (Table 1). To determine the effects of 2-bromopalmitate on GSIS, we incubated islets for 60 minutes in the presence of 2.8 mmol/L glucose or 16.7 mmol/L glucose with or without 0.5

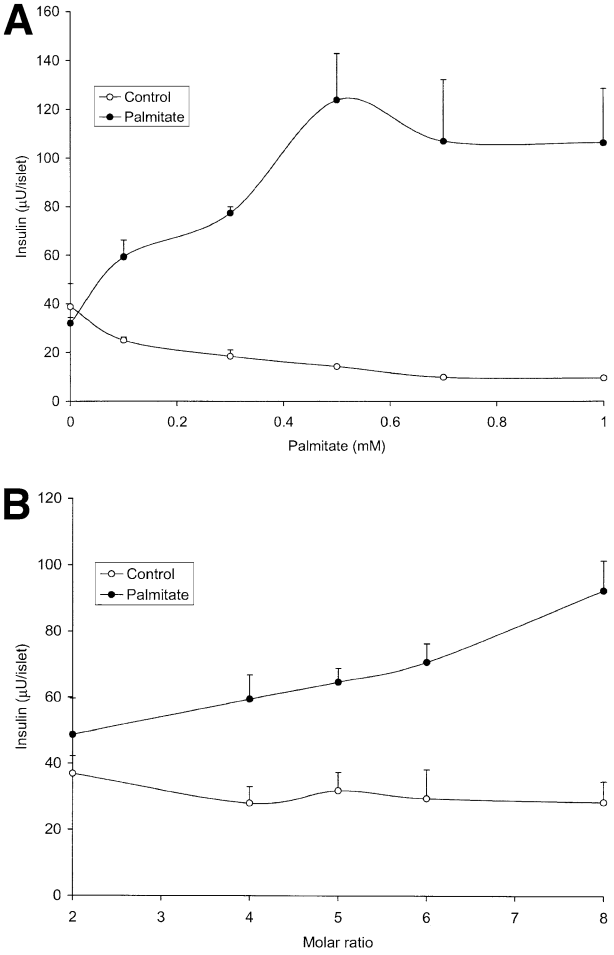

Fig 2. Concentration-dependency of palmitate potentiation of insulin secretion. Isolated islets were incubated in the presence of 11.1 mmol/L glucose and increasing concentrations of palmitate for 1 hour at 37°C as described in Materials and Methods. (A) The concentration of BSA was increased proportionally to that of palmitate so that the molar ratio of palmitate:BSA remained constant at 5:1. (B) The concentration of BSA was fixed at 0.1 mmol/L so that the molar ratio of palmitate:BSA increased from 2:1 to 8:1. Results are mean ± SE of 3 replicate experiments for both A and B.

mmol/L palmitate, 0.5 mmol/L 2-bromopalmitate, or 0.5 mmol/L methyl palmitate, a nonmetabolizable analogue of palmitate[17]) (Fig 3). As expected, palmitate markedly potentiated GSIS ($P < .0001$, n = 5), whereas neither 2-bromopalmitate nor methyl palmitate had any effect (both NS, n = 5). In the experiments shown in Fig 3, 2-bromopalmitate was added at the same time as palmitate in the incubation buffer. In a

Table 1. Effects of 2-Bromopalmitate on Palmitate Oxidation and Esterification in Isolated Rat Islets

	2-Bromopalmitate (mmol/L)	
	0 mmol/L	0.5 mmol/L
Palmitate oxidation (pmol CO_2/islet/h)	10.8 ± 0.9	10.6 ± 1.3
Palmitate esterification (pmol/islet/h)	219.7 ± 29.7	114.3 ± 16.5*

*$P < .05$.

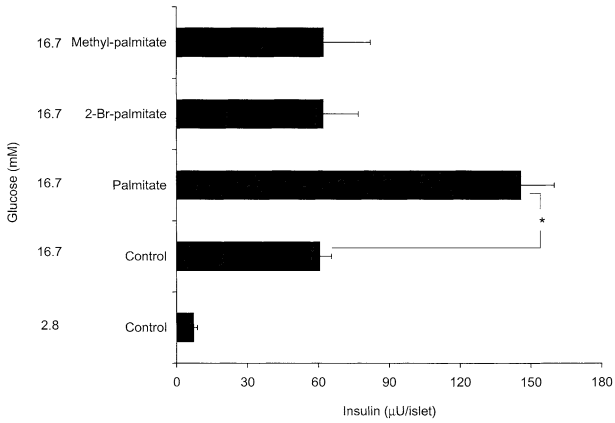

Fig 3. Effects of palmitate analogues on insulin secretion. Isolated islets were incubated in the presence of 2.8 mmol/L glucose or 16.7 mmol/L glucose with or without 0.5 mmol/L palmitate, 0.5 mmol/L 2-bromopalmitate, or 0.5 mmol/L methyl palmitate for 1 hour at 37°C as described in Materials and Methods. The BSA concentration was 0.1 mmol/L in all conditions. Results are mean ± SE of 5 replicate experiments. *$P < .0001$.

second series of experiments, islets were preincubated for 30 minutes in 2.8 mmol/L glucose + 0.5 mmol/L 2-bromopalmitate and then challenged for 60 minutes with 2.8 mmol/L glucose, 16.7 mmol/L glucose or 16.7 mmol/L glucose + 0.5 mmol/L palmitate with or without 0.5 mmol/L 2-bromopalmitate (Fig 4). As expected, palmitate strongly potentiated GSIS ($P < .0001$, n = 4). 2-bromopalmitate did not stimulate insulin secretion at either 2.8 or 16.7 mmol/L glucose (both NS, n = 4), confirming the results of Fig 3. However, 2-bromopalmitate significantly inhibited the potentiating effect of palmitate on GSIS ($P < .01$, n = 4).

DISCUSSION

The aim of this study was to characterize palmitate potentiation of insulin secretion in isolated rat islets and to assess the

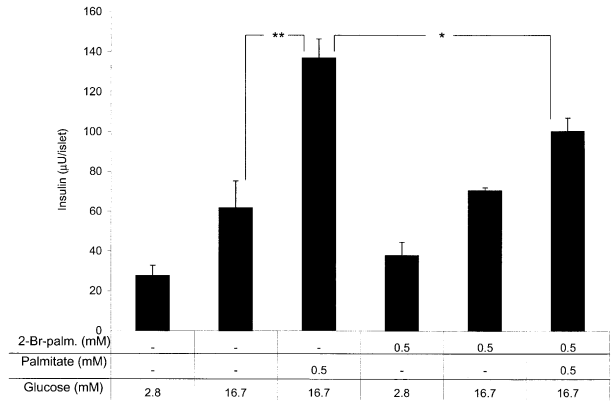

Fig 4. Effects of preincubation with 2-bromopalmitate on insulin secretion. Isolated islets were preincubated with 0.5 mmol/L 2-bromopalmitate for 30 minutes and then incubated in the presence of 2.8 mmol/L glucose, 16.7 mmol/L glucose, or 16.7 mmol/L glucose + 0.5 mmol/L palmitate, with or without 0.5 mmol/L 2-bromopalmitate, for 1 hour at 37°C as described in Materials and Methods. Results are mean ± SE of 4 replicate experiments. *$P < .01$; **$P < .0001$.

effects of 2-bromopalmitate on palmitate metabolism and insulin secretion. We found that palmitate, at a total concentration of 0.5 mmol/L complexed with 0.1 mmol/L BSA, did not initiate insulin release in the presence of substimulatory concentrations of glucose, but strongly potentiated insulin release at glucose concentrations $\geq$ 5.6 mmol/L. These results are consistent with previous studies in rat[13] and mouse[12] islets and HIT-T15 cells.[11] When the concentration of BSA was increased in parallel with that of palmitate to maintain the molar ratio constant at 5:1, the potentiating effect of palmitate at 11.1 mmol/L glucose increased up to 0.5 mmol/L total palmitate and reached a plateau thereafter. In contrast, when the BSA concentration was fixed at 0.1 mmol/L, palmitate dose-dependently potentiated 11.1 mmol/L glucose-induced insulin secretion throughout the entire concentration range (from 0.2 to 0.8 mmol/L total palmitate). This suggests that both the total concentration of palmitate and the palmitate:BSA molar ratio determine the potency of the FA for total concentrations $\leq$ 0.5 mmol/L, whereas its unbound concentration is determinant above 0.5 mmol/L. Indeed, under the conditions used in our experiments, the unbound concentration of palmitate is likely to remain constant when the molar ratio is fixed and the total palmitate concentration is less than 0.5 mmol/L.[18] The linear increase in potentiation of GSIS obtained by increasing the molar ratio of palmitate:BSA is consistent with previous observations by Warnotte et al[14] in mouse islets, who found that increasing the concentration of palmitate from 0.5 to 1 mmol/L and keeping the palmitate:BSA ratio at 3.3 did not lead to a further increase in GSIS. However, Warnotte et al[14] did not directly assess the effects of increasing the total palmitate concentration together with the BSA concentration at total palmitate concentration less than 0.5 mmol/L. Similarly, Prentki et al[11] found that increasing the palmitate:BSA ratio at total concentrations of palmitate higher than 0.5 mmol/L does not further stimulate insulin secretion from insulin-secreting HIT-T15 cells, but did not investigate lower palmitate concentrations. In contrast, Vara and Tamarit-Rodriguez[19] observed an increase in GSIS in islets isolated from fed rats in response to increasing palmitate with a constant ratio of palmitate:BSA of 3.3. Therefore, conflicting results have been reported concerning the importance of total palmitate versus unbound palmitate in determining the potency of the FA. Our results suggest that both might actually be important, especially when total palmitate concentration is $<$0.5 mmol/L. The unbound concentration of palmitate has been estimated in several studies[11,14,20] by the stepwise equilibrium method published by Spector et al,[21] which takes into account the 6 FA-binding sites of BSA. However, more direct measurements using a fluorescent FA binding protein (ADIFAB) have shown that theoretical calculations have overestimated the unbound FA concentration under most conditions.[18] Using the ADIFAB method in preliminary experiments, we have observed that a total concentration of 0.5 mmol/L palmitate complexed to 0.1 mmol/L BSA corresponds to an unbound concentration of approximately 20 nmol/L (data not shown), representing about 3 times the concentration measured in human plasma.[22]

Whether FA potentiate insulin secretion via their mitochondrial oxidation or the generation of FA-derived cytosolic signals has been debated. Initially, Malaisse et al[23] showed that blockade of FA oxidation with methyl palmoxirate inhibited insulin release induced by a variety of secretagogues, including glucose, and concluded that oxidation of endogenous FA participated in the regulation of insulin release. The issue has, however, been complicated by conflicting reports regarding the effects of brominated derivatives of long-chain FA. Two studies in perifused rat islets showed that 2-bromostearate potentiated GSIS in islets from fasted, but not from fed, animals.[24,25] Further, 2-bromostearate decreased palmitate oxidation in islets from both fed and fasted rats and increased esterification at low, but not high, glucose in fed rats and at high, but not low, glucose in fasted rats.[24] It was later reported that 2-bromostearate did not affect insulin secretion at either 2.8 or 16.7 mmol/L glucose in perifused adult rat islets.[26] Similarly, in perifused mouse islets, 2-bromopalmitate did not stimulate secretion and reduced palmitate-potentiated GSIS by half.[12] In contrast, in HIT-T15 cells, 2-bromopalmitate potentiated GSIS and decreased palmitate oxidation,[11] which led to the concept that FA potentiation of GSIS was not mediated by their mitochondrial oxidation. In the perfused rat pancreas, 0.2 mmol/L 2-bromopalmitate or 2-bromostearate potentiated GSIS.[27] Although the reasons for these discrepancies are unclear, they might be due to variable effects of the brominated derivatives on palmitate metabolism in β cells, depending on their nature, their concentration, and the concentration of glucose at which they are being used. The premise that brominated derivatives of long-chain FA inhibit palmitate oxidation comes from the initial observations that 2-bromopalmitate strongly inhibited carnitine-dependent oxidation of long-chain FA in intact isolated mitochondria,[16] and that 2-bromostearate inhibited palmitate oxidation in islets from *ob/ob* mice.[28] However, subsequent studies have shown that these compounds also inhibit overall neutral lipid synthesis in adipose,[29] intestinal,[30] and liver[31] cells. This is due to inhibition of activation of long-chain FA into their CoA esters,[32] which would be expected to decrease the overall metabolism of FA, as well as inhibition of enzymes involved in neutral lipid synthesis, such as monoglycerol-, diacylglycerol-, and glycerol-3-P acyltransferases.[33] Our results in isolated rat islets demonstrate that 2-bromopalmitate does not affect palmitate oxidation, but inhibits palmitate esterification into neutral lipids, consistent with its action in other tissues.[29-31]

Because 2-bromopalmitate is not esterified into neutral lipids[20] and inhibits esterification of endogenous FAs,[32] its lack of stimulation of insulin secretion suggests that a metabolic intermediate generated in the esterification pathway mediates FA potentiation of GSIS. This is further supported by our observation, consistent with that of Warnotte et al[12] that 2-bromopalmitate partly inhibits palmitate-potentiation of GSIS. Indeed, it is noteworthy that a 50% reduction in palmitate esterification in the presence of 2-bromopalmitate is associated with an approximately 50% decrease in the effect of palmitate on GSIS, suggesting a correlation between the rate of esterification of the FA and its potentiating effect. Although the nature of the signal generated in the esterification pathway is unknown, it has been proposed that LC-CoA can directly modulate insulin secretion via activation of protein kinase C[34] or acylation of proteins involved in insulin exocytosis.[35]

In conclusion, this study indicates that both the total concentration and molar ratio to BSA are important determinants of

the potency of palmitate in potentiating GSIS at total concentration of the FA <0.5 mmol/L, and that 2-bromopalmitate, which unexpectedly decreases palmitate esterification without affecting its oxidation in isolated rat islets, does not affect insulin secretion by itself, but inhibits the potentiating effect of palmitate. These findings support the notion that FA potentiation of insulin secretion is mediated by a cytosolic, lipid-derived signal, the nature of which remains to be determined.

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