Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 9712–9716, October 1995
Cell Biology

Dissociation between changes in cytoplasmic free Ca^{2+} concentration and insulin secretion as evidenced from measurements in mouse single pancreatic islets

(islets of Langerhans/protein phosphorylation/kinetics)

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Communicated by Rolf Luft, Karolinska Sjukhuset, Stockholm, Sweden, July 5, 1995 (received for review May 7, 1995)

ABSTRACT **Simultaneous measurements of cytosolic free Ca^{2+} concentration and insulin release, in mouse single pancreatic islets, revealed a direct correlation only initially after stimulation with glucose or K^+. Later, there is an apparent dissociation between these two parameters, with translocation of α and ε isoenzymes of protein kinase C to membranes and simultaneous desensitization of insulin release in response to glucose. Recovery of insulin release, without any concomitant changes in cytosolic free Ca^{2+} concentration, after addition of phorbol 12-myristate 13-acetate, okadaic acid, and forskolin supports the notion that the desensitization process is accounted for by dephosphorylation of key regulatory sites of the insulin exocytotic machinery.**

It is well accepted that the cytoplasmic free calcium concentration ($[Ca^{2+}]_i$) has a key regulatory role in the insulin secretory process from pancreatic β cells (1–3). Nevertheless, controversy regarding the exact interrelationship between $[Ca^{2+}]_i$ and insulin release exists. Whereas some papers suggest that there is a close relationship between changes in $[Ca^{2+}]_i$ and insulin secretion (4, 5), others support the view that insulin secretion can be regulated independently from changes in $[Ca^{2+}]_i$ (6, 7). It is possible that one of the main reasons for this apparent controversy is that previous experiments were designed to perform $[Ca^{2+}]_i$ and insulin release measurements independently of each other. To clarify whether $[Ca^{2+}]_i$ has a direct regulatory role or more of a permissive role in the insulin secretory process, it is necessary to carry out simultaneous measurements of $[Ca^{2+}]_i$ and insulin release. So far, two papers have described such measurements (8, 9). In both cases, a possible correlation between oscillations in $[Ca^{2+}]_i$ and insulin release from a mouse single islet was addressed.

The aim of the present study was to evaluate in detail the extent to which there is a direct correlation between $[Ca^{2+}]_i$ and insulin release in a mouse single pancreatic islet in the presence of physiological concentrations of extracellular Ca^{2+} after activation of stimulus-secretion coupling. We mainly focused on comparative kinetics of glucose-induced $[Ca^{2+}]_i$ and insulin responses as well as on evaluation of a possible role of phosphorylation in regulation of these processes. To minimize background $[Ca^{2+}]_i$ recordings from non-insulin-producing cells, pancreatic islets from obese hyperglycemic (*ob/ob*) mice, containing >90% β cells (10), were used. Another advantage of these islets is that they can produce, on average, enough insulin so that insulin secretion can be conveniently measured from one single islet, with good precision, by conventional RIA.

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MATERIALS AND METHODS

Isolation of Islets of Langerhans. Adult obese hyperglycemic (*ob/ob*) mice of both sexes were obtained from a local noninbred colony (11) and starved for 24 h. Islets were isolated by a collagenase technique (12) and cultured overnight in RPMI 1640 medium containing 10% (vol/vol) heat-inactivated fetal calf serum (Flow Laboratories) and 11 mM glucose at 37°C.

Perifusion of Single Islets and Measurements of $[Ca^{2+}]_i$. For perifusion, a medium supplemented with bovine serum albumin (1 mg/ml) containing 125 mM NaCl, 5.9 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgCl_2$, and 25 mM Hepes (pH 7.4) with different concentrations of glucose and other additions was used. Average-sized islets were chosen for further experiments. Islets were loaded with 5 μM fura 2 AM for 1 h in medium containing 3.3 mM glucose. After loading, a single islet was transferred to an open perifusion chamber custom built for microscopic work and maintained at a temperature of 37 ± 0.2°C. This chamber had an elongated polygonal shape, with a volume of ≈100 μl, and input and outlet cannulas put opposite one another through the side walls of the chamber. With this design, a close to laminar flow was achieved. The islet was placed under a microscopic grid, which was fixed ≈2 mm upstream apart from the outlet cannula. All other details concerning measurements of the 340/380-nm fluorescence ratio and calculations of $[Ca^{2+}]_i$ were done as described (13). Before perifusion, the islet was allowed to adhere to the glass coverslip forming the bottom of the chamber for 3 min. For the next 15 min, preperifusion with medium containing 3.3 mM glucose was performed without any recording. A flow rate of 150 μl/min was selected to be optimal not to perturb the islet and to give enough volume for insulin measurements by RIA. Experiments with methylene blue showed that the time for full exchange of solution in the area near the islet was 44 s after switching of media. Perifusate was collected with a Fractomin td collector (Stålprodukter, Uppsala, Sweden) in 30- or 48-s time intervals to give the best precision in the insulin assay. The lag time from the grid to the vials was 54 s. These delays were corrected for in the graphical representations of the data.

Insulin RIA. Special attention was paid to precision and sensitivity of insulin analysis by RIA. Screening of assays of commercially available RIAs for mouse insulin (Linco Research, St. Louis; Sigma; and Farbwerke Hoechst, Frankfurt) as well as one used in our laboratory for many years, based on charcoal separation (14) and rat insulin (Novo-Nordisk, Copenhagen) as a standard, showed that all the assays had sensitivity in the same range (1.5–5 microunits/ml or 64–214 pg/ml). The sensitivity was defined by well-accepted criteria:

Abbreviations: $[Ca^{2+}]_i$, cytoplasmic free Ca^{2+} concentration; PMA, phorbol 12-myristate 13-acetate; OA, okadaic acid; PKC and PKA, protein kinases C and A, respectively.

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insulin concentration displacing 10% of labeled insulin or insulin concentration corresponding to the mean value of the zero calibrator + 3SD. Fifty percent displacement of freshly labeled ^{125}I-labeled insulin occurred at about the same concentration range of nonradioactive insulin for all assays (15–50 microunits/ml or 0.064–2.14 ng/ml). Nevertheless, we compared results on dynamics of insulin secretion from the same *ob/ob* single islet using the Linco Research RIA and our own RIA. The results were almost identical ($r^2 = 0.95$). Therefore, all other experiments on insulin detection were done in duplicate using a procedure analogous to the one previously described (14). Intraassay precision was estimated to be about 4%, 10%, and 30% at insulin concentrations of 25, 10, and 1.5 microunits/ml, respectively. Although it was determined that none of the substances added to the perifusion buffer influenced insulin RIA determination, all assays were run using standard curves based on the very same buffer as the samples.

Immunodetection of Protein Kinase C (PKC) Isoenzymes. The distribution of α and ε isoenzymes of PKC (PKC-α and PKC-ε, respectively) was determined in isolated pancreatic islets. Briefly, dissected islets were pooled (≈250 in each group; final vol, 75 μl) and incubated with 20 mM glucose for different periods of time in the same medium used for simultaneous $[Ca^{2+}]_i$ and insulin release measurements at 37°C. The incubation period was terminated by addition of 75 μl of chilled homogenization buffer [2 mM EGTA, 2 mM EDTA, 20 mM Tris·HCl (pH 7.4 at room temperature), 10 mM 2-mercaptoethanol, and protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 0.3 μM aprotinin, leupeptin at 20 μg/ml and 10 μM pepstatin A)] and sonication. The cytosolic fraction was prepared by centrifugation at 100,000 × *g* for 1 h at 4°C. The pellet was resuspended in a medium (150 μl) identical to the homogenization buffer and with the addition of 0.1% Triton X-100. Samples were centrifuged at 100,000 × *g* for 1 h at 4°C. The supernatant contained the solubilized proteins from the membrane fraction. Proteins were analyzed on SDS/polyacrylamide gel with a Laemmli buffer system and transferred to nitrocellulose (Hybond C; Amersham). Immunodetection of PKC isoenzymes was performed with specific polyclonal antibodies (GIBCO). The proteins were identified with an ECL detection kit (Amersham) as recommended by the manufacturer.

Data Analysis. Data were analyzed by the program SIGMA PLOT, version 4.10 (Jandel, San Rafael, CA). Time average $[Ca^{2+}]_i$ and insulin concentrations were obtained by integrations of the surface areas below the corresponding curves and were normalized per min.

RESULTS

Typical results of simultaneous measurements of $[Ca^{2+}]_i$ and insulin release, after addition of 11 or 20 mM glucose to a single islet of the *ob/ob* mouse, are shown in Fig. 1. The kinetic patterns of $[Ca^{2+}]_i$ increase and insulin release could be divided into three main phases. Phase 1a, corresponding to a simultaneous increase in $[Ca^{2+}]_i$ and insulin release (Fig. 1 *A*

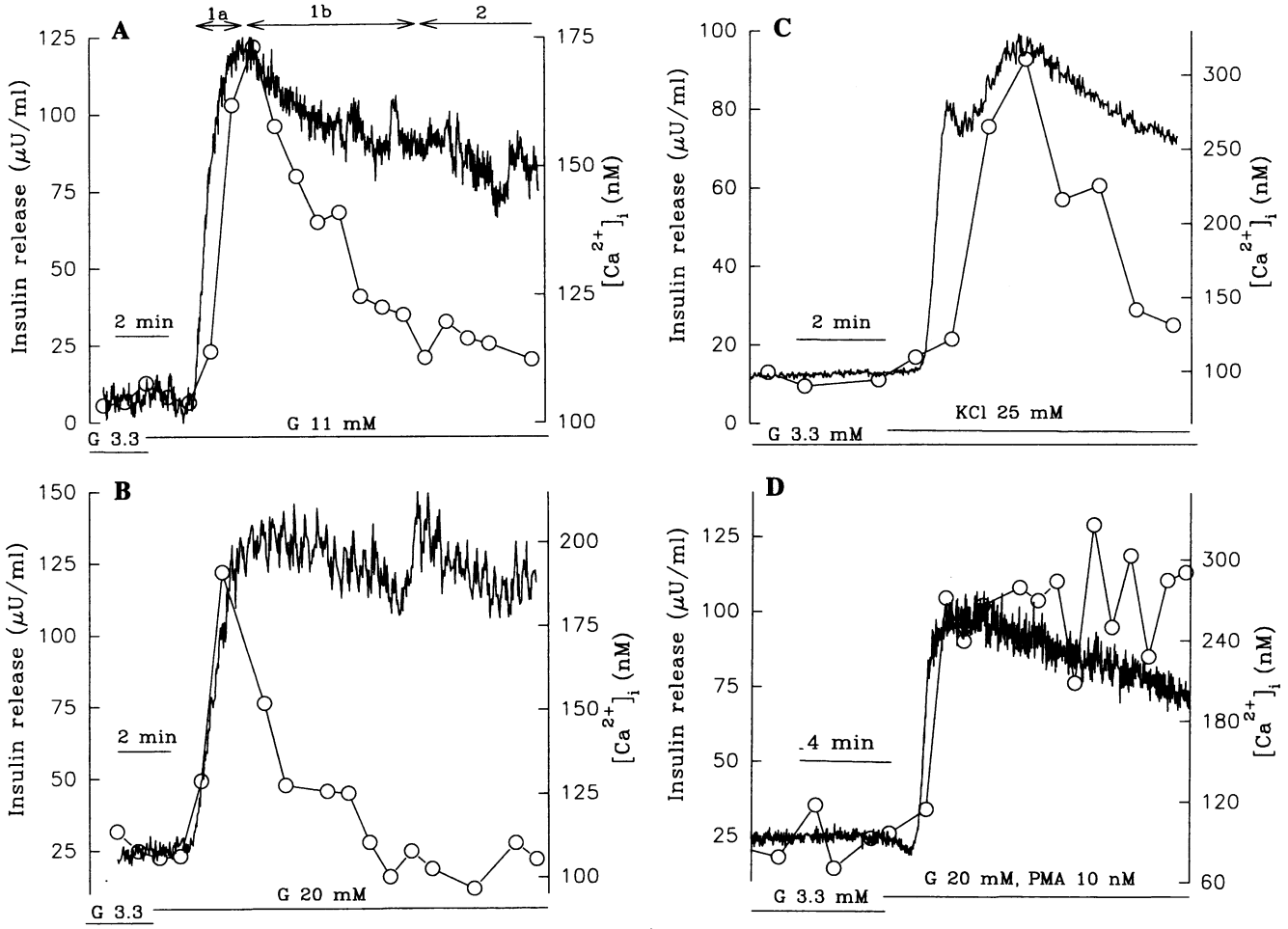

FIG. 1. Comparison of glucose- (in the absence or presence of 12-myristate 13-acetate; PMA) and K^+-induced changes in $[Ca^{2+}]_i$ (solid trace) and insulin release (open circles) in single *ob/ob* mouse islets. Glucose (G) at 11 mM (*A*) or 20 mM (*B*, *D*) in the absence (*B*) or presence of 10 nM PMA (*D*) or 25 mM KCl (*C*) was added to single islets (each of the curves shown is representative of five experiments performed under the same conditions in islets from different animals).

and *B*), was established 1.8–2.2 min after increasing the glucose concentration and lasted for 50–75 s. In all cases investigated, the insulin release curve coincides or follows $[Ca^{2+}]_i$ with a delay of not more than 50 s, which corresponds to resolution time for insulin release measurements (as mentioned above, the interval between sample collections was 30 or 48 s). This means that there is a direct correlation between $[Ca^{2+}]_i$ and insulin release at this stage. Phase 1b describes the decrease in $[Ca^{2+}]_i$ and insulin release after the peaks (Fig. 1 *A* and *B*). With regard to insulin release, this corresponds to a decrease to 5–25% of maximal elevation—i.e., a level only slightly higher than the basal one—in 2 experiments of 10 even to the basal level. Almost the same half-life ($t_{1/2}$) was obtained at 11 and 20 mM glucose. In contrast, corresponding decreases in average $[Ca^{2+}]_i$ were rather small, reaching 70–80% maximal level, in the presence of 11 mM glucose and even less in the presence of 20 mM glucose. Although small, the magnitude of $[Ca^{2+}]_i$ decrease at 11 mM glucose allowed one to estimate time characteristics of changes in this parameter. Interestingly, the $t_{1/2}$ of the average $[Ca^{2+}]_i$ decrease in any separate experiment always corresponded to the $t_{1/2}$ of insulin decline; analyses of all five experiments (mean ± SEM) with 11 mM glucose gave $t_{1/2} = 1.97 \pm 0.38$ min and $t_{1/2} = 1.73 \pm 0.28$ min for changes in $[Ca^{2+}]_i$ and insulin release, respectively. Phase 2 (Fig. 1 *A* and *B*) occurs 4–6 min after the peaks and corresponds to the situation where average $[Ca^{2+}]_i$ shows a sustained elevation (70–80% maximal level). Very small (not more than 5–10% of the $[Ca^{2+}]_i$ average concentration), but statistically significant, oscillations in $[Ca^{2+}]_i$ were seen at this stage. Average insulin release was also rather stable at this time period, being only slightly above basal level.

Data on $[Ca^{2+}]_i$ and insulin release, measured simultaneously in the same islet stimulated with depolarizing concentrations of 25 mM KCl (Fig. 1*C*), showed the same pattern as that obtained with glucose stimulation—i.e., initial increases in $[Ca^{2+}]_i$ and insulin release were directly correlated. Furthermore, in this case there was also a subsequent dramatic decline in insulin release despite the fact that corresponding $[Ca^{2+}]_i$ was still high.

Fig. 1*D* illustrates data on simultaneous measurements of $[Ca^{2+}]_i$ and insulin release in the same islet in the presence of 20 mM glucose and the PKC activator phorbol ester PMA at 10 mM. Dissociation between $[Ca^{2+}]_i$ and insulin release of another type clearly can be seen in this case. A simultaneous increase in $[Ca^{2+}]_i$ and insulin concentration, during phase 1a, is followed by a decrease in $[Ca^{2+}]_i$ with almost no change in elevated average insulin release. We have previously demonstrated that PMA also promotes insulin release at low concentrations of glucose (7). Measurements of $[Ca^{2+}]_i$ under similar conditions, but in separate experiments, showed no change (7).

Results on the effect of okadaic acid (OA), an inhibitor of serine/threonine protein phosphatases type 1, type 2A, and type 3 (15), on $[Ca^{2+}]_i$ and insulin release are shown in Fig. 2*A*. Addition of OA to islets after preperifusion with 11 mM glucose for 15 min—i.e., phase 2 of response to high glucose—causes an increase in average insulin release of ≈50%. This increase was not accompanied by changes in average $[Ca^{2+}]_i$.

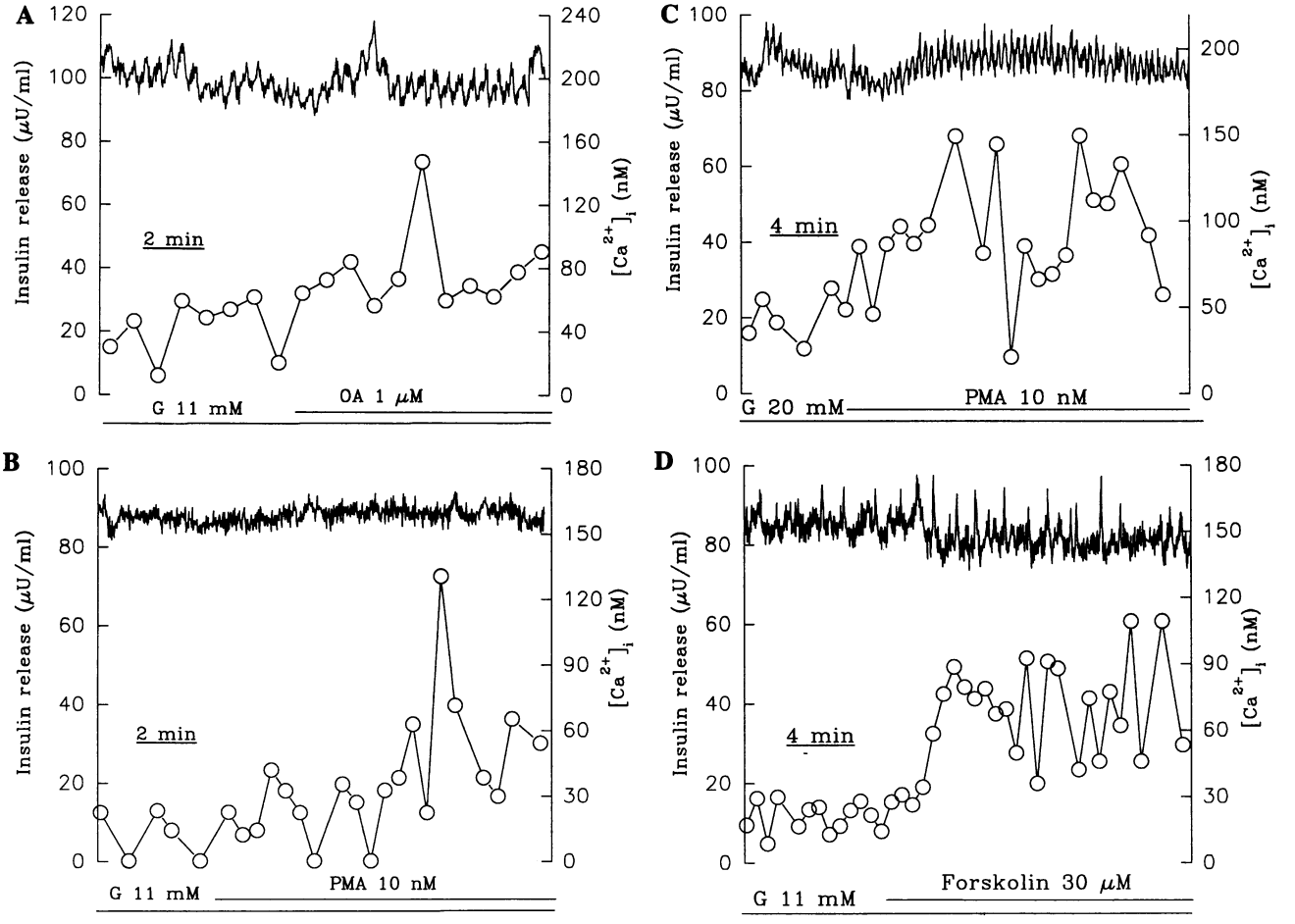

FIG. 2. Effects of OA, PMA, and forskolin on $[Ca^{2+}]_i$ and insulin response in single *ob/ob* mouse islets. OA at 1 μM (*A*), 10 nM PMA (*B* and *C*), or 30 μM forskolin (*D*) was added to a medium containing 11 mM (*A*, *B*, and *D*) or 20 mM (*C*) glucose after preperifusion in the same type of medium for 15 or 12 min, respectively (each of the curves shown is representative of four experiments performed under the same conditions in islets from different animals). Symbols are the same as in Fig. 1.

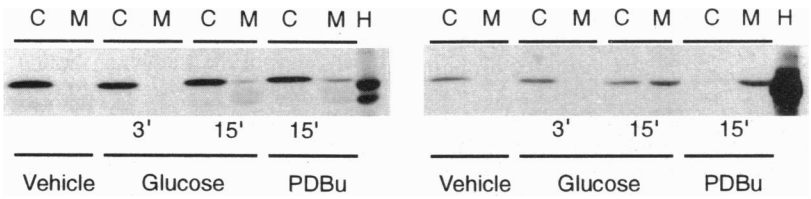

FIG. 3. Effect of glucose and phorbol 12,13-dibutyrate (PDBu) on distribution of PKC-α (*Left*) and PKC-ε (*Right*) in isolated pancreatic islets from *ob/ob* mice. Islet samples were incubated for 3 and 15 min with 20 mM glucose or for 15 min with 100 nM PDBu. Control islets were incubated under the same conditions with vehicle. Islet membrane (lanes M) and cytosolic (lanes C) fractions were isolated and identical aliquots were separated on SDS/polyacrylamide gel and immunoblotted as described. A reference sample from *ob/ob* mice homogenized striatum (lanes H) was also analyzed. A representative immunoblot of four independent experiments is shown.

A similar, although more pronounced, effect is clearly seen if protein phosphorylation is affected by PMA or the adenylate cyclase activator forskolin; the latter is known to elevate the β-cell level of cAMP (16). Data on the influence of 10 nM PMA, in the presence of 11 or 20 mM glucose—i.e., phase 2 of sugar response—on $[Ca^{2+}]_i$ and insulin release measured simultaneously are shown in Fig. 2 *B* and *C*. After 3–6 min, subsequent to addition of the PKC activator, the average level of insulin secretion is 1.5- to 4-fold higher than in the absence of PMA. This increase is more clearly seen if stimulation with PMA occurs in the presence of 11 mM glucose compared to 20 mM glucose, with mean values of increases being 2.7- and 1.7-fold, respectively. In contrast to insulin release, no significant changes in $[Ca^{2+}]_i$ were observed. Qualitatively the same type of effect could be observed in the presence of forskolin (Fig. 2*D*). Average insulin release increased up to 3-fold in comparison to the situation without forskolin. This elevation in insulin release occurred practically at the same average $[Ca^{2+}]_i$ level. Thus, results obtained with an inhibitor of protein phosphatases or activators of PKC and PKA suggest that, by changing the level of protein phosphorylation in the β cell, insulin release can be promoted independently from changes in $[Ca^{2+}]_i$. Interestingly, the addition of activators of protein phosphorylation (Figs. 1*D* and 2 *B–D*) gives rise to an increase in amplitude of oscillations in insulin release with negligible influence on corresponding $[Ca^{2+}]_i$.

It was described earlier that α and ε isoenzymes of PKC play important roles in exocytosis (17, 18). To investigate the role of these isoenzymes in the events mentioned above, translocation of PKC-α and PKC-ε from the β-cell cytoplasm to the membrane fraction was studied (Fig. 3). In glucose-stimulated β cells, neither PKC-α nor PKC-ε was translocated at 3 min after addition of sugar (phase 1a, the time of maximal $[Ca^{2+}]_i$ and insulin responses; Fig. 1 *A* and *B*). After 15 min (phase 2, corresponding to a drastic decrease in insulin release and a 20–30% decrease in $[Ca^{2+}]_i$), PKC-α as well as PKC-ε were translocated from the cytoplasm to the membrane fraction—the translocation being more accentuated with regard to the latter isoenzyme (Fig. 3). It is of interest to note that addition of phorbol 12,13-dibutyrate also induced a more pronounced translocation of PKC-ε compared to PKC-α (Fig. 3).

DISCUSSION

In the present study, the concept of a direct regulatory role of $[Ca^{2+}]_i$ in insulin release was challenged. The experimental approach was to simultaneously measure $[Ca^{2+}]_i$ and insulin release from mouse single pancreatic islets. It is of interest to note that overall there was a clear difference in glucose-induced changes in $[Ca^{2+}]_i$ and insulin release. All experiments showed a nice correlation between $[Ca^{2+}]_i$ and insulin release during phase 1a, after stimulation of single *ob/ob* mouse islets with glucose or K^+. Taking the time resolution in our insulin measurements into consideration, corresponding $[Ca^{2+}]_i$ traces and insulin release curves almost coincided. These results agree with those in a recent study concerning oscillations in $[Ca^{2+}]_i$ and insulin release in islets of NMRI mice (8). However, in the present study, after reaching maximal $[Ca^{2+}]_i$ and insulin release levels—i.e., during phase 1b—differences in patterns between these two parameters appeared. Although the period of half-decline of $[Ca^{2+}]_i$ and insulin release was about the same, the degree of these changes was very different. A 75–95% drop in average insulin release corresponded to only a 20–30% decrease in elevated $[Ca^{2+}]_i$.

The best way to prove that there is a dissociation between $[Ca^{2+}]_i$ and insulin secretion is to identify conditions where stimulation drastically influences one of these two parameters without significantly affecting the other. Some data along these lines were shown in a recent publication by Gilon *et al.* (8). In the presence of epinephrine, distinguished oscillations in $[Ca^{2+}]_i$ were observed under conditions where corresponding insulin release was almost negligible. Nevertheless, as pointed out in that study, this shows only that inhibition of secretion by α_2-adrenoceptors involves specific interaction with molecular mechanisms distal to the $[Ca^{2+}]_i$ increase. More informative would be to determine whether secretion can be triggered without any concomitant increase in $[Ca^{2+}]_i$ or whether there is no drop in average insulin secretion while at the same time $[Ca^{2+}]_i$ decreases. In our hands, both situations were obtained by activation of β-cell protein phosphorylation. Simultaneous addition of PMA and high glucose led to a situation where, after 3 min, $[Ca^{2+}]_i$ decreased but time-averaged insulin release was essentially the same. In turn, activators of PKC and PKA at phase 2 of response to high glucose markedly increased insulin release without any essential change in average $[Ca^{2+}]_i$. The protein phosphatase inhibitor OA showed the same type of effect. The latter results confirmed our previous data on the role of protein phosphorylation, based on separate measurements of $[Ca^{2+}]_i$ and insulin release in the presence of PMA, in the β-cell stimulus-secretion coupling (7, 19) and data on indirect measurement of insulin release by the cell capacitance technique (20, 21).

The results on glucose-induced insulin release from a perifused single islet, obtained in this study as well as our previous data on perifused β cells (7), clearly showed a transient pattern of insulin release with a drastic decrease in secretion after 3–4 min of addition of high glucose. As mentioned above, this decrease is accompanied by a slight drop in $[Ca^{2+}]_i$. This type of insulin release and $[Ca^{2+}]_i$ behavior give rise to the question concerning the underlying molecular mechanisms of β-cell desensitization to the sugar. Data on glucose-induced translocation of PKC-α and PKC-ε from the β-cell cytoplasm to the membrane fraction showed that translocation did not correlate with a simultaneous increase in $[Ca^{2+}]_i$ and elevation in insulin release (phase 1a). Indeed, there was no evidence of translocation of PKC isoforms at 3 min after high glucose addition, whereas both PKC-α and PKC-ε translocated 15 min after glucose addition.

Hence, it is of interest to note that the phase of both decreased $[Ca^{2+}]_i$ and insulin release, after glucose stimulation, corresponded to translocation of PKC-α as well as PKC-ε to the β-cell plasma membrane. This clearly shows that the

molecular mechanisms regulating both the initial increase in $[Ca^{2+}]_i$ and exocytosis of insulin are operating under conditions where PKC-α and PKC-ε are localized in the cytosolic compartment. Although the decrease in both $[Ca^{2+}]_i$ and insulin release (during phases 1b and 2) correlated with translocation of the PKC isoenzymes to the membrane fraction, direct activation of the kinase with PMA prevented the decrease in hormone release after glucose stimulation. Within the time period that PMA activation of PKC prevents a decrease in insulin release, the phorbol ester promotes translocation of both PKC-α and PKC-ε. These data indeed suggest a coupled modulatory role of PKC in the β-cell stimulus-secretion coupling. Despite the fact that future studies need to address more precisely how the various PKC isoenzymes interact with the molecular mechanisms involved in this complex chain of events, it is of interest to note that activation of PKC is sufficient to prevent the desensitization phase (phases 1b and 2) of insulin release after glucose stimulation without any concomitant change in $[Ca^{2+}]_i$.

Thus, simultaneous measurements of $[Ca^{2+}]_i$ and insulin release in mouse single pancreatic islets, under conditions close to physiological, clearly reveal that correlation between $[Ca^{2+}]_i$ and insulin release takes place only initially (phase 1a) after stimulation with high concentrations of glucose or K^+. Later, a dissociation between these two parameters occurs in connection with desensitization of islet response. It is noteworthy that desensitization of insulin release can be prevented under conditions where $[Ca^{2+}]_i$ remains unchanged after stimulation of either PKC or PKA as well as inhibition of serine/threonine protein phosphatase types 1, 2A, and 3. Hence, these data may suggest that the desensitization phase in glucose-induced insulin release is linked to dephosphorylation of various key regulatory sites of exocytosis, with these sites being subjected to well-depicted modulation by various kinases and phosphatases. The results obtained in the present study are also compatible with the notion that Ca^{2+} in itself can promote fusion only of secretory granules that are already positioned at the release site, whereas recruitment and transport of granules from the interior of the cell requires protein phosphorylation, a process where PKC-ε may play a central role. Such a phosphorylation is then likely to result in activation of the microtubule–microfilament system. In the latter case, $[Ca^{2+}]_i$ has a more permissive than direct regulatory role.

We thank M. Köhler for fruitful discussions, R. L. Gingerich (Linco Research) for the kind gift of the Linco insulin RIA kit, and R. Mahgouri and T. Zeccarias for excellent technical assistance. This study was supported by grants from the Swedish Medical Research Council (03X-09890 and 19X-00034), the Swedish Diabetes Association, Funds of the Karolinska Institute, Novo Industry, the Nordic Insulin Foundation, and the Juvenile Diabetes Foundation International.

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