Glyceraldehyde, but not cyclic AMP-stimulated insulin release is preceded by a rise in cytosolic free Ca^{2+}

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The dynamics of changes in membrane potential, cytosolic free Ca^{2+}, $[Ca^{2+}]_i$ and immunoreactive insulin release were assessed in RINm5F cells. Membrane depolarization and a rise in $[Ca^{2+}]_i$ preceded the stimulation of insulin release by D-glyceraldehyde. Forskolin, which raised the cellular cyclic AMP levels, stimulated insulin release without changing membrane potential or $[Ca^{2+}]_i$. It is concluded that cyclic AMP acts on insulin release not by mobilizing Ca^{2+} but by another, as yet undefined, mechanism.

Cyclic AMP *Forskolin* *Cytosolic free Ca^{2+}* *Insulin release* *Carbohydrate* *Membrane potential*

1. INTRODUCTION

Both Ca^{2+} and cyclic AMP have been proposed as intracellular mediators of glucose-induced insulin release (review, [1,2]). Recently, the mediator function of Ca^{2+} has been strengthened by the finding that the concentration of cytosolic free Ca^{2+}, $[Ca^{2+}]_i$, increases during the stimulation of insulin-secreting cells with the triose glyceraldehyde [3] or glucose itself [4,5]. In contrast, an increase of cellular cyclic AMP levels does not always accompany glucose-induced insulin release, making cyclic AMP a less likely direct mediator than Ca^{2+} [2,6]. Therefore, the main role of cyclic AMP is now generally considered to be that of potentiating insulin release [2].

It has been suggested that cyclic AMP may potentiate insulin release by mobilizing Ca^{2+} from intracellular stores. This idea was based on measurements of $^{45}Ca^{2+}$ efflux from pancreatic islets preloaded with the isotope, where theophylline, 3-isobutyl-1-methylxanthine and dibutyryl cyclic AMP were found to increase $^{45}Ca^{2+}$ efflux [7–9].

Here, we have directly measured changes in $[Ca^{2+}]_i$ and insulin release in response to forskolin or D-glyceraldehyde in the insulin-secreting cell line RINm5F [10,11]. Forskolin, a substance which activates adenylate cyclase [12], was found to raise the cellular cyclic AMP levels without increasing $[Ca^{2+}]_i$, while glyceraldehyde-induced insulin release is preceded by a rise in $[Ca^{2+}]_i$ without an accompanying change in cyclic AMP levels.

2. EXPERIMENTAL

RINm5F cells [10] were cultured, transferred to spinner cultures and loaded with quin2 acetoxymethyl ester [13] (100 μM) as in [3]. Loaded and control cells were kept at 18–20°C in medium RPMI1640 supplemented with 25 mM Hepes until use. Intracellular quin2 fluorescence was monitored on cells resuspended in a modified Krebs-Ringer-bicarbonate buffer containing 5 mM $NaHCO_3$, 1.1 mM $CaCl_2$, 25 mM Hepes, 0.1 mM EGTA and 2.8 mM glucose. The cells were resuspended in 2 nl of this buffer at a final concentration of $1–2 \times 10^6$ cells/ml. Quin2 fluorescence was measured in glass cuvettes thermostatted to 37°C under continuous stirring. The fluorescence was measured with excitation and emission wavelengths of 339 and 492 nm, respectively, in a

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Perkin-Elmer LS3 spectrofluorometer. The calibration of the signal was as in [3]. D-Glyceraldehyde was added to the cuvettes from a 1 M aqueous stock solution and forskolin from a 30 mM stock solution in dimethylsulfoxide (DMSO). The final concentration of DMSO was 0.1% (v/v) under all experimental conditions. At this concentration DMSO did not interfere with any of the measurements performed.

Membrane potential changes were assessed by using the fluorescent probe bis(1,3-dimethylthiobarbiturate) trimethineoxonol, bisoxonol [14], at a final concentration of 100 nM [3] and excitation and emission wavelengths at 540 and 580 nm, respectively.

Insulin release was measured during perifusions of suspensions of quin2 loaded and control cells at concentrations of $1.5–4 \times 10^6$ cells/chamber. The chamber volume was 700 μl and the cells were perifused with the same modified Krebs-Ringer-bicarbonate Hepes buffer as described above supplemented with 0.07% of bovine serum albumin. Perifusions were carried out at 37°C without gassing. The perifusion system has been described in [15]. The flow rate was 1 ml/min. The dead space time was 3 min and the results have been corrected accordingly. Insulin in the perifusate was measured by radioimmunoassay using rat insulin as standard. The insulin content of the cells prior to perifusion was measured following acid ethanol extraction [11] and the insulin release was expressed as a percentage of the initial cellular hormone content, which averaged 425 ± 49 ng/10^6 cells (n = 5).

For the measurement of cellular cyclic AMP levels $0.8–1.1 \times 10^6$ cells were incubated in 1 ml of the modified Krebs-Ringer-bicarbonate buffer (0.5% bovine serum albumin) for 5 min at 37°C in the presence of test substances. Thereafter, two 100-μl aliquots of the cell suspension from each tube were boiled for 20 min after the addition of 200 μl of 0.05 M acetate buffer (pH 6.2). The boiled samples were centrifuged for 10 min at 450 $\times$ g; 100 μl of the supernatants were succinylated and cyclic AMP determined by radioimmunoassay as in [6].

Results are given as means ± SE and statistical analysis was by Student's t-test for paired data ($Ca^{2+}{}_i$) or unpaired data (insulin released and cyclic AMP).

The materials and their sources were the same as those reported in [3,6].

3. RESULTS

In fig.1 the results are shown of an experiment investigating the effect of D-glyceraldehyde (10 mM) on membrane potential. $[Ca^{2+}]_i$ and insulin release was tested in parallel on aliquots from the same batch of quin2 loaded cells. Glyceraldehyde started depolarizing the cells after a lag of 10–15 s. The membrane potential was maximally depolarized at 2 min, whereafter it gradually repolarized. $[Ca^{2+}]_i$ increased more slowly than the membrane depolarization, but also reached its highest value after 2 min. A definite increase of insulin release in this experiment only began 2.5 min after the exposure of the cells to

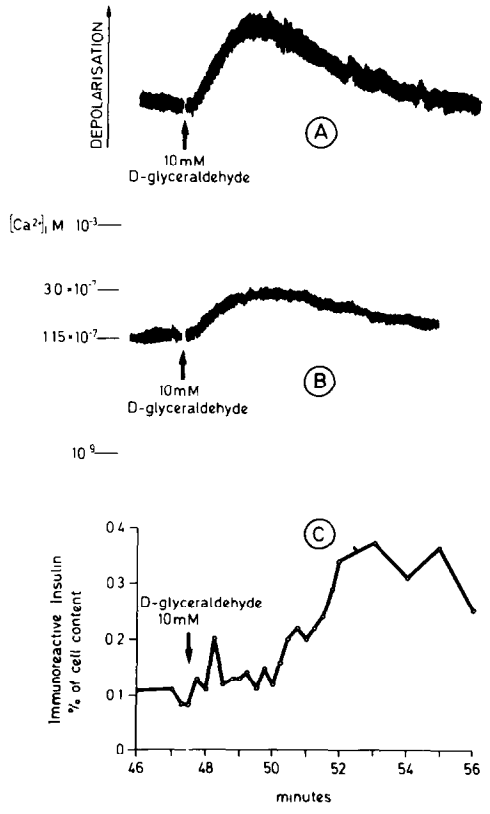

Fig.1. Effect of D-glyceraldehyde (10 mM) on membrane potential (A), $[Ca^{2+}]_i$ (B) and immunoreactive insulin release (C) in RINm5F cells. Aliquots from the same batch of quin2 loaded cells were used.

glyceraldehyde. Mean values of $[Ca^{2+}]_i$ and insulin release from several experiments are shown in fig.2. At 2 min, glyceraldehyde raised $[Ca^{2+}]_i$ from 123 ± 11 to 210 ± 15 nM. $[Ca^{2+}]_i$ returned to prestimulatory levels again within 5 min. In the quin2 loaded cells the rate of insulin release increased after 2.5 min and thereafter reached a plateau about twice the basal rate beyond 3.5 min. The insulin release rate remained elevated even when $[Ca^{2+}]_i$ had returned to basal values.

Insulin release was also measured from control cells not loaded with quin2 in the same experiments (fig.2). Although the rate of insulin release was slightly lower from quin2 loaded relative to control cells during the prestimulatory period, this difference was not significant. Glyceraldehyde caused an almost 3-fold stimulation of insulin release from control cells. The pattern of the stimulated release was fairly similar in both groups of cells and in both cases the insulin release rate returned towards basal upon cessation of the stimulus.

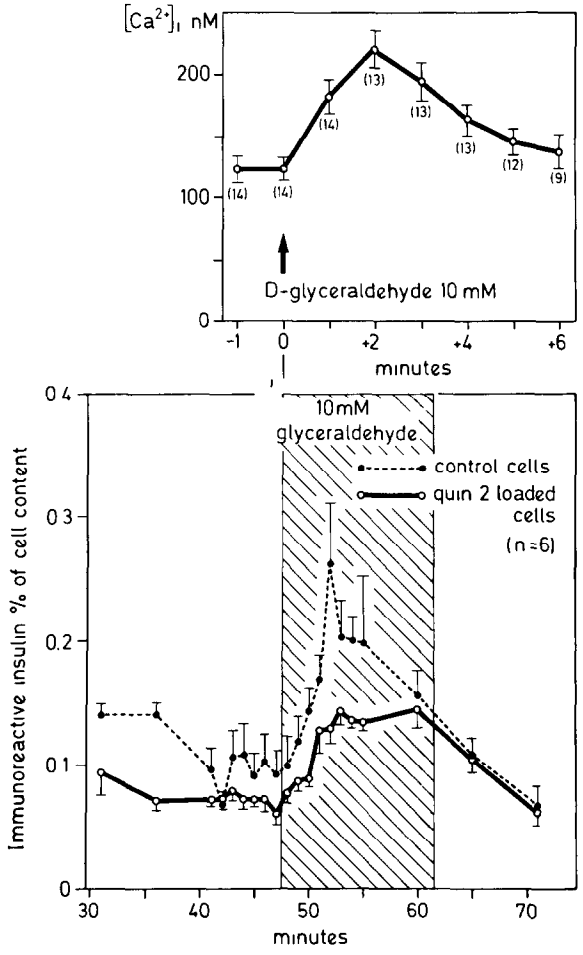

Fig.2. Mean values of $[Ca^{2+}]_i$ and immunoreactive insulin release in response to D-glyceraldehyde. Quin2 loaded and control cells were exposed to glyceraldehyde (10 mM) from minute 47.5 to minute 62.5 of a 70 min perfusion (3 separate experiments).

In fig.3 the effects are shown of forskolin on membrane potential, $[Ca^{2+}]_i$ and insulin release from aliquots of the same batch of quin2 loaded cells. Forskolin (30 μM) did not alter membrane potential or $[Ca^{2+}]_i$ but stimulated insulin release. Fig.4 gives the results of several experiments where forskolin was added to quin2 loaded cells for the assessment of $[Ca^{2+}]_i$ and to both loaded and control cells for the measurement of insulin release. There was no rise in $[Ca^{2+}]_i$ whereas the rate of insulin release was stimulated significantly in both groups of cells. As $[Ca^{2+}]_i$ in some experiments showed a slight drift upwards, we examined whether $[Ca^{2+}]_i$ would increase with time. In two experiments where the cells were exposed to forskolin during 10 min, no increase in $[Ca^{2+}]_i$ occurred.

As can be seen in fig.3A,B, glyceraldehyde still elicited membrane depolarization and a rise in

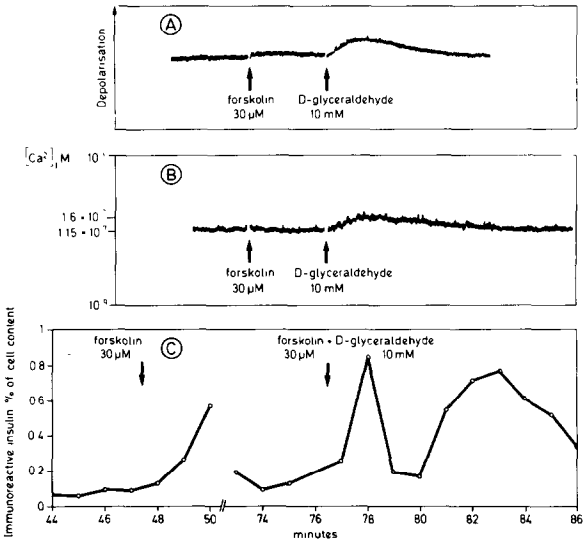

Fig.3. Effect of forskolin (30 μM) and D-glyceraldehyde (10 mM) on membrane potential (A), $[Ca^{2+}]_i$ (B) and immunoreactive insulin release (C). Aliquots from the same batch of quin2 loaded cells were used.

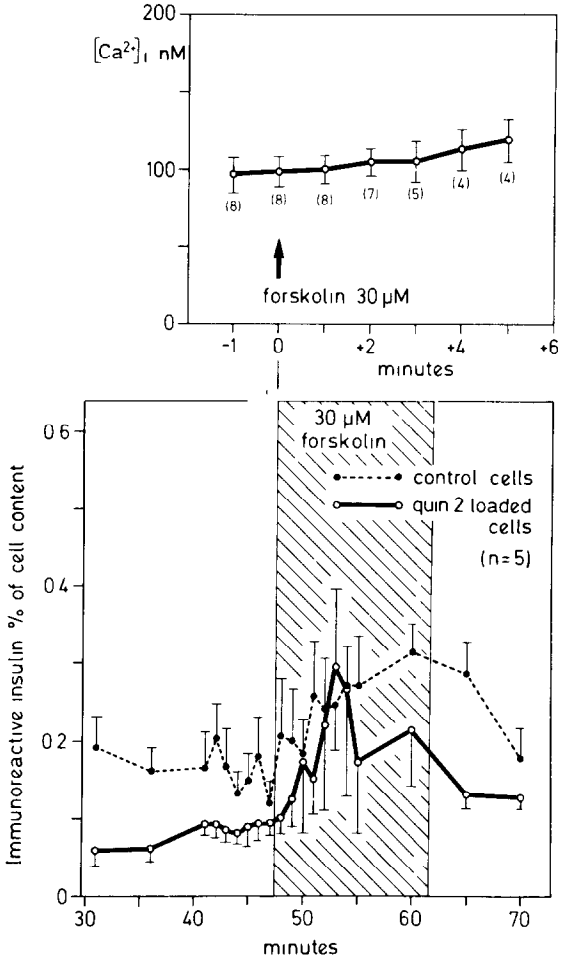

Fig.4. Mean values of $[Ca^{2+}]_i$ and immunoreactive insulin release in response to forskolin (30 μM). The perifusion protocol was the same as in fig.2 (3 separate experiments).

$[Ca^{2+}]_i$ when added to a cuvette already containing forskolin. The combination of forskolin and glyceraldehyde elicits insulin release which is greater than that seen with either substance alone (fig.3C). This is indicated in table 1, where 3 perifusion experiments of quin2 loaded cells are summarized. The cells were exposed to forskolin, to glyceraldehyde and to the combination of the two. In the presence of forskolin, glyceraldehyde-induced insulin release was potentiated significantly at 50 and 51 min, i.e., 2.5 and 3.5 min after the beginning of the stimulation.

Finally, cellular cyclic AMP levels were measured after a 5 min exposure to forskolin and glyceraldehyde. Forskolin (30 μM) increased the cyclic AMP levels from 5.0 ± 0.3 to 14.4 ± 1.1 pmol/10^6 cells ($n = 10$, $p < 0.001$). The cellular cyclic AMP levels in the presence of glyceraldehyde (10 mM) were 5.0 ± 0.3 pmol/10^6 cells ($n = 10$) and forskolin still increased cyclic AMP, in this case to 12.4 ± 0.8 ($n = 5$) which was not significantly different from the level observed with forskolin alone.

Table 1

Immunoreactive insulin release during perifusion of quin2 loaded RINm5F cells with forskolin, D-glyceraldehyde or the two substances combined

Perifusion time (min)	(I) Forskolin (30 μM)	(II) D-Glyceraldehyde (10 mM)	(III) Forskolin + D-glyceraldehyde	*p* III *vs* II
	(Immunoreactive insulin % of cell content/min)			
47	0.14 ± 0.03	0.12 ± 0.02	0.16 ± 0.02	>0.20
49	0.16 ± 0.03	0.21 ± 0.03[a]	0.29 ± 0.04[b]	>0.10
50	0.18 ± 0.01	0.23 ± 0.03[b]	0.39 ± 0.04[d]	<0.01
51	0.18 ± 0.02	0.29 ± 0.03[c]	0.46 ± 0.04[d]	<0.02
53	0.24 ± 0.03	0.41 ± 0.03[d]	0.53 ± 0.07[d]	>0.10
55	0.28 ± 0.03[b]	0.39 ± 0.04[d]	0.55 ± 0.06[d]	>0.05

[a] $p < 0.05$
[b] $p < 0.02$
[c] $p < 0.005$
[d] $p < 0.001$ *vs* prestimulatory values at 47 min
Cells were perifused for 47.5 min with basal buffer and then exposed to the test substances (see also figs.2,4)

4. DISCUSSION

To measure $[Ca^{2+}]_i$ and insulin secretion in parallel large amounts of cells in suspension are required. Therefore, the cell line RINm5F was used rather than cells from pancreatic islets. This cell line has retained the capacity of increasing the rate of insulin release to a variety of secretagogues including glyceraldehyde, amino acids and glucagon [3,11]. Because of an abnormal glucose metabolism, the cells are no longer sensitive to the secretagogue function of glucose [11,16]. However, RINm5F cells provide a valid model system for the study of insulin release and the use of glyceraldehyde as a glucose-like means of stimulating release has recently been validated. Thus, it was found that glucose causes a similar rise in $[Ca^{2+}]_i$ in pancreatic islet cells [4,5] as that seen with glyceraldehyde here, and in [3].

The rise in $[Ca^{2+}]_i$ evoked by glyceraldehyde precedes the stimulation of insulin release but is transient, while the rate of insulin release remains elevated during continuous exposure to the stimulus. This finding suggests that a rise in $[Ca^{2+}]_i$ is necessary for the triggering of insulin release but not for the maintenance of elevated release rates. As membrane depolarization occurs early, it is reasonable to assume that Ca^{2+} enters the cells through voltage-dependent Ca^{2+} channels. However, the channel blocker, verapamil, only inhibits part of the rise in $[Ca^{2+}]_i$ and the insulin release response [3,5]. Glyceraldehyde may thus raise $[Ca^{2+}]_i$ by an additional mechanism not involving Ca^{2+} channels but perhaps the mobilization of stored calcium [5].

The 3-fold elevation of cellular cyclic AMP levels induced by forskolin resulted in an increase of insulin release without changing $[Ca^{2+}]_i$. Two other agents which raise cyclic AMP, glucagon (1 μM) a secretagogue in these cells [11], and theophylline (2 mM) were also ineffective in elevating $[Ca^{2+}]_i$ (not shown). It can thus be concluded that an increase in cyclic AMP does not mobilize calcium from cellular stores in insulin secreting cells. This result is not due to the failure of detecting Ca^{2+} mobilization with quin2, since carbamylcholine-evoked mobilization is clearly seen in these cells [17].

Forskolin, which also raises cyclic AMP levels in pancreatic islets [6,18] has been reported to increase glucose-induced electrical activity and $^{45}Ca^{2+}$ uptake in mouse pancreatic islets [19,20]. We did not observe any depolarization of the average membrane potential nor a change in the capacity of glyceraldehyde to raise $[Ca^{2+}]_i$. If anything, the effect of glyceraldehyde was smaller in the presence than in the absence of forskolin, despite the potentiation of insulin release. There is, however, no fundamental difference between RINm5F cells and pancreatic islets, since, at low glucose, forskolin-stimulated insulin release from islets was not accompanied by depolarization or $^{45}Ca^{2+}$ uptake [19,20].

It was of interest to note that forskolin enhanced glyceraldehyde-induced insulin release without a further rise in cyclic AMP levels. This situation is very similar to the interaction between high glucose concentrations and forskolin in pancreatic islets [6]. Thus, the most likely interpretation of the action of cyclic AMP in insulin release is that the nucleotide sensitizes the release machinery to other factors, such as the concentration of cytosolic free Ca^{2+}.

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