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Single islet β-cell stimulation by nutrients: relationship between pyridine nucleotides, cytosolic Ca^{2+} and secretion

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It is generally believed that the initiation of insulin secretion by nutrient stimuli necessitates the generation of metabolic coupling factors, leading to membrane depolarization and the gating of voltage-sensitive Ca^{2+} channels. To establish this sequence of events, the kinetics of endogenous fluorescence of reduced pyridine nucleotides [NAD(P)H], reflecting nutrient metabolism, were compared to those of cytosolic calcium ($[Ca^{2+}]_i$) rises in single cultured rat islet β-cells. In preliminary experiments, the loss of quinacrine fluorescence from prelabelled cells was used as an indicator of secretion. This dye is concentrated in the acidic insulin-containing secretory granules. Both glucose and 2-ketoisocaproate (KIC) raised $[Ca^{2+}]_i$ in a dose-dependent manner. There was marked cellular heterogeneity in the $[Ca^{2+}]_i$ response patterns. The two nutrient stimuli also increased NAD(P)H fluorescence, again showing cell-to-cell variations. In combined experiments, where the two parameters were measured in the same cell, the elevation of the NAD(P)H fluorescence preceded the rise in $[Ca^{2+}]_i$, confirming the statistical evaluation performed on separate cells. The application of two consecutive glucose challenges revealed coordinated changes in $[Ca^{2+}]_i$ and NAD(P)H fluorescence. Finally, quinacrine secretion was stimulated by two nutrients with onset times similar to those recorded for $[Ca^{2+}]_i$ elevations. These results clearly demonstrate that increased metabolism occurs during the lag period preceding Ca^{2+} influx via voltage-sensitive Ca^{2+} channels, a prerequisite for the triggering of insulin secretion by nutrient stimuli.

Key words: cytosolic Ca^{2+}/insulin secretion/pancreatic β-cell/pyridine nucleotides/quinacrine

Introduction

The nutrient agonists, glucose and the leucine deamination product, 2-ketoisocaproate (KIC), which are metabolized through different pathways (Hutton *et al.*, 1980; Panten and Lenzen, 1988), cause membrane depolarization with electrical activity and insulin secretion from the pancreatic β-cell (Henquin and Meissner, 1981). One of the earliest events following β-cell stimulation by nutrients, is the depolarization of the plasma membrane, thought to be due to the closure of the ATP-sensitive K^+ channels (Ashcroft *et al.*, 1984; Cook and Hales, 1984; Petersen and Findlay, 1987). This, in turn, leads to influx of extracellular Ca^{2+} through voltage gated Ca^{2+} channels (Rorsman *et al.*, 1988). It has been hypothesized that increased metabolic flux modulates ATP-sensitive K^+ channels through alterations in the cytoplasmic ATP/ADP ratio (Petersen and Findlay, 1987; Ashcroft *et al.*, 1987). However, such changes in the level of the adenine nucleotides have been difficult to demonstrate in intact cells following elevations of glucose within the physiological range (Ashcroft *et al.*, 1987; Malaisse and Sener, 1987). Other metabolic factors including pyridine nucleotides (Hutton *et al.*, 1980; Malaisse *et al.*, 1984; Panten and Lenzen, 1988), glutathione (Ammon *et al.*, 1983), and *de novo* synthesized diacylglycerol (Dunlop and Larkins, 1985; Peter-Riesch *et al.*, 1988; Vara *et al.*, 1988; Wollheim *et al.*, 1988) and acyl CoA derivatives (Prentki and Matschinsky, 1987) have been proposed to participate in coupling the β-cell metabolism to insulin secretion. Their effect could also in part be exerted on the ATP-sensitive K^+ channels, since pyridine nucleotides (Dunne *et al.*, 1988) and activators of protein kinase C (Wollheim *et al.*, 1988) have been shown to affect the open state probability of the channel in an insulin-secreting cell line.

It is generally accepted that the rise in cytosolic Ca^{2+} ($[Ca^{2+}]_i$) is a prerequisite for the triggering of insulin release by nutrients (Wollheim *et al.*, 1984; Hoenig and Sharp, 1986; Arkhammer *et al.*, 1987) and by certain receptor agonists (Wollheim and Biden, 1986; Prentki and Matschinsky, 1987; Grapengiesser *et al.*, 1989). However the temporal relationship between fuel metabolism, $[Ca^{2+}]_i$ and insulin release has not been investigated so far at the single cell level.

In the present study we examined the precise kinetics of the changes in reduced pyridine nucleotides (NAD(P)H) and those in $[Ca^{2+}]_i$ following stimulation of single pancreatic β-cells with the two nutrient secretagogues, glucose and KIC. The detailed analysis of the initial alterations in NAD(P)H and $[Ca^{2+}]_i$ was extended, in preliminary experiments, to the monitoring of quinacrine secretion from single β-cells to better define the sequence of events in β-cell activation. This fluorescent dye has been shown to label the acidic secretory granule compartment in the β-cell (Lundquist *et al.*, 1985).

The present approach allowed us to demonstrate a cellular heterogeneity in the response patterns elicited by glucose and KIC for pyridine nucleotide fluorescence, $[Ca^{2+}]_i$ and secretion. In addition, we observed that the rise in NAD(P)H levels precedes the rise in $[Ca^{2+}]_i$ and evidence for a correlation between the response profile of the two parameters is presented. Finally, our results indicate that the initiation of secretion occurs after time lags compatible with those observed for the $[Ca^{2+}]_i$ response.

Results

The single cell superfusion system used in this study is schematized in Figure 1A. The close apposition of a wide-

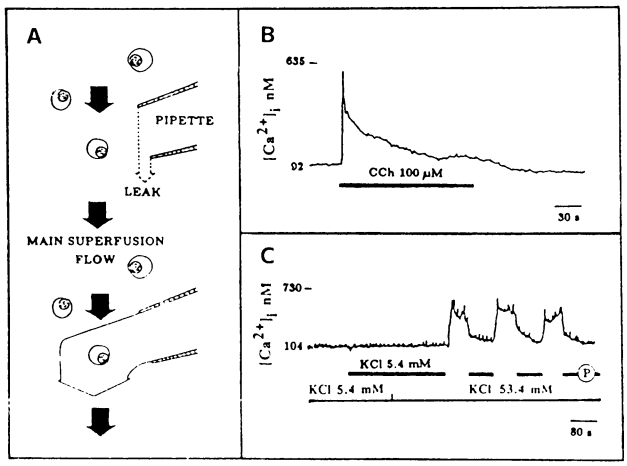

Fig. 1. Scheme of the superfusion system and its evaluation by $[Ca^{2+}]_i$ measurements in fura-2 loaded single β-cells. (**A**) Relationship between the main superfusion and 'side-arm' through a perifusion micropipette. (**B**) CCh increases $[Ca^{2+}]_i$ within 1 s after start of 'side-arm' perifusion. (**C**) Demonstration of successful isolation of the single cell from the main superfusion by 'side-arm' infusion. The main superfusion contained a depolarizing K^+ concentration, whereas the micropipette (P) contained normal buffer. Every time the 'side-arm' superfusion was stopped, an immediate increase in $[Ca^{2+}]_i$ was observed.

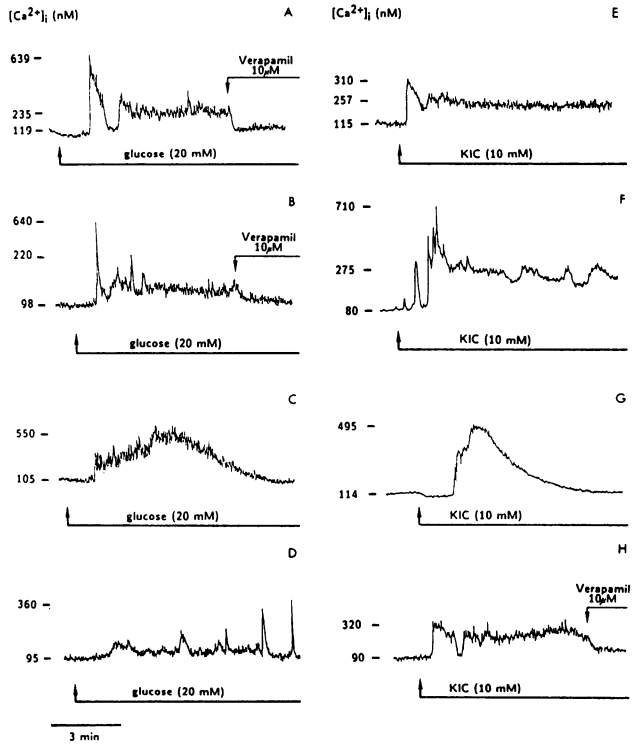

Fig. 2. Changes in $[Ca^{2+}]_i$ in single fura-2 loaded β-cells stimulated either by glucose (**A–D**) or by KIC (**E–H**). The traces are representative of 55 and 41 experiments for glucose and KIC respectively.

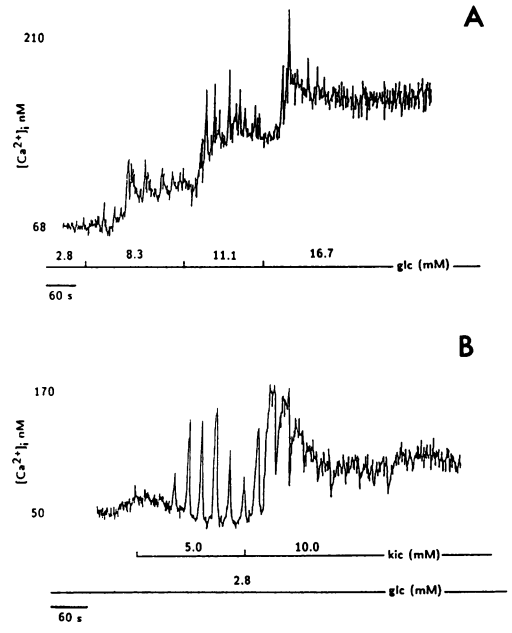

Fig. 3. Dose-related elevation in $[Ca^{2+}]_i$ in response to a stepwise increase in glucose (**A**) or KIC (**B**) measured in single fura-2 loaded β-cells.

mouthed micropipette (tip diameter = ~50 μm) to the cell under study, permits its selective superfusion and isolation from the main superfusion medium as illustrated in Figure 1A. The pipette solutions were infused with a Harvard pump. To determine the time lag for the 'side-arm' superfusate to reach the cell under study, we used carbachol (CCh) at a high concentration (100 μM) which is known to bind to muscarinic receptors on the β-cell and to cause a rise in $[Ca^{2+}]_i$ (Wollheim and Biden, 1986; Grapengiesser *et al.*, 1989). Despite the close proximity of the micropipette containing CCh, no spontaneous effect on resting $[Ca^{2+}]_i$ produced by the probable leak of the agonist could be detected. Starting the superfusion with the muscarinic agonist produced an increase in $[Ca^{2+}]_i$ after ~1 s and the peak response was reached after 3.9 s (Figure 1B). Therefore, we have considered this lag as negligible for the evaluation of the time of onset for nutrient secretagogue stimulation. Further, when a β-cell was suddenly exposed to normal buffer introduced from the micropipette, no artifactual change in $[Ca^{2+}]_i$ was detected (Figure 1C). Switching the main superfusion to a medium containing 53 mM KCl failed to elicit a rise in $[Ca^{2+}]_i$ as long as the superfusion with medium from the 'side-arm' micropipette continued. Thus, the 'side-arm' infusion is capable of effectively isolating the cell from the main superfusion medium. This is clearly illustrated by the rapid rise in $[Ca^{2+}]_i$ seen upon switching off the 'side-arm' infusion which exposes the cell to a depolarizing K^+ concentration, and by the rapid return of $[Ca^{2+}]_i$ to near basal levels after reinitiation of the 'side-arm' superfusion. This manoeuvre could be repeated several times (Figure 1C).

First we investigated the changes in $[Ca^{2+}]_i$ evoked by 20 mM glucose, a concentration that stimulates insulin secretion maximally. Glucose stimulation displayed marked heterogeneity in the response patterns. Figure 2 (left-hand panels) illustrates four representative traces obtained under these conditions. In Figure 2A and B, glucose caused a biphasic increase in $[Ca^{2+}]_i$ with a large initial phase followed by a nadir and second plateau phase. The sustained rise in $[Ca^{2+}]_i$ is due to gating of voltage-sensitive Ca^{2+} channels, as addition of the channel blocker verapamil (10 μM) reduced $[Ca^{2+}]_i$ to prestimulatory levels. In contrast, the response seen in Figure 2C, was characterized

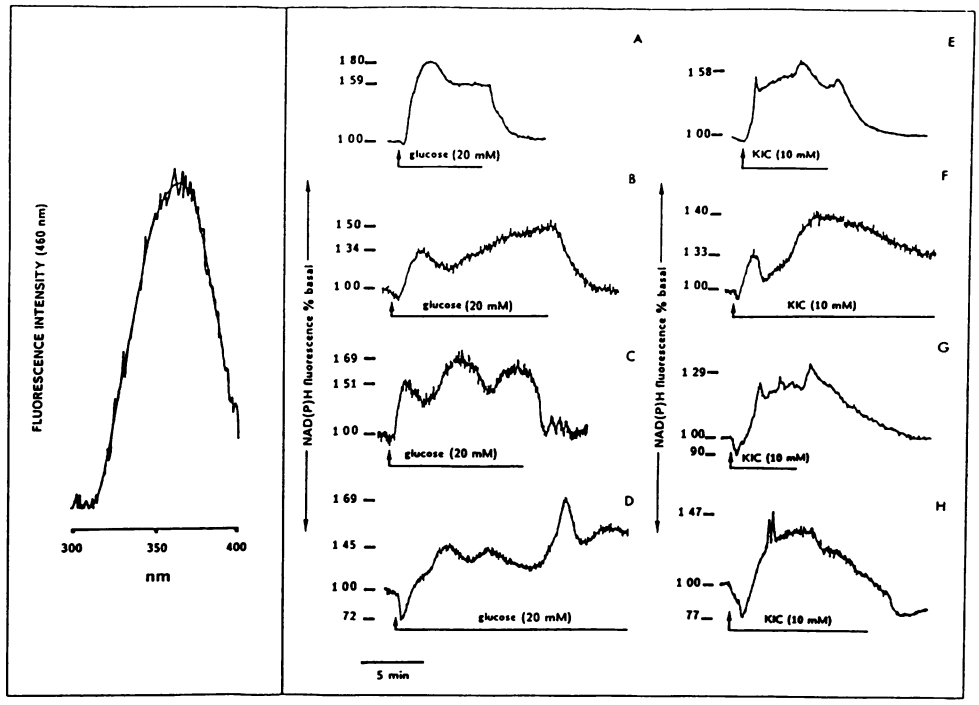

Fig. 4. Endogenous fluorescence changes of reduced pyridine nucleotides [NAD(P)H] in single β-cells. Left-hand panel shows a representative excitation spectrum of NAD(P)H with a maximum intensity at 358 nm. The right-hand panels show representative traces of 32 and 21 experiments where single β-cells were stimulated with glucose or KIC respectively.

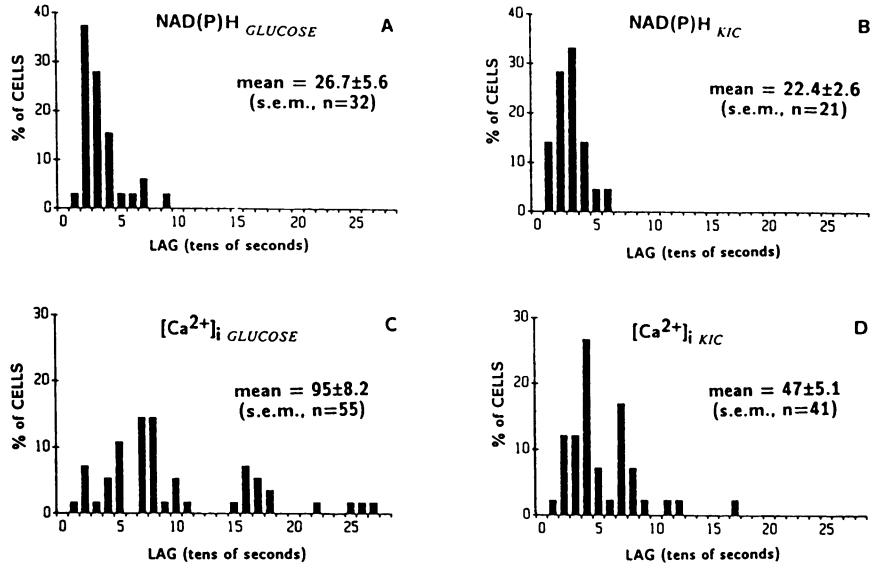

Fig. 5. Distribution histogram of the lags of onset in NAD(P)H and $[Ca^{2+}]_i$ rises above basal. The data are expressed as percentage of cells responding in the same tens of seconds to either a glucose or a KIC challenge. The latencies of onset were calculated by defining the first fluorescence value above basal. If the first response was a decrease, the time of onset was measured at the first fluorescence value above the extrapolated base line preceding the stimulus.

by a gradual increase of $[Ca^{2+}]_i$ followed by a spontaneous return to basal levels. The cell studied in Figure 2D responded with a moderate increase in $[Ca^{2+}]_i$ on which superimposed spiking activity was observed. It is noteworthy that not only the shape of the $[Ca^{2+}]_i$ response, but also the maximal amplitude and onset time of the initial $[Ca^{2+}]_i$ rise, varied among cells. Cells selected according to the criteria outlined in the Materials and methods section responded to glucose in 50–70% of the cases. KIC (10 mM), like glucose, elicited rises in $[Ca^{2+}]_i$ displaying different patterns (Figure 2E–H). Again, the effect of KIC could be abolished by verapamil (Figure 2H).

Next we investigated the relationship between the rise in $[Ca^{2+}]_i$ and secretagogue concentration. In view of the heterogeneity of the responses, this had to be performed by exposing the same cell to stepwise increases in the concentration of the nutrients. An increase of glucose from 2.8 to 8.3 mM resulted in a rise in $[Ca^{2+}]_i$ and, in the trace shown

in Figure 3A, oscillations occurred. A further increase of glucose to 11.1 mM raised $[Ca^{2+}]_i$ further with marked superimposed spiking activity. At 16.7 mM glucose, there was a further increase in $[Ca^{2+}]_i$ without discernible oscillations, possibly due to confluence of the spikes and maximal gating of the Ca^{2+} channels. In other cells subjected to similar protocols or to reversal of the order of glucose infusion, glucose always evoked a dose-related rise in the amplitude of the $[Ca^{2+}]_i$ signal, whereas oscillations were often irregular or sometimes absent.

KIC also elicited a dose-dependent rise in $[Ca^{2+}]_i$, as illustrated in Figure 3B. While the example shown in Figure 3B demonstrates regular $[Ca^{2+}]_i$ oscillations, some cells displayed spikes appearing at random, others simple changes in $[Ca^{2+}]_i$ levels without oscillations. The percentage of cells responding with an elevation of $[Ca^{2+}]_i$ to a KIC challenge was similar to that obtained with glucose.

The endogenous fluorescence of reduced pyridine nucleotides, which is an index of early metabolic activation of the β-cell (Panten and Ishida, 1975; Panten and Lenzen, 1988) was measured under the same experimental conditions as for fura-2. The left panel in Figure 4 represents the autofluorescence excitation spectrum (from 300 to 400 nm) of a single β-cell (see Materials and methods). The right-hand panels illustrate representative traces of stimulation with either glucose or KIC. An increase of glucose from 2.8 to 20 mM elicited in ~60% of the cells a biphasic increase in NAD(P)H fluorescence (Figure 4A) which was sometimes preceded by a small transient decrease (Figure 4B) in fluorescence. When the glucose concentration was restored to 2.8 mM, NAD(P)H fluorescence returned towards pre-stimulatory levels. Another pattern encountered is depicted in Figure 4D, where glucose caused a more pronounced decrease followed by an increase displaying slow oscillations.

KIC (10 mM), as glucose, enhanced the fluorescence of reduced pyridine nucleotides (Figure 4E–H). For both stimuli the steady state response was ~50% above basal levels. It is apparent from the traces shown that the patterns of reduced pyridine nucleotide fluorescence varied greatly among cells either in response to glucose or KIC.

The temporal relationship between the oxidation state of pyridine nucleotides and elevation of $[Ca^{2+}]_i$ in nutrient-stimulated cells was then determined. Therefore the kinetics of the initial changes in NAD(P)H and fura-2 fluorescence in response to glucose and KIC were compared. Further, as the initial metabolism of glucose and KIC differs, comparison of the two nutrients should yield important information on the origin of metabolic coupling factors and their impact on the triggering of Ca^{2+} influx. Figure 5 shows the lags of onset of the increase in NAD(P)H fluorescence and $[Ca^{2+}]_i$ in β-cells stimulated by glucose or KIC. When considering the first elevation of fluorescence above basal, this occurred in the average at 26 and 95 s for the effect of glucose on NAD(P)H fluorescence and $[Ca^{2+}]_i$ respectively. The corresponding values for KIC were 22 and 47 s. Thus, the metabolic events reflected by NAD(P)H fluorescence precede the rise in $[Ca^{2+}]_i$ for both stimuli. Although there was a tendency for a more rapid onset of the rise in NAD(P)H fluorescence in response to KIC relative to that of glucose, the difference was not significant. Interestingly, KIC caused a more rapid elevation of $[Ca^{2+}]_i$ than glucose. It is noteworthy that the distribution of the onset in $[Ca^{2+}]_i$ in response to glucose showed a greater spread than that induced by KIC.

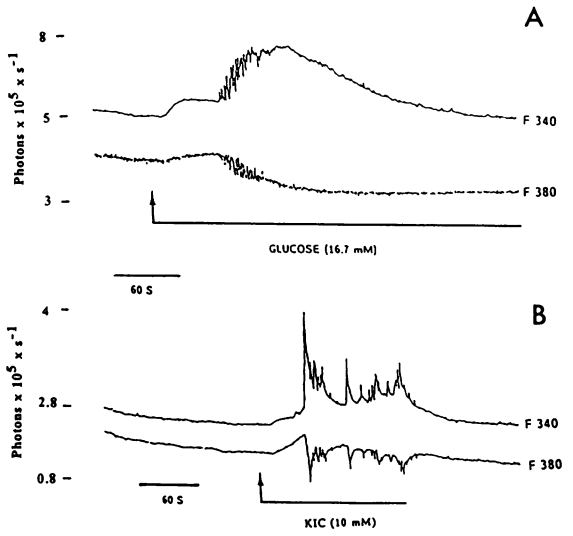

Fig. 6. Combined measurements of NAD(P)H and fura-2 fluorescence in single fura-2 loaded β-cells. The changes in NAD(P)H are indicated by parallel alterations in the fluorescence signals and the antiparallel modifications denote $[Ca^{2+}]_i$ elevations.

As these results were obtained from separate single β-cells, it was felt important to record both parameters from the same cell. To this end, we took advantage of the overlapping excitation spectra of fura-2 and NAD(P)H. We monitored the fluorescence observed at the optimal excitation wavelength for fura-2 (340 and 380 nm) with the interference filter used for NAD(P)H measurements (see Materials and methods). Under these conditions, a change in the NAD(P)H fluorescence results in a parallel modification of the recorded signals, whereas alterations of $[Ca^{2+}]_i$ are indicated by antiparallel changes in the emitted fluorescence. Figure 6 illustrates combined recordings of NAD(P)H and $[Ca^{2+}]_i$ from single fura-2 loaded cells. Glucose stimulation caused parallel increases of the fluorescence after a lag of ~15 s (Figure 6A). After an additional period of 60 s, antiparallel changes in the emitted light were seen, indicating a rise in $[Ca^{2+}]_i$. These results strengthen the conclusion that a rise in NAD(P)H upon glucose stimulation precedes the rise in $[Ca^{2+}]_i$. Similar results were obtained in five independent experiments displaying various lag times. Likewise, exposure of a single β-cell to 10 mM KIC (Figure 6B) elicited first a parallel increase in fluorescence after ~12 s followed by a rise in $[Ca^{2+}]_i$ after an additional period of ~33 s. The trace is representative of four different cells.

To examine further the potential relationship between the increase in reduced pyridine nucleotides and $[Ca^{2+}]_i$, we had to find a condition where the response pattern to secretagogue stimulation could be anticipated in order to overcome cellular heterogeneity. This was made possible by the finding that, when present, a biphasic increase in NAD(P)H fluorescence elicited by 16.8 mM glucose was invariably followed by a monophasic one (Figure 7A) in response to a second glucose challenge (n = 6). Figure 7B illustrates the corresponding pattern in $[Ca^{2+}]_i$ in a fura-2 loaded cell subjected to the same protocol. In addition to displaying a monophasic pattern, the second response occurred more rapidly for both NAD(P)H and $[Ca^{2+}]_i$. Similar results were obtained for two consecutive additions of 10 mM KIC (not shown).

The obvious next step was to search for a method allowing

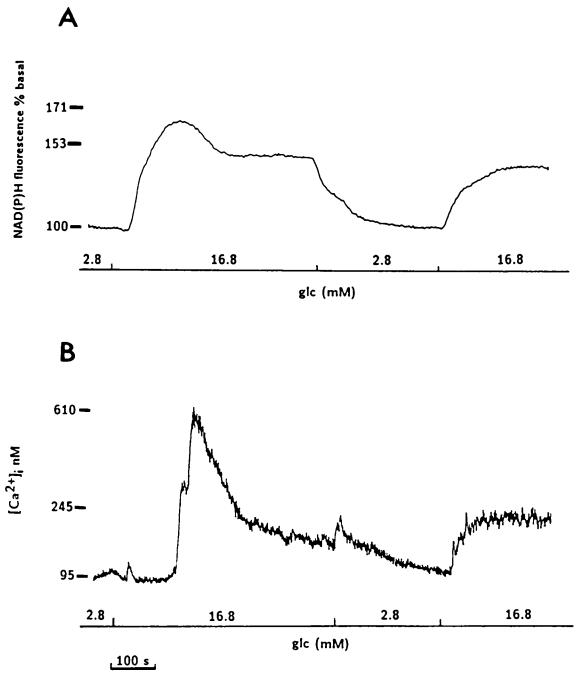

Fig. 7. Correlation between the response profiles in NAD(P)H and $[Ca^{2+}]_i$ in single β-cells submitted to two consecutive glucose challenges. The cell in A was not loaded with fura-2.

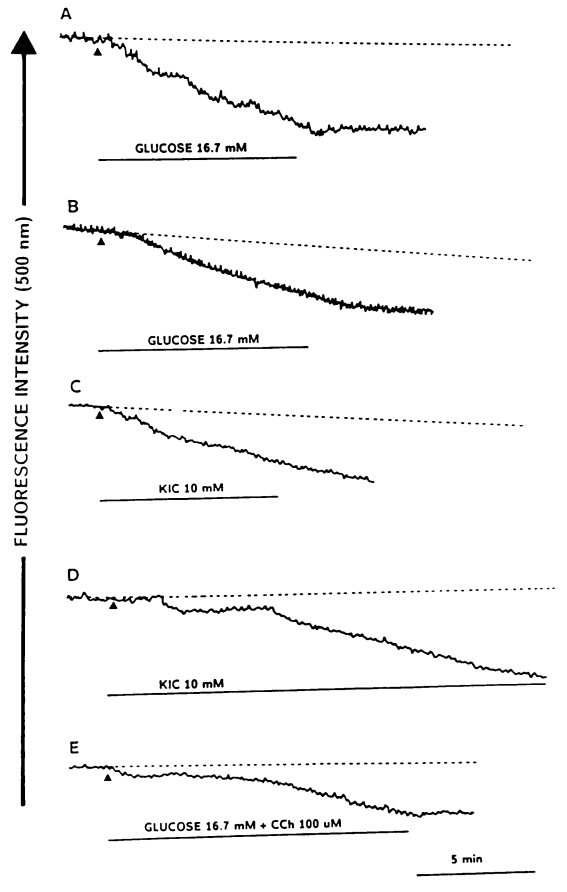

Fig. 8. Loss of quinacrine fluorescence from prelabelled single β-cells after stimulation with glucose (**A,B**), KIC (**C,D**) or glucose and CCh (**E**). The arrows indicate the start of the 'side-arm' superfusion with the different stimuli. These traces are representative of 15 different experiments.

the assessment of the kinetics of insulin secretion at the single cell level. Such a fluorescence method, measuring the disappearance of the fluorescence localized in giant granules prelabelled with quinacrine has been used in parallel to the recording of membrane capacitance changes in mast cells (Breckenridge and Almers, 1987). Furthermore, a previous report has shown that it is possible to load the insulin-containing granules in the β-cell with quinacrine, due to their acidic pH (Lundquist *et al.*, 1985). In a limited series of experiments, the decrease of quinacrine fluorescence from single loaded β-cells was monitored during stimulation with either glucose or KIC. In Figure 8, five representative experiments with separate single cells are shown. Glucose stimulation (16.7 mM) resulted in either a stepwise (Figure 8A) or a continuous (Figure 8B) decrease in cellular quinacrine fluorescence, seen after a lag time of 42 and 60 s respectively. When the glucose concentration was returned to pre-stimulatory levels (2.8 mM), there was a clear decrease in the rate of quinacrine loss which occurred more rapidly in the first trace (A) than in the second one (B). KIC also caused an increased loss of quinacrine fluorescence with an onset time of 120 and 30 s in the examples shown in Figure 8C and D. Due to the limited number of experiments, differences in lag of onset between glucose and KIC stimulations could not be established.

Finally, cells were stimulated with a combination of carbachol (100 μM) and glucose (16.7 mM) to test whether carbachol, which raises $[Ca^{2+}]_i$ more rapidly than glucose (cf. Figures 1 and 5), could promote quinacrine release displaying a corresponding shorter lag of onset. This was indeed the case, since the combined stimulation caused a decrease of the fluorescence starting after only 5 s (Figure 8E). As for $[Ca^{2+}]_i$, between 30 and 50% of the cells did not show any decrease in fluorescence after stimulation with either glucose or KIC. Thus, these preliminary experiments demonstrate that the rate of quinacrine release can be altered by insulin secretagogues with similar heterogeneity in the patterns, frequency of response and time kinetics as those encountered in NAD(P)H and $[Ca^{2+}]_i$ measurements (cf. Figure 5).

Discussion

The measurement of $[Ca^{2+}]_i$ at the single cell level has, in many cell systems, permitted the definition of phenomena not discernible in whole cell populations (Tsien and Poenie, 1986; Schlegel *et al.*, 1987). Using the single cell approach, we attempted to delineate the initial sequence of events in nutrient-stimulated islet β-cells. In parallel to $[Ca^{2+}]_i$ measurements, the estimation of NAD(P)H fluorescence offers a convenient kinetic parameter of the activation of cellular metabolism at the single cell level (Panten and Ishida, 1975; Cohen *et al.*, 1979; Balaban and Blum, 1982; Panten and Lenzen, 1988). In some experiments we also labelled the acidic insulin secretory granules with quinacrine (Lundquist *et al.*, 1985) to probe for hormone secretion from single cells. This method, which has been validated in mast cells (Breckenridge and Almers, 1987) for the assessment of exocytosis, would be simpler than the measurement of membrane capacitance changes performed thus far in single chromaffin cells, mast cells (Neher and Marty, 1982; Penner and Neher, 1988) and single β-cells (Penner and Neher, 1988).

The single cell superfusion system permitted the demon-

stration of rapid increases in $[Ca^{2+}]_i$ in response to either K^+ depolarization or to the Ca^{2+} mobilizing agonist carbachol which binds to muscarinic receptors on the β-cell (Prentki and Matschinsky, 1987). In contrast, much longer lag times preceding the rise in $[Ca^{2+}]_i$ were seen with the nutrient stimuli, glucose and KIC. This was to be expected, as the initiation of membrane depolarization and the rhythmical electrical activity elicited by these nutrient secretagogues also occur after a lag period (Henquin and Meissner, 1981). There was agreement between the latency in the rise of $[Ca^{2+}]_i$ and the decrease of quinacrine fluorescence in cells stimulated with carbachol, glucose or KIC. It thus appears that quinacrine provides a promising tool for monitoring the release of secretory granule contents in the β-cell. Furthermore, a decrease of quinacrine content of pancreatic β-cells following the stimulation of insulin secretion *in vivo* by different secretagogues, has been demonstrated (Lundquist *et al.*, 1985).

The main purpose of the present study was to examine the relationship between the nutrient-induced changes in β-cell metabolism and $[Ca^{2+}]_i$. The recordings of NAD(P)H fluorescence revealed that the reduction of pyridine nucleotides is an early event in β-cell activation preceding the rise in $[Ca^{2+}]_i$. It is noteworthy that, in contrast to the rise in $[Ca^{2+}]_i$, the latency of the reduction was similar for glucose and KIC. The NAD(P)H responses were often biphasic, a pattern observed previously in intact islets for KIC, but not for glucose which was monophasic (Panten and Ishida, 1975). In our experiments, the nutrient increased NAD(P)H fluorescence by ~50% above basal (2.8 mM glucose). Biochemical estimation of steady-state levels of NAD(P)H in glucose or KIC stimulated islets have shown increases in either NADH or NADPH (Hutton *et al.*, 1980; Ammon *et al.*, 1983; Sener *et al.*, 1984; Matschinsky *et al.*, 1986; Hedeskov *et al.*, 1987). In specimens of micro-dissected islets from the perfused rat pancreas, glucose evoked a rise in NADH at 36 s (Matschinsky *et al.*, 1986), a finding in good agreement with our results.

The conclusion that the generation of reduced pyridine nucleotides precedes the rise in $[Ca^{2+}]_i$ in nutrient-stimulated cells was borne out by the experiments where the two parameters were measured simultaneously. Thus, the latencies for the increase in NAD(P)H in fura-2 loaded cells were similar to those recorded in non-loaded cells for both glucose and KIC stimulation. These experiments also suggest that fura-2 loading does not exert untoward effects on the β-cells under the present conditions.

The response patterns with both nutrient stimuli exhibited marked cellular heterogeneity. While NAD(P)H increases were often clearly biphasic, this was less pronounced for $[Ca^{2+}]_i$ rises. The reason for this is unclear. The mechanism underlying the biphasic response profile remains to be established. However, a correlation was observed between the changes in β-cell redox state and the $[Ca^{2+}]_i$ patterns. This was clear from the experiments in which the cells were exposed to two consecutive stimulations with glucose, separated by a resting period of ~5 min. Invariably, in cells displaying initial biphasic patterns, these were altered into monophasic responses, a finding applicable both to NAD(P)H and $[Ca^{2+}]_i$. The more rapid responses in the 'primed' cells, a phenomenon also reported for insulin secretion (Grill *et al.*, 1978), suggests metabolic adaptation as a possible explanation for these findings. The overshooting first phase of NAD(P)H might then reflect the period when essential coupling factors are generated, permitting the sustained activation of the cell necessary to maintain the second phase of insulin release.

It is noteworthy that in some experiments a decrease of either NAD(P)H fluorescence (Figure 4) or $[Ca^{2+}]_i$ (Figure 2) was detected immediatley upon nutrient addition. *Ob/ob* mouse β-cell suspensions display a similar initial decrease in either $[Ca^{2+}]_i$ or insulin release upon stimulation with glucose. It was suggested that this phenomenon was associated with fuel depletion of the β-cells prior to the application of the nutrient stimulus and could indeed be obliterated by raising the resting glucose concentration to 5 mM glucose (Nilsson *et al.*, 1988). In our experiments, the initial decrease was most often seen in cells exhibiting, on the one hand, a lower initial NAD(P)H level or, on the other, a higher resting $[Ca^{2+}]_i$. Both conditions are compatible with a decreased availability of endogenous energy sources evoked by prolonged superfusion with 2.8 mM glucose. Finally, in cells which showed reduced viability at the end of the experiments, prolonged stimulation with glucose or KIC elicited only decreases in NAD(P)H fluorescence (not shown).

The heterogeneity of the response patterns displayed by the single β-cells could be explained, in part, by the lack of cell-to-cell communication normally present in pancreatic islets and thought to determine the synchronization of electrical (Bruzzone and Meda, 1988) and possibly metabolic events within the communicating cell territories (Cohen *et al.*, 1979). Alternatively, the heterogeneity could be representative of discrete subpopulations of β-cells as suggested previously *in vivo* (Stefan *et al.*, 1987) or *in vitro* for insulin release (Salomon and Meda, 1986) and proinsulin biosynthesis (Schuit *et al.*, 1988). Finally, the observation that some single cells display oscillatory rises in $[Ca^{2+}]_i$ reminiscent of membrane potential oscillations recorded with microelectrodes in the islet core (Hutton *et al.*, 1980; Henquin and Meissner, 1981) suggests the possibility that differentiated subpopulations of β-cells could pace the rhythmical electrical activity in the whole islet. When present, the glucose-induced oscillations in $[Ca^{2+}]_i$ were of higher frequency than those reported previously by others (Grapengiesser *et al.*, 1989). However, the frequency of bursts of electrical activity in glucose-stimulated β-cells has been shown to differ from islet to islet within the same pancreas (Atwater, 1980).

As is the case for insulin secretion, nutrient-evoked rises in $[Ca^{2+}]_i$ steady-state varied with the concentration of the stimulus, confirming findings in islet cell suspensions (Gylfe, 1988) and in some limited experiments in single islet cells (Grapengiesser *et al.*, 1989). In addition, the $[Ca^{2+}]_i$ increase was abolished in the presence of the Ca^{2+} channel blocker, verapamil. This confirms the notion that nutrients raise $[Ca^{2+}]_i$ primarily by promoting Ca^{2+} influx via voltage-sensitive Ca^{2+} channels.

How could the changes in reduced pyridine nucleotides be related to the changes in ionic conductance at the plasma membrane? Nutrient secretagogues increase islet oxygen consumption with a time lag similar to the reduction of pyridine nucleotides (Hutton and Malaisse, 1980). It follows that the increase in NAD(P)H fluorescence can closely reflect enhanced ATP production (Hutton and Malaisse, 1980; Panten and Lenzen, 1988). Thus, during the exposure of

the β-cell to stimulatory glucose concentrations, both NAD(P)H and ATP are formed. It is well established that a rise in free ATP or ATP/ADP ratio reduces the open state probability of the ATP-sensitive K^+ channel which initiates β-cell membrane depolarization (Ashcroft *et al.*, 1984; Cook and Hales, 1984; Arkhammer *et al.*, 1987; Ashcroft *et al.*, 1987; Petersen and Findlay, 1987). Alternatively, pyridine nucleotides themselves might also modulate K^+ channel activity as reported in an insulin-secreting cell line (Dunne *et al.*, 1988).

Our results do not favour the conclusion that the recorded degree of pyridine nucleotide reduction correlates directly with membrane depolarization and Ca^{2+} channel gating, since there was a discrepancy between glucose and KIC in this regard. Thus, despite a similar lag of onset and degree of reduction of the pyridine nucleotides, KIC promoted a much more rapid elevation of $[Ca^{2+}]_i$ compared to glucose. This is in agreement with the findings that KIC depolarizes the β-cell and evokes Ca^{2+} action potentials more rapidly than glucose (Henquin and Meissner, 1981). Taken together, this indicates that the critical coupling factors are generated more rapidly via KIC than via glucose metabolism. The explanation for this remains to be established. Nevertheless, it is possible that the fluorescence recorded when the cells are stimulated by glucose or by KIC reflects metabolical events in different cellular compartments, at least initially. The findings suggest that during glucose stimulation the NAD(P)H fluorescence could be raised before the increase, e.g. of the ATP/ADP ratio. The build-up of ATP at the plasma membrane would be retarded relative to the NAD(P)H fluorescence because of marked cytosolic ATP consumption during glucose phosphorylation (Meglasson and Matschinsky, 1986). In contrast, KIC metabolism consumes mitochondrial ATP. This occurs only after the generation of the reducing equivalents, NADH and $FADH_2$, at the first degradation steps of KIC inside the organelle (Stryer, 1988). Therefore, the early metabolism of KIC may rapidly increase the ATP production of mitochondrial origin without primarily affecting the cytosolic pool of the adenine nucleotide.

Although ATP clearly plays a role in the coupling of fuel metabolism to membrane depolarization, other factors such as pyridine nucleotides and diacylglycerol may also act separately or in combination with ATP to modulate ionic conductance, including not only K^+ channels (Dunne *et al.*, 1988; Wollheim *et al.*, 1988) but also Ca^{2+} channels (Velasco and Petersen, 1989). In this context, it should be noted that KIC, like pyruvate, (derived from glucose metabolism) provides acetyl CoA to the Krebs cycle. This would in turn cause the shuttling from mitochondria of intermediates increasing the redox state in the cytosol (Meglasson and Matschinsky, 1986) which would be again of slower onset for glucose than for KIC. The secondary increase in cytosolic NADPH is critical for the *de novo* synthesis of lipids including diacylglycerol (Dunlop and Larkins, 1985; Peter-Riesch *et al.*, 1988; Vara *et al.*, 1988; Wollheim *et al.*, 1988). In an insulin-secreting cell line, diacylglycerol has been shown to depolarize the membrane potential by closing the ATP-sensitive K^+ channel (Wollheim *et al.*, 1988). However, the demonstration of such a mechanism in normal β-cells has not yet been accomplished.

The present findings demonstrate that the reduction of pyridine nucleotides is an obligatory early event in stimulus-response coupling of nutrient-stimulated β-cells. This reduction precedes Ca^{2+} channel gating and the rise in $[Ca^{2+}]_i$. The latter is a reflection of membrane potential depolarization and is a prerequisite for the triggering of insulin secretion. Further, the biphasic nature of the changes in NAD(P)H fluorescence and $[Ca^{2+}]_i$ strongly suggest a causal relationship between the generation of metabolic coupling factors, the triggering of Ca^{2+} entry and insulin secretion.

Materials and methods

Islet cell isolation and primary culture

Pancreatic islets were isolated from *ad lib.* fed male Wistar rats weighing ~200 g. The islets were prepared from one pancreas removed after a ductal injection (Sutton *et al.*, 1986) under anaesthesia (25 mg Pentothal/100 g body weight) of 6 ml of ice cold Hanks buffer containing 3 mg/ml collagenase (Serva, Heidelberg, FRG). After 20 min digestion at 37°C, the tissue was dispersed by gentle shaking and repeated aspirations through a 14 gauge needle. The minced pancreas was then passed through a tissue sieve and collected in a siliconized Erlenmeyer. After several centrifugations and resuspensions in ice cold Hanks solution, the tissue was separated into two pellets. These were resuspended in 10 ml of Histopaque 1077 (Sigma, St Louis, MO, USA) on top of which 10 ml of Hanks buffer was carefully layered. The tubes were centrifuged for 20 min at 700 *g* at room temperature. The interphase containing the islets was collected and washed twice by centrifugation.

The islets were pooled in 10 ml of a Ca^{2+} and Mg^{2+} free phosphate-buffered saline containing 5 mM EDTA and 0.25 mg/ml trypsin (Difco, Detroit, MI, USA) and incubated for 10 min at 37°C with occasional mixing. The digestion was stopped by chilling the tube on ice. The isolated cells were centrifuged, washed in culture medium RPMI 1640 (Animed, Basel, Switzerland) and finally resuspended at a concentration of ~625 000 cells/ml. This procedure yielded ~0.8 to 1 × 10^6 islet cells per pancreas. The culture medium contained 8.3 mM glucose, 10% fetal calf serum (Animed), 100 IU/ml penicillin G and 200 μg/ml streptomycin. Forty microliters of the cell suspension was then plated onto glass cover slips which had been coated with 2 μg/ml polyornithine (Sigma) contained in a Petri dish and placed into a tissue culture incubator. The cells were allowed to adhere for 24 h and were thereafter replenished with 1 ml RPMI 1640 medium with the same supplements as above. After 3 days, the islet cells formed either small clusters or stayed in a single cell configuration. Immunostaining of the islet cells with guinea pig anti-insulin serum raised against porcine insulin and a FITC-labelled second antibody revealed that ~75% of the cell population were insulin-containing β-cells. In addition it was found, as previously reported (Pipeleers *et al.*, 1985) that the rat non-insulin containing islet cells were characterized by their smaller size (<10 μm in diameter). The experiments were always performed on individual cells >10 μm and not in contact with other cells. Furthermore, recordings obtained from cells showing manifestation of reduced viability (blebs, major shape alterations) at the end of the experiment were discarded. The same selection criteria were used for the different fluorescence measurements.

Dual wavelength excitation microfluorimetry

Microfluorimetry was performed with a SPEX modular fluorimeter (Glenn Spectra Limited, London, UK) coupled to an inverted microscope (DIAPHOT-TMD, Nikon, Tokyo, Japan) used in the epifluorescence mode (objective: Nikon F100). The system has been described in detail previously (Schlelgel *et al.*, 1988). The microscope was placed in a thermostatted box and kept at 37°C. By the use of an electronic device, the solutions were precisely maintained at 37°C through a thermosensor and a feed-back loop controlled resistance. The integration time per point varied between 0.3 and 1 s for fura-2 and 2 to 4 s for reduced pyridine nucleotide and quinacrine fluorescence.

Fluorescence measurements

$[Ca^{2+}]_i$ measurements. Islet cell monolayers were loaded for 20 min at 37°C in the presence of 0.25–0.5 μM final concentration of fura-2 acetoxymethylester (Molecular Probes, Eugene, OR, USA), kept as a 1 mM stock solution in Me_2SO. The medium was a modified Krebs–Ringer–bicarbonate–HEPES buffer (KRBH) containing in mM: 134 NaCl, 4.7 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 1.0 $CaCl_2$, 25 HEPES (Sigma), 4% bovine

serum albumin (BSA) and 2.8 glucose, pH 7.4 at 37°C. The cells were then mounted in a perifusion device and allowed to equilibrate for 15 min in the KRBH medium containing 0.1% BSA.

In the first part of this study the excitation wavelengths were set at 350 and 380 nm and later changed to 340 and 380 nm when the system was equipped with quartz lenses. The emission wavelength was selected with an interference filter (500 nm, Nikon). In the former setting the calibration of $[Ca^{2+}]$ (obtained from the ratio 350/380) was as described by Schlegel *et al.* (1987, 1988). In the latter part of the study, calibration was repeated and R_{min}, R_{max} and B were obtained as in the former procedure, yielding values (mean ± SD) of 0.4 ± 0.1, 8 ± 0.6 and 3.4 ± 0.2 (n = 6) respectively.

Reduced pyridine nucleotide fluorescence. The reduced forms of NAD and NADP, referred to as NAD(P)H, were measured using the same experimental set-up as for fura-2 fluorescence. After optical alignment of the microfluorimeter to obtain optimal detection of the cell autofluorescence, emission wavelength was fixed for NAD(P)H fluorescence by an interference filter (470 nm, Zeiss, Oberkochen, FRG) and the excitation was chosen at the maximal intensity obtained afer running an excitation spectrum, usually 360 nm (see also Figure 4).

The assessment of secretion. The secretory granules of the β-cell were labelled for 15 min prior to the transfer of the cells to the microfluorimeter with the fluorescent dye, quinacrine (Sigma) at a final concentration of 100 nM. Fluorescence was recorded at an excitation wavelength of 350 nm and emission was recorded with the same interference filter as for fura-2. The same criteria for selection of the cells as those used for fura-2 recording were applied, to avoid experimentation on non-β-cells. Control experiments were performed on the same cells to compare the distribution of the quinacrine fluorescence with the labelling of the β-cell with anti-insulin serum following the addition of a rhodamine-conjugated second antibody. There was a good correlation of the immune staining with the distribution of quinacrine fluorescence.

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