Correlation between Cytosolic Free Ca\textsuperscript{2+} and Insulin Release in an Insulin-secreting Cell Line*

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Changes in the concentration of free cytosolic Ca\textsuperscript{2+} (\([\text{Ca}^{2+}]_i\)), membrane potential, and immunoreactive insulin release were measured in suspensions of RINm5F cells. \([\text{Ca}^{2+}]_i\), was monitored with the intracellularly trapped fluorescent Ca\textsuperscript{2+} indicator quin 2. Changes in membrane potential were assessed with the fluorescent probe bisoxonol. The effects of depolarizing K\textsuperscript{+} concentrations and of the Ca\textsuperscript{2+} ionophore ionomycin were compared with those of the metabolizable stimulants glyceroldehyde and alanine.

The mean resting \([\text{Ca}^{2+}]_i\) was 105 nM ± 6, \(n = 35\). Of the substances tested, ionomycin caused the most marked increases of \([\text{Ca}^{2+}]_i\), and insulin release. Membrane depolarization was evoked by K\textsuperscript{+}, glycerolphosphate, or alanine, and the agent stimulated insulin release and \([\text{Ca}^{2+}]_i\), to a similar degree. However, the mechanism by which glycerolphosphate, on the one hand, and K\textsuperscript{+} and alanine, on the other, elevate \([\text{Ca}^{2+}]_i\), appears different. Thus, verapamil, a blocker of voltage-sensitive Ca\textsuperscript{2+} channels, almost abolished the effects of K\textsuperscript{+} and alanine on \([\text{Ca}^{2+}]_i\), and insulin release, whereas the responses to glycerolphosphate were only blocked by approximately 50%. Glycerolphosphate may thus, in addition to opening voltage-sensitive Ca\textsuperscript{2+} channels, alter cellular Ca\textsuperscript{2+} handling at another step. The present findings provide direct experimental evidence that secretagogue-induced insulin release is accompanied by a rise in \([\text{Ca}^{2+}]_i\).

The importance of Ca\textsuperscript{2+} in stimulus-secretion coupling in the pancreatic β cell is well established (for review, see Ref. 1). An increase in the concentration of \([\text{Ca}^{2+}]_i\) is thought to mediate the action of glucose and that of certain other stimulators of insulin release. However, direct and quantitative measurements of \([\text{Ca}^{2+}]_i\) in the β cell during stimulation of insulin release are not available.

With the introduction of the fluorescent Ca\textsuperscript{2+} indicator, quin 2, it has now become possible to monitor \([\text{Ca}^{2+}]_i\), in small cells (2). By the use of this compound, it has been shown that \([\text{Ca}^{2+}]_i\), increases during cellular activation of lymphocytes (2-4), platelets (5), and neutrophils (6). Quin 2 binds Ca\textsuperscript{2+} with high selectivity and its fluorescence increases more than 5-fold upon saturation with Ca\textsuperscript{2+} (2). The fluorescence signal has its greatest sensitivity to changes in \([\text{Ca}^{2+}]_i\) in the range 20-500 nM. As \([\text{Ca}^{2+}]_i\), has been found to vary in this concentration range, when measured by other direct means (7-9), quin 2 is well suited to monitor \([\text{Ca}^{2+}]_i\). The tetracarboxylate anion quin 2 is water soluble and does not readily enter living cells. However, the tetracarboxylate ester quin 2/AM is lipophilic and is thus taken up by cells. The quin 2/AM does not bind Ca\textsuperscript{2+} and for successful application of the method, hydrolysis of quin 2/AM in the cells is a necessity. The quin 2 thus generated remains trapped in the cell cytosol without binding to cytoplasmic proteins or uptake into organelles (2, 6).

As the recording of quin 2 fluorescence from loaded cells in a fluorimeter requires more cells than could be conveniently purified from normal endocrine pancreas, the present study employs an insulin-producing permanent cell line, RINm5F. This cell line, originally established from an x-ray induced rat insulinoma (10, 11), has been shown to retain the capacity to store and release immunoreactive insulin (11, 12). The release of insulin from RINm5F cells can be stimulated by a variety of secretagogues, with the exception of glucose (12, 13). Although the cells display higher rates of glucose utilization than normal rat islets of Langerhans, glucose phosphorylation is abnormal, in that the high \(K_m\) phosphorylating activity (glucokinase) normally present in islets (14) appears to be absent (13). Nonetheless, RINm5F cells are capable of secreting insulin in response to the triose glyceraldehyde (12, 13), which enters glycolysis at a later step than glucose. As glyceraldehyde mimics all effects of glucose on islet function (15, 16), its use as a “glucose-like” substance in the study of stimulus-secretion coupling in insulin-producing cells seems warranted. In the present report, the effects of glyceraldehyde, alanine, potassium and the Ca\textsuperscript{2+}-ionophore ionomycin (17) on \([\text{Ca}^{2+}]_i\), and insulin release were examined in RINm5F cells. In addition, membrane potential was monitored with bis(1,3-diethyl thiobarbiturate)-trimethineoxonol (18) and the involvement of voltage-sensitive Ca\textsuperscript{2+} channels in the control of \([\text{Ca}^{2+}]_i\), and insulin release was investigated by using the Ca\textsuperscript{2+} channel blocker verapamil (18).

EXPERIMENTAL PROCEDURES

Cell Culture—RINm5F cells were cultured as described in detail elsewhere (12, 13) using 75-cm\textsuperscript{2} culture flasks containing 20 ml of medium RPMI 1640 supplemented with 10% (v/v) fetal calf serum, 100 IU penicillin/ml, 100 μg streptomycin/ml and 0.25 μg fungizone/ml. The medium was changed every 3 days. The morning of the...
trypsin (0.025%) in Ca2+/Mg2+-free
transferred to a spinner culture flask containing
with
the emission of nonloaded cells. By using the differential sensitivity
tion, all cells were washed and resuspended in RPMI
25 mM Hepes and
cell concentration was 20-30
according to the same equation (2). The same

calibrated at the end of each individual trace essentially as described
standard and of loaded cells was measured at
binding to quin
(6),
2 (excitation wavelength,
5-15%.

decrease of the fluorescence of the extracellular dye. Alternatively,
equations 20-50% of total quin 2 was extracellular. This is probably due
ing 20-50% of total quin 2 was extracellular. This is probably due
to cell damage during centrifugation and resuspension of the cells,
extracellular quin 2 was measured after centrifugation
concentration of
(4)
and
580 nm, respectively. The slit width was
excitation (40
mm (excitation) and 20 nm (emission) for both quin 2 and bisoxonol
standard cuvette holder. The cells were suspended in 2 ml of buffer
339
measured following addition of 1 ml of acid ethanol (ethanol/water/con-
concentrated HCl, 140:57:3 by volume). Insulin in incubation media and
determined as described previously (12) using rat insulin as standard.

Results are expressed as mean ± S.E. Statistical analysis was by

The materials and their sources were as described previously (12) except that quin 2 and quin 2/AM were initially supplied by Dr. R. Y. Tsien, Univ. of California, Berkeley, later purchased from Lancas-
ter Synthese (Morecamb, Lancashire, U. K.), bisoxonol (Dr. Tsien), and bovine serum albumin and d-glyceraldehyde were from Sigma
Chemical Co. Ionomycin was a generous gift of Dr. Liu, Hoffman
LaRoche, and diltiazem-HCl was kindly provided by Dr. H. Bahr-
mann, Gödcke, Freiburg (F. R. G.).

RESULTS

The ability of quin 2-loaded cells to release insulin in
response to glyceraldehyde, K+, or ionomycin was first tested
(Fig. 1). In the loaded cells, an increase of the external K+
concentration from 6 to 30 mM increased insulin release over
10 min by 75%, whereas 10 mM glyceraldehyde elicited a
doubling of the release (p < 0.001 for both secretagogues
relative to the 2.8 mM glucose control). Ionomycin (2 μM)
stimulated insulin release by 131% (p < 0.001 relative to the
control in the presence of 0.1% Me2SO). For control cells
from the same cultures incubated in parallel, the results were
qualitatively similar although basal insulin release was higher
(p < 0.02). K+ caused an increase of 87% (p < 0.001). Glyceral-
aldyde was less potent in control compared to loaded cells,
since the release rate was augmented by 49% (p < 0.001). The
increase obtained with ionomycin in control cells was 124%
(p < 0.001). The quin 2-loaded cells are thus still able to
respond to stimulators of insulin release.

In Fig. 2 are shown the changes in [Ca2+]i, induced either by
depolarizing the cells with K+ or by using the selective
Ca2+ ionophore ionomycin. Resting [Ca2+]i in the experi-
ment was around 100 nM. The addition of 24 mM K+ resulted in
a rapid increase of [Ca2+]i, which reached a peak of about 350
nM within 15 s after addition of the stimulus. [Ca2+]i, then fell
but still remained twice basal levels. Verapamil (20 μM), a
blocker of voltage-sensitive Ca2+ channels added 5 min after
K+, caused a rapid return of [Ca2+]i, to basal level, which was
completed within 30 s. Finally, the addition of 2 μM ionomycin
induced a more than 4-fold rise in [Ca2+]i, reaching its peak
value in less than 10 s. The steady state level reached there-
after was not altered by a second addition of 20 μM verapamil.

<ref>Y. Tsien, unpublished.</ref>

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When verapamil was added prior to K* no increase in [Ca2+]i was observed.

In parallel experiments using the membrane potential-sensitive probe bisoxonol, we have demonstrated that K* depolarized the cell membrane while ionomycin did not when added either before or after KCl. Verapamil at 20 μM had no effect on membrane potential (not shown).

The effects of two metabolic stimuli, glyceraldehyde and alanine, on membrane potential and [Ca2+]i are shown in Fig. 3. The addition of 10 mM glyceraldehyde depolarized the cells (Fig. 3A). There was a short lag phase and maximal depolarization was reached in approximately 2 min; thereafter, the cells slowly repolarized again. Alanine (10 mM) also depolarized the cells but the depolarization was sustained (Fig. 3B).

In Fig. 3C is shown that glyceraldehyde caused an increase of [Ca2+]i from about 130 to about 300 nM, measured in cells from the same preparation as those used in Fig. 3A. Alanine (Fig. 3D) caused a rapid, approximately 3-fold increase in [Ca2+]i. Addition of verapamil accelerated the return of [Ca2+]i toward basal levels. In a large series of experiments resting [Ca2+]i was 105 ± 6 nM (n = 35).

Thus, in contrast to ionomycin, the three stimuli K*, glyceraldehyde, and alanine both depolarize the cell membrane and increase [Ca2+]i. In order to determine whether the increase in [Ca2+]i, was due only to opening of voltage-sensitive Ca2+ channels, verapamil was employed. In Fig. 4A, it is shown that glyceraldehyde raised [Ca2+]i with a time course and to an extent similar to that described for Fig. 3C, and that alanine, added after glyceraldehyde, was still capable of raising [Ca2+]i. In contrast, pretreatment of cells from the same batch with verapamil, 20 μM (Fig. 4B), nearly completely inhibited the alanine-induced rise of [Ca2+]i, while only partially blocking the [Ca2+]i response to glyceraldehyde. In the presence of verapamil, ionomycin was still capable of raising [Ca2+]i. To investigate whether blockade of Ca2+ channels indeed does not completely abolish the glyceraldehyde-induced rise in [Ca2+]i, verapamil was used at several concentrations. In addition, diltiazem, another Ca2+ channel blocker not structurally related to verapamil (18) was employed. In Table 1 are given the mean peak increases in [Ca2+]i obtained in these experiments. At 20 μM, verapamil inhibited the effect of glyceraldehyde by about 50%. In the presence of both 60 and 100 μM verapamil, the glyceraldehyde-evoked rise in [Ca2+]i was inhibited by approximately 70%, while diltiazem (20 μM) caused a 53% inhibition. Under all these experimental conditions, glyceraldehyde was still able to raise [Ca2+]i significantly.

To examine whether the effect of verapamil on [Ca2+]i, is accompanied by a parallel change in insulin release, the release was measured during a 10-min incubation with and without verapamil. The results are given in Table II. Verapamil (20 μM) did not reduce basal insulin release significantly (p > 0.05). Insulin release stimulated by K* or alanine was inhibited by more than 90% in the presence of 20 μM verapamil. In contrast, this concentration of verapamil only inhibited glyceraldehyde-stimulated release by 54%. Moreover, the higher concentrations of verapamil were even less effective in inhibiting insulin release stimulated by glyceraldehyde (32 and 26% inhibition at 60 and 100 μM verapamil, respectively). With diltiazem, the corresponding inhibition was 44%. It should be noted that the two higher verapamil concentrations and diltiazem increased the basal rate of insulin release (Table II). This was associated with membrane depolarization (not shown) and may reflect nonspecific actions of the Ca2+ channel-blocking agents when used at very high concentrations (23).

As expected, verapamil (20 μM) did not affect ionomycin-stimulated insulin release (Table II). Ionomycin effects, however, largely depend on the presence of extracellular Ca2+, since the addition of 2 mM EGTA to the buffer containing 1 mM Ca2+ reduced the stimulation of insulin release by 68% (not shown).

All these experiments were performed in the presence of 2.8 mM glucose. Membrane potential and [Ca2+]i, were not altered when the glucose concentration was increased from 0 to 16.7 mM (not shown), a condition associated with an increase in glucose metabolism by RINm5F cells but no increase in insulin release (13).

**DISCUSSION**

Measurement of [Ca2+]i with quin 2 involves the intracellular trapping of millimolar concentrations of the fluorescent...
**Ca**²⁺ indicator. This might have toxic actions and/or affect resting or stimulated [Ca**²⁺**], levels. Therefore, it was important to demonstrate that quit 2-loaded RINm5F cells are still functionally intact. Functional integrity was demonstrated by an increase in mitogenic stimulation (4). On the other hand, in neutrophils (6) and pheochromocytoma cells (21), as in RINm5F cells, quit 2 loading does not result in any stimulation of secretion. Furthermore, receptor-mediated cell activation is still possible in a number of cell types including lymphocytes (2-4), platelets (5), neutrophils (6), adrenal medullary cells (27), and pheochromocytoma cells (21). Thus, although this method still has its drawbacks (discussed in Ref. 2), it allows direct monitoring of [Ca**²⁺**] in functionally intact cells. It should be noted that quin 2 measures the average [Ca**²⁺**], in the whole cell population. The use of this approach does thus permit neither the assessment of homogeneity of the response between cells nor changes of [Ca**²⁺**], in different parts of the cell cytosol. The buffering ability of quit 2 would, if anything, tend to dampen sharp variations in [Ca**²⁺**]. Working with a cloned cell line should minimize intercellular variations. Moreover, resting [Ca**²⁺**], levels obtained with quit 2 in various cell types are similar to those found in individual cells using intracellular Ca**²⁺**-select-
tive electrodes (7, 8). In addition, [Ca\textsuperscript{2+}] elevation in response to physiological stimuli in secretory cells also appears to occur in the same range (8).

The main purpose of this study was to test directly the hypothesis that an elevation of [Ca\textsuperscript{2+}], mediates the action of physiological stimuli of insulin release. Glucose itself could be a good correlation between the inhibitory effects on glyceraldehyde-stimulated insulin release preparations. Advantage was thus taken of the retained sensitivity of RINm5F cells to the glucose-like stimulus, glyceraldehyde (12). In addition, alanine was used as an example of amino acid-induced insulin release (25, 26). Glyceraldehyde and alanine both caused an average increase of approximately 2-fold in [Ca\textsuperscript{2+}]. For both agents, the onset of the response required to elucidate whether an increase of [Ca\textsuperscript{2+}] always

<table>
<thead>
<tr>
<th>Condition</th>
<th>Control</th>
<th>20 μM verapamil</th>
<th>40 μM verapamil</th>
<th>100 μM verapamil</th>
<th>20 μM diltiazem</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>100 ± 7 (12)</td>
<td>88 ± 5 (12)</td>
<td>173 ± 17 (14)</td>
<td>244 ± 34 (15)</td>
<td>190 ± 13 (12)</td>
</tr>
<tr>
<td>10 mM glyceraldehyde</td>
<td>253 ± 16 (12)</td>
<td>167 ± 16 (12)</td>
<td>277 ± 27 (13)</td>
<td>359 ± 44 (15)</td>
<td>291 ± 36 (15)</td>
</tr>
<tr>
<td>24 mM K\textsuperscript{+}</td>
<td>293 ± 13 (12)</td>
<td>102 ± 11 (12)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 mM alanine</td>
<td>233 ± 23 (10)</td>
<td>98 ± 10 (10)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 mM ionomycin</td>
<td>304 ± 22 (12)</td>
<td>325 ± 20 (12)</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

The previous indirect approaches (1), combined with the present direct demonstration that glyceraldehyde raises cytosolic free Ca\textsuperscript{2+} in an insulin-producing cell line, further support the hypothesis that glycolytic breakdown of glucose in the β cell leads to an increase in [Ca\textsuperscript{2+}], and the subsequent triggering of insulin release. In the present study, three agents which depolarized the cell membrane of the insulin-producing cells as well as the Ca\textsuperscript{2+} ionophore ionomycin caused a parallel increase in [Ca\textsuperscript{2+}], and insulin release. Further studies are required to elucidate whether an increase of [Ca\textsuperscript{2+}], always accompanies the stimulation of insulin release or whether certain stimulators induce release without such increase.

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