Activation of insulin-secreting cells by pyruvate and halogenated derivatives

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Addition of pyruvate to rat islets perfused in the presence of 5 mM-glucose elicited an immediate pronounced biphasic stimulation of insulin secretion. At lower concentrations of glucose (2.5 mM), only the initial, transient, phase of secretion was observed. Pyruvate inhibited $^{45}\text{Ca}^{2+}$ efflux from islets at 2.5 mM-glucose and stimulated efflux at 5 mM-glucose. Pyruvate also decreased the rate of efflux of $^{86}\text{Rb}^{+}$ from perfused islets. A marked stimulation of insulin secretion and $^{45}\text{Ca}^{2+}$ efflux rate was observed in response to 3-fluoropyruvate and 3-bromopyruvate, compounds which inhibited oxidative metabolism of [^{14}C]glucose and [^{14}C]pyruvate in islets. The stimulatory effects of 3-fluoro- and 3-bromo-pyruvate were associated with enhanced $^{86}\text{Rb}^{+}$ efflux. Withdrawal of pyruvate or halogenated analogues from the perfusate resulted in a secondary stimulation of insulin release, $^{45}\text{Ca}^{2+}$ efflux and, to some extent, $^{86}\text{Rb}^{+}$ efflux rates. Pyruvate, 3-fluoropyruvate and 3-bromopyruvate were all effective in promoting intracellular acidification and a rise in cytosolic Ca^{2+} concentration, as judged from fluorescence measurements in HIT-T15 cells loaded with 2',7'-biscarboxyethyl-5'(6')-carboxyfluorescein and Quin 2 respectively. It is proposed that oxidative metabolism of pyruvate is not a prerequisite for its stimulatory actions on pancreatic β -cells. An alternative mechanism of activation by pyruvate and its halogenated derivatives is proposed, based on the possible electrogenic flux of these anions across the cell membrane.

INTRODUCTION

It is generally accepted that the ability of glucose and certain other nutrients to initiate the secretion of insulin is dependent on the metabolism of that nutrient in the islet β -cell (Malaisse *et al.*, 1979; Ashcroft, 1980; Täljedal, 1981), although the precise nature of the coupling mechanism between nutrient metabolism and exocytosis remains uncertain. For hexoses and some α -oxo acids, there appears to be a close correlation between oxidative metabolism and secretory activity (Malaisse *et al.*, 1979; Hutton *et al.*, 1980), suggesting that a factor derived from mitochondrial metabolism could be involved in stimulus-secretion coupling. The demonstration of an adenine-nucleotide-regulated K^{+} channel in the plasma membrane of β -cells (Cook & Hales, 1984; Rorsman & Trube, 1985) suggested that a rise in cellular ATP concentration, resulting from oxidative metabolism, could be responsible for depolarization and enhanced secretory activity (Henquin, 1987; Cook *et al.*, 1988).

In view of this proposed role for mitochondrial metabolism in the control of insulin-secretory activity, it is at first surprising that pyruvate is a relatively poor secretagogue (Coore & Randle, 1967; Montague & Taylor, 1969), despite being well metabolized by islet cells (Sener *et al.*, 1978; Lenzen & Panten, 1981; Best & Tomlinson, 1988). In order to explain this discrepancy, Sener *et al.* (1978) pointed out that pyruvate, unlike glucose, impaired the rate of oxidation of endogenous nutrients in islet cells. However, the reasons for this effect of pyruvate, and whether it fully accounts for the fact that pyruvate stimulates secretion in the presence, but not in the absence, of glucose, are unclear.

The results of the present study suggest that the insulinotropic action of pyruvate might not entirely depend on its mitochondrial oxidation, but could result from depolarization of the β -cell plasma membrane via an additional mechanism.

MATERIALS AND METHODS

Pancreatic islets were isolated from fed adult rats by collagenase digestion (Lacy & Kostianovsky, 1967). Insulin secretion, $^{45}\text{Ca}^{2+}$ and $^{86}\text{Rb}^{+}$ fractional outflow rates were measured essentially as described by Best *et al.* (1989). Previously published methods were also used for measuring oxidation of [$\text{U-}^{14}\text{C}$]glucose and [$2\text{-}^{14}\text{C}$]pyruvate to $^{14}\text{CO}_2$ (Best & Tomlinson, 1988).

Cytosolic pH and [Ca^{2+}] were monitored in stirred suspensions of insulinoma (HIT-T15) cells loaded with the fluorescent dyes BCECF (Lynch *et al.*, 1989) or Quin 2 (Meats *et al.*, 1989). Briefly, cells were incubated at 37 °C for 15 min with 4 μM -BCECF-AM (acetomethoxy ester) or for 60 min with 20 μM -Quin 2-AM, then washed and resuspended at a density of 4×10^6 cells/ml. A 2.5 ml sample of this cell suspension was placed in a quartz cuvette and stirred at 37 °C in a Perkin-Elmer LS5B luminescence spectrophotometer.

Where sodium salts of pyruvate or other acids were added, the Na^{+} content of the medium was correspondingly decreased.

^{125}I -insulin, $^{45}\text{CaCl}_2$ and $^{86}\text{RbCl}$ were obtained from Amersham International, and [$2\text{-}^{14}\text{C}$]pyruvate was from New England Nuclear. Collagenase (*Clostridium histolyticum*) was supplied by Cambridge Biosciences, Cam-

Abbreviation used: BCECF, 2',7'-biscarboxyethyl-5'(6')-carboxyfluorescein.

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Fig. 1. Effects of pyruvate (a), 3-fluoropyruvate (b) and 3-bromopyruvate (c) on insulin secretion from rat pancreatic islets

Groups of 30 islets were perfused in the presence of either 2.5 mM- (●) or 5 mM- (○, □, △) glucose and exposed to pyruvate or halogenated derivative during the period designated by the vertical broken lines. Each point represents the mean $\pm$ S.E.M. of 4–5 determinations.

bridge, U.K., BCECF-AM was from Calbiochem, San Diego, CA, U.S.A., and the sodium salts of pyruvate, 3-fluoropyruvate and 3-bromopyruvate were from Sigma Chemical Co., Poole, Dorset, U.K.

RESULTS

The addition of pyruvate (20 mM) to perfused rat islets resulted in an immediate stimulation of insulin release (Fig. 1a). In the presence of a threshold concentration of glucose (5 mM), a pronounced biphasic secretory response was observed, whereas at a lower glucose concentration pyruvate exerted only a modest, transient, effect. A marked potentiation of insulin secretion was also observed in response to 3-fluoropyruvate and 3-bromopyruvate (both 20 mM; Figs. 1b and 1c), these effects again being dependent on the presence of 5 mM-glucose. In all three cases, the withdrawal of the acid from the perfusion medium produced a subsequent transient increase in secretory rate ('off' effect).

The effects of the three agents upon the fractional outflow rate of $^{45}\text{Ca}^{2+}$ are shown in Fig. 2. In the presence of 2.5 mM-glucose, pyruvate caused a modest decrease in $^{45}\text{Ca}^{2+}$ efflux rate (Fig. 1a). At a higher threshold glucose concentration (5 mM), pyruvate produced an initial decrease, followed by a pronounced rise, in $^{45}\text{Ca}^{2+}$ outflow. In both cases, removal of pyruvate

Fig. 2. Effects of pyruvate (a), 3-fluoropyruvate (b) and 3-bromopyruvate (c) on the fractional outflow rate (FOR) of $^{45}\text{Ca}^{2+}$ from pre-loaded perfused rat pancreatic islets

Groups of 100 islets were perfused in the presence of 2.5 mM- (●) or 5 mM- (○, □, △) glucose and exposed to pyruvate or halogenated derivative during the period designated by the vertical broken lines. Each point represents the mean $\pm$ S.E.M. of 3–4 determinations.

from the medium was associated with a small rebound stimulation of $^{45}\text{Ca}^{2+}$ efflux. Exposure of islets to 3-fluoropyruvate or 3-bromopyruvate in the presence of 5 mM-glucose was also found to result in a stimulation of $^{45}\text{Ca}^{2+}$ efflux (Figs. 2b and 2c), the 'off' effect being particularly prominent with the bromo derivative.

The stimulation of islets with pyruvate resulted in a decreased rate of $^{86}\text{Rb}^{+}$ efflux (Fig. 3a). This effect was also dependent on the ambient glucose concentration, it being less pronounced at 5 mM-glucose. Treatment of islets with either 3-fluoropyruvate or 3-bromopyruvate produced a marked increase, rather than a fall, in $^{86}\text{Rb}^{+}$ outflow rate (Fig. 3b).

Both 3-fluoropyruvate and 3-bromopyruvate were found to inhibit the oxidation by islets of $[2-^{14}\text{C}]$ pyruvate (5 mM) to $^{14}\text{CO}_2$ (Table 1). In addition, preincubation of islets for 20 min with 20 mM-3-fluoropyruvate or 3-bromopyruvate, followed by washing, resulted in a subsequent inhibition of $[U-^{14}\text{C}]$ glucose oxidation (Table 1).

The effects of pyruvate and 3-fluoropyruvate on cytosolic pH and $[\text{Ca}^{2+}]$ in cultured HIT-T15 insulinoma cells

Fig. 3. Effects of pyruvate (a), 3-fluoropyruvate ($\square$) and 3-bromopyruvate ($\triangle$) (b) on the fractional outflow rate (FOR) of $^{86}\text{Rb}^+$ from pre-loaded perfused rat pancreatic islets

Groups of 100 islets were perfused in the presence of 2.5 mM- ($\bullet$) or 5 mM- ($\circ$, $\square$, $\triangle$) glucose and exposed to pyruvate or halogenated derivative during the period designated by the vertical broken lines. Each point represents the mean $\pm$ S.E.M. of 4 determinations.

are illustrated in Fig. 4. Both agents caused a marked intracellular acidification, presumably as a result of proton co-transport or diffusion of the undissociated acid, together with a rise in cytosolic $[\text{Ca}^{2+}]$. The fall in cytosolic pH was followed by recovery and subsequent 'overshoot' to more alkaline values. This recovery was

prevented by amiloride (Fig. 4), suggesting that it occurred by a process of Na^+/H^+ exchange (Lynch *et al.*, 1989). 3-Bromopyruvate had qualitatively similar effects (results not shown), although reliable calibration could not be obtained in this case, owing to some interference by this compound with the fluorescence recordings.

DISCUSSION

The results of the present study confirm previous observations that pyruvate can stimulate insulin release from perfused pancreatic islets, this effect being dependent on the glucose concentration of the medium (Sener *et al.*, 1978). Similarly, the effects of pyruvate on $^{45}\text{Ca}^{2+}$ efflux were critically dependent on concentration of the hexose (Sener *et al.*, 1978; the present study). Since islet cells have a high capacity for oxidizing pyruvate, even compared with glucose (Sener *et al.*, 1978; Lenzen & Panten, 1981; Best & Tomlinson, 1988), the poor correlation between oxidative flux and secretion questions whether these processes are causatively related. The present finding that the stimulatory effects of pyruvate could be largely mimicked by comparable concentrations of 3-fluoropyruvate and 3-bromopyruvate, compounds which, presumably by inactivating pyruvate dehydrogenase (Apfel *et al.*, 1984; Flournoy & Frey, 1986), were found to impair the mitochondrial metabolism of pyruvate and glucose in islet tissue, makes it further probable that an alternative mechanism is responsible for the insulinotropic actions of these compounds.

One possibility is that the intracellular acidification resulting from exposure to pyruvate or the halogenated derivatives may in some way enhance secretion. In support of this idea, it has been demonstrated that agents which lower islet-cell pH_i also potentiate electrical activity and insulin release (Malaisse *et al.*, 1980; Pace *et al.*, 1983; Best *et al.*, 1988; Lynch *et al.*, 1989), possibly by restricting Ca^{2+} outflow from islet cells (Malaisse *et al.*, 1980; Best *et al.*, 1988). Indeed, at low concentrations of glucose, pyruvate did cause a diminution in the rate of $^{45}\text{Ca}^{2+}$ efflux (Sener *et al.*, 1978; the present paper). However, at higher concentrations of glucose, a subsequent increase in $^{45}\text{Ca}^{2+}$ outflow rate, probably reflecting increased $^{40}\text{Ca}^{2+}$ uptake, was observed, which was clearly not the result of intracellular acidification,

Table 1. Effects of 3-fluoropyruvate and 3-bromopyruvate on the oxidation of pyruvate and glucose in rat pancreatic islets

(a) Groups of 50 islets were incubated for 90 min with $[2\text{-}^{14}\text{C}]$ pyruvate (5 mM) in the presence or absence of halogenated derivative (2 mM). (b) Groups of 50 islets were preincubated for 20 min in the presence of pyruvate or one of the halogenated derivatives (all 20 mM), then washed and incubated for a further 90 min with $[\text{U-}^{14}\text{C}]$ glucose (5 mM). All values are means $\pm$ S.E.M. of three determinations: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Conditions	Substrate oxidation (pmol/90 min per 50 islets)
(a) Pyruvate oxidation	
1. Pyruvate	579 $\pm$ 55
2. Pyruvate + 3-fluoropyruvate	180 $\pm$ 14 (** versus line 1)
3. Pyruvate + 3-bromopyruvate	135 $\pm$ 17 (***) versus line 1)
(b) Glucose oxidation	
4. Pyruvate-preincubated	555 $\pm$ 80
5. 3-Fluoropyruvate-preincubated	230 $\pm$ 35 (* versus line 4)
6. 3-Bromopyruvate-preincubated	160 $\pm$ 20 (** versus line 4)

Fig. 4. Effects of pyruvate and 3-fluoropyruvate on cytosolic pH (pH_i) and $[Ca^{2+}]$ in HIT-T15 cells loaded with BCECF or Quin 2

See the Materials and methods section for experimental details.

since it was not observed upon treatment with weak acids (Best *et al.*, 1988).

The present study has demonstrated an inhibition of K^+ ($^{86}Rb^+$) efflux by pyruvate, an effect which could theoretically result either from increased islet-cell ATP concentrations and subsequent closure of ATP-sensitive K^+ channels (Cook *et al.*, 1988) or from an H^+ -mediated inhibition of Ca^{2+} -sensitive K^+ channels (Cook *et al.*, 1984). Such a decrease in membrane K^+ permeability would be expected to cause depolarization, which would be consistent with a potentiation of secretory activity. However, the fact that an inhibitory effect of pyruvate on $^{86}Rb^+$ efflux was much less apparent at 5 mM-glucose, where the insulinotropic effect was maximal, is not fully consistent with this mechanism. More strikingly, the secretory responses to fluoropyruvate and bromopyruvate were associated with a marked increase, rather than a decrease, in $^{86}Rb^+$ outflow, again suggesting an additional mechanism to decrease K^+ permeability. It is uncertain how the halogenated compounds induce this increase in K^+ permeability, although one possibility is impaired oxidative metabolism, decreased islet-cell ATP levels and consequent opening of ATP-sensitive K^+ channels. Alternatively, inhibition of mitochondrial metabolism could result in loss of Ca^{2+} from the mitochondria into the cytosol, and hence activation of Ca^{2+} -sensitive K^+ channels. Either mechanism could also explain why the secretory responses to the halogenated derivatives

are rather less pronounced and prolonged than those to pyruvate.

The exact mechanism whereby pyruvate, fluoropyruvate and bromopyruvate promote Ca^{2+} mobilization and potentiate glucose-induced insulin secretion is not entirely clear. However, a model with which the present findings are entirely consistent is based on that previously proposed to explain the stimulation of islet-cell function elicited by the addition, and by the subsequent withdrawal, of lactate (Best *et al.*, 1989; Meats *et al.*, 1989). It is suggested that the islet β -cell membrane could be permeable to lactate ions, such that the outward movement of lactate constitutes an electrogenic mechanism which could lead to cell membrane depolarization.

Thus, for pyruvate and its analogues, we propose that the compound enters the cell in conjunction with a proton, resulting in the observed intracellular acidification. Recovery from this acidification is prevented by amiloride, suggesting that it occurs largely through operation of the electroneutral Na^+/H^+ exchange system. It is likely that a certain proportion of this pyruvate will be reduced to lactate, an active pathway in islet cells (Sener *et al.*, 1978; Best *et al.*, 1989). The subsequent exit from the cell of lactate or pyruvate, in anionic form, would represent a net loss of negative charge which could be responsible for depolarization of the plasma membrane. The relative contributions of lactate and pyruvate efflux to such a depolarization would clearly depend on several factors, including the permeability of the plasma membrane to these anionic species and their relative intracellular concentrations. Such considerations could explain the quantitative differences observed between the actions of pyruvate, 3-fluoropyruvate and 3-bromopyruvate. Detailed studies of the transport and metabolism of the halogenated derivatives should help elucidate this point.

The model for pyruvate activation of islet cells described above would account for a number of additional observations regarding the effects of pyruvate on islet-cell function. For example, it is likely that the presence of pyruvate in the incubation medium restricts to a large extent the exit of lactate (or pyruvate) from the cell, thus limiting the degree of depolarization. This would explain why pyruvate, although well metabolized, is a poor secretagogue and is only effective in the presence of threshold concentrations of glucose. It would also explain the stimulatory effect observed upon withdrawal of pyruvate from the medium, which would permit a transient acceleration of lactate or pyruvate efflux from the cell.

Clearly, the proposed model for the activation of insulin-secreting cells by pyruvate and its analogues depends on the presence of an electrogenic pathway for the flux of such organic anions across the plasma membrane, for which there is, as yet, no direct evidence. However, it is noteworthy that there is indirect evidence for anionic transport pathways in liver and striated muscle (Edlund & Halestrap, 1988). Further work is required to investigate fully the importance of anionic fluxes in the coupling of nutrient metabolism to stimulated insulin secretion.

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