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MODULATION BY CYTOSOLIC pH OF CALCIUM AND RUBIDIUM FLUXES IN RAT PANCREATIC ISLETS

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Abstract—Cytosolic pH (pH_i) of pancreatic islet cells was assessed using the fluorescent dye 2'7'-biscarboxyethyl-5'(6')-carboxyfluorescein (BCECF). pH_i was rapidly lowered by addition of the sodium salt of a weak acid or by treatment with amiloride. In the latter case, no recovery of pH_i occurred. NH_4Cl produced a rise in pH_i. Stimulation of islet cells with glyceraldehyde produced a sustained fall in pH_i, whereas glucose and α-ketoisocaproate caused a small, gradual rise in pH_i.

Intracellular acidification, particularly with amiloride, resulted in an immediate potentiation of glucose-induced insulin secretion from perifused islets. In the case of weak acid treatment, subsequent removal of the weak acid produced a paradoxical stimulation of insulin release which was not observed upon removal of amiloride. NH_4Cl produced a transient stimulation followed by a reduction in the rate of glucose-induced insulin secretion.

A reduction in pH_i, either in response to weak acid or amiloride treatment, was associated with a diminution in the rate of efflux of $^{86}Rb^+$ and of $^{45}Ca^{2+}$. Removal of weak acid produced a marked "rebound" stimulation of $^{86}Rb^+$ and $^{45}Ca^{2+}$ efflux. Treatment of islets with NH_4Cl, either in the presence or absence of glucose or Ca^{2+}, resulted in a marked stimulation of efflux of $^{86}Rb^+$ and $^{45}Ca^{2+}$. The stimulatory effect of NH_4Cl on $^{45}Ca^{2+}$ efflux was markedly impaired in the absence of Na^+. It is concluded that pH_i can influence the secretory activity of pancreatic islets, possibly via effects on potassium permeability and sodium–calcium exchange across the plasma membrane, resulting in altered mobilisation of calcium in the islet cell. However, it is unlikely that glucose or other nutrient stimuli activate islets solely via an effect on pH_i.

A considerable amount of attention has focussed on the possibility that changes in cytosolic pH (pH_i) in islet cells may provide a coupling mechanism between the oxidation of nutrients, such as glucose, and the subsequent stimulation of secretory activity. Thus, it has been suggested that the generation of protons as a result of nutrient metabolism in islet cells could enhance the secretion of insulin [1]. In support of this hypothesis, it has been found that intracellular acidification, either by treatment with a weak acid or amiloride, can reproduce certain effects upon islets of glucose, including a reduction in the efflux of potassium [rubidium; 2–4] and calcium [3, 5] and the subsequent modification of electrical activity [6, 7]. Conversely, the activation of islets has been shown to be impaired by intracellular alkalinisation using NH_4Cl [7, 8] or omission of bicarbonate [9]. There is uncertainty, however, regarding the mechanism by which pH_i is able to influence cationic fluxes. Malaisse and colleagues [1] have proposed that intracellular protons may inhibit sodium–calcium exchange across the islet cell plasma membrane. An alternative suggestion has been a primary inhibition by protons of potassium conductance, with a consequent depolarisation of the β-cell [6]. Both of these mechanisms would be expected to result in a rise in cytosolic calcium concentration, which could explain the enhanced secretory activity. Further uncertainty regarding the role of pH_i in the regulation of insulin release arises from the study of Lindstrom and Sehlin [10] who reported that a rise in pH_i in mouse islets using NH_4Cl enhanced insulin secretion, whereas intracellular acidification by lowering the pH of the incubation medium or by addition of bicarbonate had no effect.

In an attempt to clarify the above issues, we have investigated the effects of alterations in pH_i by amiloride, weak acids and NH_4Cl on the secretion of insulin from perifused islets. In order to further investigate the mechanism by which pH_i influences secretory activity, we have studied the effects of these substances on $^{86}Rb^+$ and $^{45}Ca^{2+}$ fluxes in islets.

MATERIALS AND METHODS

Isolated islets were prepared from adult rat pancreases by collagenase digestion [11]. Incubations were performed in gassed bicarbonate-buffered medium (pH 7.4) containing 0.25% (w/v) bovine serum albumin. Where the sodium salts of weak acids were used, these were substituted for NaCl. The secretion of insulin was measured using an islet perifusion technique as described previously [12]. The insulin content of the perifusate was estimated by radioimmunoassay [13].

$^{86}Rb^+$ and $^{45}Ca^{2+}$ efflux was monitored in groups of 100 islets which were pre-incubated for 90 min with 20 μCi of isotope. The islets were then washed and perifused at a rate of 1 ml/min. Following a period of 20 min, samples of perifusate were collected at 1 min intervals and the radioactivity counted using a liquid scintillation counter. Results were

expressed as fractional outflow rate over the period 20–50 min. Changes in cytosolic pH were assessed using a pH-sensitive dye, 2′,7′-biscarboxyethyl-5′-(6′)-carboxyfluorescein (BCECF). Batches of 1000 islets were dispersed into individual cells or small aggregates by mechanical agitation in a calcium-free medium followed by filtration through nylon mesh. Cells were incubated at 37° for 1–2 hr prior to loading with the dye (5 μM) for 20 min. The cells were then washed three times and resuspended at a density of 100,000–300,000/ml. 1.9 ml of cell suspension was placed in a quartz cuvette and incubated at 37° in a Perkin–Elmer LS5 fluorescence spectrophotometer, modified to permit continuous stirring. Fluorescence intensity was measured at 500 nm excitation, 530 nm emission. Test substances were added in 100 μl of medium. The traces were calibrated by lysing the cells by the addition of 10 μl digitonin. The pH of the lysate was measured using a microelectrode and plotted against fluorescence following the addition of each of several 10 μl aliquots of 1 M HCl.

^{125}I-insulin, ^{86}RbCl and $^{45}CaCl_2$ were obtained from Amersham International, Amersham, U.K., and anti-insulin antisera from Wellcome Reagents Ltd., Beckenham, U.K. Amiloride, D-glyceraldehyde and α-ketoisocaproate were purchased from Sigma Chemical Co. Ltd., Poole, Dorset, and 2′7′-biscarboxyethyl-5′(6′)carboxyfluorescein from Calbiochem, Cambridge Bioscience Ltd., Cambridge, U.K.

RESULTS

Treatment of islet cells with 40 mM sodium acetate produced a rapid fall in pH_i followed by a gradual recovery, as assessed by BCECF fluorescence (Fig. 1). Amiloride (1 mM) also caused an intracellular acidification, although in this case, no recovery occurred. Addition of 10 mM NH_4Cl resulted in intracellular alkalinisation. Exposure of islet cells to nutrient secretagogues had no consistent effect on pH_i. Glyceraldehyde produced a sustained lowering in pH_i, whereas glucose and α-ketoisocaproate resulted in an apparent gradual rise in pH_i.

Exposure of perifused islets to 40 mM sodium acetate (Fig. 2a) or sodium propionate (Fig. 2b), or to 1 mM amiloride (Fig. 3a) caused an immediate enhancement of insulin secretion. Only amiloride produced a significant effect at substimulatory glucose concentrations. Following the removal of weak acid, an immediate, pronounced stimulation of secretion was observed, again in the presence of 5.6 mM glucose. No such stimulation of insulin

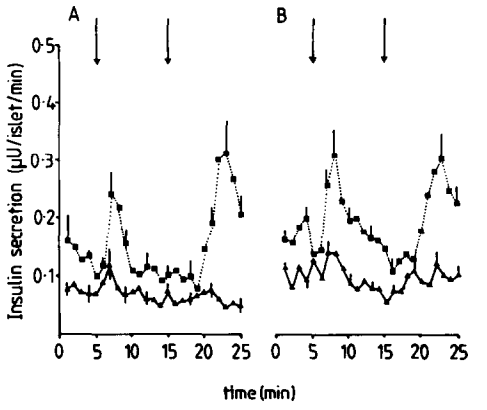

Fig. 2. Insulin secretion from perifused islets. Effects of 40 mM sodium acetate (a) and propionate (b) in the presence of either 2.5 mM (▲——▲) or 5.6 mM (■---■) glucose. In all cases, islets were exposed to test agents between the 5th and 15th minute of perifusion. Each point represents the mean ±SEM of 4–6 determinations.

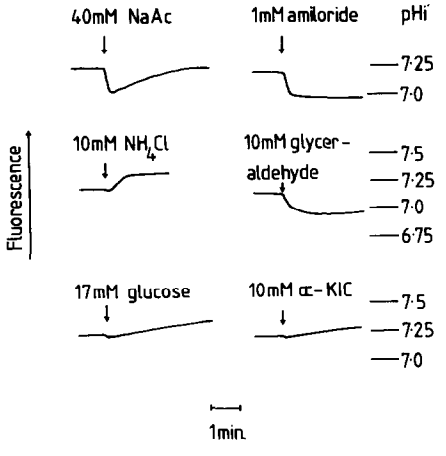

Fig. 1. Effects of various agents on pH_i in dispersed islet cells. Cells were loaded with 5 μM BCECF for 20 min, washed and resuspended at a density of 100,000–300,000/ml. Fluorescence was measured at 500 nm excitation, 530 mm emission, using a Perkin–Elmer LS5 spectrophotometer.

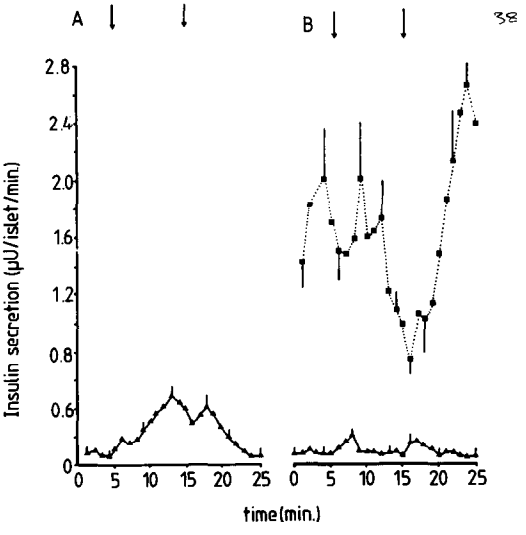

Fig. 3. Insulin secretion from perifused islets. Effects of 1 mM amiloride (a) and 10 mM NH_4Cl (b) in the presence of either 2.5 mM (▲——▲) or 20 mM (■---■) glucose. Islets were exposed to test agents between the 5th and 15th minute of perifusion. Each point represents the mean ± SEM of 4–6 determinations.

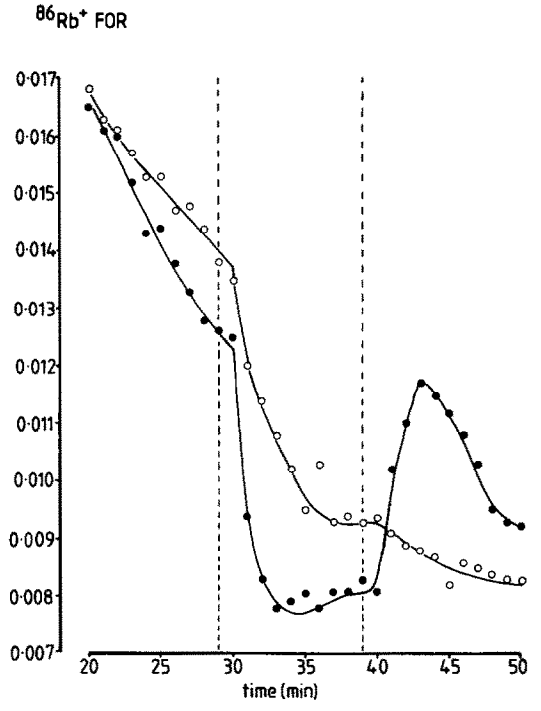

Fig. 4. Fractional outflow rate (FOR) of $^{86}Rb^+$ from perifused islets, exposed to either 40 mM sodium propionate (●) or 1 mM amiloride (○) between the 29th and 39th minute of perifusion. The glucose concentration was 2.5 mM.

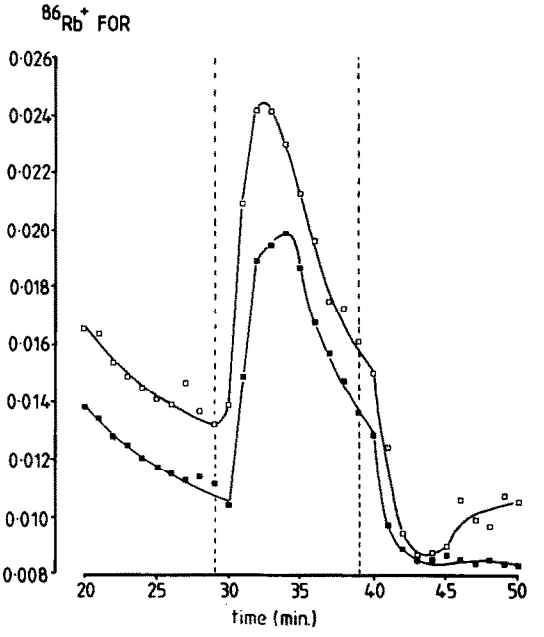

Fig. 5. Fractional outflow rate (FOR) or $^{86}Rb^+$ from perifused islets either in the preence (□) or absence (■) of calcium. Islets were exposed to 10 mM NH_4Cl between the 29th and 39th minute of perifusion. The glucose concentration was 2.5 mM.

release occurred upon removal of amiloride from the perifusate. Exposure of perifused islets to NH_4Cl produced a reversible inhibition of glucose-induced insulin release (Fig. 3b). At lower glucose concentrations, this inhibition was preceded by a transient stimulation of secretion.

Intracellular acidification, either in response to sodium propionate or amiloride treatment, was associated with an immediate reduction in the rate of efflux of $^{86}Rb^+$ (Fig. 4) and of $^{45}Ca^{2+}$ (Fig. 6). Sodium acetate evoked similar responses to those seen with propionate (not shown). The subsequent withdrawal of the weak acid prompted a marked "rebound" stimulation of efflux of these isotopes, which was not observed upon withdrawal of amiloride.

Exposure of islets to NH_4Cl resulted in a pronounced stimulation of efflux of $^{86}Rb^+$ (Fig. 5) and $^{45}Ca^{2+}$ (Fig. 7). Removal of NH_4Cl from the perifusate caused a subsequent reduction in the rate of efflux of these isotopes. The stimulatory effects of NH_4Cl on cationic fluxes did not require the presence of Ca^{2+} in the incubation medium, although the stimulation of $^{45}Ca^{2+}$ efflux by NH_4Cl was greatly impaired in the absence of sodium from the medium (Fig. 7). Essentially similar results were observed in the presence or absence of 2.5 mM glucose.

DISCUSSION

Changes in pH_i are one possible mechanism by which the oxidative metabolism of glucose and other

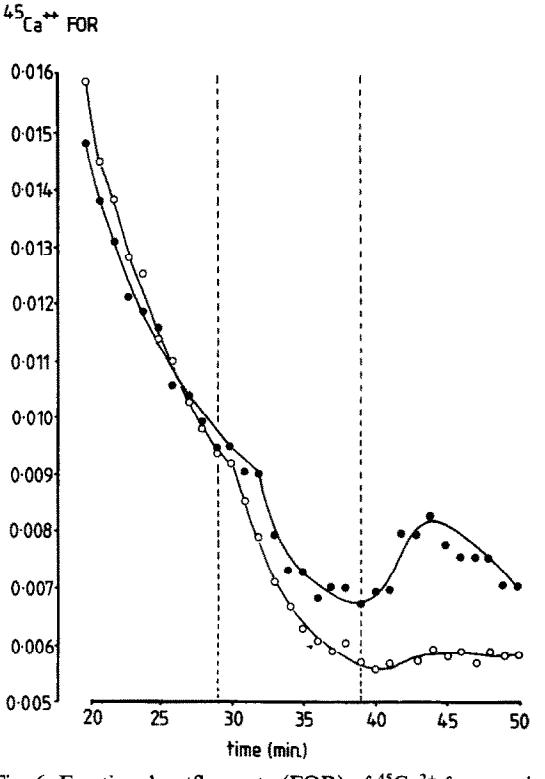

Fig. 6. Fractional outflow rate (FOR) of $^{45}Ca^{2+}$ from perifused islets, exposed to either 40 mM sodium propionate (●) or 1 mM amiloride (○) between the 29th and 39th minute of perifusion. The glucose concentration was 2.5 mM.

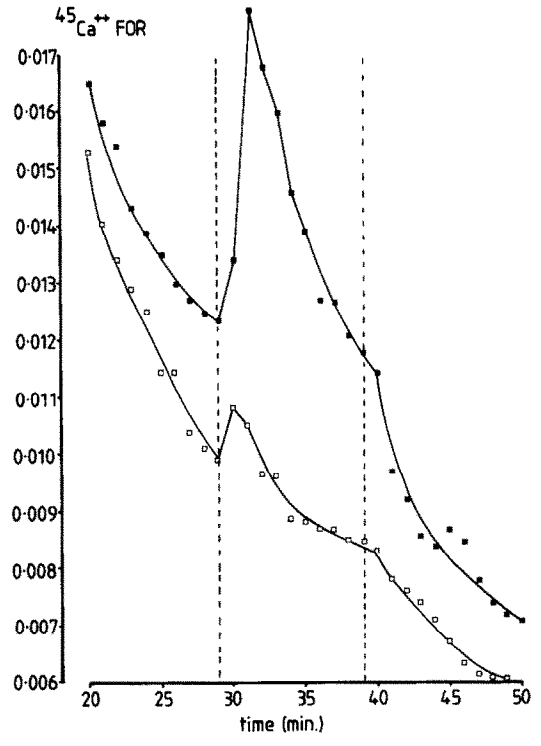

Fig. 7. Fractional outflow rate (FOR) of $^{45}Ca^{2+}$ from perifused islets in normal medium containing NaCl (■) or with sodium substituted by sucrose (□). Islets were exposed to 10 mM NH_4Cl between the 29th and 39th minute of perifusion. The glucose concentration was 2.5 mM.

nutrients in islet cells might be coupled to altered ionic fluxes thought to be a prerequisite for the secretion of insulin. We have directly monitored pH_i in islet cells and examined the effects of manipulating pH_i upon the handling of $^{86}Rb^+$ and $^{45}Ca^{2+}$ and on insulin release. It has been demonstrated that treatment of islet cells with the sodium salts of weak acids causes an immediate but transient fall in pH_i. Amiloride, an inhibitor of Na^+/H^+ exchange, also resulted in a reduction in pH_i, although no subsequent recovery occurred in this case, presumably reflecting the importance of this exchange system in the maintenance of pH_i. The observation that NH_4Cl resulted in a sustained rise in pH_i suggests that the islet cells do not have an efficient mechanism for the correction of intracellular alkalinization.

Intracellular acidification of perifused islets with either weak acids or amiloride was associated with a rapid enhancement of insulin secretion in the presence of a stimulatory glucose concentration. Amiloride, unlike weak acid treatment, was also found to be effective at substimulatory glucose concentrations. The reasons for this difference are not known, but could be related to the transience of the fall in pH_i in response to weak acids. In addition, it has been reported that amiloride is able to inhibit Na^+/Ca^{2+} exchange in rat brain [14], an action which would result in a rise in cytosolic calcium and thus contribute further toward an enhancement of insulin secretion.

The potentiation of insulin secretion by a lowering of pH_i supports the previous observations of Pace [6] and Lebrun *et al.* [3] but is inconsistent with the findings of Lindstrom and Sehlin [10] who found no stimulation of secretion following intracellular acidification of mouse islets. Indeed, the latter reported enhanced insulin release when pH_i was raised by NH_4Cl treatment. It was therefore of interest that, in the present study, NH_4Cl produced an immediate, transient potentiation of glucose-induced insulin secretion prior to the inhibition which has been previously observed under static incubation conditions [8, 15]. Whether this initial, transient potentiation of secretion is an early result of intracellular alkalinization or some other action of NH_4Cl is not known, but this biphasic effect could, in part, explain some of the conflicting studies of the involvement of pH_i in islet function. An initial stimulation of secretion upon intracellular alkalinization could also explain an unexpected finding in the present secretion studies; namely the marked but paradoxical increase in secretion upon withdrawal of weak acid from the perifusate, which could also possibly result in intracellular alkalinization.

The influence of altered pH_i on insulin release was closely associated with opposing actions upon the efflux of $^{86}Rb^+$ and $^{45}Ca^{2+}$. Thus, a fall in pH_i resulted in reduced rates of efflux of both isotopes, whilst NH_4Cl and to a lesser extent removal of weak acid, produced a marked enhancement in efflux.

These effects of pH_i on $^{86}Rb^+$ and $^{45}Ca^{2+}$ efflux could be causally related or could represent independent actions of protons in the cell. It appears unlikely that the influence of pH_i on $^{86}Rb^+$ efflux is secondary to altered calcium mobilization, since NH_4Cl-stimulated efflux was unaffected in the absence of calcium from the medium. Similarly, the stimulation of $^{45}Ca^{2+}$ efflux by NH_4Cl persisted in calcium-free conditions, again suggesting that this increase in efflux rate was not a reflection of $^{40}Ca^{2+}$ entry into the islet cells. Furthermore, the depolarisation likely to result from reduced $^{86}Rb^+$ efflux in response to a fall in pH_i would not explain reduced $^{45}Ca^{2+}$ efflux.

The simplest explanation for the present observations is that intracellular protons exert two separate actions, namely inhibition of potassium (Rb^+) efflux as suggested by previous studies [4, 16], and inhibition of calcium efflux. It seems likely that the latter action involves an inhibition of Na^+/Ca^{2+} exchange, as proposed by Malaisse and colleagues [1], since NH_4Cl-stimulated $^{45}Ca^{2+}$ efflux was markedly impaired in the absence of sodium from the medium. Both of these actions would be predicted to result in an increase in cytosolic $[Ca^{2+}]$, and could thus explain the enhancement of insulin secretion which accompanies a reduction in pH_i.

A number of authors have proposed that the stimulation of secretion evoked by nutrient stimuli such as glucose could be due, at least in part, to the production of H^+ as a result of oxidation of that nutrient [1, 3, 6]. The measured effects of nutrients on islet cell pH_i in the present study do not, however, support this hypothesis. Of the three nutrients examined, only glyceraldehyde produced an intracellular acidification. In contrast, glucose and α-ketoiso-

caproate resulted in a small apparent rise in pH_i. Such an effect of glucose was also detected, using fluorescein diacetate, by Deleers *et al.* [17] who suggested that a stimulation of Na^+/H^+ exchange by glucose masked the predicted fall in pH_i. In any case, the apparent increase in pH_i elicited by glucose is unlikely to account for the stimulation of insulin release evoked by the sugar, judging from the secretory response to NH_4Cl which produced a more marked rise in pH_i. Nevertheless, it is clear that changes in pH_i can indeed modify cationic fluxes and hence the secretory activity of pancreatic islets, although whether certain types of agonist regulate insulin release, or other aspects of islet function, via altered pH_i remains to be established.

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