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GLUCOSE-STIMULATED EFFLUX OF FURA-2 IN PANCREATIC β-CELLS IS PREVENTED BY PROBENECID

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Fura-2 loaded pancreatic β-cells, isolated from obese hyperglycemic mice, were studied with respect to cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$), insulin release and efflux of indicator. In the absence of glucose there was a continuous efflux of fura-2, which was markedly increased by stimulation with a high concentration of the sugar. Probenecid both reduced basal efflux of fura-2 and prevented that promoted by glucose. There was no interference of the drug with glucose-induced either insulin release or rise in $[Ca^{2+}]_i$. When applying fura-2 in pancreatic β-cells, the use of probenecid markedly improves the measurements of $[Ca^{2+}]_i$.

Fluorescent Ca^{2+} indicators, e.g. quin-2 and fura-2 (1), are routinely used when investigating the intracellular Ca^{2+} metabolism in various cell systems. However, in some cell types, the information provided from such measurements is of limited value due to significant leakage of the dye into the extracellular space (2-4). Apart from a decreasing signal-to-noise ratio, the loss of indicator into Ca^{2+} containing media will lead to its saturation and thereby an increase in fluorescence. When measuring in a finite volume, such as in a cuvette, accumulating extracellular dye will constitute a substantial part of the total fluorescence signal and present a serious problem in the determination of the cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$). Under conditions where loss of indicator can be considered linear with time, it might be corrected for. However, in situations where leakage of indicator is enhanced in response to test substances, and/or is non-linear, it will be difficult to make reliable estimations of absolute changes in $[Ca^{2+}]_i$. Such a leakage pattern of fura-2 was recently demonstrated in pancreatic β-cells stimulated with the fuel secretagogue glucose (5), a phenomenon not related to insulin release.

It has been suggested that efflux of fura-2 is mediated by a carrier system since leakage is reduced by lowering the temperature (4) and by the addition of probenecid

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(6), a blocker of organic anion transport. In the present study we investigated whether this drug could be used to reduce leakage of fura-2 also in pancreatic β-cells and thereby improve the measurements of $[Ca^{2+}]_i$. A special interest was then focused on the ability of probenecid to reduce glucose-induced efflux of indicator, without interfering with glucose-stimulated insulin release.

MATERIALS AND METHODS

Media: The basal medium used for the preparation of cells as well as in all experiments, was a Hepes buffer, pH 7.4, with Cl^- as the sole anion (7), containing 1.28 mM Ca^{2+} and 1 mg/ml bovine serum albumin.

Animals and preparation of cells: Adult obese hyperglycemic mice (ob/ob) of both sexes were taken from a local non-inbred colony (8) and starved overnight. The islets were isolated with collagenase and a cell suspension was prepared essentially as described by Lernmark (9). The cell suspension was incubated overnight, as previously described (10).

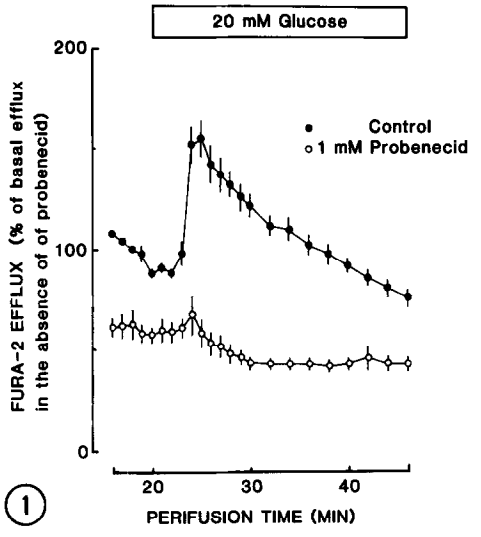
Measurements of insulin release and efflux of indicator: Cell suspensions were incubated with 1 μM fura-2/acetoxymethylester (AM) (Calbiochem, La Jolla, CA.) for 45 min. The dynamics of insulin release and efflux of indicator were subsequently studied by perifusing 1 x 10^6 fura-2 loaded cells mixed with Bio-Gel P-4 polyacrylamide beads (Bio-Rad Laboratories, Richmond, CA.) in a 0.5 ml column at 37°C (11). The flow rate was 0.3 ml/min and 1 or 2 min fractions were collected and analysed for insulin radioimmunologically, using crystalline rat insulin as the standard. The indicator content in each fraction was measured fluorometrically and the results corrected for background fluorescence.

Measurements of $[Ca^{2+}]_i$: For measurements of $[Ca^{2+}]_i$, freshly prepared cell suspensions were seeded onto coverslips and cultured overnight. After loading with 2 μM fura-2/AM for 45 min, the coverslips were rinsed in buffer, fixed in a specially built holder and placed in a cuvette at a 30° angle towards the excitation light beam (12), in a Perkin-Elmer LS-5 spectrofluorometer. $[Ca^{2+}]_i$ was monitored by switching the excitation wavelengths between 340 and 380 ± 5 nm and recording the emitted light at 510 ± 10 nm. After subtraction of autofluorescence, the 340/380 nm ratios were calculated at time intervals of 1 and 2 min. Measurements were performed at 37°C and the medium was continuously stirred with a magnetic bar. Test substances were added from concentrated stock solutions. At the concentration used, there was no interference of probenecid with fura-2 fluorescence.

RESULTS AND DISCUSSION

When fura-2 loaded β-cell aggregates were perifused in a column system, there was continuous efflux of indicator, as detected by measuring fluorescence in samples of the perifusates (Fig. 1). Stimulation of the β-cells with high glucose induced a marked increase of fura-2 efflux (closed symbols), in accordance with what has previously been demonstrated (5). However, in cells run in parallel, the continuous presence of 1 mM probenecid (open symbols) reduced not only the basal efflux of fura-2, but also prevented that promoted by glucose. Thus, it seems that the same mechanism is responsible for part of the basal as well as the stimulated extrusion of indicator.

The extent to which probenecid can be adopted routinely for reducing fura-2 efflux, is a matter of its interference with important physiological processes of



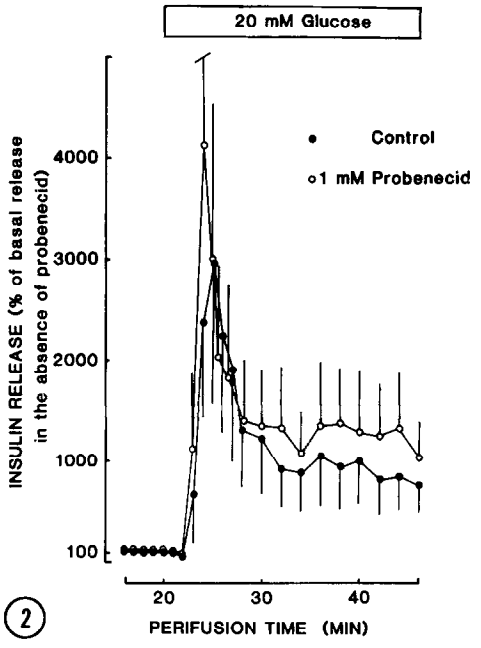


Fig. 1. Effects of glucose on the efflux of fura-2 in the absence (●) or in the presence (○) of 1 mM probenecid. Mean ± S.E.M. for four separate experiments.

Fig. 2. Insulin release from fura-2 loaded β-cells in response to glucose in the absence (●) or in the presence (○) of 1 mM probenecid. Mean ± S.E.M. for four separate experiments.

the respective cell system. A sensitive parameter of β-cell function is secretion of insulin in response to glucose, since this process demands a well-functioning metabolism of the sugar as well as an intact secretory machinery. As evident from figure 2 (open symbols) and in accordance with a previous study (13), 1 mM probenecid had no effect on the stimulatory action of glucose on insulin release. Neither did the drug change the basal rate of hormone secretion, when compared to the control (closed symbols). In agreement with previous results (5), our data confirm that glucose-stimulated fura-2 efflux is distinct from insulin release and also suggest that the mechanism of indicator loss in pancreatic β-cells is similar to that in J744.2 macrophages (6). In the latter cells probenecid, at a concentration of 2.5 mM, did neither affect the response to platelet-activating factor nor phagocytosis.

Since the probenecid-sensitive carrier transports fura-2, indicator efflux can be used as a measure of its activity. The natural substrate(s) for this transport process is sofar not defined in the β-cell, but from our data it is tempting to speculate that metabolic intermediates are involved. Indeed, glucose, which is readily metabolized by the β-cell, initiated an increased efflux of fura-2, whereas the non-metabolizable glucose analogue 3-O-methylglucose was without effect (5). The postulated carrier

could either be stimulated directly by the change in concentration of the metabolite or indirectly by its binding to a regulatory site. One might argue that an increased formation of a substrate for the transporter should compete with fura-2 and thereby decrease the efflux of indicator. However, if the specificity is low and the rate of transport is much increased, there would still be a net stimulation of fura-2 efflux. Insulin-producing RINm5F cells produce more lactate compared to normal β-cells (14) and also demonstrate a higher rate of fura-2 efflux than their normal counterparts (unpublished observations). It could thus be speculated that lactate serves as a substrate for the probenecid-sensitive carrier (6). In this context, it should be remembered that stimulation of pancreatic islets with nutrient secretagogues evokes an efflux of phosphate by an hitherto unknown mechanism (15,16). Against this background we were interested in whether glucose stimulated the efflux of other Ca^{2+} indicators as well from the β-cell. Indeed, there was a substantial loss of the fluorescent Ca^{2+} indicator indo-1 (1) (data not shown), whereas we have previously failed to demonstrate a glucose-induced leakage of quin-2 (5). This does not

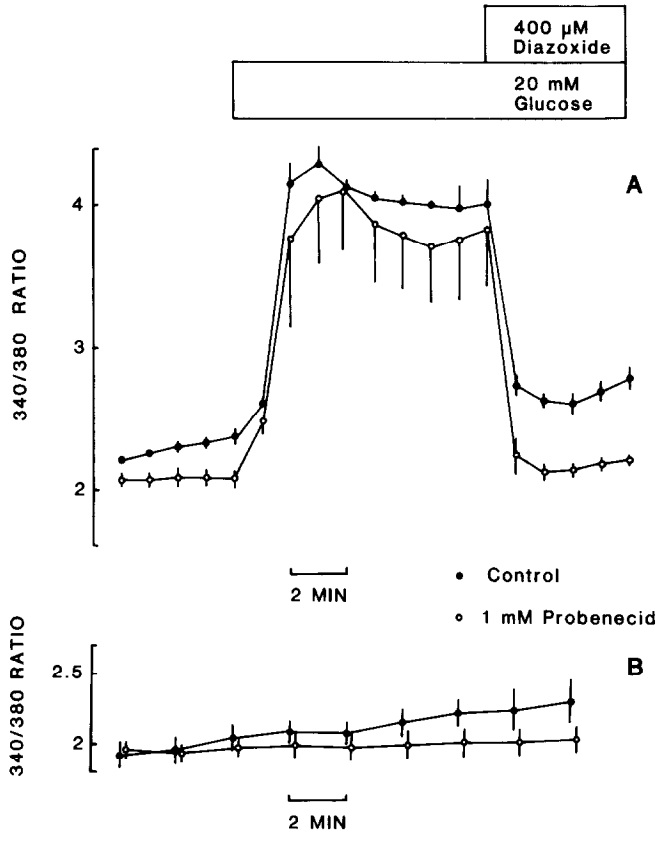


Fig. 3. Measurements of the 340/380 nm fluorescence ratio of fura-2 loaded β-cells. A. Effects of glucose and diazoxide in the absence (●) or in the presence (O) of 1 mM probenecid. Mean ± S.E.M. for three separate experiments. B. Measurements performed under non-stimulatory conditions in the absence (●) or in the presence (O) of 1 mM probenecid. Mean ± S.E.M. for four to five separate experiments.

necessarily imply that quin-2 is not transported by such a mechanism, but might rather be accounted for by difficulties in detecting this dye in the perifusates, due to its considerably weaker fluorescence (1). The fact that leakage of more than one type of indicator is stimulated under conditions where metabolism is increased, should be considered also when studying other cells than β-cells.

When measuring in cell suspensions in a cuvette, it is obvious that leakage of fura-2 might seriously interfere with estimations of $[Ca^{2+}]_i$. To avoid the problems caused by fura-2 leakage, we measured $[Ca^{2+}]_i$ in a small number of β-cells attached to coverslips placed in a cuvette. Under these conditions extracellular dye is extensively diluted and the source of error consequently reduced. As shown in figure 3A (filled symbols), there was a slow increase in the calculated 340/380 nm ratio even in the absence of glucose. Stimulation with a high glucose concentration increased $[Ca^{2+}]_i$ by depolarizing the cells with subsequent opening of voltage-activated Ca^{2+} channels (17,18). Addition of diazoxide rapidly lowered $[Ca^{2+}]_i$ by completely repolarizing the cells, as has previously been demonstrated (18,19). It should be noted that the basal level did not return to its initial value implying that, despite the large cuvette volume and the small number of cells, extracellular fura-2 did interfere with the measurements of $[Ca^{2+}]_i$. In the presence of 1 mM probenecid (open symbols) the effects of glucose and diazoxide were similar to those obtained in the absence of the drug, but in this case the resting level returned to near its initial value. Moreover, when continuously monitoring the 340/380 nm ratio under non-stimulatory conditions (panel B), the slope of the baseline was less pronounced in the presence of the drug (open symbols) compared to the control (closed symbols). Hence, probenecid can be succesfully employed in pancreatic β-cells to reduce the problems associated with fura-2 leakage and thus markedly improve the measurements of $[Ca^{2+}]_i$.

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