Synchronous Oscillations of Cytoplasmic Ca²⁺ and Insulin Release in Glucose-stimulated Pancreatic Islets

(Received for publication, May 18, 1993, and in revised form, January 7, 1994)

Peter Bergsten, Eva Grapengiesser, Erik Gylfe, Anders Tengholm, and Bo Hellman

From the Department of Medical Cell Biology, Uppsala University, Biomedicum, Box 571, S-751 23 Uppsala, Sweden

The cytoplasmic Ca²⁺ concentration ([Ca²⁺]i) was measured in single pancreatic mouse islets superfused in a system allowing concomitant recordings of insulin release. When glucose was raised from 3 to 11 mM, [Ca²⁺]i responded by a transient lowering followed by a rise to an average level of 192 ± 11 nM. In 77% of the islets the rise was associated with the gradual appearance of oscillations, which were either fast (2-7/min), slow (0.3-0.9/min), or a combination of both types. The characteristics of the fast [Ca²⁺]i oscillations were those expected from a relationship with the electrical burst activity in islets. Accordingly, in most cases the fast oscillations were remarkably regular. The slow [Ca²⁺]i oscillations had characteristics similar to the large amplitude ones in individual β-cells. Whereas glucagon and dibutyryl cAMP could transform slow islet oscillations into fast ones, the α₂-adrenergic agonist clonidine had the opposite effect. The rapid islet oscillations were also facilitated by elevated concentrations of extracellular Ca²+. Reinforcing the arguments for [Ca²⁺]i oscillations as responsible for a pulsatile insulin secretion it was possible to demonstrate that the release of the hormone from single islets is synchronized with the slow [Ca²⁺]i oscillations.

Stimulation of insulin release by glucose and other nutrients is mediated by a rise of the cytoplasmic Ca²⁺ concentration ([Ca²⁺]i) following increased entry of the ion into the pancreatic β-cells (1). A characteristic feature of the [Ca²⁺]i response to glucose is its oscillatory nature observed both in individual β-cells (2-6) and in intact pancreatic islets (7-10). These oscillations may have important physiological implications in being responsible for a pulsatile release of insulin. In analogy to what has been proposed for the periodic release of other biologically active peptides it can be supposed that the cyclic variations of circulating insulin prevent down-regulation of the peripheral receptors (11).

It has been reported that glucose triggers the appearance of both fast (7, 8, 10) and slow (7, 9) oscillations of [Ca²⁺]i, in intact pancreatic islets. In the present study these oscillatory events have been characterized in the attempt to understand the mechanisms for their generation and significance for insulin secretion. The observations support the concept that the fast type of oscillations reflects the bursts of action potentials observed in β-cells within pancreatic islets. In contrast, the slow [Ca²⁺]i oscillations can be regarded as resulting from a coordination of [Ca²⁺]i cycles already present in the individual β-cells. When [Ca²⁺]i and insulin secretion were measured simultaneously we found that the release of the hormone from a single islet is synchronized with the slow [Ca²⁺]i oscillations.

EXPERIMENTAL PROCEDURES

Materials—Reagents of analytical grade and deionized water were used. Collagenase, HEPES, and bovine serum albumin (fraction V) were obtained from Boehringer Mannheim GmbH (Mannheim, Germany). Sigma supplied poly-L-lysine (P-8899), sulfinpyrazone, dibutyryl cAMP, [3H]-dibutylbenzine, and insulin peroxidase from Gibco Ltd. ( Paisley, United Kingdom) provided fetal calf serum. Clonidine and glucagon were gifts from Boehringer Ingelheim GmbH (Ingelheim/Rhein, Germany) and Novo Nordisk A/S ( Bagsvaerd, Denmark), respectively. The acetoxyethyester of fura-2 and Pluronic F-127 were from Molecular Probes Inc. (Eugene, OR), and IgG-certified microtiter plates were from NCID (Roskilde, Denmark).

Loading with Ca²⁺ Indicator and Superfusion of Cells—Pancreatic islets were isolated by collagenase using 10-month-old ob/ob mice taken from a non-inbred colony (12). These islets consist of more than 90% β-cells, which respond normally to glucose and other regulators of insulin release (13). The isolated islets (1-2 μg, dry weight) were kept overnight at 37 °C in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 30 μg/ml gentamicin. Further experimental handling was performed with a basal medium physiologically balanced in cations with Cl⁻ as the sole anion (14) and containing 0.5 mg/ml albumin and unless otherwise stated 2.56 mM Ca²+.

To whom correspondence should be sent: Dept. of Medical Cell Biology, Biomedicum, Box 571, S-751 23 Uppsala, Sweden. Tel.: 46-18-174059.
which was <15%. Neither was there any effect of blocking the anion channels in the islets with 10 μM sulfipyrazone, indicating that leakage of fura-2 into the extracellular space did not interfere with the present measurements.

Measurements of Insulin Release—The perifusate was collected in 18-s fractions, which were immediately put on ice. Insulin was measured adhering to a previous protocol (19) by competitive enzyme-linked immunosorbent assay with the insulin antibody immobilized directly onto the solid phase (20). The results were expressed in terms of dry weight after freeze-drying and weighing of the islet on a quartz fiber balance.

RESULTS

A resting [Ca^{2+}]i of 63 ± 3 nM (n = 44) was recorded at 3 mM glucose. Increase of the glucose concentration to 11 mM resulted in a slight lowering of [Ca^{2+}]i, followed by a rise to an average plateau level of 192 ± 11 nM. Different types of responses to glucose are shown in Fig. 1. In 77% of the islets (34 out of 44) the rise of [Ca^{2+}]i was associated with a gradual appearance of oscillations from an elevated level. Three major categories of oscillation with a frequency of 0.3-0.9/min was seen in 27% of the islets (34 out of 44). They displayed a remarkable regularity, there were also irregular patterns with fast oscillations superimposed on the slow ones. In the latter case the durations of the cycles varied periodically with a frequency similar to that of the slow oscillations (Fig. 2).

The type of oscillatory [Ca^{2+}]i response was influenced by agents modulating cAMP (Fig. 3). In islets with slow [Ca^{2+}]i oscillations the addition of 10 nM glucagon resulted in the gradual appearance of a rapid pattern in 3 out of 8 cells (panel A). Dibutyryl cAMP had a similar action in 7 out of 11 cells (panel B). The reverse effect was more reproducible, spontaneous rapid oscillations being transformed into slow ones in 5 out of 6 cells by 100 nM α2-adrenergic agonist clonidine (panel C). In the remaining cell the rapid pattern was replaced by sustained increase of [Ca^{2+}]i. With 10 nM clonidine the transformation of fast oscillations into slow ones was less prompt (not shown). Also the extracellular Ca^{2+} concentration affected the type of response. Lowering Ca^{2+} from 2.56 to 1.28 mM during superfusion with 11 mM glucose sometimes resulted in immediate replacement of the uniform fast [Ca^{2+}]i oscillations with a pattern of irregular small ones from the elevated level (Fig. 4, panel A). The effect of raising glucose from 11 to 20 mM in a medium containing 2.56 mM Ca^{2+} is shown in the lower panels of Fig. 4. In this experimental situation time-average [Ca^{2+}]i increased by 47 ± 7 nM (p < 0.001, n = 17). Whereas the rapid oscillations always disappeared, the slow ones were maintained in 2 out of 9 experiments.

The [Ca^{2+}]i oscillations at 11 mM glucose were synchronized in different parts of the islets. Examples of this synchrony are given in Fig. 5. Whereas the fast oscillations had a similar appearance in different regions (panel A), the amplitudes of the slow ones varied considerably. Indeed, it was possible to identify regions with pronounced slow waves and others almost devoid of oscillatory activity (panel B).

Fig. 6 illustrates the results of simultaneous measurements of [Ca^{2+}]i, and insulin secretion from a single islet superfused with 11 mM glucose. It is apparent that the hormone is released in synchrony with the slow [Ca^{2+}]i oscillations. Whereas further elevation of glucose to 20 mM makes the cyclic variations of [Ca^{2+}]i less pronounced, the amplitudes of the insulin pulses increase.
Discussion

In recent years dual wavelength fluorometry with indicators available as membrane-permeable esters has been widely employed for measuring [Ca\textsuperscript{2+}]. Although this approach is suited for detecting variations of [Ca\textsuperscript{2+}], it is a matter of discussion as to how accurate are the calculated concentrations. The methodological uncertainties can be expected to be more apparent in analyses of intact pancreatic islets than with single cells. It is unclear to what extent methodological complications can explain why the glucose-induced slow oscillations of [Ca\textsuperscript{2+}] in islets occur from a plateau rather than from the basal level as in individual β-cells or monolayer clusters (17). The geometry of the measurements may also be a factor of importance for the considerable variation of the amplitudes of the synchronized [Ca\textsuperscript{2+}] oscillations in different islet regions. Whereas autofluorescence has been insignificant in our previous studies of [Ca\textsuperscript{2+}] in β-cells (2, 6, 17), the contribution of this factor to the total fluorescence has been estimated to be 15–20% in studies of islets using indo-1 (7) or fura-2 (10) as indicator. Under the present excitation conditions autofluorescence was less significant (<15%) and did not change when altering the glucose concentration. The error introduced by not compensating for this autofluorescence was an underestimate of basal [Ca\textsuperscript{2+}] by 3–5% and elevated [Ca\textsuperscript{2+}] by 6–14%. There is a slow leakage of fura-2 from the β-cell cytoplasm by extrusion via an anion transporter stimulated by glucose (21). It is therefore pertinent to consider whether Ca\textsuperscript{2+}-saturated fura-2 retained within the extracellular space of the islet may contribute to the Ca\textsuperscript{2+} signal. This does not seem to be the case, since the glucose-induced increase of [Ca\textsuperscript{2+}] was unaffected by blocking anion transport with sulfinpyrazone. Moreover, the present levels of [Ca\textsuperscript{2+}], were not higher than those recorded in individual β-cells under conditions when extruded fura-2 is rapidly diluted and washed away by the superfusion medium (2, 6, 17).

Although reacting to 11 mM glucose with a rise of [Ca\textsuperscript{2+}], the islets differed considerably with regard to the response pattern. In most islets the rise of [Ca\textsuperscript{2+}], was associated with a gradual appearance of oscillations, referred to as fast (2–7/min) or slow (0.3–0.9/min). In agreement with the observations of Valdeomillogo et al. (7) there were also islets responding with mixed oscillations. In this case we did not observe any obvious clustering of the superimposed fast oscillations to specific phases of the slow ones.

It has been convincingly demonstrated that the fast [Ca\textsuperscript{2+}], oscillations in the intact islets reflect the electrical burst activity of the β-cells. This is indicated not only by the presence of such oscillations under a number of situations known to induce electrical activity (10) and their prolongation with an increase of the glucose concentration (7) but also by the observation of a perfect synchronization between the two phenomena (8).
phenomenon with regularly varying periods at the plateau of depolarization has previously been observed in 20–50% of islets (22, 23). Interestingly, the frequency of these periodic variations was similar to that of the slow oscillations of [Ca$^{2+}$].

The dominating type of [Ca$^{2+}$], oscillations in glucose-stimulated individual β-cells also reflects their electrical burst activity (24, 25), but the frequency is about 10-fold lower than recorded in islets and similar to the presently observed slow oscillations. The mechanism behind the faster bursts of action potentials in the islets remains to be clarified. It has been shown that intracellular mobilization of Ca$^{2+}$ by muscarinic activation can interrupt firing of action potentials by evoking transient hyperpolarization (26). In the physiological situation a feedback mechanism involving intracellular mobilization of Ca$^{2+}$ by purinergic activation has been anticipated (27), since ATP is released from the secretory granules together with insulin. If a Ca$^{2+}$-activated hyperpolarization mediated by activation of K$^+$ channels is involved in the repolarization during burst activity in islets (26, 28), attention should be paid also to the influx of extracellular Ca$^{2+}$. It is therefore pertinent to note that practically all studies of the burst activity and [Ca$^{2+}$], in

islets, including most of the present experiments, have been performed at 2.5–2.6 mM ambient Ca$^{2+}$, representing a doubling of the physiological concentration. High external Ca$^{2+}$ concentrations have indeed been found to trigger membrane potential oscillations in mouse β-cells during stimulation with sulfonylureas (29). Like Gilon and Henquin (10) we found that elevated external Ca$^{2+}$ favors the occurrence of the fast regular [Ca$^{2+}$], oscillations in the islets.

It was evident from the present study that additions of both glucagon and dibutyryl cAMP to islets with low [Ca$^{2+}$], oscillations sometimes induce the rapid pattern and that spontaneous rapid oscillations are transformed into slow ones with the α$\beta$-adrenergic agonist clonidine. Indeed, epinephrine, which activates β-cell α$\beta$-adrenoceptors, has previously been found to induce a strikingly parallel transformation of the rapid electrical burst pattern in mouse islets into a slow one (30). The cAMP-elevating effect of glucagon is well established, and concentrations of clonidine similar to the present ones cause pronounced depression of this cyclic nucleotide (31). It has also been argued that clonidine activates a low conductance G protein-dependent K$^+$ channel in the β-cells (32). However, the relevance of this effect is questionable, since it was observed at 50–500-fold higher concentrations of the agonist. The results therefore indicate that cAMP is an important factor for the establishment of the rapid regular [Ca$^{2+}$], oscillations in the islets. It is likely that secretion of glucagon from the α$\beta$-cells is significant for the glucose induction of the fast [Ca$^{2+}$], oscillations in islets. In isolated β-cells deprived of adjacent α$\beta$-cells the dominating pattern in response to glucose is slow oscillations. Also in single β-cells glucagon induces rapid [Ca$^{2+}$], oscillations, which have a similar frequency but lack the striking regularity observed in islets (33).

We have previously proposed that the burst activity recorded in intact pancreatic islets may be an artifact from the physiological point of view due to insufficient exchange of the extracellular medium in islets lacking a capillary circulation (17). Autocrine and paracrine effects can be expected to be accentuated in this situation. It is therefore pertinent to note that comparative studies of hormone secretion from the pancreas perfused in the anterograde and retrograde directions have indicated that the β-cells are perfused before the α$\beta$- and δ-cells (34). The importance of paracrine effects of the other islet hormones in the regulation of the β-cell activity can consequently be questioned.

In about 50% of the islets exposure to 11 mM glucose resulted in the appearance of slow [Ca$^{2+}$], cycles with a frequency of 0.3–0.9/min. These cycles were synchronized in different parts of the islets but differed from the slightly slower large amplitude oscillations of individual β-cells in being superimposed upon an elevated plateau rather than occurring from the basal level (2, 6, 17). Nevertheless, it seems probable that the slow

---

**Fig. 5.** Fast (panel A) and slow (panel B) oscillations of cytoplasmic Ca$^{2+}$ in different regions of mouse islets. Recordings in different areas of 1–3 cells (upper traces) are shown together with that of the entire field of 60–80 cells (lower trace).

**Fig. 6.** Simultaneous recordings of cytoplasmic Ca$^{2+}$ (panel A) and insulin release (panel B) during superfusion of an individual mouse islet. Increase of the glucose concentration from 11 to 20 mM is indicated by an arrow. The rate of insulin release is presented as the 3-point moving average. This figure is representative of five experiments.
cycles in the islets represent a coordination of the large amplitude oscillations in individual β-cells, since functional coupling of the β-cells somewhat increases the oscillatory rate (17). As in individual β-cells (2, 6) we observed transformation of the glucose-induced slow oscillations of [Ca²⁺], into a sustained increase in islets exposed to high glucose concentrations.

The question arises as to whether the oscillations of [Ca²⁺], can account for the pulsatile release of insulin. So far the arguments in favor of this concept have relied on the established role of [Ca²⁺], in stimulating insulin secretion and the fact that the frequencies of the slow β-cell oscillations of [Ca²⁺], are similar to those reported for insulin (5). We have now obtained direct evidence for such a relationship by demonstrating that insulin release from the glucose-stimulated single islet occurs in synchrony with the slow [Ca²⁺], oscillations. However, such a synchrony should not be taken as indicating that only 50% of the islets, in which slow [Ca²⁺], oscillations were detected, have a periodic release. Actually, all islets studied in our laboratory (>100) have been found to respond to 11 mM glucose with similar insulin pulses. It may seem puzzling that insulin release from the glucose-stimulated single islet occurs aperiodically, reflecting the concentration in a submembrane space directly involved in the regulation of the secretory activity. There are reasons for believing that glucose stimulation of insulin release is mediated not only by a rise of [Ca²⁺], in the β-cells but also by an increased sensitivity of the secretory machinery to the Ca²⁺ signal (1). It will be a matter for further studies to decide whether such a sensitization explains why raising the glucose concentration from 11 to 20 mM increases the amplitudes of the insulin oscillations while damping those of [Ca²⁺].

REFERENCES
1. Hellman, B., Gyfle, E., Grapengiesser, E., and Lund, P. E. (1989) in Nutrient Regulation of Insulin Secretion (Flatt, P. R., ed) pp. 213–246, Portland Press, London
2. Grapengiesser, E., Gyfle, E., and Hellman, B. (1989) Arch. Biochem. Biophys. 268, 404–407
3. Wang, J. L., and McDaniel, M. L. (1990) Biochem. Biophys. Res. Commun. 169, 813–818
4. Thaler, J.-M., Mollard, P., Guerreiro, N., Vacher, P., Pralong, W., and Wollheim, C. B. (1992) J. Biol. Chem. 267, 18110–18117
5. Hellman, B., Gyfle, E., Grapengiesser, E., Lund, P.-E., and Berts, A. (1992) Biochem. Biophys. Acta 1139, 295–305
6. Hellman, B., Gyfle, E., and Hellman, B. (1992) Cell Calcium 13, 219–226
7. Vallomolinos, M., Santos, R. M., Contreras, D., Soria, B., and Rosario, L. M. (1989) FEBS Lett. 259, 19–23
8. Santos, R. M., Rosario, L. M., Nadal, A., Garcia-Sanchez, J., Soria, B., and Valdeolmillos, M. (1991) Pflügers Arch. Eur. J. Physiol. 416, 417–422
9. Longo, E., Tornheim, K., Donne, J. T., Varmus, B. A., Tillotson, I., Premkitt, M., and Corkey, B. E. (1991) J. Biol. Chem. 266, 9314–9319
10. Gilen, P., and Henquin, J.-C. (1992) J. Biol. Chem. 267, 20713–20720
11. Lefebvre, P. J., Paulsson, G., Scheen, A. J., and Henquin, J. C. (1987) Diabetologia 30, 445–452
12. Hellman, B. (1985) Ann. N. Y. Acad. Sci. 131, 541–558
13. Hahn, H.-J., Hellman, B., Lernmark, A., Schlinn, J., and Taljedal, I. B. (1974) J. Biol. Chem. 249, 450–456
14. Hellman, B. (1975) Endocrinology 97, 392–398
15. Sykes, J. A., and Moore, E. B. (1959) Proc. Soc. Exp. Biol. Med. 100, 125–127
16. Gryniewicz, G., Poenie, M., and Tsien, R. Y. (1985) J. Biol. Chem. 260, 3440–3450
17. Gyfle, E., Grapengiesser, E., and Hellman, B. (1991) Cell Calcium 12, 249–250
18. Chance, B., Legallais, V., Surge, J., and Graham, N. (1976) Anal. Biochem. 66, 496–514
19. Børglum, P., and Hellman, B. (1983) Diabetes 32, 670–674
20. Webster, H. V., Bone, A. J., Webster, K. A., and Wilkin, T. J. (1990) J. Immunol. Methods 134, 85–105
21. Arkhammar, P., Nilsson, T., and Berggren, P. O. (1989) Biochem. Biophys. Res. Commun. 159, 223–226
22. Hohns, J. C., Matson, L. P., and Schmeer, W. (1982) Pflügers Arch. Eur. J. Physiol. 393, 322–327
23. Cook, D. L. (1983) Metabolism 32, 681–686
24. Hellman, B., Gyfle, E., Grapengiesser, E., Panten, U., Schwanstecher, C., and Heipol, C. (1990) Cell Calcium 11, 415–419
25. Smith, P. A., Ashcroft, F. M., and Rorsman, P. (1990) FEBS Lett. 261, 187–190
26. Ammala, C., Larsson, O., Berggren, P.-O., Bokvist, K., Juntti-Berggren, L., Kindmark, H., and Borman, P. (1991) Nature 353, 849–852
27. Gyfle, E., and Hellman, B. (1987) Br. J. Pharmacol. 92, 281–289
28. Gyfle, E. (1988) J. Biol. Chem. 263, 5044–5048
29. Santos, R. M., Barbosa, R. M., Silva, A. M., Antunes, C. M., and Rosario, L. M. (1992) Biochem. Biophys. Res. Commun. 187, 872–878
30. Cook, D. L., and Perrara, E. (1982) Diabetes 31, 985–990
31. García-Morales, P., Dufrane, S. F., Sener, A., Valverde, I., and Malaisse, W. J. (1989) Ricerca Sci. 429, 521–528
32. Rorsman, P., Bokvist, K., Ammala, C., Arkhammar, P., Berggren, P. O., Larsen, O., and Wåhländer, K. (1991) Nature 348, 77–79
33. Grapengiesser, E., Gyfle, E., and Hellman, B. (1991) J. Biol. Chem. 266, 12207–12210
34. Stagner, J. L., and Samols, E. (1992) Diabetes 41, 95–97