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Research Article

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A Fatty Acid–dependent Step Is Critically Important for Both Glucose- and Non–Glucose-Stimulated Insulin Secretion

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Abstract

Lowering of the plasma FFA level in intact fasted rats by infusion of nicotinic acid (NA) caused essentially complete ablation of insulin secretion (IS) in response to a subsequent intravenous bolus of arginine, leucine, or glibenclamide (as previously found using glucose as the β-cell stimulus). However, in all cases, IS became supranormal when a high FFA level was maintained by co-infusion of lard oil plus heparin. Each of these secretagogues elicited little, if any, IS from the isolated, perfused "fasted" pancreas when tested simply on the background of 3 mM glucose, but all became extremely potent when 0.5 mM palmitate was also included in the medium. Similarly, IS from the perfused pancreas, in response to depolarizing concentrations of KCl, was markedly potentiated by palmitate. As was the case with intravenous glucose administration, fed animals produced an equally robust insulin response to glibenclamide regardless of whether their low basal FFA concentration was further reduced by NA. In the fasted state, arginine-induced glucagon secretion appeared to be independent of the prevailing FFA concentration.

The findings establish that the essential role of circulating FFA for glucose-stimulated IS after food deprivation also applies in the case of nonglucose secretagogues. In addition, they imply that (*i*) a fatty acid–derived lipid moiety, which plays a pivotal role in IS, is lost from the pancreatic β-cell during fasting; (*ii*) in the fasted state, the elevated level of plasma FFA compensates for this deficit; and (*iii*) the lipid factor acts at a late step in the insulin secretory pathway that is common to the action of a wide variety of secretagogues. (*J. Clin. Invest.* 1998. 101:2370–2376.) Key words: fatty acids • insulin secretion • insulin secretagogues

Introduction

Although early studies from a number of laboratories pointed to an important role of fatty acids in the regulation of pancreatic β-cell function (1–3), the phenomenon was largely overlooked until recently. Three new observations have served to emphasize the potent interaction between fatty acids and glucose in stimulating insulin secretion. First, when the elevated level of circulating FFA in 18–24-h–fasted rats was acutely lowered by infusion of the antilipolytic agent nicotinic acid (NA),[1] subsequent glucose-stimulated insulin secretion (GSIS) was completely ablated, but became supranormal when the FFA concentration was maintained high by co-infusion of a lipid emulsion plus heparin (4, 5). Qualitatively similar results have been obtained in 48-h–fasted humans (6). Second, GSIS in fed rats was equally robust regardless of whether their already low level of plasma FFA was further reduced by NA (4); here again, however, artificial elevation of the FFA concentration caused a dramatic enhancement of GSIS. Third, the insulinotropic potency of fatty acids was found to be influenced profoundly by their chain length and degree of saturation, the longer chain and more saturated species being the most effective (5).

Of immediate interest is how, in biochemical terms, FFAs exert such a remarkable synergism with glucose to activate β-cell activity. As a first step toward answering this question, we asked whether the effect of FFA is restricted to the situation in which glucose drives insulin secretion. The studies outlined below, carried out both in vivo and in the isolated perfused pancreas, show that this is not the case. It turns out that in the fasted rat, FFAs are indispensable for insulin secretion in response to secretagogues as different in their action as leucine, glibenclamide, potassium, and arginine. A common and presumably late site of action of FFA in the insulin secretory process is thus implied.

Methods

Animals. Male Sprague-Dawley rats weighing ~300 g (Harlan Sprague-Dawley Co., Indianapolis, IN) were used throughout. The rats were maintained on a light–dark cycle (lights on from 1000 to 2200 h) with ad libitum access to standard rodent chow containing 4% fat (Harlan Teklad, Madison, WI). For in vivo studies, we fitted the animals with carotid and jugular vein catheters 5–9 d before use as described previously (4); in most studies the rats were deprived of food from 1800 h the night before experiments.

Preparation of solutions. [L]arginine (Cal Biochem-Nova Biochem International, La Jolla, CA) and [L]leucine (Sigma Chemical Co., St. Louis, MO) were dissolved in water in amounts close to the limit of their solubility to give a final concentration of 0.72 and 0.16 M, respectively (pH adjusted to 7.0). Similarly, we prepared glibenclamide (Sigma Chemical Co.) at a final concentration of 1.0 mM in saline (pH adjusted to 10.2 with NaOH). We diluted potassium chloride (Sigma Chemical Co.) to 0.72 M in saline with pH adjusted to 7.0. The nicotinic acid and heparin solutions, as well as the lard oil emulsion and blood for replacement during infusion studies, were prepared as described previously (4, 5).

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1. *Abbreviations used in this paper:* CPT I, carnitine palmitoyltransferase I; GSIS, glucose-stimulated insulin secretion; NA, nicotinic acid.

Euglycemic clamp studies. On the morning of experiments at ~ 1000 h, we flushed catheters with 25 U/ml heparinized saline. We connected syringes containing the insulin secretagogue, lard emulsion, glucose, heparin, NA, and blood replacement to the venous catheter, while the arterial sampling catheter was kept patent with heparinized saline. Each experiment consisted of a 90-min equilibration phase followed by a 30-min experimental period initiated with a 2-min bolus of 0.46 mmol/kg leucine, 2 μmol/kg glibenclamide, or 2.1 mmol/kg arginine. We took arterial blood samples at −90, 0, 5, 10, 15, 20, 25 and 30 min into cold heparin–lithium-coated microfuge tubes and rapidly centrifuged them. The plasma glucose concentration was determined immediately. We separated the remainder of each plasma sample into aliquots that were snap-frozen in liquid N_2 and stored at −70°C pending further analysis. In some studies, ~ 0.6 ml of blood was mixed with 270 KIU of aprotinin and 0.7 mg of Na EDTA and centrifuged to obtain plasma, which was subsequently assayed for glucagon.

The response to each secretagogue was measured under three different conditions: (*i*) continuous infusion of vehicle from −90 min onward (control); (*ii*) continuous infusion of nicotinic acid from −75 min onward (NA); and (*iii*) continuous infusion of lard oil plus heparin from −90 min and of nicotinic acid from −75 min onward (NA + lard), as described in Stein et al. (5). We maintained baseline plasma glucose concentrations by exogenous infusion of 25% dextrose.

Pancreas perfusion. Pancreases were perfused as described in Chen et al. (7). The basal perfusate, maintained at 37°C under 95% O_2/5% CO_2, contained Krebs–Ringer bicarbonate buffer, pH 7.4, 3 mM glucose, 4.5% (wt/vol) Dextran T-70, and 1% (wt/vol) fatty acid–free BSA. When used, palmitate was present throughout the perfusion as a sodium salt at a concentration of 0.5 mM. We began sample collection after a 15-min equilibration period (designated time zero). 10 min later, a concentrated solution of leucine, glibenclamide, potassium chloride, or arginine was infused into the perfusate to provide a final concentration of 10 mM, 2 μM, 16 mM, or 10 mM respectively. At the 40-min time-point, we stopped this infusion and collected samples for a final 10 min. The flow rate was ~ 1.5 ml/min. The effluent, collected every 2 min in chilled tubes, was stored at −20°C until it was assayed for insulin or glucagon (generally within 3–5 d). We carried out perfusions in the absence and presence of palmitate in random order.

Analyses on plasma and perfusion fluid. We determined glucose concentrations with a Glucose Analyzer II (Beckman Instruments, Fullerton, CA). Fatty acids were measured using the colorimetric kit from Boehringer Mannheim Corp. (Indianapolis, IN); however, we used our own palmitate standard (5). Stein et al. (4) described the insulin assay in detail. We determined glucagon concentrations using a RIA kit (Linco Inc., St. Charles, MO). Arginine and leucine were measured by ion exchange chromatography (8).

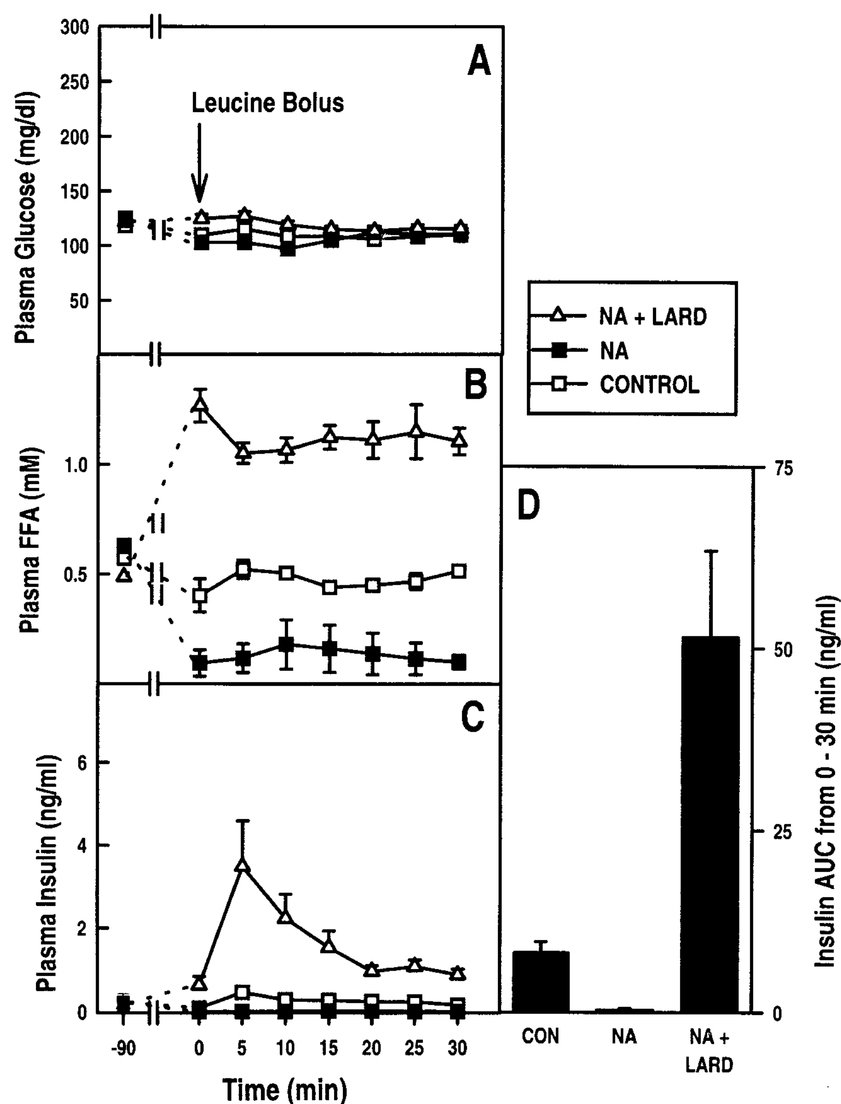

Figure 1. Effect of NA and lard oil infusion on the plasma insulin response to leucine in fasted rats. Animals received the indicated infusions from −75 min (NA) or −90 min (lard oil) as described in Methods. At 0 min, a bolus of leucine (0.46 mmol/kg) was given intravenously. Values are means±SEM for four to five animals in each group. *A*, changes in plasma glucose (mg/dl); *B*, plasma FFA (mM); *C*, plasma insulin (ng/ml); *D*, stimulated insulin area under curve (*AUC*) from 0–30 min (ng/ml). When not shown, SE bars are contained within the symbol. *Lard*, lard oil.

Statistical analysis. We made statistical comparisons between groups using Bonferroni-corrected *t*-tests with the help of SigmaStat software (Jandel Scientific, San Rafael, CA).

Results

In vivo studies. We have previously shown that in the fasted rat an elevated level of plasma FFA is essential for insulin secretion after a glucose challenge (4, 5). To determine whether this also holds in the case of nonglucose secretagogues, we chose three such compounds for study: (*i*) leucine, a nutrient secretagogue; (*ii*) glibenclamide, a commonly prescribed sulfonylurea drug; and (*iii*) arginine, a cationic amino acid that causes β-cell depolarization. Preliminary studies established that an intravenous bolus of leucine at 0.46 mmol/kg resulted in an increase in its plasma concentration from 100±4 μM at baseline to 1,070±5 and 670±5 μM (means±SEM, $n = 3$) at 5 and 10 min, respectively. The equivalent values for plasma arginine after its administration in a dose of 2.1 mmol/kg were 140±4, 5,960±300, and 2,930±1,805 μM, respectively. Neither amino acid affected the circulating concentration of the other. The glibenclamide bolus used here (2 μmol/kg) was based on studies by others (9), but we did not measure the plasma concentration achieved.

As Fig. 1 *C* shows, the leucine bolus caused a significant increase in the plasma insulin concentration, from 0.09±0.02 ng/ml at 0 min to 0.47±0.11 ng/ml at +5 min ($P < 0.05$); the increase, however, subsequently waned. The plasma FFA level changed reciprocally with that of insulin, as expected (*B*), while the glucose concentration was held essentially constant with exogenous infusion (*A*). In keeping with earlier findings (4, 5), infusion of NA greatly reduced the FFA level and lowered the 0-min insulin concentration to 0.01±0.001 ng/ml (*B* and *C*). Importantly, this maneuver caused complete suppression of leucine-stimulated insulin secretion. By contrast, co-infusion of lard oil plus heparin together with NA, which maintained a high circulating FFA level (*B*), increased the 0-min insulin concentration to 0.64±0.21 ng/ml and dramatically boosted the +5 min response to leucine to 3.49±1.10 ng/ml ($P < 0.01$ compared with the control and NA groups; *C*). The integrated insulin concentration from 0 to 30 min (*D*) was 8±1, 0.3±0.2, and 51±12 ng/ml in the control, NA, and NA plus lard groups, respectively, all differences being highly significant.

The acute insulin response to glibenclamide (0.12±0.02 to

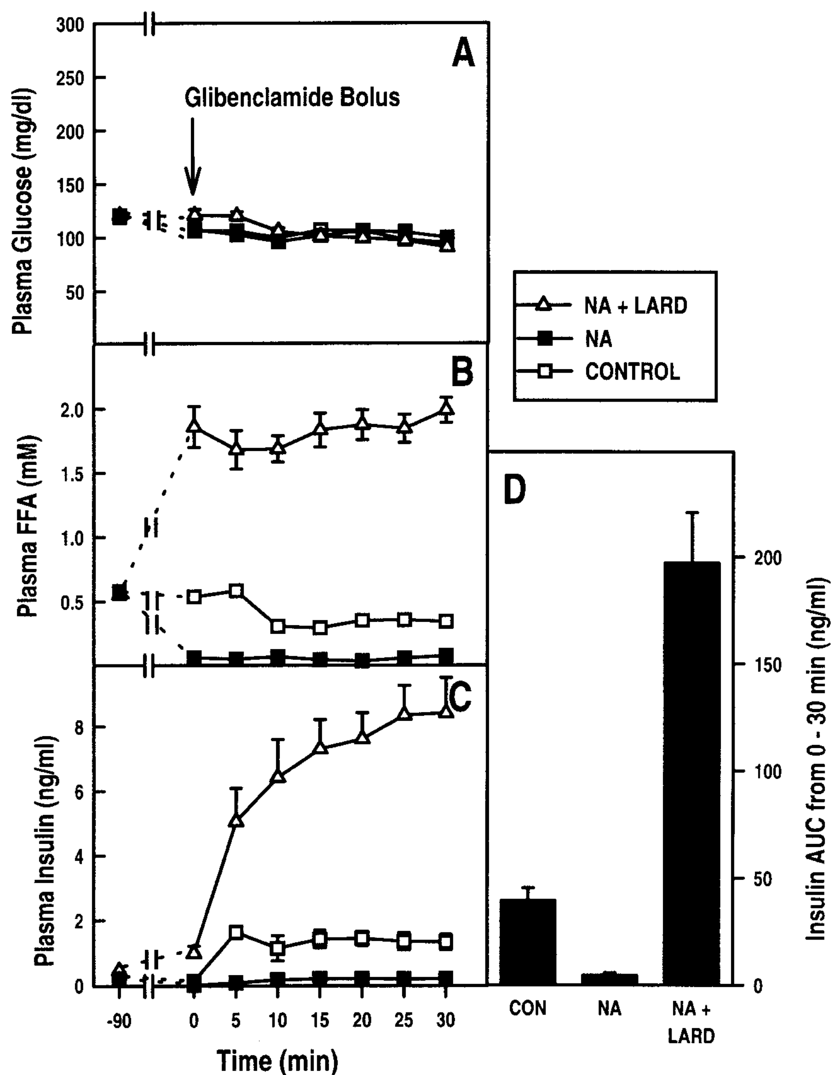

Figure 2. Effect of NA and lard oil infusion on the plasma insulin response to glibenclamide in fasted rats. The procedure was as in Fig. 1 except that an intravenous bolus of glibenclamide (2 μmol/kg) was given at 0 min. Values are means±SEM for four to five animals in each group. *A–D* as in Fig. 1; abbreviations as in Fig. 1.

1.62±0.19 ng/ml at 0 and +5 min, respectively; Fig. 2 *C*) was greater than that seen with leucine (Fig. 1 *C*). Furthermore, after the sulphonylurea bolus, the insulin concentration did not fall appreciably over the next 25 min and, as a result, suppression of lipolysis continued throughout this time (Fig. 2 *B*). Once again, prior lowering of the FFA concentration by NA totally abrogated insulin secretion, but this was greatly stimulated, and in sustained fashion, by the simultaneous infusion of lard oil plus heparin. The integrated insulin concentration between 0 and 30 min for the control, NA, and NA plus lard groups was 39±6, 4.4±1, and 197±24 ng/ml, respectively (Fig. 2 *D*), all differences being highly significant.

The results with arginine infusion (Fig. 3) were qualitatively similar to those obtained when leucine was used as the insulin secretagogue. Although the +5-min insulin response (1.49±0.40 compared with 0.11±0.03 ng/ml at 0 min) was greater than that seen with leucine, it was almost entirely blocked by NA treatment, but was greatly exaggerated in the NA plus lard group to 4.75±0.68 ng/ml; (*C*). The integrated insulin concentration from 0 to 30 min (*D*) was 12±3, 1.5±0.2, and 43±5 ng/ml in the control, NA, and NA plus lard groups, respectively, all differences being highly significant. Again, plasma FFA levels changed reciprocally with those of insulin in control animals (*B*). The glucagon response to arginine was not appreciably affected by NA or lard oil infusion (data not shown).

All of the experiments described above were carried out in fasted rats. Because we had previously observed that GSIS in fed animals is equally robust in the absence or presence of NA (4), the question arose as to whether this also applied in the case of nonglucose secretagogues. Accordingly, we examined the effect of glibenclamide with and without NA in fed animals. The plasma insulin profiles in control and NA-infused rats (whose plasma FFA levels before the sulphonylurea bolus were ~ 0.40±0.03 and 0.13±0.02 mM, respectively) were now found to be indistinguishable, as were the areas under the 0–30-min insulin curves (104±12 and 108±2 ng, respectively [*n* = 3]). Similar studies were attempted with arginine and leucine. However, interpretation of these experiments, in which agents are administered to fed animals, is complicated by the fact that in both cases the insulin response is a composite of two events. One is a direct stimulation of the β-cell by the amino acid itself; the second arises indirectly after the simultaneous enhancement of glucagon secretion, which, in turn, pro-

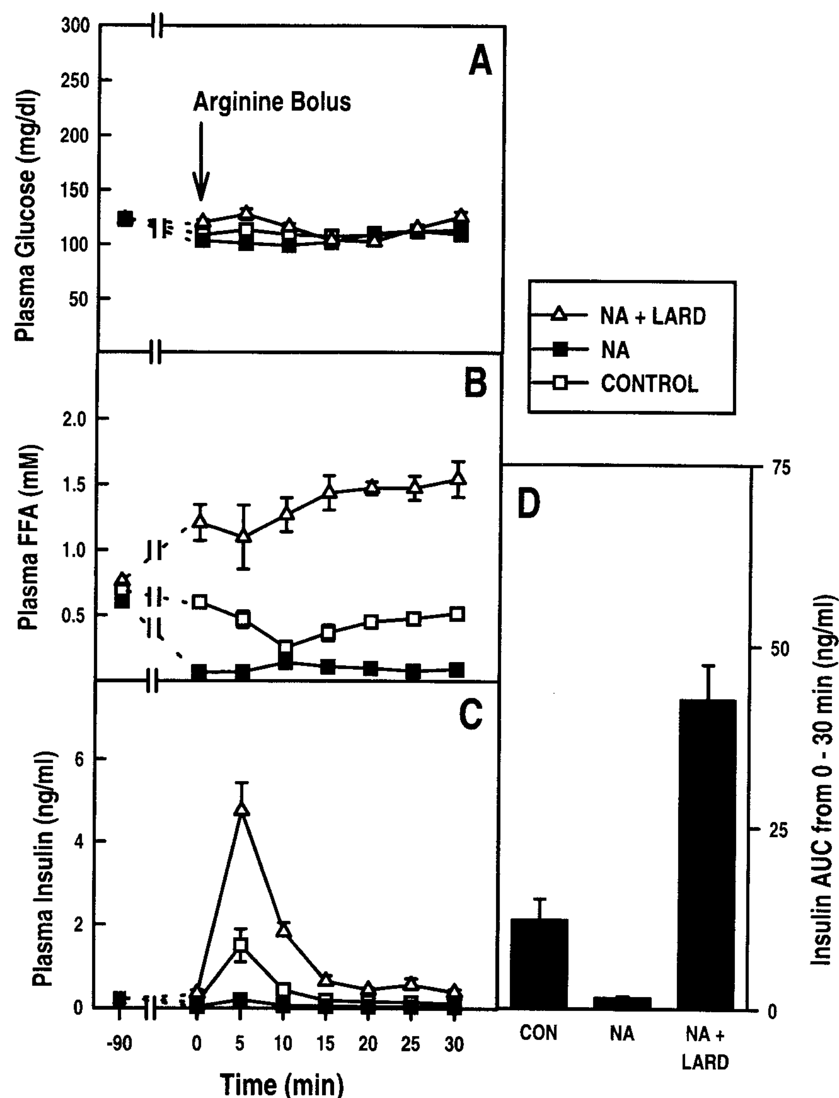

Figure 3. Effect of NA and lard oil infusion on the plasma insulin response to arginine in fasted rats. The procedure was as in Fig. 1 except that an intravenous bolus of arginine (2.1 mmol/kg) was given at 0 min. Values are means±SEM for five to six animals in each group. *A–D* as in Fig. 1; abbreviations as in Fig. 1.

motes hepatic glycogenolysis and elevation of the blood glucose level, adding a further stimulus to insulin secretion.

Studies with the perfused pancreas. The in vivo studies described above imply that in the fasted rat, an elevated plasma FFA level is an absolute requirement for insulin secretion in response to intravenous administration of leucine, glibenclamide, or arginine, reminiscent of the situation when glucose was used to stimulate the β-cell (4, 5). To exclude the possibility that the different plasma insulin profiles observed were simply the result of changes in clearance of the hormone, we examined the ability of each agent to elicit insulin release from the perfused pancreas in a setting of a nonstimulatory level of glucose (3 mM) and in the absence or presence of palmitate (0.5 mM). We also studied potassium chloride, which cannot be administered safely in vivo but has been used extensively in in vitro systems.

When used alone, 10 mM leucine was ineffective in triggering insulin release, but in the presence of palmitate it produced a robust and biphasic response (Fig. 4 *A*), such that total hormone output during the stimulatory period increased from 0.2±0.1 to 58.9±12.6 ng (Fig. 4 *B*).

Glibenclamide, at a 2-μM concentration, also failed to stimulate insulin release, but when palmitate was added to the medium we observed a brisk first phase and a sustained second phase of insulin release (Fig. 4 *C*), resulting in an increase of total hormone output from 0.1±0.03 to 24.0±7.9 ng in the stimulated interval (Fig. 4 *D*). In this case, insulin secretion continued unabated until the last sample collected after the cessation of secretagogue infusion, unlike the pattern observed with leucine. This lingering insulin stimulatory effect of glibenclamide paralleled that observed in vivo (Fig. 2 *C*), suggesting a slow removal of the sulfonylurea from its receptor on the β-cell membrane (now believed to be a subunit of the K^{+}_{ATP} channel [10]).

With the addition of 10 mM potassium to the perfusion medium, which was already 5.9 mM in this cation, we readily demonstrated pancreatic insulin release, and again the response was greatly exaggerated when palmitate was also present (Fig. 4 *E*); stimulated insulin release rose from 33.4±11.1 to 188.2±18.1 ng; (Fig. 4 *F*).

Arginine alone, at a 10-mM concentration, produced a low-grade, slow-onset output of insulin. However, with inclusion of

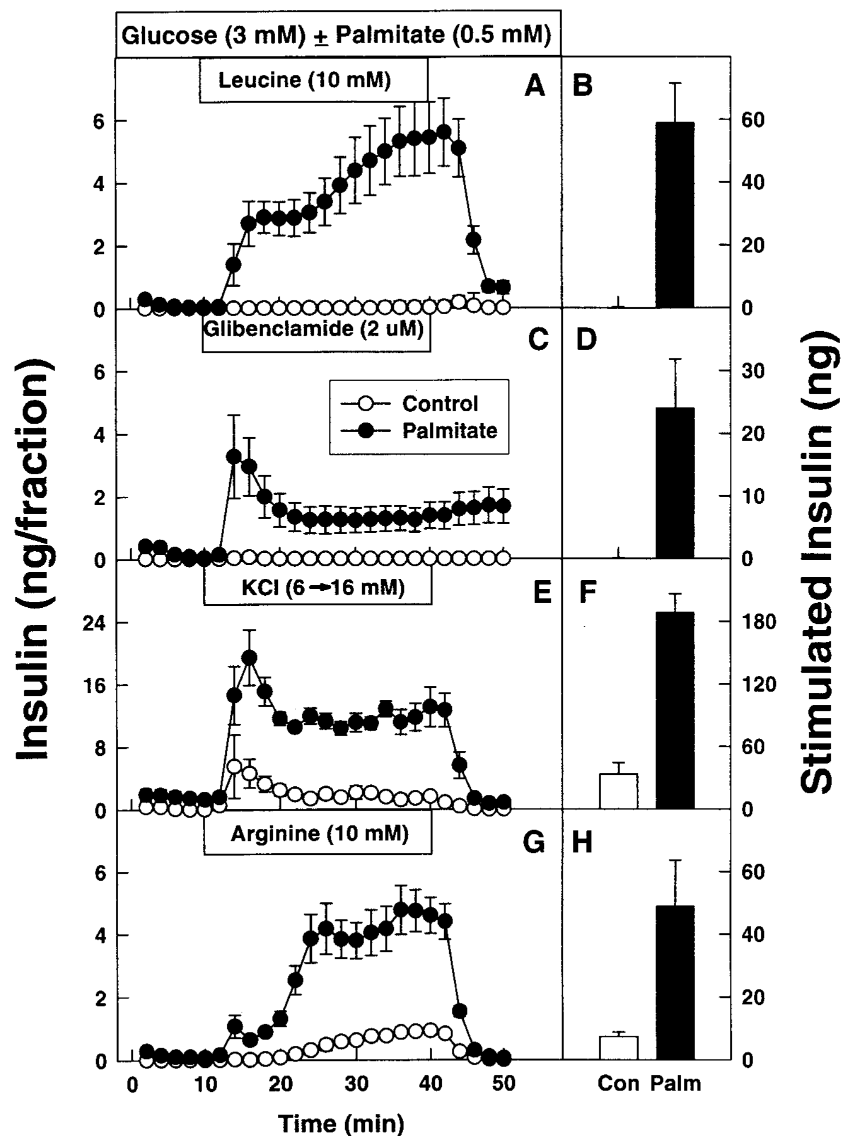

Figure 4. Effect of palmitate on the insulinotropic potency of various secretagogues in the perfused pancreas from fasted rats. The perfusion medium contained 3 mM glucose with or without 0.5 mM palmitate throughout. Secretagogues were infused to give the indicated concentrations from 10–40 min. *B*, *D*, *F*, and *H* show the total insulin output during the stimulated period. Values are means±SEM for three to six perfusions in each group.

the fatty acid, an acute first-phase response was clearly evident, and this was followed by a greatly enhanced second phase of insulin release (Fig. 4 *G*), such that total hormone output during the period of arginine infusion rose from 7.4±1.4 to 48.9±6.6 ng (Fig. 4 *H*).

Because arginine is a potent glucagon secretagogue, we also measured immunoreactive glucagon concentrations in the perfusate. Glucagon secretion at 3 mM glucose was ~ 0.5 ng/fraction and exhibited a sustained fourfold increase after arginine infusion (Fig. 5). Unlike β-cell function, that of the α-cell appeared to be unaffected by perfusion with 0.5 mM palmitate, since stimulated glucagon secretion was 24.1±6.0 and 25.7±3.7 ng, in the vehicle and palmitate groups, respectively. We cannot be certain that FFA had no impact on α-cell function; however, it is conceivable that a FFA-induced enhancement of arginine-stimulated glucagon secretion would have been seen had there not been a simultaneous boost of insulin release, which might have had a suppressive effect on the α-cell.

Discussion

The newly appreciated indispensability of fatty acids for GSIS in the fasted state has been reviewed in the Introduction. This study establishes that the same principle is at work in the case of nonglucose secretagogues. Thus, in the 18–24-h–fasted rat an elevated circulating FFA level proved to be an absolute requirement for normal insulin secretion in response to agents as diverse as arginine, leucine, and glibenclamide. Moreover, as was found in the experiments with glucose (4, 5), both of the amino acids and the sulfonylurea elicited a supranormal rate of insulin secretion if the plasma FFA concentration was maintained high by artificial means. In keeping with the in vivo findings, the aforementioned stimuli promoted little, if any, insulin release from the isolated perfused “fasted” pancreas when present simply on a background of a basal glucose concentration (3 mM). However, when a physiological concentration of palmitate (0.5 mM in the context of 1% albumin) was also included, all three secretagogues became extremely effective, paralleling our earlier results where 12.5 mM glucose acted as the β-cell stimulus (4, 5). Qualitatively similar results were obtained when a high K^+ concentration was used as the stimulant for insulin release. Interestingly, the availability of FFA appeared to have no net effect on glucagon secretion either in vivo or from the isolated, fasted rat pancreas, at least in the case where arginine was used as the stimulus.

Although the mechanism of this fatty acid augmentation of insulin secretion remains to be established, certain points emerge from a consideration of how the various secretagogues we have tested are thought to act on the β-cell. In the case of glucose, it is generally believed that the primary mechanism of stimulus–secretion coupling requires the metabolism of the hexose causing elevation of the ATP/ADP ratio, closure of the plasma membrane K^+_{ATP} channels, depolarization of the membrane, and opening of voltage-dependent Ca^{2+} channels, which triggers insulin release (11). However, a separate calcium-independent mechanism requiring activation of protein kinase A and protein kinase C to allow insulin release has recently been described (12). A possible additional component, at least in the fed state, is the suppression of mitochondrial carnitine palmitoyltransferase I (CPT I) by glucose-derived malonyl-CoA. This is expected to cause an increase in the concentration of cytosolic long-chain fatty acyl-CoA, which, in turn, is thought to serve as a coupling factor (mechanism unknown) in insulin secretion (7, 13). As to why GSIS becomes absolutely dependent upon circulating FFA in the fasted state, we have suggested two potential explanations (5). One would be that during starvation the β-cell loses its ability to convert glucose into malonyl-CoA, possibly because of a lowered activity of pyruvate dehydrogenase and/or acetyl-CoA carboxylase (as in liver). The expected result might be failure to suppress CPT I activity, resulting in insufficient elevation of the cytosolic fatty acyl-CoA concentration to activate the insulin secretory process, thus rendering the β-cell dependent upon a high external FFA concentration to make up the deficit. Alternatively, starvation might simply cause depletion of a critical lipid moiety that is needed to synergize with glucose for activation of hormone release and whose concentration can only be maintained provided that an adequate supply of FFA is available from the circulation.

The present findings with nonglucose secretagogues might help to discriminate between these two scenarios. Leucine is converted by transamination into α-ketoisocaproate that can be metabolized in mitochondria to form acetyl-CoA, a process expected to generate ATP and citrate (a precursor of malonyl-CoA) (11). As noted above, both of these products are implicated in stimulus-secretion coupling. Glibenclamide is be-

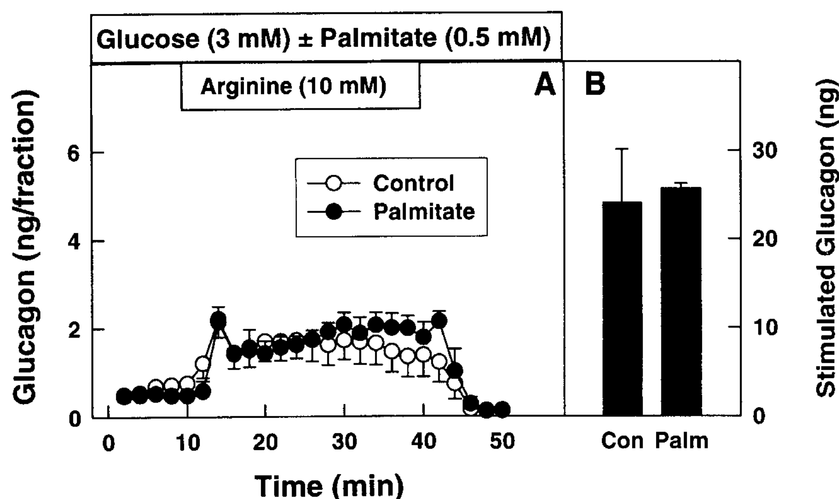

Figure 5. Lack of effect of palmitate on arginine-stimulated glucagon release from the perfused pancreas of fasted rats. The procedure was as described for Fig. 4. Values are means±SEM for three perfusions in each group.

lieved to bind to the sulphonylurea receptor protein associated with the K^+_{ATP} channel, causing its closure and subsequent events leading to insulin secretion as outlined above (10). A high potassium concentration appears to disrupt the K^+_{ATP} channel, resulting in the accumulation of cations in the β-cell and opening of the voltage-sensitive Ca^{2+} channel (12). Arginine, on the other hand, is thought to enter the β-cell via an amino acid transporter and accumulates positive charge within the cell, eventually causing depolarization (14). That in the fasted rat all four of these agents, just like glucose, showed an absolute or very strong dependence on FFA for their insulinotropic action, strongly suggests that fatty acids are involved in activation of a common and permissive step in the secretory process that is independent of the nature of the secretagogue. Important in this regard is the additional finding that in nonfasted rats, NA treatment had no impact on insulin secretion driven by glibenclamide (this work) or glucose (4). We suspect that the same is true for arginine and leucine (see above). This would be consistent with the notion that in the fed state, the β-cell contains enough of the fatty acid–derived entity to allow insulin secretion regardless of the secretagogue used, whereas with food deprivation this lipid pool is dissipated, rendering circulating FFA mandatory for hormone release. In both nutritional states, however, an abnormally high plasma FFA level will promote hypersecretion of insulin. In keeping with this view is the finding that depletion of islet triglyceride stores in leptin-treated rats resulted in total loss of glucose and arginine-stimulated insulin secretion, which was immediately corrected by the provision of an oleate–palmitate mixture (15).

Finally, we must now determine precisely where in the insulin secretory process fatty acids exert their dramatic effect. The current data indicate that the site of fatty acid interaction is late in the secretory cascade and likely occurs at the level of Ca^{2+} entry into the β-cell or at a point distal to this step (perhaps at the stage of fusion of the insulin granule with the plasma membrane or exocytosis). We must also discern in what form the fatty acids act, whether as free acids, CoA esters, or esterified products, and why their efficacy increases so profoundly with chain length and degree of saturation (5).

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