MEASUREMENTS OF CYTOPLASMIC pH IN INSULIN-RELEASING CELLS WITH INTRACELLULARLY TRAPPED PHENOL RED

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ABSTRACT

Cytoplasmic pH was measured in insulin-releasing clonal RINm5F cells using a new technique. The cells were permeabilized by electric discharges and loaded with phenol red. After resealing, changes in the absorbance of the intracellular indicator was monitored by dual wavelength spectrophotometry. The phenol red loading did not affect glucose metabolism, which was characterized by an oxidation to utilization ratio as low as 0.076. The basal cytoplasmic pH of the RINm5F cells was 7.25, and exposure to glucose caused intracellular acidification by 0.10 pH units. Since the RINm5F cells are characterized by a defective insulin response to glucose, the data do not support ideas that intracellular acidification is a link in the stimulus-secretion coupling of glucose-induced insulin release.

Up to now estimates of the intracellular pH of pancreatic β -cells have been based on measurements of the distribution of radiolabelled 4,4-dimethyloxazolidine-2,4-dione (DMO; 14, 18, 19), and the fluorescence from intracellularly trapped probes (3, 9, 10, 28). Whereas DMO will permeate into several cellular compartments, the fluorescent dyes have been believed to be specific for the cytoplasm, since their intracellular trapping require the action of esterases on the membrane-permeable ester derivatives (26). However, recent data indicate a substantial accumulation of such indicators also in secretory granules (2), which in β -cells have an acid interior (1). It is consequently not clear to what extent the results obtained are representative for the cytoplasm. In the present study a new approach has been made. Clonal insulin-releasing cells were exposed to intense electrical fields which is known to selectively permeabilize the plasma membrane to small molecules (17). After loading with the indicator phenol red the cells were allowed to reseal, and the cytoplasmic pH was then meas-

ured by dual wavelength spectrophotometry.

MATERIALS AND METHODS

Materials

Reagents of analytical grade and deionized water were used. Phenol red was from E. Merck, Darmstadt, F.R.G.; arsenazo III from Fluka AG, Buchs, Switzerland; and Hepes from Sigma Chemical, St. Louis, MO, U.S.A. D-[5-³H]glucose and D-[U-¹⁴C]glucose were purchased from Amersham International, Amersham, Bucks, U.K.; and Hyamine from Packard Instrument International S.A., Zurich, Switzerland. Statens Veterinärmedicinska Anstalt, Uppsala, Sweden, supplied RPMI 1640 culture medium, whereas Hanks' balanced salt solution and trypsin were obtained from Gibco, Paisley, U.K.. NUSERUMTM was supplied by Collaborative Research Inc., Lexington, MA, U.S.A., and fetal bovine serum, penicillin and streptomycin by Flow Laboratories, Irvine, U.K. Nunclon culture flasks (200 ml) were from Nunc A/S, Kamstrup, Denmark.

Cell Culture

The studies were performed with the clonal insulin-producing rat cell line RINm5F (20). The cells were grown in 200 ml Nunc flasks containing 25 ml of RPMI 1640 medium supplemented with 10% NU-SERUMTM, 1% fetal bovine serum, 100 units/ml penicillin and 100 µg/ml streptomycin. Only flasks with a confluent cell layer were used for the experiments. The cells were detached by incubation for 3 min at 37°C with 2 ml of Hanks' balanced salt solution (pH 7.4) supplemented with 2.5 mg/ml trypsin and 10 mg/ml EDTA. At the end of the enzyme treatment the cells were suspended by gentle shaking, and 10 ml RPMI 1640 medium was added to dilute the enzyme. After pelleting and two successive washes the cells were incubated for 60 min in RPMI medium and were then resuspended in 1 ml of a medium containing 1 mg/ml phenol red, 125 mM M⁺, 17.9 mM Na⁺, 1.2 mM Mg²⁺, 133.3 mM Cl⁻ and 25 mM Hepes (pH 7.4).

Loading with Phenol Red

To permeabilize the RINm5F cells the suspension was subjected to two successive electrical discharges (2.75 kV/cm; time constant 200 µsec) at 2°C with an interval of 10 sec. The suspension was then incubated for 10 min on ice and subsequently transferred to a culture flask containing 10 ml RPMI 1640 medium supplemented with phenol red (2 mg/ml) for 90 min incubation at 37°C. After this period the cells resealed as judged by their ability to exclude trypan blue. The cells were washed three times in a medium containing 137 mM Na⁺, 5.9 mM K⁺, 1.3 mM Ca²⁺, 1.2 mM Mg²⁺, 135.9 mM Cl⁻ and 25 mM Hepes (pH 7.4), and were finally suspended in 1 ml of this medium.

Measurements of Cytoplasmic pH

The cell suspension was transferred to a disposable standard cuvette (10 mm) placed in a thermostatted (37°C) cuvette holder of a time-sharing multi-channel spectrophotometer (8). Gentle magnetic stirring prevented sedimentation. The relative absorbance difference $A_{525}-A_{499}$ of phenol red was monitored as well as extracellular pH (pH_e) using a BETRODETM micro pH electrode (W-P Instruments, New Haven, CT, U.S.A.) and a miniature reference electrode. The produc-

tion of H⁺ equivalents by the cells or acidification by different supplements were neutralized by automatic 5 nl additions of 2 M NaOH (12). In order to calculate intracellular pH (pH_i), it is necessary to take the influence of extracellular phenol red into account. Assuming that the rapid change in absorbance upon a slight acidification of the medium with HCl (~0.04 pH units) originates from extracellular phenol red, it was possible to determine the distribution of the indicator. The quotients between the changes in absorbance and pH_e after HCl additions were calculated at two time points during the experiment and compared to that obtained after disintegration of the cells with Triton X-100. The release of the indicator was assumed to be linear with time until addition of Triton X-100.

When phenol red was titrated photometrically ($A_{525}-A_{499}$) with NaOH at 37°C, pK_a was found to be 7.68 irrespective of whether a normal (Na⁺-rich) or an inverted (K⁺-rich, Ca²⁺-deficient) incubation medium was used. Since the instrument does not measure the absolute absorbance difference $A_{525}-A_{499}$ but changes from an arbitrary reference point, the minimal absorbance difference (all indicator in the acid form) was set to zero. The measured changes in absorbance difference $A_{525}-A_{499}$ during a titration can now be described by the function

$$[1] \quad A_m = \frac{A_t \cdot 10^{(pH-pK_a)}}{1 + 10^{(pH-pK_a)}}$$

where $A_t = (A_{525}-A_{499})_{\max} - (A_{525}-A_{499})_{\min}$. Derivation yields

$$[2] \quad \frac{dA_m}{dpH} = \frac{A_t \cdot \ln 10 \cdot 10^{(pH-pK_a)}}{(1 + 10^{(pH-pK_a)})^2}$$

A_t was obtained from function 2 using the measured dA_m/dpH at HCl addition after cell disintegration with Triton X-100. The value of A_m was then calculated from function 1 for the situation immediately after addition of Triton X-100, and this value (A_{ref}) was used for the calculations of pH_i. The following relation can now be established

$$[3] \quad A_{\text{ref}} = f_e \cdot \frac{A_t \cdot 10^{(pH_e-pK_a)}}{1 + 10^{(pH_e-pK_a)}} + f_i \cdot \frac{A_t \cdot 10^{(pH_i-pK_a)}}{1 + 10^{(pH_i-pK_a)}} + A_d$$

where A_d is the negative absorbance deviation from A_{ref} (Fig. 1), and f_e and f_i the fractions of extra- and intracellular indicator. Using function 3, pH_i was calculated for every time point.

Measurements of Glucose Metabolism

Glucose metabolism was studied at 20 mM of the sugar. The production of [$^3\text{H}_2$]O from D-[5- ^3H]glucose and [^{14}C]O $_2$ from D-[U- ^{14}C]glucose were used to indicate the combined flux through the glycolytic and phosphogluconate pathways and oxidative degradation, respectively. The RINm5F cell suspensions (0.25–1.50 mg protein/ml) had a total volume of 100 μl and contained 0.9 and 0.5 Ci/mol of [^{14}C]- and [^3H]-labelled glucose, respectively. The incubations were performed in small glass vials placed inside 20 ml glass vessels used for liquid scintillation counting. After incubation for 60 min at 37°C, 50 μl of 0.2 M HCl was added to the medium to stop the reaction. [^{14}C]O $_2$ was then collected in Hyamine (13) or the [$^3\text{H}_2$]O produced was allowed to equilibrate overnight at 37°C with 1 ml of water (4). The radioactivities of the Hyamine or water in the outer vials were determined by liquid scintillation spectrophotometry.

Measurements of Protein

After the experiments all cell suspensions were frozen and retained for measurements of protein using a fluorescamine method (27).

Evaluation of Results

Results are expressed as mean values $\pm$ SE. Statistical significances were calculated from the differences between paired test and control data using Student's *t* test.

RESULTS

Table 1 shows glucose metabolism in untreated and phenol red loaded RINm5F cells. At 20 mM glucose the cells were characterized by an oxidation-to-utilization ratio as low as 0.076. It is apparent that neither glucose utilization nor oxidation were affected by the loading procedure.

Among the different experiments, the fraction of phenol red that was intracellular varied between 28 and 60% and decreased slowly with time. Fig. 1 shows recordings of phenol red absorbance and extracellular pH as well as the calculated cytoplasmic pH. At the first HCl addition, 34% of the indicator was shown to be intracellular as compared with 32% at the second addition 12 min later. Because of the large proportion of extracellular indicator

Table 1 *Effect of Phenol Red Loading on Glucose Oxidation and Utilization in RINm5F Cells*

Metabolic parameter	Glucose equivalents metabolized (mmol/kg protein)	
	Untreated cells	Phenol red loaded cells
Glucose oxidation	90 $\pm$ 8(12)	93 $\pm$ 31(7)
Glucose utilization	1,190 $\pm$ 65(5)	1,198 $\pm$ 100(5)

Glucose oxidation and utilization were measured as the formation of [^{14}C]O $_2$ and [$^3\text{H}_2$]O from 20 mM of D-[U- ^{14}C]glucose and D-[5- ^3H]glucose respectively during 60 min of incubation at 37°C. The results are expressed as mean values $\pm$ SE for the number of experiments indicated in parentheses.

in this experiment the effect of compensating for changes in extracellular pH is particularly clear. Addition of 20 mM glucose induced a decrease in cytoplasmic pH, which reached a nadir after $\sim$ 1 min and then slowly recovered. The introduction of 10 mM NH_4^+ caused rapid alkalization, which tended to be transient. In a series of 6 experiments the basal pH was 7.25 $\pm$ 0.05 and the maximal reduction by glucose 0.10 $\pm$ 0.04 ($P < 0.05$).

DISCUSSION

With the present technique the intracellular pH indicator should be restricted to the cytoplasm, since exposure of cells to intense electrical fields is known to selectively permeabilize the plasma membrane to small molecules (17). The latter conclusion seems valid also for the RINm5F cells, which do not appear to lose cytoplasmic enzymes as judged from the unchanged rates of glucose metabolism. The fact that metabolism was unaltered by the phenol red loading, indicates that the dye is essentially non-toxic. The use of an absorbance indicator eliminates some of the problems associated with fluorescence measurements such as fading and quenching. A difficulty common to all methods using intracellular indicators is leakage from damaged cells. However, the present measurements are unique in compensating also for very minute changes in extracellular pH.

Although the phenol red approach may be superior for measuring pH in the cytoplasm, parallel studies with quene-1 gave almost identical results. In six experiments with this fluorescent probe, the basal pH was found to

Fig. 1 Effect of glucose and NH_4^+ on intracellular pH (pH_i) of RINm5F cells. Cells were loaded with phenol red and incubated in a disposable standard cuvette (10 mm) placed in a thermostatted (37°C) cuvette holder of a time-sharing multi-channel spectrophotometer. Gentle magnetic stirring prevented sedimentation of the cells which corresponded to 8.6 mg of protein/ml of cell suspension. Extracellular pH (upper trace) was monitored using electrodes as well as the relative absorbance difference $A_{525}-A_{499}$ of phenol red (middle trace). During the periods indicated by the horizontal bars the production of H^+ equivalents by the cells or acidification by different supplements were neutralized by automated 5 nl additions of 2 M NaOH. The calculation of cytoplasmic pH (lower trace) is described in the Materials and Methods section.

be 7.27 ± 0.02 (P. Arkhammar and P.-O. Berggren, to be published). The influence of quene-1 within the acid insulin granules may virtually be negligible in the RINm5F cells, which are only sparsely granulated (11). When Wollheim *et al.* (28) used 2',7'-bis(carboxyethyl)-5,6-carboxyfluorescein to estimate the intracellular pH of the RINm5F cells, it was found to be only 6.95 ± 0.01 . It is not clear whether the discrepancy between the two laboratories represent altered characteristics of the RINm5F cells or is due to differences in techniques. However, the basal intracellular pH of the presently used RINm5F cells is considerably higher than the 6.71 ± 0.06 observed with normal β -cells using the same quene-1 technique as for the RINm5F cells (3). As discussed above, it cannot be excluded that the quene-1 method underestimates cytoplasmic pH in the well-granulated normal β -cells due to accumulation of the dye in the acid granules. However, since an increase in cytoplasmic pH is a typical event in cell growth stimulation (5), it is also possible that a high pH is a characteristic feature of the tumourous RINm5F cells.

Exposure of pancreatic β -cells to glucose concentrations stimulating insulin release results in markedly increased production of H^+ equivalents (12, 19). Intracellular acidification by glucose has therefore been predicted (16) and suggested to be an important event in stimulus-secretion coupling either by inhibiting the outward transport of Ca^{2+} (6, 15, 19) or decreasing the K^+ permeability (7, 21–23). Nevertheless, at concentrations which stimulate insulin secretion maximally, glucose increased the intracellular pH of normal β -cells by 0.05–0.20 units (3, 9, 10, 18). Such data indicate that glucose stimulates the outward transport of H^+ equivalents by more than that required to balance the raised acid production. In the present measurements on the RINm5F cells the opposite action of glucose on cytoplasmic pH was observed. The acidification was not unexpected considering the rapid glycolysis of these cells. However, since glucose decreases the cytoplasmic Ca^{2+} activity of the RINm5F cells (25), and unlike depolarizing agents, fails to release their insulin (11, 24), the available data do not support a role of acidification in the stimulus-secretion coupling of glucose-induced insulin release.

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