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Enhanced activation of phospholipase C and insulin secretion from islets incubated in fatty acid–free bovine serum albumin

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Abstract

Incubation in 100 μmol/L fatty acid–free bovine serum albumin (FAF-BSA) significantly amplifies insulin secretion from isolated, perifused rat islets. When compared with the responses of control islets incubated in 100 μmol/L radioimmunoassay-grade BSA, insulin secretion rates were increased 2- to 3-fold when these islets were stimulated with 10 mmol/L glucose alone or with the combination of 10 mmol/L glucose, 15 mmol/L KCl, and 100 μmol/L diazoxide. These amplified secretory responses were paralleled by significant increases in the phospholipase C (PLC) activation monitored by fractional increases in ^{3}H-inositol efflux from these same islets. Amplified PLC responses were also observed with the cholinergic agonist carbachol (50 μmol/L). No differences in the secretory responses to the protein kinase C activator phorbol 12-myristate 13-acetate (200 nmol/L) could be detected between control and FAF-BSA–pretreated rat islets. Mouse islets were also immune to the amplifying impact of this treatment protocol. These findings demonstrate that short-term incubation in FAF-BSA significantly augments the activation of PLC in rat islets by a number of agonists. This proximal event provides the impetus for the distal activation of protein kinase C. If applicable to human islets, this manipulation may provide a mechanism to enhance the secretory responses from islets destined for transplantation, thus improving their in vivo secretory capacity.
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1. Introduction

Glucose-induced insulin secretion is a complex biochemical event regulated by a host of potential second-messenger molecules acting alone or in concert [1-3]. These events include not only the cation calcium, which gains access to the β-cell via the opening of voltage-regulated channels, but also cyclic adenosine monophosphate and phosphoinositide-derived second-messenger molecules, generated as a consequence of phospholipase C (PLC) activation. Because of the emerging consensus [4,5] that β-cell failure plays a major pathogenic role in the emergence of type 2 diabetes mellitus, attention has been focused on elucidating the nature of the pathways that so elegantly control insulin secretion. Moreover, their identification may also provide insight into maneuvers that might improve their function.

Recently, Straub and Sharp [6] reported that a brief 3- to 6-hour exposure of isolated rat islets to fatty acid–free bovine serum albumin (FAF-BSA) dramatically potentiated the subsequent insulin secretory response to 16.7 mmol/L glucose. This amplified response was not confined to glucose; it could be observed in response to α-ketoisocaproate alone and from islets stimulated with the combination of glucose and potassium in the presence of diazoxide. However, this amplified response could not be duplicated in mouse islets or in 2 clonal cell lines, INS-1 and INS 832/13. They further demonstrated that the activation of protein kinase C (PKC) appeared to be playing a preeminent role in this amplification process because a number of PKC inhibitors proved effective in thwarting its development and evocation.

The species specificity of the amplification induced by incubating islets in FAF-BSA is reminiscent of the divergent short-term stimulatory impact that glucose exerts on perfused or freshly isolated perifused rat or mouse islets [7-9]. We have previously demonstrated that the smaller second-phase response observed from mouse islets by a number of investigators including ourselves might be attributable to the less robust activation of PLC in this species as opposed to rat islets [1,10,11]. Because the agonists used in the comprehensive study by Straub and Sharp [6] activate PLC in addition to PKC, we decided to explore how incubation of islets in FAF-BSA influences

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PLC activation in both species. In addition, we also examined how the response to phorbol 12-myristate 13-acetate (PMA) might be influenced by this manipulation because this is a direct activator of PKC. Our findings confirm the observation that prior exposure to FAF-BSA markedly augments glucose-induced secretion from rat but not from mouse islets. Our studies also suggest that enhanced activation of PLC appears to account for this response.

2. Materials and methods

2.1. Islet isolation

The detailed methodologies used to assess insulin output from collagenase-isolated islets have been previously described [12,13]. Male Sprague-Dawley rats (weighing 325-425 g at the time of study) and male CD-1 mice (weighing 28-45 g at the time of study) were purchased from Charles River (Wilmington, MA) and used in all studies. All animals were treated in a manner that complied with the National Institutes of Health's *Guidelines for the Care and Use of Laboratory Animals*. The animals were fed ad lib. After pentobarbital sodium (Nembutal, 50 mg/kg; Abbott, North Chicago, IL)–induced anesthesia, 20 to 30 mL of cold Hanks solution (without any added glucose) was used to distend the pancreas via the biliary system. Islets were isolated by collagenase digestion and handpicked, using a glass loop pipette, under a stereomicroscope into Krebs-Ringer bicarbonate (KRB) supplemented with 3 mmol/L glucose. They were free of exocrine contamination.

2.2. Islet incubation

After isolation, a process lasting no more than 90 minutes after initial surgical removal of the pancreas, groups of 18 to 32 islets were loaded onto nylon filters (Sefar America, Kansas City, MO). The larger groups of islets were used for the ^{3}H-inositol efflux studies. The islets on nylon filters were then placed in Corning 3513 12-well, cell culture cluster, flat-bottom containers. Two milliliters of CMRL culture medium 1066 (Gibco, Carlsbad, CA, 11530, without added L-glutamine) was gently added to each well. This medium contains 5.6 mmol/L (100 mg/dL) glucose. Control islets were incubated in CMRL 1066 supplemented with 100 μmol/L BSA (Sigma, St Louis, MO; albumin A7888, radioimmunoassay [RIA] grade, fraction V, batch 044KO460). Experimental islets were incubated in CMRL 1066 supplemented with 100 μmol/L FAF-BSA (Roche, Indianapolis, IN; heat shock, FAF, lot 93433222). All groups of islets were then incubated for 4 hours at 37°C in an incubator with 5% CO_2/95% humidified air.

In those experiments where PLC activation was monitored, ^{3}H-inositol was included during the incubation period in CMRL 1066 to label phosphoinositide pools [14]. This solution was prepared as follows. To 20 μL of stock myo-(2-^{3}H[N])inositol (PerkinElmer Life Sciences, Boston, MA; 1 mCi/mL), 2.1 mL of CMRL 1066 was added. After mixing, 2 mL of this solution was gently added to the incubation well with the islets.

2.3. Perifusion studies

After the incubation period, all islets were washed gently with 5 mL of warmed, fresh KRB perifusion medium. They were then perifused in a KRB buffer at a flow rate of 1 ± 0.1 mL/min for 30 minutes in the presence of 3 mmol/L glucose unless otherwise indicated. Sigma BSA (RIA grade), 25 μmol/L, was included during the entire perifusion period with all groups of islets irrelevant of their pretreatment. This is the standard BSA concentration we have used in many previous reports [15,16]. After this 30-minute stabilization, period islets were then perifused with the appropriate agonist or agonist combinations as indicated in the figure legends and the Results section. To prevent the reincorporation of ^{3}H-inositol back into phosphoinositide pools and to facilitate the measurements of ^{3}H-inositol efflux from these small groups of islets, 0.1 mmol/L cold myoinositol was included in the perifusion where efflux was monitored.

Perifusate solutions were gassed with 95% O_2/5% CO_2 and maintained at 37°C. Insulin released into the medium was measured by RIA [17]. From the same collection tubes, the amount of ^{3}H-inositol effluxing from the islets was also determined [18,19]. At the termination of the efflux experiments, the number of counts remaining in the islets was assessed; and efflux of ^{3}H-inositol was expressed as fractional efflux rates [19-21].

2.4. Reagents

Ice-cold Hanks solution without any added glucose was used for the islet isolation. The perifusion medium consisted of 115 mmol/L NaCl, 5 mmol/L KCl, 2.2 mmol/L $CaCl_2$, 1 mmol/L $MgCl_2$, 24 mmol/L $NaHCO_3$, and 0.17 g/dL (25 μmol/L) BSA (Sigma A7888, RIA grade). The ^{125}I-labeled insulin used for the insulin assay and the ^{3}H-inositol used to assess PLC activation were purchased from PerkinElmer Life Sciences. Glucose, carbachol, LiCl, diazoxide, PMA, and the salts used to make the Hanks solution and perifusion medium were purchased from Sigma. Rat insulin standard (lot 615-ZS-157) was the generous gift of Dr Gerald Gold, Eli Lilly (Indianapolis, IN). Collagenase (type P) was obtained from Roche Applied Science.

2.5. Statistics

Statistical significance was determined using the Student *t* test for unpaired data or analysis of variance. A P value $\leq$ .05 was taken as significant. Values presented in the figures and the Results section represent means ± SEMs of at least 3 observations.

3. Results

In the initial series of studies, we first confirmed the observations made previously by Straub and Sharp [6]

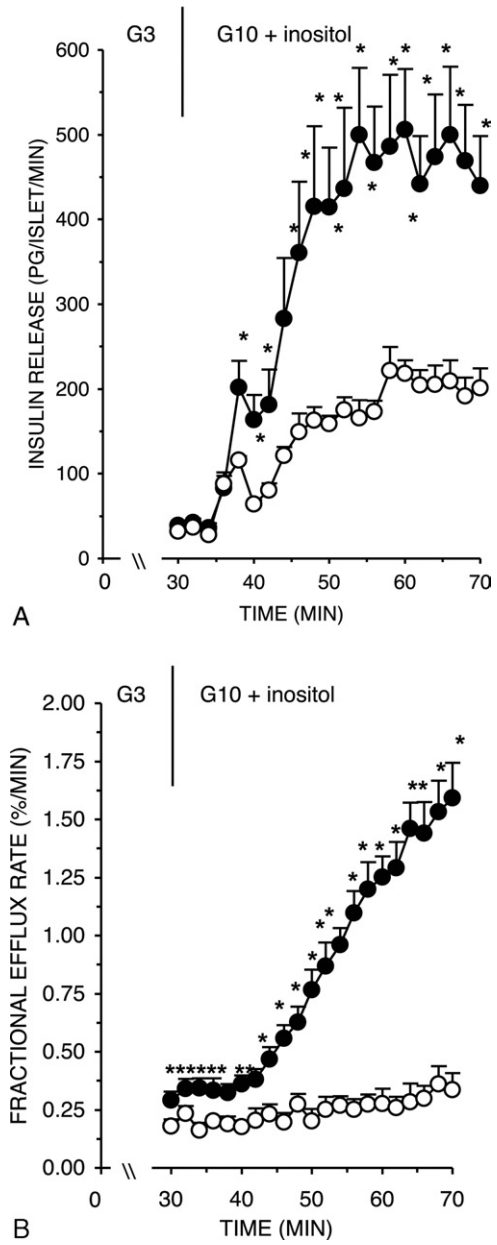

Fig. 1. Effects of FAF-BSA on insulin secretion and ^{3}H-inositol efflux from isolated, perifused rat islets stimulated with 10 mmol/L glucose. Groups of islets were isolated and subjected to a 4-hour incubation in CMRL 1066 medium supplemented with 100 μmol/L Sigma 7888 RIA-grade BSA (open circles) or 100 μmol/L Roche FAF-BSA (closed circles) and ^{3}H-inositol. After washing to remove unincorporated label, the islets were then perifused. For the initial 30 minutes of the perifusion, the glucose level was maintained at 3 mmol/L (G3). For the next 40 minutes, onset indicated by the vertical line, both groups of islets were stimulated with 10 mmol/L glucose (G10) plus 0.1 mmol/L cold inositol to restrain reincorporation of labeled ^{3}H-inositol back into phosphoinositide pools. Insulin secretion rates (A) were measured, and fractional rates of ^{3}H-inositol efflux rates (B) were calculated. This and subsequent figures have not been corrected for the dead space, about 2.5 mL or 2.5 minutes with a flow rate of 1 mL/min, in the perifusion apparatus. Mean values ± SEM of at least 7 experiments are shown. The asterisk indicates a significant difference ($P \leq .05$) between groups at the indicated time points. With regard to fractional efflux rates, significant differences between groups were noted during the entire perifusion.

that a 4-hour incubation period in culture medium containing 100 μmol/L FAF-BSA enhances glucose-induced insulin secretion. Because it is more physiologic than the 16.7-mmol/L glucose concentration used in this previous report, we decided to use a lower glucose level, 10 mmol/L. In addition, to facilitate the assessment of PLC activation, monitored by the efflux of ^{3}H-inositol [19,22], 0.1 mmol/L inositol was also included with glucose. In response to 10-mmol/L glucose stimulation, a modest biphasic insulin secretory response was evoked from control islets (Fig. 1A). Peak first-phase secretory rates averaged 116 ± 5 pg per islet per minute, whereas peak second-phase release rates were 221 ± 28 pg per islet per minute (n = 7). When compared with prestimulatory secretion of about 30 pg per islet per minute, release rates were increased about 6- to 7-fold.

Prestimulatory secretion rates from FAF-BSA–preincubated islets were comparable with those measured from control islets. However, islets incubated for 4 hours in FAF-BSA exhibited markedly enhanced insulin release rates (Fig. 1A). Both phases of secretion were amplified. Peak first- and second-phase responses to 10 mmol/L glucose from FAF-BSA–incubated islets averaged 202 ± 31 and 506 ± 71 pg per islet per minute, respectively (n = 8). When compared with prestimulatory secretion rates of about 30 pg per islet per minute, release rates were increased about 16- to 17-fold.

A closer analysis of the data presented by Straub and Sharp [6] suggested to us that the proximal activation of PLC by glucose might result in the distal, enhanced activation of PKC as they suggested. To address this issue, the efflux of ^{3}H-inositol from control and FAF-BSA–incubated islets was next determined. As shown in Fig. 1B (open circles), the efflux of ^{3}H-inositol in response to 10 mmol/L glucose was modestly increased from control islets in the presence of 0.1 mmol/L inositol. Efflux rates measured in the presence of 3 mmol/L glucose alone increased from 0.16% ± 0.03%/min to 0.32% ± 0.07%/min after 40 minutes of stimulation with 10 mmol/L glucose.

A different result emerged when similar studies were conducted using islets incubated in FAF-BSA. A small increase in efflux rates was measured in the presence of 3 mmol/L glucose from these islets (Fig. 1B, closed circles). However, the addition of 10 mmol/L glucose to these islets resulted in a significant enhancement of ^{3}H-inositol efflux. Rates of ^{3}H-inositol efflux increased rapidly from these islets and averaged 1.63% ± 0.15%/min after 40 minutes of stimulation.

In their seminal report on this topic, Straub and Sharp [6] also reported that in the presence of diazoxide, the combination of glucose plus potassium also evoked amplified secretion from FAF-BSA–incubated islets. We confirmed this as well, as shown in Fig. 2A, and also demonstrated that PLC activation is similarly enhanced from these islets (Fig. 2B). In islets perifused with 100 μmol/L diazoxide to maintain the patency of the ATP–sensitive potassium

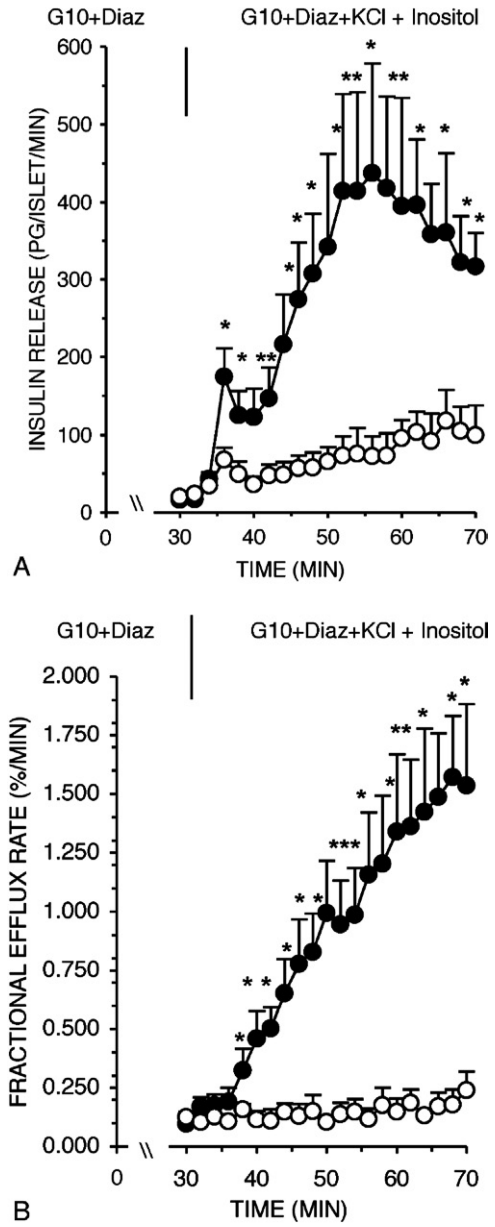

Fig. 2. Effects of FAF-BSA on insulin secretion and ^{3}H-inositol efflux from isolated, perifused rat islets stimulated with KCl. Groups of islets were isolated and subjected to a 4-hour incubation in CMRL 1066 medium supplemented with 100 μmol/L Sigma 7888 RIA-grade BSA (open circles) or 100 μmol/L Roche FAF-BSA (closed circles) and ^{3}H-inositol. After washing to remove unincorporated label, the islets were then perifused for 70 minutes with the combination of 10 mmol/L glucose (G10) plus 100 μmol/L diazoxide. After 30 minutes, 15 mmol/L KCl was then added to the perifusion medium to stimulate the islets. Inositol (0.1 mmol/L) was also included during this time to prevent reincorporation of labeled ^{3}H-inositol back into phosphoinositide pools. Insulin secretion (A) and fractional rates of ^{3}H-inositol efflux (B) were calculated. At least 3 experiments were performed under each condition. The asterisk indicates a significant difference ($P \leq .05$) between groups at the indicated time points.

channel, the stimulatory combination of 10 mmol/L glucose plus 15 mmol/L KCl evoked amplified secretory and PLC responses from FAF-BSA–incubated islets.

In the next set of experiments, we determined the impact of FAF-BSA incubation on PLC activation by the cholinergic agonist carbachol (50 μmol/L). In the presence of 3 mmol/L glucose, the addition of 50 μmol/L carbachol to control islets modestly increased ^{3}H-inositol efflux (Fig. 3). This response was dramatically enhanced in FAF-BSA–pretreated islets. In additional experiments, we included 10 mmol/L LiCl in the perifusion medium. This compound blocks the phosphatases responsible for the metabolism of inositol phosphates into membrane-permeable free inositol [23]. This effect facilitates cellular measurements of inositol phosphates. It reduces the efflux of free inositol from the cell, and studies with lithium have been used to confirm the activation of PLC as well [19,24,25]. Its inclusion in the perifusion medium significantly reduced the efflux of ^{3}H-inositol from FAF-BSA–incubated islets (Fig. 3). Although not shown, the addition of LiCl also significantly reduced ^{3}H-inositol efflux in response to 10 mmol/L glucose

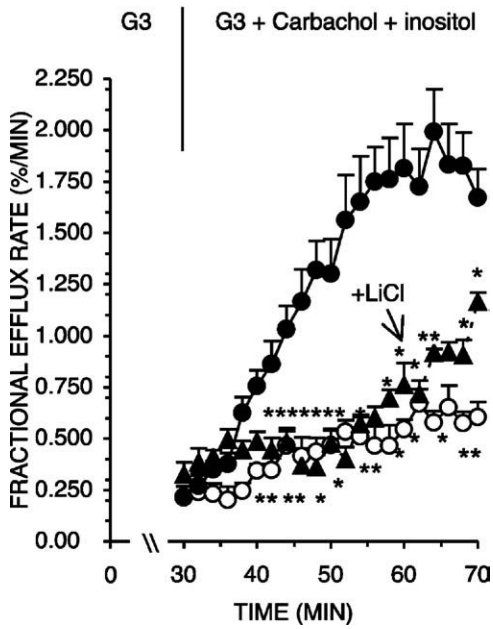

Fig. 3. Effects of FAF-BSA on ^{3}H-inositol efflux from isolated, perifused rat islets stimulated with carbachol. Groups of islets were isolated and subjected to a 4-hour incubation in CMRL 1066 medium supplemented with 100 μmol/L Sigma 7888 RIA-grade BSA (open circles) or 100 μmol/L Roche FAF-BSA (closed circles, closed triangles) and ^{3}H-inositol. After washing to remove unincorporated label, the islets were then perifused with 3 mmol/L glucose. For the next 40 minutes, onset indicated by the vertical line, all groups of islets were stimulated with 50 μmol/L carbachol in the presence of 3 mmol/L glucose plus 0.1 mmol/L cold inositol to restrain reincorporation of labeled ^{3}H-inositol back into phosphoinositide pools. In one group of FAF-BSA–incubated islets (closed triangles), 10 mmol/L LiCl was included during the stimulatory period with carbachol. At least 3 experiments were performed under each condition. The asterisk indicates a significant difference ($P \leq .05$) in efflux from islets incubated for 4 hours in CMRL 1066 (closed circles) when compared with the 2 other groups at the indicated time points. At all time points after minute 40 of the perifusion, these differences were significant.

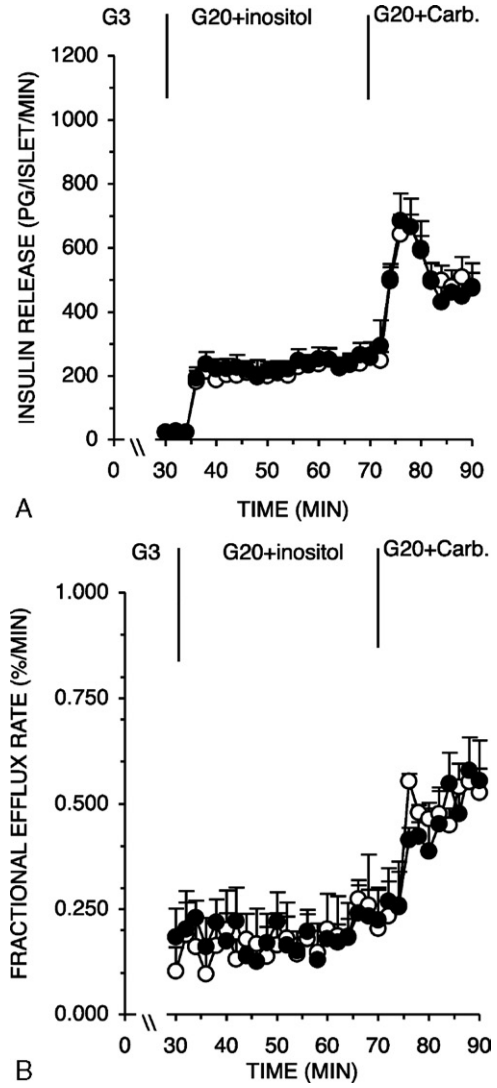

Fig. 4. Effects of FAF-BSA on insulin secretion and ^{3}H-inositol efflux from isolated, perifused mouse islets. Groups of mouse islets were isolated and subjected to a 4-hour incubation in CMRL 1066 medium supplemented with 100 μmol/L Sigma 7888 RIA-grade BSA (open circles) or 100 μmol/L Roche FAF-BSA (closed circles) and ^{3}H-inositol. After washing to remove unincorporated label, the islets were then perifused. For the initial 30 minutes of the perifusion, the glucose level was maintained at 3 mmol/L (G3). For the next 40 minutes, onset indicated by the vertical line, both groups of islets were stimulated with 20 mmol/L glucose (G20) plus 0.1 mmol/L cold inositol. For the final 20 minutes, 50 μmol/L carbachol, in the continued presence of both 20 mmol/L glucose plus inositol, was added to the medium. Insulin secretion rates (A) were measured, and fractional rates of ^{3}H-inositol efflux (B) were calculated. At least 4 experiments were performed under each condition.

alone or to the combination of 10 mmol/L glucose, KCl, and diazoxide from FAF-BSA–incubated islets as well.

The amplified ^{3}H-inositol responses observed in response to glucose, glucose plus KCl, and carbachol stimulation could not be accounted for by any preferential increase in ^{3}H-inositol incorporation during the 4-hour incubation period. Total amounts of ^{3}H-inositol incorporation—the amount effluxing from the β-cell during the perifusion and the amount of label remaining at the end of the experiment—were not significantly different from control or FAF-BSA–incubated islets. They averaged 20 768 ± 1565 cpm/28 islets (n = 17) and 22 800 ± 1216 cpm/28 islets (n = 25) in control and FAF-BSA–incubated islets, respectively.

Confirming that glucose-induced insulin secretion is indeed enhanced by prior exposure to FAF-BSA, a response that was paralleled by a marked enhancement of PLC activation as well, allowed us to make several predictions that could be experimentally tested. First, Straub and Sharp [6] reported that mouse islet secretory responses to glucose were immune to the enhancing impact of incubation in FAF-BSA. If PLC activation underlies amplification, then mouse islet PLC responses should also be unaffected as well. Second, if the proximal activation of PLC results in the distal activation of PKC, then rat islet responses to PMA stimulation should not be significantly enhanced as a result of incubation in FAF-BSA. Experiments were designed to explore these 2 issues.

We first confirmed that mouse islet responses to 20-mmol/L glucose stimulation are indeed immune to the enhancing impact of a prior 4-hour exposure to 100 μmol/L FAF-BSA. This particular glucose level was chosen in this species because it is approximately equipotent, in terms of insulin secretion, to the 10-mmol/L glucose concentration used for rat islet studies (Fig. 1). The first- and second-phase insulin secretory responses from control BSA and FAF-BSA–pretreated groups were comparable (Fig. 4A). Basal secretion rates from both groups averaged 20 to 25 pg per islet per minute. In response to 20-mmol/L glucose stimulation (plus 0.1 mmol/L cold inositol), peak first-phase release rates increased to 215 ± 37 pg per islet per minute (n = 4) from control islets and to 238 ± 38 pg per islet per minute (n = 5) from FAF-BSA–incubated islets. After 40 minutes of stimulation, release rates from control BSA islets now averaged 263 ± 41 pg per islet per minute (n = 4), whereas those from FAF-BSA islets were 259 ± 29 pg per islet per minute (n = 5). Consistent with the minimal impact of glucose stimulation on inositol phosphate accumulation in mouse islets [10,11], only small, minor increments in ^{3}H-inositol efflux rates were noted from control islets during the final minutes of the perifusion (Fig. 4B). There was no enhancement of ^{3}H-inositol efflux after incubation of mouse islets in FAF-BSA. Both groups responded to the further addition of 50 μmol/L carbachol with comparable increments in ^{3}H-inositol efflux, and these were accompanied by enhanced insulin release as well.

The conclusion that PKC activation played a major role in the amplified secretory responses from FAF-BSA–incubated islets was supported by the findings that a number of PKC antagonists inhibited this response [6]. However, the findings presented above suggest that although PKC is playing an important role, its involvement may be more distal to the proximal activation of PLC. If this is indeed the case, it might be predicted that rat islets should be insensitive to the secretory impact of the direct PKC activator PMA. By

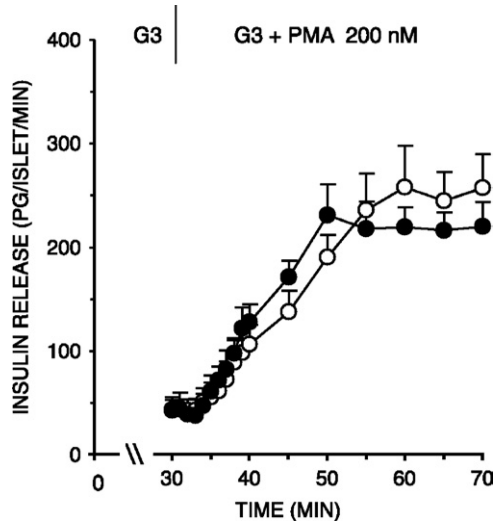

Fig. 5. Effects of FAF-BSA on insulin secretion in response to phorbol ester stimulation. Groups of 18 rat islets were isolated and subjected to a 4-hour incubation in CMRL 1066 medium supplemented with 100 μmol/L Sigma 7888 RIA-grade BSA (open circles) or 100 μmol/L Roche FAF-BSA (closed circles). They were then washed and perifused with 3 mmol/L glucose for 30 minutes to establish stable and basal secretion rates. They were then stimulated, onset indicated by the vertical line, for the next 40 minutes with 200 nmol/L PMA. Four experiments were performed under each condition.

substituting for PLC-derived diacylglycerol (DAG), exposure of islets to PMA culminates in a PLC-independent secretory response [26]. We used a 200-nmol/L concentration of PMA because it is approximately equipotent to the secretory impact of 10 mmol/L glucose in rat islets and 20 mmol/L glucose in mouse islets. In the presence of 3 mmol/L glucose, the further addition of 200 nmol/L PMA resulted in modest secretion of insulin from control BSA-pretreated rat islets. Peak release rates of about 200 to 250 pg per islet per minute were noted 30 to 40 minutes after the onset of stimulation (Fig. 5). Islets incubated for 4 hours in FAF-BSA, a pretreatment condition that significantly amplifies secretion in response to 10 mmol/L glucose from this species, displayed similar responses to 200-nmol/L PMA stimulation. Thus, our prediction that the proximal activation of PLC results in the distal activation of PKC was supported by the findings presented in Fig. 5.

4. Discussion

When confronted with a hyperglycemic challenge, a carefully titrated insulin secretory response from the β-cell is necessary to maintain fuel homeostasis. Failure to activate the secretory apparatus commensurate with the elevation in glucose culminates in diabetes [27]. Thus, the factors that control insulin exocytosis, although of interest from a purely scientific perspective, have important clinical implications as well. A burgeoning number of studies have probed the nature of the signaling apparatus that so elegantly regulates insulin secretion. Our working hypothesis is that information flow in the PLC/PKC cascade plays a pivotal role in stimulus-response coupling. This concept is supported by a number of observations. First, a tight coupling exists between the ambient glucose level and the calcium-dependent activation of both PLC and insulin secretion [1]. Second, glucose-induced insulin release from perfused or freshly studied perifused mouse islets [7-10,28] is less robust than that from rat islets, a response pattern paralleled by reduced activation of PLC as well [1,11]. Third, the further addition of the PLC-activating cholinergic agonist carbachol converts the minimal mouse islet response to glucose into one that now simulates the robust response of rat islets to glucose alone [10]. We have attributed this amplified response to the established ability of carbachol to activate PLC in mouse islets, a finding also supported by muscarinic receptor type 3 knockout mice [29-31]. Finally, in rat islets desensitized by long-term exposure to glucose, reduced activation of PLC parallels the secretory defect [1,32-34].

The concept that PLC/PKC activation plays an important regulatory role in glucose-induced insulin secretion would, however, be substantially strengthened if the PLC response to physiologic glucose concentrations could be enhanced. In theory, at least, this should amplify secretion as well. Recently, Straub and Sharp [6] reported that a 3- to 6-hour incubation of rat islets in FAF-BSA culminated in a markedly enhanced insulin secretory response to a number of agonists including glucose. Of particular interest was their finding that mouse islet responses to glucose were immune to this manipulation. They focused their attention on the activation of PKC or a DAG response element as the pertinent biochemical alteration and used a variety of PKC inhibitors to substantiate the concept that PKC activation was important in this response. It is of interest to note, however, that all the stimulatory conditions used in this detailed study also activate PLC as well. Thus, not only high glucose but also α-ketoisocaproate [35,36], leucine [35], tolbutamide [37,38], and high potassium in the presence of high glucose and diazoxide [15] all activate PLC. Thus, the observations of Straub and Sharp are also compatible with the concept that the proximal activation of PLC results in the distal activation of PKC. We decided to use rat islets incubated in FAF-BSA to assess the potential role of PLC in these amplified insulin secretory responses. Comparable studies were also conducted with mouse islets. Our findings strengthen the concept that PLC activation plays a critically important role in the regulation of glucose-induced release, and further comment on our findings are appropriate.

We first confirmed the findings previously reported by Straub and Sharp [6] that incubation of rat islets in FAF-BSA markedly enhances glucose-induced insulin secretion. While we used a lower glucose level in our studies (10 mmol/L as opposed to 16.7 mmol/L), our results clearly support the finding that this type of manipulation markedly amplifies both phases of glucose-induced secretion.

We also demonstrated that, in parallel with augmented secretion, the activation of PLC was also dramatically enhanced in these islets. We thus confirmed the hypothesis that provided the motivating force for these experiments. It is not clear from our studies how incubation in FAF-BSA amplifies glucose-induced activation of PLC and, pari passu, PKC activation and insulin secretion. Initially proposed by Straub and Sharp [6] was the idea that fatty acids in the β-cell exert a tonic inhibitory effect on β-cell signal transduction events. If this is an accurate portrayal of events occurring after exposure to FAF-BSA, then the action of fatty acids would appear to involve the tonic inhibition of PLC because this manipulation markedly amplifies the activation of this enzyme and subsequent secretion as well. These findings also suggest that conditions that elevate fatty acids in vivo might result in impaired PLC activation and a decompensation of insulin secretion. The lipotoxic impact of elevated fatty acids, like glucotoxicity, might thus be mediated by altered activation of PLC as well. Whatever the exact mechanism involved, it is quite clear that from a quantitative perspective, this inhibition may play a crucial and previously unappreciated role in the regulation of secretion. Unraveling the biochemical identity of the perturbation that so dramatically enhances secretion promises to be a fruitful, from both a scientific and clinical perspective, avenue of investigation and warrants future study.

We monitored the activation of PLC by measuring the efflux of ^{3}H-inositol from ^{3}H-inositol–labeled islets. The strength of this methodology is that secretion along with both the time course and extent of PLC activation can be assessed in the same islets. Many groups have used this approach [14,19,39-41]. There are, however, limitations with this method as well. First, the nature of the inositol phosphates that give rise to the membrane-permeant free inositol cannot be determined. Second, reincorporation of free inositol occurs, resulting in an underestimation of PLC activation with this approach. The inclusion of cold inositol circumvents this last problem and increases efflux rates of the label. These methodological considerations have been discussed in detail elsewhere [1,24]. Our observations that similar amounts of label are incorporated into both control and FAF-BSA–incubated islets and that lithium significantly reduces efflux from stimulated islets support the concept that the activation of PLC accounts for the increase in efflux.

We also confirmed that secretion in response to the combination of glucose and potassium in the presence of diazoxide is also enhanced from FAF-BSA–pretreated islets. A parallel potentiation of PLC stimulation also occurred. Augmented PLC activation also occurred in response to carbachol, an agonist thought to activate an isozyme of PLC distinct from that activated by glucose [42]. Thus, whatever the nature of the effect produced by incubation in FAF-BSA, it is not confined to glucose but extends to other PLC activators as well.

Several other observations made herein also implicate PLC as the seminal alteration in the amplified insulin secretory response observed from islets incubated in FAF-BSA. Although the inhibitor studies used by Straub and Sharp [6] clearly suggest the involvement of PKC, they do not exclude that PLC activation may also be involved as a more upstream event or as the principal pathway involved in response to FAF-BSA. If enhanced sensitivity of PKC to stimulation underlies amplified secretion from these islets, then we predicted that the response to the phorbol ester PMA should be enhanced. This did not occur and suggests to us that the provision of greater amounts of PKC-activating moieties, in particular DAG derived from PLC-mediated hydrolysis of islet phosphoinositide pools, and not the enhanced sensitivity of PKC itself, accounts, in large part, for the amplification of exocytosis. This does not mean that PKC is not involved in the amplified response that results from this manipulation, but only that it is a downstream participant whose activation culminates in enhanced secretion from these cells.

Species differences separate mouse and rat islet insulin secretory responses to glucose stimulation [1,7-9,43,44]. When compared with the robust rising second-phase response to glucose stimulation from freshly studied rat islets, freshly studied mouse islet responses from both perfused and perifused islet preparations are reduced and flat. This secretory dichotomy is also paralleled by reduced activation of PLC in mouse islets as well [10]. Freshly studied mouse islets also fail to exhibit time-dependent potentiation to prior glucose stimulation [13,45] and are also markedly less sensitive to the desensitizing effect of prolonged glucose exposure as well [46]. We have proposed that PLC activation is involved in all of these disparate time-dependent effects of glucose on the β-cell [1]. We confirmed herein that mouse islets are also immune to the amplifying effect of incubation in FAF-BSA, an anomaly previously noted by Straub and Sharp [6] as well. Our earlier studies suggested that the underexpression of a nutrient-activated PLC isozyme, perhaps PLCδ, accounts for this species dichotomy [10,11]. A similar explanation may account for the failure of mouse islets to augment secretion after incubation in FAF-BSA. Thus, because they express less of the PLC isozyme that couples glucose to secretion, mouse islets are also less sensitive to the amplifying effect induced by incubation in FAF-BSA. Whatever the nature of the exact underlying mechanism involved in these differences, comparative studies using both mouse and rat islet should prove instrumental in its identification.

In the initial report by Straub and Sharp [6], they concluded that a novel PKC isoform or a DAG-binding protein was involved. With regard to PKC, a number of inhibitors were used to establish its involvement in the amplification process. Our data support the involvement of PKC as a distal response element in these augmented responses. However, it remains to be established how the activation of PLC might account for their findings with these inhibitors. Several points pertinent to this issue deserve

comment. First, multiple isozymes of PKC are present in islets; and their actions may be either excitatory or inhibitory to the secretory process. For example, the inhibitor of conventional PKC isoforms Go 6976 has a small inhibitory effect on second-phase glucose-induced secretion from rat islets but actually potentiates secretion from mouse islets [47]. Second, the dose-response effects of calphostin and Ro 31-8220 on each of these have not been established. Third, the time-dependent actions of these inhibitors on amplified responses add an additional tier of complexity as initially noted. Finally, it should also be noted that the fatty acid composition of membrane phospholipids synthesized during the 4-hour incubation period may be altered as well; and this might change the nature of the DAG generated during subsequent stimulation. It is clear that major issues remain to be addressed and explored in more detail. However, considering the magnitude of the amplification process induced by this manipulation, their identification should proceed as expeditiously as possible.

Before concluding, several other points should be made. From a clinical perspective at least, 2 future applications of the findings made using this approach should be entertained. First, considering the vulnerability of PLC to desensitization, a process that culminates in impaired secretion as well, pharmacologic manipulation of PLC might be a reasonable therapeutic approach to enhance secretion from a failing β-cell, a defect that appears to result in the emergence of type 2 diabetes mellitus. Second, before their transplantation, incubation of isolated human islets in FAF-BSA might be considered a useful adjunct to improve or retain their physiologic integrity. In this context, it might allow the more rapid restoration of insulin secretion and even enhance their therapeutic potential, thus lowering the often prohibitively large numbers of islets necessary to restore normoglycemia [48]. The implementation of these 2 suggestions would seem to depend, however, on the more precise identification of the exact biochemical mechanisms involved. Future studies should thus focus on this exciting biochemical area replete with potentially important clinical overtones.

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References

[1] Zawalich WS, Zawalich KC. Regulation of insulin secretion by phospholipase C. Am J Physiol 1996;271:E409-16.
[2] Zawalich WS, Yamazaki H, Zawalich KC. Phosphoinositides, phospholipase C and the regulation of insulin secretion: physiologic and pathophysiologic implications [Online]. Cell Science Reviews; July 2006.
[3] Henquin JC, Bozem M, Schmeer W, et al. Distinct mechanisms for two amplification systems of insulin release. Biochem J 1987;246:393-9.
[4] Godsland IF, Jeffs JAR, Johnston DG. Loss of beta cell function as fasting glucose increases in the non-diabetic range. Diabetologia 2004;47:1157-66.
[5] Gastadelli A, Ferrannini E, Miyazaki Y, et al. Beta-cell dysfunction and glucose intolerance: results from the San Antonio metabolism (SAM) study. Diabetologia 2004;47:31-9.
[6] Straub SG, Sharp GWG. Massive augmentation of stimulated insulin secretion induced by fatty acid–free BSA in rat pancreatic islets. Diabetes 2004;53:3152-8.
[7] Lenzen S. Insulin secretion by isolated perfused rat and mouse pancreas. Am J Physiol 1979;236:E391-E400.
[8] Berglund O. Different dynamics of insulin secretion in the perfused pancreas of the mouse and rat. Acta Endocrinol 1980;93:54-60.
[9] Ma MYH, Wang J, Rodd GG, et al. Differences in insulin secretion between rat and mouse islets: role of cAMP. Eur J Endocrinol 1995; 132:370-6.
[10] Zawalich WS, Zawalich KC, Kelley GG. Regulation of insulin release by phospholipase C activation in mouse islets: differential effects of glucose and neurohumoral stimulation. Endocrinology 1995;136: 4903-9.
[11] Zawalich WS, Bonnet-Eymard M, Zawalich KC. Insulin secretion, inositol phosphate levels and phospholipase C isozymes in rodent pancreatic islets. Metabolism 2000;49:1156-63.
[12] Zawalich WS, Zawalich KC, Kelley GG. Effects of short-term culturing on islet phosphoinositide and insulin secretory responses to glucose and carbachol. Acta Diabetologia 1995;32:158-64.
[13] Zawalich WS, Zawalich KC. Species differences in the induction of time-dependent potentiation of insulin secretion. Endocrinology 1996; 137:1664-9.
[14] Zawalich WS, Takuwa N, Takuwa Y, et al. Interactions of cholecystokinin and glucose in rat pancreatic islets. Diabetes 1987; 36:426-33.
[15] Zawalich WS, Zawalich KC. Regulation of insulin secretion via ATP-sensitive K^+ channel independent mechanisms: role of phospholipase C activation. Am J Physiol 1997;35:E671-7.
[16] Zawalich WS, Zawalich KC. A link between insulin resistance and hyperinsulinemia: inhibitors of phosphatidylinositol 3-kinase augment glucose-induced insulin secretion from islets of lean, but not obese, rats. Endocrinology 2000;141:3287-95.
[17] Albano JDM, Ekins RP, Maritz G, et al. A sensitive, precise radioimmunoassay of serum insulin relying on charcoal separation of bound and free hormone moieties. Acta Endocrinol 1972;70: 487-509.
[18] Zawalich WS, Diaz VA, Zawalich KC. Influence of cAMP and calcium on [^{3}H]inositol efflux, inositol phosphate accumulation, and insulin release from isolated rat islets. [published erratum appears in Diabetes 1989 Mar;38(3):3]Diabetes 1988;37:1478-83.
[19] Vadakekalam J, Rabaglia ME, Chen QH, et al. Role for GTP in glucose-induced phospholipase C activation in pancreatic islets. Am J Physiol 1996;271:E85-E95.
[20] Borle AB, Uchikawa T, Anderson JH. Computer simulation and interpretation of ^{45}Ca efflux profile patterns. J Membr Biol 1982;68: 37-46.
[21] Zawalich WS, Zawalich KC. Phosphoinositide hydrolysis and insulin release from isolated perifused rat islets. Studies with glucose. Diabetes 1988;37:1294-300.
[22] Zawalich WS. Modulation of insulin secretion from beta-cells by phosphoinositide-derived second-messenger molecules [Review]. Diabetes 1988;37:137-41.
[23] Majerus PW, Connolly TM, Bansal VS, et al. Inositol phosphates: synthesis and degradation. J Biol Chem 1988;263:3051-4.
[24] Zawalich WS. Multiple effects of increases in phosphoinositide hydrolysis on islets and their relationship to changing patterns of insulin secretion. Diabetes Res 1990;13:101-11.
[25] Berridge MJ, Downes CP, Hanley MR. Lithium amplifies agonist-dependent phosphatidylinositol responses in brain and salivary glands. Biochem J 1982;206:587-95.
[26] Zawalich WS, Zawalich KC, Ganesan S, et al. Effects of the phorbol ester phorbol 12-myristate 13-acetate on islet-cell responsiveness. Biochem J 1991;278:49-56.

[27] DeFronzo RA. The triumvirate: β-cell, muscle, liver. A collusion responsible for NIDDM. Diabetes 1988;37:667-87.
[28] Noda M, Komatsu M, Sharp GWG. The BHC-9 pancreatic B-cell line preserves the characteristics of progenitor mouse islets. Diabetes 1996;45:1766-73.
[29] Zawalich WS, Zawalich KC, Tesz GJ, et al. Effects of muscarinic receptor type 3 knockout on mouse islet secretory responses. Biochem Biophys Res Commun 2004;315:872-6.
[30] Gautam D, Han SJ, Hamdan FF, et al. A critical role for β cell M3 muscarinic acetylcholine receptors in regulating insulin release and blood glucose homeostasis in vivo. Cell Metabolism 2006;3:449-61.
[31] Duttaroy A, Zimlike CL, Gautam D, et al. Muscarinic stimulation of pancreatic insulin and glucagon release is abolished in M3 muscarinic acetylcholine receptor-deficient mice. Diabetes 2004;53:1714-20.
[32] Zawalich WS, Zawalich KC, Rasmussen H. The conditions under which rat islets are labelled with [^{3}H]inositol alter the subsequent responses of these islets to a high glucose concentration. Biochem J 1989;259:743-9.
[33] Yamazaki H, Philbrick W, Zawalich KC, et al. Acute and chronic effects of glucose and carbachol on insulin secretion and phospholipase C activation: studies with diazoxide and atropine. Am J Physiol 2006; 290:E26-E33.
[34] Yamazaki H, Zawalich KC, Zawalich WS. Desensitization of the pancreatic β-cell: effects of physiologic hyperglycemia and hyperkalemia. Am J Physiol 2006;291:E1381-7.
[35] Zawalich WS. Time-dependent potentiation of insulin release induced by α-ketoisocaproate and leucine in rats: possible involvement of phosphoinositide hydrolysis. Diabetologia 1988;31:435-42.
[36] Best L. A role for calcium in the breakdown of inositol phospholipids in intact and digitonin-permeabilized pancreatic islets. Biochem J 1986;238:773-9.
[37] Laychock SG. Identification and metabolism of polyphosphoinositides in isolated islets of Langerhans. Biochem J 1983;216:101-6.
[38] Zawalich WS, Zawalich KC. Induction of memory in rat pancreatic islets by tolbutamide. Dependence on ambient glucose level, calcium, and phosphoinositide hydrolysis. Diabetes 1988;37:816-23.
[39] Blachier F, Segura MC, Malaisse WJ. Unresponsiveness of phospholipase C to the regulatory proteins Ns and Ni in pancreatic islets. Res Commun Chem Pathol Pharmacol 1987;55:335-55.
[40] Axen KV, Schubart UK, Blake AD, et al. Role of Ca^{2+} in secretagogue-stimulated breakdown of phosphatidylinositol in rat pancreatic islets. J Clin Invest 1983;72:13-21.
[41] Mathias PCF, Best L, Malaisse WJ. Stimulation by glucose and carbamylcholine of phospholipase C in pancreatic islets. Cell Biochem Funct 1985;3:173-7.
[42] Kelley GG, Zawalich KC, Zawalich WS. Synergistic interaction of glucose and neurohumoral agonists to stimulate islet phosphoinositide hydrolysis. Am J Physiol 1995;269:E575-82.
[43] Maechler P, Gjinovci A, Wollheim CB. Implication of glutamate in the kinetics of insulin secretion in rat and mouse perfused pancreas. Diabetes 2002;51(Suppl 1):S99-S102.
[44] Shiota C, Larsson O, Shelton KD, et al. Sulfonylurea receptor type 1 knock-out mice have intact feeding-stimulated insulin secretion despite marked impairment in their response to glucose. J Biol Chem 2002; 277:37176-83.
[45] Berglund O. Lack of glucose-induced priming of insulin release in the perfused mouse pancreas. J Endocrinol 1987;114:185-9.
[46] Zawalich WS, Bonnet-Eymard M, Zawalich KC. Glucose-induced desensitization of the pancreatic β-cell is species dependent. Am J Physiol 1998;275:E917-24.
[47] Zawalich WS, Zawalich KC. Effects of protein kinase C inhibitors on insulin secretory responses from rodent pancreatic islets. Mol Cell Endocrinol 2001;177:95-105.
[48] Shapiro AM, Lakey JRT, Ryan EA, et al. Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. N Engl J Med 2000;343:230-8.