

# The Role of Cytosolic Phospholipase A<sub>2</sub> in Insulin Secretion

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**Cytosolic phospholipase A<sub>2</sub> (cPLA<sub>2</sub>) comprises a widely expressed family of enzymes, some members of which have the properties required of signal transduction elements in electrically excitable cells. Thus,  $\alpha$ - and  $\beta$ -isoforms of cPLA<sub>2</sub> are activated by the increases in intracellular Ca<sup>2+</sup> concentration ([Ca<sup>2+</sup>]<sub>i</sub>) achieved in depolarized cells. Activation is associated with a redistribution of the enzyme within the cell; activation of cPLA<sub>2</sub> generates arachidonic acid (AA), a biologically active unsaturated fatty acid that can be further metabolized to generate a plethora of biologically active molecules. Studies using relatively nonselective pharmacological inhibitors have implicated cPLA<sub>2</sub> in insulin secretory responses to stimuli that elevate  $\beta$ -cell [Ca<sup>2+</sup>]<sub>i</sub>; therefore, we have investigated the role of cPLA<sub>2</sub> in  $\beta$ -cell function by generating  $\beta$ -cell lines that under- or overexpress the  $\alpha$ -isoform of cPLA<sub>2</sub>. The functional phenotype of the modified cells was assessed by observation of cellular ultrastructure, by measuring insulin gene expression and insulin protein content, and by measuring the effects of insulin secretagogues on cPLA<sub>2</sub> distribution, on changes in [Ca<sup>2+</sup>]<sub>i</sub>, and on the rate and pattern of insulin secretion. Our results suggest that cPLA<sub>2</sub> is not required for the initiation of insulin secretion from  $\beta$ -cells, but that it plays an important role in the maintenance of  $\beta$ -cell insulin stores. Our data also demonstrate that excessive production of, or exposure to, AA is deleterious to normal  $\beta$ -cell secretory function through metabolic dysfunction. *Diabetes* 53 (Suppl. 1):S172–S178, 2004**

**T**here is a body of literature suggesting that the long-chain polyunsaturated fatty acid arachidonic acid (AA) is involved in the regulation of insulin secretion from pancreatic  $\beta$ -cells, either as intact AA or as biologically active metabolites generated by the action of oxygenase enzymes (1). The abundance of AA in islets suggests an important role—AA constitutes at least 30% of the fatty acyl content of islet

glycerophospholipids, compared with only 15% in the exocrine pancreas (2)—and exogenous AA can initiate an insulin secretory response from intact and permeabilized islets (3–5). One obvious generator of AA as a second messenger is the phospholipase A<sub>2</sub> (PLA<sub>2</sub>) family of proteins. These enzymes catalyze the hydrolysis of membrane glycerophospholipids to release AA and are ubiquitously expressed in mammalian cells. However, earlier studies implicating PLA<sub>2</sub> and AA in stimulus-response coupling in  $\beta$ -cells could not explain how the PLA<sub>2</sub> activity was regulated by insulin secretagogues, since the PLA<sub>2</sub> enzymes identified at that time required millimolar concentrations of Ca<sup>2+</sup> for activation (1). These PLA<sub>2</sub> enzymes, also known as secretory PLA<sub>2</sub>, were classified into two types (I and II) on the basis of their primary structure (6). However, the more recent identification of other  $\beta$ -cell PLA<sub>2</sub> activities that are sensitive to intracellular concentrations of Ca<sup>2+</sup> (5,7–9) or ATP (10) offered effector molecules through which regulated PLA<sub>2</sub> activities could control the insulin secretory process by changes in intracellular AA.

The ATP-sensitive group VIA PLA<sub>2</sub> enzyme, now designated iPLA<sub>2</sub> $\beta$ , is an important islet PLA<sub>2</sub> species that is expressed in  $\beta$ -cells (10,11). The iPLA<sub>2</sub> $\beta$  enzyme does not require Ca<sup>2+</sup> for catalytic activity and is inhibited by a bromoenol lactone (BEL) suicide substrate (11–14). It is activated by millimolar concentrations of ATP (in the presence of Mg<sup>2+</sup>) and inhibited by ADP (15); therefore, it is possible that the increased ATP/ADP ratio after nutrient metabolism may activate this enzyme, much in the same way as the ATP-sensitive K<sup>+</sup> channels are thought to be sensitive to the ATP/ADP ratio rather than to ATP levels per se. The majority of studies on the role of iPLA<sub>2</sub> $\beta$  in  $\beta$ -cell function have been performed using rat islets and the HIT-T15  $\beta$ -cell line, but iPLA<sub>2</sub> $\beta$  activity has also been quantified in human islets (10). An absolute requirement for iPLA<sub>2</sub> $\beta$  in the physiological regulation of insulin secretion has not been established, but there are several lines of evidence to suggest that it plays an important role. Thus, the secretory response to glucose in rat islets and  $\beta$ -cell lines is inhibited by BEL (11,16). Loss of glucose responsiveness of  $\beta$ -cell lines is associated with a decline in iPLA<sub>2</sub> $\beta$  activity (16), and potentiation of insulin release by cholecystokinin may be mediated, at least in part, through the activation of iPLA<sub>2</sub> $\beta$  (17).

The type IV cytosolic phospholipase A<sub>2</sub> (cPLA<sub>2</sub>) enzymes are also phospholipase activities that can be regulated by intracellular signals known to be important in regulating  $\beta$ -cell function (18,19). Thus, cPLA<sub>2</sub> is a family

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AA, arachidonic acid; BEL, bromoenol lactone; [Ca<sup>2+</sup>]<sub>i</sub>, intracellular Ca<sup>2+</sup> concentration; cPLA<sub>2</sub>, cytosolic phospholipase A<sub>2</sub>; PLA<sub>2</sub>, phospholipase A<sub>2</sub>; UPC2, uncoupling protein 2.

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of enzymes comprised of proteins of the predicted molecular masses of 85 kDa (cPLA<sub>2</sub>α), 114 kDa (cPLA<sub>2</sub>β), and 61 kDa (cPLA<sub>2</sub>γ), all of which selectively hydrolyze membrane phospholipids to generate AA (20). Most importantly, the enzyme activity of the α- and β-isoforms of cPLA<sub>2</sub> is sensitive to micromolar concentrations of intracellular Ca<sup>2+</sup> ([Ca<sup>2+</sup>]<sub>i</sub>), suggesting that they may act as downstream effectors for the well-defined initial stages in β-cell stimulus-secretion coupling, which result in increases in [Ca<sup>2+</sup>]<sub>i</sub>. Furthermore, the discovery that cPLA<sub>2</sub> activity can also be regulated through phosphorylation (18,19) by protein serine/threonine kinases that have been implicated in stimulus-response coupling in β-cells (21) offered multiple levels for the control of β-cell cPLA<sub>2</sub> activity by insulin secretagogues. Given the importance of both [Ca<sup>2+</sup>]<sub>i</sub> and protein phosphorylation in stimulus-response coupling in β-cells, this article will focus on the possible role of cPLA<sub>2</sub> in the regulation of insulin secretion.

Most studies of cPLA<sub>2</sub> have focused on the physiological roles of the α-isoform, which was the first member of the cPLA<sub>2</sub> family to be identified and which has been implicated in a range of cellular processes, including mitogenesis, allergic responses, fertility, and cytotoxicity (18,19). There is considerable circumstantial evidence that cPLA<sub>2</sub> plays an important role in regulating insulin secretion. Thus, cPLA<sub>2</sub>α expression has been identified in islets and β-cells by Western blotting, Northern blotting, and PCR (5,7,8), and β-cell cPLA<sub>2</sub> enzyme activity has been shown to be sensitive to Ca<sup>2+</sup> in the micromolar range (22). Glucose and other stimuli that increase β-cell [Ca<sup>2+</sup>]<sub>i</sub> activate Ca<sup>2+</sup>-dependent cytosolic PLA<sub>2</sub> in islets (22), and pharmacological inhibitors of cPLA<sub>2</sub> inhibit glucose-induced insulin secretion (5).

Studies using pharmacological inhibitors of PLA<sub>2</sub> cannot be considered as conclusive because of the overlapping specificities of the inhibitors for the many different classes of PLA<sub>2</sub> enzymes expressed in most cells. For example, earlier studies on insulin secretion using the then available PLA<sub>2</sub> inhibitors are difficult to interpret because many of these compounds have multiple effects, not always related to PLA<sub>2</sub> activity (1). Similarly, the newer classes of AA-like PLA<sub>2</sub> inhibitors do not differentiate between cPLA<sub>2</sub> and the Ca<sup>2+</sup>-independent PLA<sub>2</sub> activity, nor do they discriminate between the cPLA<sub>2</sub> isoforms (19). Furthermore, the BEL inhibitor of iPLA<sub>2</sub>β suppresses the effect of glucose on the ATP:ADP ratio in mouse islets (23), which may account for some of its inhibitory effects on glucose-induced insulin secretion (11,16). To circumvent the use of pharmacological inhibitors, we chose to investigate the role of cPLA<sub>2</sub>α in β-cells by generating β-cell lines in which cPLA<sub>2</sub>α was permanently underexpressed (23) or overexpressed by stable transfection with vectors encoding cPLA<sub>2</sub>α in the antisense or sense orientation, respectively. Our studies using these modified β-cells suggest that the activation of cPLA<sub>2</sub>α is not required for glucose-induced insulin secretion, in contrast to the available circumstantial evidence.

## RESEARCH DESIGN AND METHODS

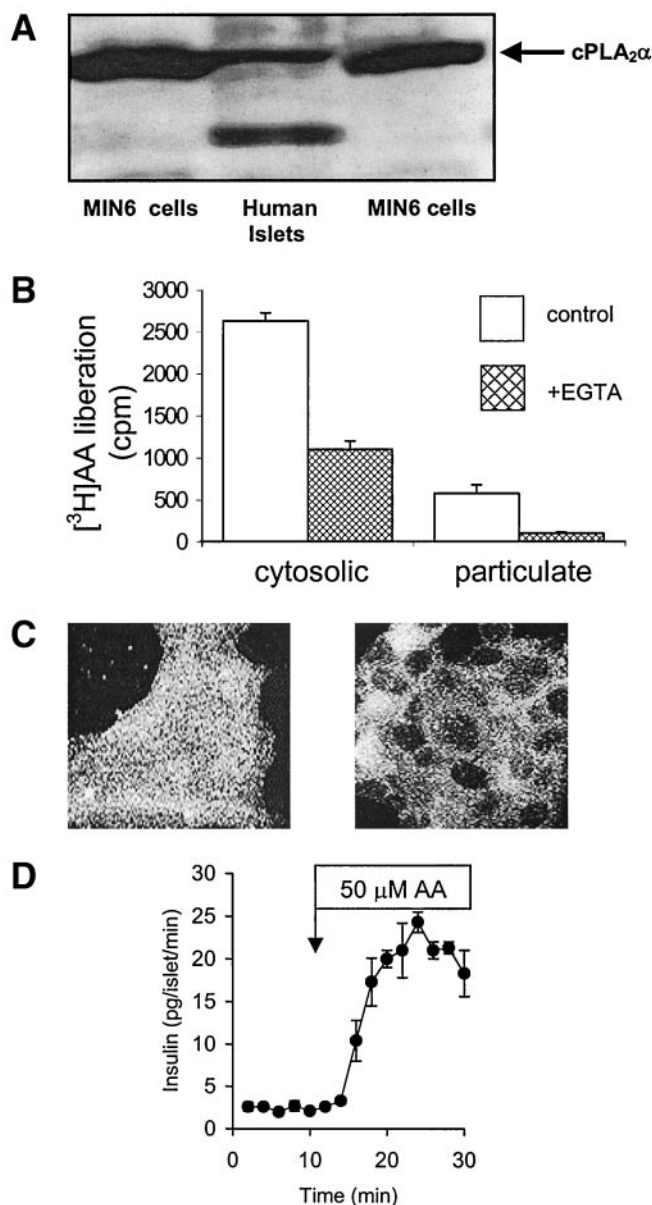
Human islets of Langerhans were obtained with appropriate consent from the Human Islet Transplantation Unit at King's College London (via Dr. G.-C. Huang and Prof. S. Amiel). Rat islets were isolated by collagenase digestion of whole rat pancreas. MIN6 cells were obtained from Dr. Y. Oka and Prof. J.-I.

Miyazaki (University of Tokyo) and stably transfected with vectors encoding the full-length mouse cPLA<sub>2</sub>α cDNA (a gift from Dr. B. Kennedy, Merck Frosst, PQ, Canada) in the pcDNA3.1 vector (Stratagene Europe, Amsterdam, the Netherlands) as described (24). cPLA<sub>2</sub> immunoreactivity was detected by Western blotting (24) or fluorescence immunocytochemistry (25) using an anti-cPLA<sub>2</sub> antibody (Genetics Institute, Cambridge, MA). PLA<sub>2</sub> enzyme activity was measured by quantifying the release of [<sup>3</sup>H]-AA from the *sn-2* position of phosphatidylcholine *La*-stearoyl-2-[<sup>3</sup>H]-arachidonyl (<sup>3</sup>H-PC). Briefly, <sup>3</sup>H-PC (2 μmol/l, 4 Ci/mmol) was incubated (10 min, 37°C) with tissue extracts in a final volume of 25 μl Tris buffer (pH 8.0) containing 50% glycerol (vol/vol), 1 mmol/l dithiothreitol, 0.5 mmol/l NaVO<sub>4</sub>, 0.25 mmol/l NaF, 0.1 mmol/l EDTA, 0.1 mmol/l EGTA, 20 μmol/l E64, 20 μmol/l tosyl-L-lysine chloromethyl ketone, 10 μmol/l okadaic acid, 0.1% β-mercaptoethanol (vol/vol), and 50 μg/ml leupeptin and supplemented with either 0.8 mmol/l CaCl<sub>2</sub> or 1 mmol/l EGTA. Reactions were terminated, and products were extracted by the addition of butanol (40 μl, 4°C). [<sup>3</sup>H]-AA was separated from an uncleaved substrate by thin-layer chromatography on silica thin-layer gels using petroleum ether: diethyl ether:glacial acetic acid 70:30:1 (vol/vol/vol) and quantified by liquid scintillation counting. Changes in [Ca<sup>2+</sup>]<sub>i</sub> in MIN6 cells were assessed by single-cell microfluorimetry of Fura-2 loaded cells (26). Changes in intracellular NAD(P)H were assessed by measuring NAD(P)H autofluorescence in MIN6 cell suspensions. The rate and pattern of insulin release was assessed using a multi-chamber perfusion system at 37°C in a temperature-controlled environment (24). To maximize secretory responses, MIN6 cells were configured as pseudoislets for secretion experiments (27). Insulin content was measured by radioimmunoassay (28), and quantitative measurements of (pre)proinsulin mRNA were obtained using real-time quantitative PCR with GAPDH as an internal standard (29). Electron micrographs were prepared by the Electron Microscope Unit at King's College London using standard techniques. Data are expressed as means ± SE and were analyzed using ANOVA, Student's *t* test, and Bonferroni's multiple comparison test as appropriate. Differences between treatments were considered significant at *P* < 0.05.

## RESULTS AND DISCUSSION

**Expression, distribution, and translocation of cPLA<sub>2</sub> in β-cells.** As has been reported previously (5,7,8), islets and β-cell lines express cPLA<sub>2</sub> immunoreactivity and enzyme activity. Figure 1A shows immunoreactive cPLA<sub>2</sub> in extracts of the mouse MIN6 insulin-secreting cell line (*lanes 1 and 3*) and human islets (*lane 2*). Whereas MIN6 cells contain only one immunoreactive protein migrating as cPLA<sub>2</sub>α, human islets also express another lower molecular weight immunoreactivity that may represent the recently identified cPLA<sub>2</sub>γ isoform (20). At present, it is not known whether this lower molecular weight immunoreactivity is expressed in human β-cells or in another islet cell type. The cPLA<sub>2</sub> enzymes are so named because, in many cell types, they are primarily localized in the cytosol, and enzyme activity is regulated through Ca<sup>2+</sup>-dependent translocation to membrane compartments enriched in the phosphatidylcholine substrate (18,30). Figure 1B shows Ca<sup>2+</sup>-dependent and Ca<sup>2+</sup>-independent PLA<sub>2</sub> enzyme activities in fractions prepared from unstimulated rat islets, where the EGTA-inhibited activity most likely reflects the Ca<sup>2+</sup>-dependent type IV cPLA<sub>2</sub> activity. The Ca<sup>2+</sup>-insensitive PLA<sub>2</sub> activity, detected in the presence of EGTA, was largely confined to the cytosol, whereas the Ca<sup>2+</sup>-sensitive cPLA<sub>2</sub> activity was found in both the cytosolic and particulate fractions.

These biochemical measurements are supported by our fluorescence immunocytochemical localization of cPLA<sub>2</sub> in monolayer MIN6 cells, as shown in Fig. 1C. Under unstimulated conditions (2 mmol/l glucose), cPLA<sub>2</sub> immunoreactivity was distributed throughout the cell, including the nuclear compartment, as shown in the left panel of Fig. 1C, and elevations in [Ca<sup>2+</sup>]<sub>i</sub> caused a redistribution of the cPLA<sub>2</sub> immunoreactivity in MIN6 β-cells. In these experi-



**FIG. 1.** cPLA<sub>2</sub> expression, activity, and distribution in  $\beta$ -cells. **A:** Western blotting with an anti-cPLA<sub>2</sub>α antiserum confirmed the expression of the protein in a mouse insulin-secreting cell line (MIN6 cells) and in an extract of whole human primary islets. **B:** The generation of AA from phosphatidylcholine was measured to estimate the Ca<sup>2+</sup>-dependent and Ca<sup>2+</sup>-independent PLA<sub>2</sub> activity in fractions prepared from whole rat islets. Type I and II PLA<sub>2</sub> activities were inhibited by the presence of DDT and  $\beta$ -mercaptoethanol in the assay. □, Total activity (Ca<sup>2+</sup>-dependent and Ca<sup>2+</sup>-independent) in the presence of Ca<sup>2+</sup>. ▨, Ca<sup>2+</sup>-independent activity measured in the absence of Ca<sup>2+</sup> and the presence of EGTA (1 mmol/l). The Ca<sup>2+</sup>-dependent type activity is the difference between these values. Bars show means  $\pm$  SE ( $n = 4$ ). **C:** Visualizing cPLA<sub>2</sub> distribution by fluorescence immunocytochemistry with an anti-cPLA<sub>2</sub>α antiserum demonstrated that the enzyme was distributed throughout the cytosolic and nuclear compartments in unstimulated MIN6 cells (left panel, 2 mmol/l glucose) and translocated from the nucleus in response to the increases in [Ca<sup>2+</sup>]<sub>i</sub> caused by exposure to tolbutamide (100  $\mu$ mol/l, 30 min). **D:** AA (50  $\mu$ mol/l, arrow) stimulated insulin secretion from human islets of Langerhans being perfused (0.5 ml/min, 37°C) with a physiological salt solution supplemented with 0.5 mg/ml BSA and 2 mmol/l glucose. Points show means  $\pm$  SE for four separate perfusion channels.

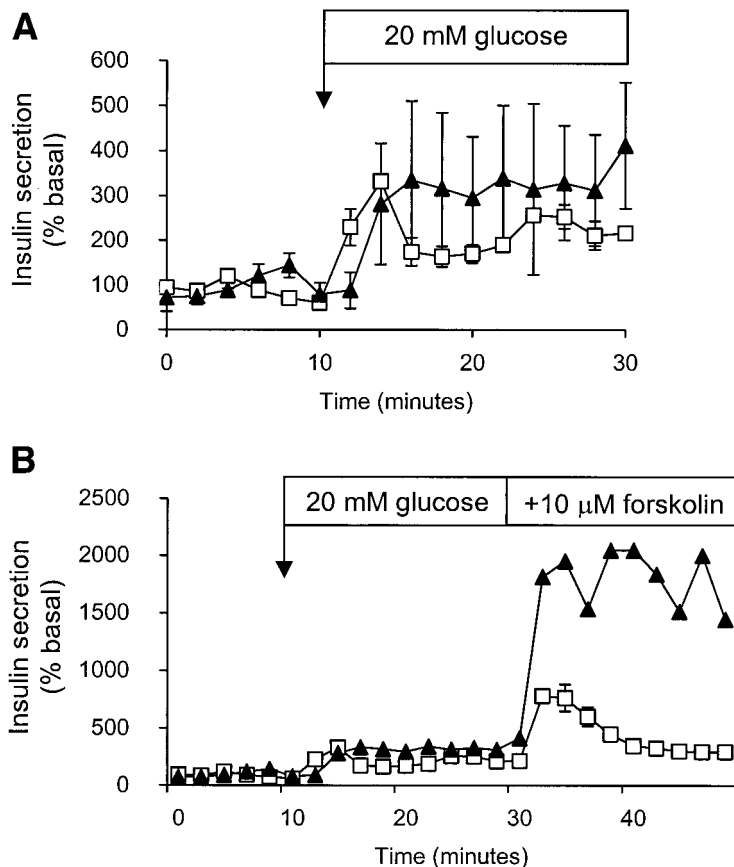
ments, exposure to tolbutamide (100  $\mu$ mol/l) caused a time-dependent redistribution of cPLA<sub>2</sub> immunoreactivity from the nuclei of MIN6 cells to the cytoplasm (Fig. 1C, right panel), and a similar response was caused by expo-

sure to a depolarizing concentration of KCl and, to a lesser extent, to a stimulatory concentration of glucose (25).

Although unusual, this distribution of cPLA<sub>2</sub> is not unique to  $\beta$ -cells because bovine endothelial cells are reported to exhibit a basal nuclear localization of cPLA<sub>2</sub>, which translocates from the nucleus in response to Ca<sup>2+</sup>-mobilizing stimuli (31). We suggest that the redistribution of cPLA<sub>2</sub> in stimulated  $\beta$ -cells may allow agents that increase [Ca<sup>2+</sup>]<sub>i</sub> to rapidly increase cPLA<sub>2</sub> activity in the cytoplasm by promoting its translocation from the nucleus, in a similar manner to the regulation of hepatic glucokinase by its export from the nucleus to the cytoplasm (32,33). Whatever the functional significance of the Ca<sup>2+</sup>-induced translocation of  $\beta$ -cell cPLA<sub>2</sub> from the nucleus, it is consistent with a stimulus-dependent activation of the enzyme, and the subsequent generation of AA, being involved in stimulus-secretion coupling. In support of this chain of events, Fig. 1D shows that exogenously applied AA (50  $\mu$ mol/l) stimulated insulin secretion from human islets in the presence of a substimulatory concentration of glucose (2 mmol/l), suggesting that elevated levels of AA are sufficient to initiate a secretory response.

**Functional phenotype of cPLA<sub>2</sub>-deficient  $\beta$ -cells.** MIN6 cells deficient in cPLA<sub>2</sub>α were generated by transfection with a vector coding cPLA<sub>2</sub> in the antisense orientation and selection for G418-resistant clones. A number of stably transfected clones showing a loss of cPLA<sub>2</sub> expression by immunoblotting were expanded and used for functional studies, showing a stable cPLA<sub>2</sub>-deficient phenotype for a least 1 year in continuous culture (24). The cPLA<sub>2</sub>-deficient cells had a much lower basal (2 mmol/l glucose) rate of insulin secretion per cell ( $8.6 \pm 2\%$  of passage-matched controls,  $P < 0.01$ ,  $n = 4$ ). However, the ability of the cells to recognize and respond to elevations in the extracellular glucose concentration was not markedly affected by the loss of cPLA<sub>2</sub>, as shown in Fig. 2A. In these perfusion experiments, the secretory responses of cPLA<sub>2</sub>-deficient cells to increased glucose were similar to those of control cells when expressed relative to the basal secretory rate, showing a rapid increase in insulin release upon elevation of glucose from 2 to 20 mmol/l and maintaining an elevated rate of secretion thereafter. The cPLA<sub>2</sub>-deficient cells also showed normal patterns of insulin secretion in response to nonnutrient stimuli that elevate [Ca<sup>2+</sup>]<sub>i</sub>, such as tolbutamide and KCl (24). These data imply that cPLA<sub>2</sub>α is not required for pancreatic  $\beta$ -cells to show an initial secretory response to nutrient and nonnutrient secretagogues, suggesting that it is not a pivotal Ca<sup>2+</sup>-sensitive sensor in  $\beta$ -cell stimulus-secretion coupling. It has been reported previously that the amplifying (ATP-dependent K<sup>+</sup> channel-independent) effect of glucose on insulin secretion from mouse islets does not require PLA<sub>2</sub> activation and AA generation (23); therefore, it seems unlikely that cPLA<sub>2</sub> plays an important role in glucose-induced insulin secretion.

Although they appeared able to respond normally to nutrient and nonnutrient stimuli, the cPLA<sub>2</sub>-deficient cells were unable to mount prolonged secretory responses to maintained stimulation, as shown in Fig. 2B. Thus, a subsequent exposure to the adenylate cyclase activator forskolin in the presence of 20 mmol/l glucose caused a further increase in the rate of insulin secretion that was



**FIG. 2.** Functional phenotype of cPLA<sub>2</sub>-deficient  $\beta$ -cells. **A:** MIN6 pseudoislets formed from cPLA<sub>2</sub> $\alpha$ -deficient (□) or passage-matched control (▲) cells were perfused (0.5 ml/min, 37°C) with a physiological salt solution supplemented with 0.5 mg/ml BSA and 2 mmol/l glucose to establish a stable basal rate of secretion. Exposure to 20 mmol/l glucose (arrow) produced a rapid and maintained elevation in insulin secretion from both populations. Points show means  $\pm$  SE ( $n = 3$ ). **B:** After the exposure to 20 mmol/l glucose in **A**, the pseudoislets were exposed to the adenylate cyclase activator forskolin (10  $\mu$ mol/l in the presence of 100  $\mu$ mol/l isobutylmethylxanthine), as shown, in the continued presence of 20 mmol/l glucose. □, Means  $\pm$  SE,  $n = 3$ ; where error bars are not shown, SEs were smaller than the symbols. ▲, The means of two experiments.

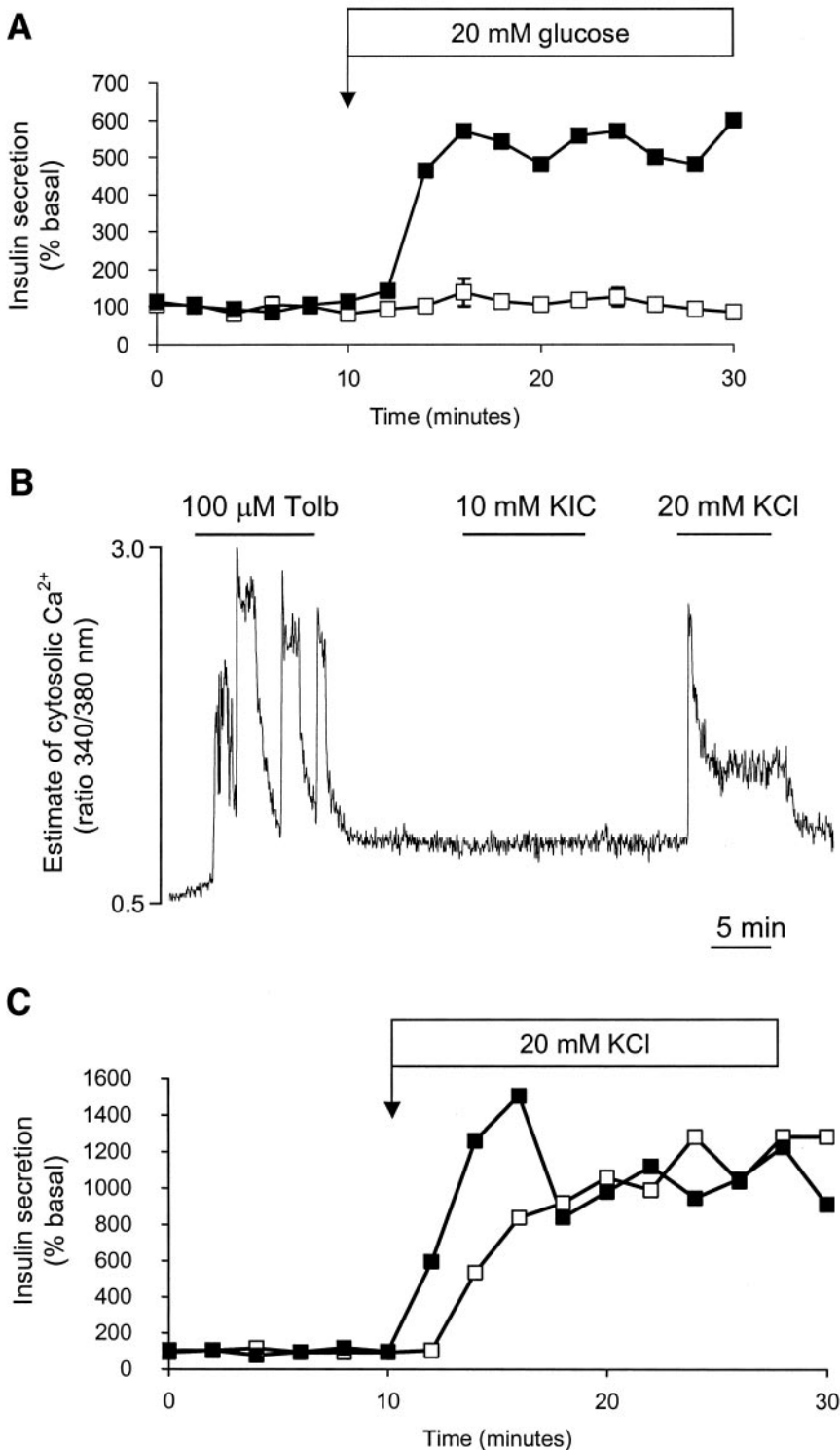
maintained in control cells, but was both lesser ( $\sim$ 8-fold vs. 18-fold) and transient in cPLA<sub>2</sub>-deficient cells, returning to preforskolin rates within  $\sim$ 10 min. The inability to maintain a high rate of secretion was independent of the stimulus, and similar results were obtained with the protein kinase C activator phorbol myristate acetate in combination with glucose, tolbutamide, or KCl (24). The lower basal rate of insulin secretion and the inability to maintain secretion correlated well with the severely reduced insulin content of the cPLA<sub>2</sub>-deficient MIN6 cells: the clone used for the secretion experiments in Fig. 2 contained only  $8.1 \pm 1.6\%$  ( $n = 5$ ) of the insulin content of control MIN6 cells transfected with an empty vector. We therefore suggest that the cPLA<sub>2</sub> underexpressing  $\beta$ -cells have sufficient intracellular stores of insulin for an initial secretory response (albeit reduced in mass in accordance with the reduced insulin content) but insufficient to maintain secretion in response to a strong prolonged stimulus.

It is not immediately apparent why cPLA<sub>2</sub> deficiency should cause such marked reductions in insulin content, but it cannot be accounted for by reduced insulin gene transcription because quantitative RT-PCR demonstrated similar levels of preproinsulin mRNA in cPLA<sub>2</sub>-deficient and control cells (cPLA<sub>2</sub>-deficient  $84 \pm 16\%$  control,  $n = 3$ ), suggesting a defect in translation, processing, or packaging of (pre)proinsulin. In other tissues, cPLA<sub>2</sub> has been implicated in maintaining the stability of the Golgi apparatus to enable appropriate intracellular vesicle trafficking (34), and this would be consistent with reported changes in ultrastructure of cPLA<sub>2</sub>-deficient MIN6 cells (24). Thus, in accordance with their reduced insulin content, these cells contained very few typical dense-cored insulin-con-

taining secretory vesicles and large numbers of nonelectron dense vesicles (24). Further studies are required to determine whether the reduced content of insulin-containing dense-cored secretory vesicles is due to defective packaging of the vesicles or to defective processing and crystallization of insulin.

In summary, the functional phenotype of  $\beta$ -cells deficient in cPLA<sub>2</sub> $\alpha$  does not support a vital role for cPLA<sub>2</sub> as a Ca<sup>2+</sup>-sensitive transduction element in the  $\beta$ -cell stimulus-secretion coupling pathway, but implicates cPLA<sub>2</sub> in the maintenance of insulin stores in dense-cored secretory vesicles. These conclusions are not entirely inconsistent with some of the earlier studies that provided the rationale for the current work. Thus, physiological stimuli that enhance insulin secretion must also enhance the production and storage of more insulin to maintain the  $\beta$ -cell secretory capacity. The activation/redistribution of cPLA<sub>2</sub> in response to insulin secretagogues may be involved in this element of stimulus-response coupling, rather than in the immediate regulation of the secretory process.

**Functional phenotype of cPLA<sub>2</sub>-overexpressing  $\beta$ -cells.** To complement our studies on cPLA<sub>2</sub>-depleted  $\beta$ -cells, we have started to investigate the functional consequences of constitutive overexpression of cPLA<sub>2</sub> $\alpha$  by generating stably transfected MIN6 cells expressing the full-length mouse cDNA inserted into the pcDNA3.1 expression vector in the sense orientation. Overexpression of cPLA<sub>2</sub> $\alpha$  was driven by the cytomegalovirus promoter and produced high and stable levels of expression of immunoreactive cPLA<sub>2</sub>, as assessed by Western blotting. As for the cPLA<sub>2</sub>-deficient MIN6 cells, the functional phenotype of the overexpressing clones was not what we had expected.



**FIG. 3.** Functional phenotype of cPLA<sub>2</sub>-overexpressing  $\beta$ -cells. **A:** MIN6 pseudoislets formed from cPLA<sub>2</sub> $\alpha$ -overexpressing ( $\square$ ) or passage-matched control ( $\blacksquare$ ) cells were perfused (0.5 ml/min, 37°C) with a physiological salt solution supplemented with 0.5 mg/ml BSA and 2 mmol/l glucose to establish a stable basal rate of secretion. Exposure to 20 mmol/l glucose (arrow) produced a rapid and maintained elevation in insulin secretion from control pseudoislets as shown in this representative experiment ( $\blacksquare$ ), but the cPLA<sub>2</sub> $\alpha$ -overexpressing cells failed to respond ( $\square$ , means  $\pm$  SE,  $n = 3$ ). **B:** Microfluorimetric measurements of [Ca<sup>2+</sup>]<sub>i</sub> in Fura2-loaded cPLA<sub>2</sub> $\alpha$ -overexpressing MIN6 cells showed a loss of the normal rapid increases in [Ca<sup>2+</sup>]<sub>i</sub> induced by nutrients such as ketoisocaproic acid (KIC, 10 mmol/l), although the cells showed normal [Ca<sup>2+</sup>]<sub>i</sub> responses to tolbutamide (Tolb, 100  $\mu$ mol/l) and to KCl (20 mmol/l). **C:** Representative experiments in which a depolarizing concentration of KCl (20 mmol/l arrow) stimulated insulin secretion from pseudoislets formed from cPLA<sub>2</sub> $\alpha$ -overexpressing ( $\square$ ) or passage-matched control ( $\blacksquare$ ) MIN6 cells.

Thus, overexpression of cPLA<sub>2</sub> had no marked effect on insulin content nor on the basal rate of insulin secretion at a substimulatory glucose concentration (2 mmol/l). However, the cPLA<sub>2</sub>-overexpressing clones failed to show insulin secretory responses to glucose, as shown in Fig. 3A. The lack of secretory responses to glucose was accompanied by a loss of the normal rapid increases in [Ca<sup>2+</sup>]<sub>i</sub> induced by glucose (data not shown) and by mitochondrial substrates such as ketoisocaproic acid, as shown in Fig. 3B, perhaps suggesting a mitochondrial

defect. The cells retained normal [Ca<sup>2+</sup>]<sub>i</sub> responses to depolarizing concentrations of KCl (Fig. 3B) and tolbutamide (data not shown), localizing the defect in the stimulus-response coupling pathway at a stage beyond glucose entry and phosphorylation but before depolarization of the  $\beta$ -cells and the subsequent influx of extracellular Ca<sup>2+</sup>. This conclusion was supported by the demonstration that cPLA<sub>2</sub>-overexpressing cells had relatively normal, although slightly delayed, onset and secretory responses to depolarizing [Ca<sup>2+</sup>]<sub>i</sub>-elevating stimuli, as

shown in Fig. 3C. Incidentally, the normal pattern of both basal secretion and of secretion in response to elevated  $[Ca^{2+}]_i$  in cells that greatly overexpress cPLA<sub>2</sub>α further reinforces the conclusion from the studies with cPLA<sub>2</sub>-deficient MIN6 cells that cPLA<sub>2</sub>α is not an important transducer of Ca<sup>2+</sup>-induced secretory responses in β-cells.

Measurements of NAD(P)H autofluorescence as an indirect assessment of oxidative phosphorylation revealed a major metabolic defect in the cPLA<sub>2</sub>-overexpressing cells. Thus, exposure to 25 mmol/l glucose caused a rapid and reversible increase in NAD(P)H autofluorescence in control MIN6 cells that was almost completely absent in the cPLA<sub>2</sub>-overexpressing cells (data not shown). These observations may explain the defective secretory phenotype of the cPLA<sub>2</sub>-overexpressing cells, since uncoupling nutrient metabolism from ATP production will prevent the ATP-dependent depolarization that initiates the insulin secretory response. The underlying cause of this defective metabolism is likely to be the chronic overproduction of the long-chain unsaturated fatty acid AA by the overexpressed cPLA<sub>2</sub>α in the sense transfects. There is a growing body of evidence that prolonged exposure to fatty acids can compromise β-cell function (35,36), with inhibition of glucose-induced insulin secretion being caused by sustained exposure to high levels of free fatty acids (37,38). The inhibitory effects on secretion appear to be confined to nutrient stimuli, with normal responses being maintained to nonnutrients (37,38), suggesting that excessive exposure to fatty acids perturbs β-cell metabolic pathways proximal to elevated  $[Ca^{2+}]_i$ , in accordance with the secretory phenotype of our cPLA<sub>2</sub>-overexpressing MIN6 cells. One link between fatty acid excess and mitochondrial dysfunction is uncoupling protein 2 (UCP2), which dissipates mitochondrial proton gradients, thus increasing the activity of respiratory chain complex enzymes and the oxidation of NADH. High concentrations of fatty acids, including AA (39,40), can upregulate expression of UCP2 in a number of tissues, including β-cells (41,42), and UCP2 upregulation is associated with reduced nutrient-induced insulin secretion (43–45). Our measurements of UCP2 mRNA demonstrate an upregulation of UCP2 expression in the cPLA<sub>2</sub>-overexpressing cells, with mRNA levels of up to  $256 \pm 28\%$  control values ( $P < 0.001$ ), presumably in response to the high local concentrations of AA in these cells. These results suggest that the uncoupling of oxidative phosphorylation from mitochondrial ATP generation is responsible for the defective secretory phenotype of the cPLA<sub>2</sub>-overexpressing cells and are consistent with UCP2 upregulation being involved in β-cell dysfunction in type 2 diabetes (44,45). The cPLA<sub>2</sub>-overexpressing MIN6 cells may offer a useful experimental model in which to study the effects of chronic exposure to fatty acids on β-cell (dys)function.

In conclusion, our expectation at the outset of these studies was to confirm an important role for cPLA<sub>2</sub>α as a Ca<sup>2+</sup> sensor in β-cell stimulus-secretion coupling. However, our results did not support our preconceptions and suggest that cPLA<sub>2</sub>α is not required for Ca<sup>2+</sup>-dependent insulin secretion, although it may play an important, but poorly defined, role in the maintenance of insulin stores in dense-cored secretory vesicles. The unexpected phenotype associated with excessive cPLA<sub>2</sub> activity may give

further insights into fatty acids, mitochondrial dysfunction, and the failure of β-cell secretory responses.

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