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Mechanism of 3-phenylpyruvate-induced insulin release from isolated pancreatic islets

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3-Phenylpyruvate evoked a monophasic insulin release from perifused mouse islets. L-Phenylalanine was not an insulin secretagogue and was oxidized by islets at a very low rate, suggesting that 3-phenylpyruvate does not trigger insulin release by enhancing production of reducing equivalents. Moreover, allosteric activation of glutamate dehydrogenase does not play a role in 3-phenylpyruvate-induced insulin secretion.

The fuel hypothesis [an analogous term for the substrate site model of Randle *et al.* (1968)] assumes that glucose and other nutrients are recognized as insulin-releasing signals as a result of their metabolism in the pancreatic B-cells (Malaisse *et al.*, 1979). The latter authors reject the existence of specific fuel receptors, presumably located at the B-cell plasma membrane (Cerasi & Luft, 1970; Matschinsky *et al.*, 1975). The insulin secretory potency of the non-metabolizable leucine analogue (−)-BCH was a major argument against the fuel hypothesis (Christensen *et al.*, 1971). However, we have summarized evidence which invalidates the use of (−)-BCH as a tool for deciding between the fuel and membrane-receptor hypotheses: (−)-BCH may enhance intramitochondrial hydrogen production from endogenous fuels by activation of glutamate dehydrogenase (Panten *et al.*, 1980). This view was supported by data from Sener & Malaisse (1980).

In the present paper we tested the fuel hypothesis with 3-phenylpyruvate. This 2-oxocarboxylic acid was reported to trigger insulin release in the absence of other fuels (Matschinsky *et al.*, 1975). Oxidative decarboxylation of 3-phenylpyruvate by islet cells yielded only minor amounts of reducing equivalents (Lenzen & Panten, 1981). The possibility remains, however, that metabolism of 3-phenylpyruvate via phenylalanine supplies islet cells with considerable amounts of reducing equivalents. Therefore we compared the insulin-releasing effects of 3-phenylpyruvate and L-phenylalanine, and measured uptake and oxidation of L-phenylalanine by isolated pancreatic islets. In addition, we investigated whether allosteric activation of glutamate dehydrogenase plays a role in 3-phenylpyruvate-induced insulin secretion.

Abbreviations used: BCH, 2-*endo*-aminonorbornane-2-carboxylic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

Experimental

Chemicals and media

The following substances were used: sodium 3-phenylpyruvate, sodium 2-oxoglutarate, L-norvaline, L-valine, L-phenyl-lactic acid, *o*-hydroxyphenylacetic acid, phenylacetyl-CoA and Hepes from Sigma, St. Louis, MO, U.S.A.; L-phenylalanine, L-norleucine and phenylacetic acid from Fluka, Buchs, Switzerland; L-isoleucine, L-leucine, sucrose and bovine serum albumin (fraction V) from Serva, Heidelberg, Germany; NAD^+ and NADH from Boehringer, Mannheim, Germany; crystalline mouse insulin from Novo, Bagsvaerd, Denmark; collagenase (type IV) from Worthington, Freehold, NJ, U.S.A.; Aqualuma from Lumac, Meise, Belgium; ^{125}I-labelled bovine insulin from Behringwerke, Frankfurt, Germany; L-[1-^{14}C]leucine, L-[U-^{14}C]phenylalanine and [6,6′-^{3}H]sucrose from The Radiochemical Centre, Amersham, Bucks., U.K.; silicone oils AR 20 and AR 200 from Wacker, Munich, Germany. (±)-BCH was synthesized by running the Bucherer–Lieb reaction in the cold (Tager & Christensen, 1972); 99% of the final dry residue was *endo*-isomer. All other reagents were analytical grade from Merck, Darmstadt, Germany.

Basal medium for perifusions or incubations was a modified Krebs–Ringer solution (Krebs & Henseleit, 1932) containing 20mM-$NaHCO_3$, 10mM-Hepes, 0.2% (w/v) albumin, and NaOH to give pH 7.4 after equilibration with O_2/CO_2 (19:1) at 37°C.

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Isolation, perifusion and incubation of islets

Pancreatic islets were isolated from fed male albino mice (NMRI, 11–15 weeks old) by collagenase digestion (Lernmark, 1974) in basal medium supplemented with 4 mM-D-glucose. Batches of 50 islets were perifused at 0.9 ml/min at 37°C as described by Panten *et al.* (1977). No corrections for the dead space of the system (about 0.4 ml) or the sodium content of 3-phenylpyruvate were made. Insulin was immunoassayed with mouse insulin as reference, by using $(NH_4)_2SO_4$ to precipitate the antibody-bound insulin (Joost, 1979).

Batches of 40 islets were preincubated and incubated at 37°C in 40 μl of medium as described by Panten *et al.* (1980). $^{14}CO_2$ production from L-[1-^{14}C]leucine (10 mM; 1.4 Ci/mol) or L-[U-^{14}C]-phenylalanine (0.5–10 mM; 48 to 3 Ci/mol) was determined as described by Holze & Panten (1979). For each test incubation a blank value was obtained by incubating the medium without islets. The radioactivity-counting error was below 2% and corrections for incomplete recovery of $^{14}CO_2$ were made.

For uptake studies the media contained L-[1-^{14}C]leucine (10 mM; 2.4 Ci/mol) or L-[U-^{14}C]-phenylalanine (1 or 10 mM; 24 or 2.4 Ci/mol) besides [^{3}H]sucrose (1 mM; 24 Ci/mol). Incubations were stopped by pipetting the medium together with the islets on top of 200 μl of silicone oil (sp.gr. 1.025, layered over 20 μl of 2 M-NaOH in a Beckman Microfuge tube) and sedimenting the islets into the NaOH (30 s at 10000 *g*). The tubes were cut on a level with the lower third of the oil layer, and the islets were dissolved by heating for 30 min at 60°C. Then the NaOH with the adjacent oil was transferred to counting vessels containing 1 ml of 0.1 M-NaOH, and 5 ml of Aqualuma was added to each. Over 90% of the radioactivity in the tip of each tube was transferred. After mixing, the ^{3}H and ^{14}C contents were measured by dual-channel liquid-scintillation counting. The intracellular amino acid content was calculated after correction for the extracellular contamination indicated by [^{3}H]-sucrose.

The DNA content of islets as prepared in the present study was measured as described by Beckmann *et al.* (1981). The total content was 0.77 ± 0.07 μg of DNA/40 islets (mean ± S.E.M.; $n = 9$).

Glutamate dehydrogenase activity

The enzyme was assayed as described by Hutton *et al.* (1979) with minor modifications. Groups of 100 islets were homogenized in 1 ml of distilled water and stored frozen up to 1 week. Then 50 μl of the homogenate was mixed with 50 μl of reaction mixture (pH 8.0; 100 mM-ammonium acetate, 14 mM-2-oxoglutarate, 0.2 mM-NADH, no ADP) and incubated for 30 min at 37°C.

Effects of test substances are given as relative enzyme activities because in each separate incubation series controls without test compound were included.

Presentation of results

Results are presented as means ± S.E.M. for independent experiments. Significances were calculated by two-tailed non-parametric tests. A P value below the 5% level was considered significant. Paired or unpaired comparisons were performed by Wilcoxon's matched-pairs signed rank test or the U-test of Wilcoxon, Mann and Whitney.

Results and discussion

As reported previously (Matschinsky *et al.*, 1975; Lenzen & Panten, 1981), 3-phenylpyruvate triggered insulin release in the absence of any other fuel (Fig. 1). This effect reflected true exocytosis because it was suppressed by omission of Ca^{2+} from the media (results not shown). We could not confirm, however, that 3-phenylpyruvate sustains a typical second phase of insulin release (Matschinsky *et al.*, 1975). A non-stimulatory glucose concentration (5 mM) enhanced the total amount of insulin released by 10 mM-3-phenylpyruvate (Fig. 1; $P < 0{,}01$). But again insulin release decreased to rather low rates during exposure to the 2-oxocarboxylic acid.

The failure of 3-phenylpyruvate to induce a distinct second phase of insulin secretion could be due to a poor fuel function of 3-phenylpyruvate. This view was supported by the lack of insulin-releasing capacity of L-phenylalanine (Fig. 1) and the very low oxidation rate of L-phenylalanine (0.5–10 mM) in pancreatic islets, though this major metabolite was taken up by islet cells (Table 1). $^{14}CO_2$ production from L-[U-^{14}C]phenylalanine by islets was barely detectable. Counting times up to 100 min were necessary to secure in each single experiment amounts of $^{14}CO_2$ higher than the corresponding blank. When using the oxidation of L-leucine as an indicator of oxidative capacity, L-phenylalanine is oxidized even less than L-arginine, which is not a fuel for islet cells (Table 1; Hellman *et al.*, 1971). The very low rates of $^{14}CO_2$ production from L-[U-^{14}C]phenylalanine in the present study may reflect exclusively oxidative decarboxylation of 3-phenylpyruvate. The results are consistent with the view that, like other tissues, islet cells lack phenylalanine hydroxylase (McGee *et al.*, 1972).

The demonstration that L-phenylalanine is not a fuel for islet cells, together with the low decarboxylation rate of 3-phenylpyruvate (Lenzen & Panten, 1981), rule out the possibility that reducing equivalents derived from degradation of 3-phenylpyruvate might trigger insulin release. Therefore other mechanisms must reconcile the fuel hypothesis with the

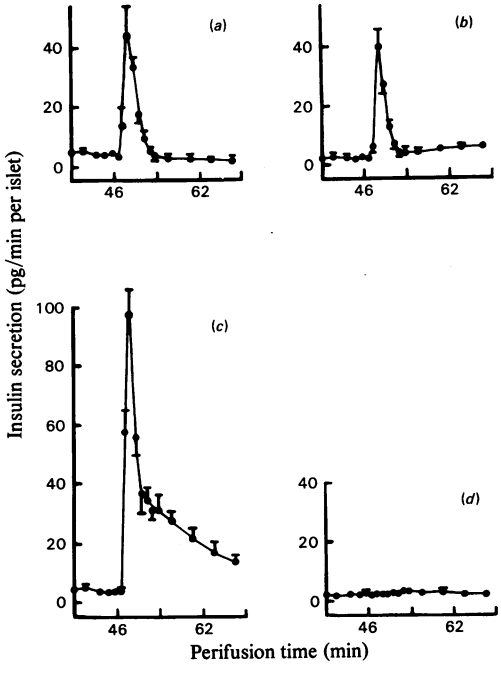

Fig. 1. *Effects of 3-phenylpyruvate, L-phenylalanine or BCH on insulin secretion from perifused pancreatic islets*
Insulin release was measured at 1–4 min intervals as described in the Experimental section. Values shown are means ± S.E.M. for results from five or six separate experiments. For (*a*) and (*b*) media contained no substrate except the test compounds; for (*c*) and (*d*) all media contained 5 mM-D-glucose. At 46 min the distribution valve of the system was switched to media with the following additions: (*a*) 10 mM-3-phenylpyruvate; (*b*) 20 mM-(±)-BCH; (*c*) 10 mM-3-phenylpyruvate; (*d*) 10 mM-L-phenylalanine. As compared with the preceding 8 min control period, insulin secretion was always higher ($P < 0.01$) during the first 8 min of exposure to (±)-BCH or 3-phenylpyruvate.

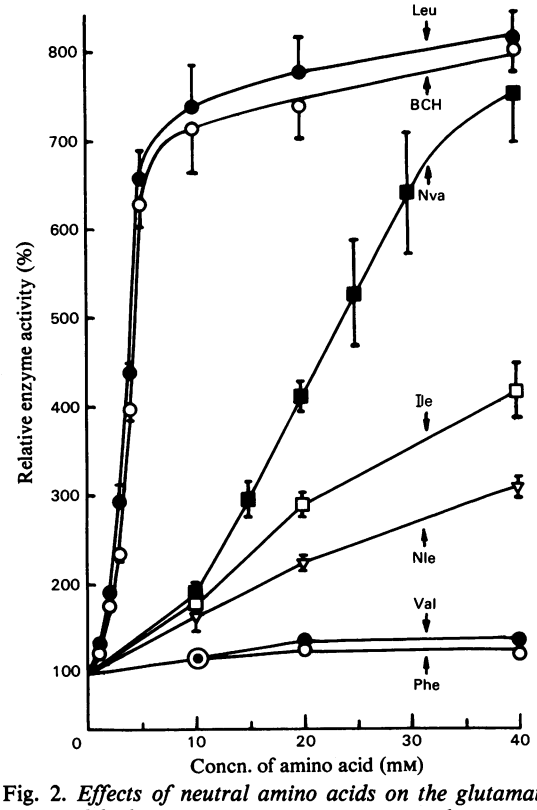

Fig. 2. *Effects of neutral amino acids on the glutamate dehydrogenase activity in pancreatic islets*
Islet homogenates were prepared and assayed as described in the Experimental section. The reaction mixture contained amino acids to give the indicated concentrations in the final incubations. The concentration of the (−) isomer of the (±)-BCH used is given. Values shown are means ± S.E.M. for results from six to ten separate experiments. The 100% value represents the enzyme activities recorded in the absence of test compound. This control activity was 240 ± 10 pmol/h per islet ($n = 45$).

Table 1. *Uptake and oxidation of phenylalanine or leucine by pancreatic islets*
Islets from fed mice were preincubated for 45 min without substrates or with 5 mM-glucose and then incubated for 30 (uptake studies) or 60 min (oxidation studies) in media containing in addition L-[U-^{14}C]phenylalanine or L-[1-^{14}C]-leucine. Media for uptake studies were supplemented with [6,6′-^{3}H]sucrose (1 mM) to correct for extracellular contamination. Further details of the methods are described in the Experimental section. Values shown are means ± S.E.M. for results from separate incubations (total numbers of observations in parentheses).

Additions (mM)	Intracellular ^{14}C-labelled amino acid content (pmol/40 islets)	Amino acid oxidation (pmol/40 islets per 60 min)
L-Phenylalanine (0.5)	—	3.1 ± 0.4 (8)
L-Phenylalanine (1.0) + D-glucose (5)	50.9 ± 4.9 (8)	5.2 ± 0.9 (8)
L-Phenylalanine (10)	484 ± 51 (8)	13.2 ± 1.2 (12)
L-Leucine (10)	321 ± 35 (7)	1292 ± 157 (7)

insulin-releasing effect of 3-phenylpyruvate. Fig. 1 shows similar insulin secretory profiles in response to 3-phenylpyruvate or BCH. The latter non-metabolizable amino acid and L-leucine could enhance oxidation of endogenous fuels in islets by activation of glutamate dehydrogenase (Fig. 2; Panten *et al.*, 1980; Sener & Malaisse, 1980). This mechanism, however, does not work with 3-phenyl-

pyruvate, because this compound activated glutamate dehydrogenase only slightly, to 156±4 or 149±3% of control activity at 10mM or 20mM respectively ($n = 9$). Moreover, L-phenylalanine was even less effective (Fig. 2), and the following metabolites did not activate glutamate dehydrogenase in islet homogenates (94–105% of control activity, $n = 6$): L-phenyl-lactate (2 or 10mM), phenylacetate (1 or 10mM), *o*-hydroxyphenylacetate (1mM) and phenylacetyl-CoA (0.02mM). Higher concentrations of phenylacetyl-CoA (0.1 or 1mM) or *o*-hydroxyphenylacetate (10mM) were inhibitory (76–89% of control activity, $n = 6$–9).

There are several reports of inhibitory effects of 3-phenylpyruvate or its metabolites on enzyme activities (e.g. Land & Clark, 1973; Benavides *et al.*, 1976). But our search in the literature did not reveal mechanisms by which 3-phenylpyruvate could increase catabolic fluxes in islet cells. Thus specific B-cell membrane receptors must be considered as possible mediators of the insulin-releasing capacity of fuels and their analogues.

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