Stimulation of Insulin Release *in Vitro* by Non-metabolized Amino Acid Analogues[1] (35581)

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The studies of Floyd *et al.* (1) have established that administration of several natural amino acids increases the circulating levels of insulin in man. The magnitude of the increases in plasma insulin varied with the individual amino acid tested while a synergistic effect was evidenced *in vivo* for some amino acid pairs but not for others (2). A direct stimulatory effect of amino acids on insulin release *in vitro*, first demonstrated for leucine and arginine (3, 4), has been confirmed for other natural amino acids (5–8). Again, large differences in the effectiveness of different amino acids were observed.

The mechanism(s) by which amino acids stimulate B cells to release insulin remain(s) unknown. It has, however, been suggested that amino acids themselves rather than their metabolites are involved in this action (9, 10) and that different stimulatory mechanisms may exist for different amino acids (11). Recently, Christensen and Cullen (12) have shown that a nonmetabolized but transported amino acid analogue, 2-aminobicyclo[2,2,1]heptane-2-carboxylic acid (BCH) lowered the blood sugar and raised plasma insulin when injected into normal or tolbutamide pretreated rats. These results received preliminary confirmation *in vivo* in the dog (13) and *in vitro* in the fetal rat pancreas where it was suggested that BCH and another nonmetabolized amino acid analogue, α-aminoisobutyric acid (AIB) directly stimulated insulin release in the absence of exogenous substrate (14).

The present studies were undertaken to establish the effect of AIB and BCH on insulin release *in vitro* from a preparation of cultured fetal rat pancreas. AIB uptake by the same preparation was also measured. It was found that both AIB-induced insulin release and AIB uptake were modified in the same direction by changing experimental conditions, therefore suggesting a relationship between the two phenomena.

Materials and Methods. Fetal rat pancreases were explanted on day 18 of gestation and cultured over 4 days as described previously (8, 15). The explants were then washed and incubated for 15 min at 37° in a Krebs–Ringer bicarbonate buffer (pH 7.4) containing 2% bovine serum albumin (KRB). They were subsequently incubated for 2 hr at 37° in fresh KRB containing BCH or AIB with or without caffeine and glucose. The osmolarity of the incubation medium was kept constant by decreasing isoosmotically the NaCl concentration. At the end of the incubation, the explants were blotted and weighed and the immunoreactive insulin (IRI) released into the medium was measured by a double antibody assay (16). The results were expressed as microunits (μU) rat IRI released per milligram of wet tissue weight per 2-hr incubation. For the study of AIB uptake, the explants were incubated as described above in a medium containing 20 m*M* ^{3}H-labeled AIB (25 μCi/ml). The incubation vials were then immediately placed in a water bath at 4°. After removal of the medium, the explants were quickly washed 4 times with a cold Tyrode's solution containing no albumin but supplemented with 20 m*M* unlabeled AIB. The explants were again blotted, weighed,

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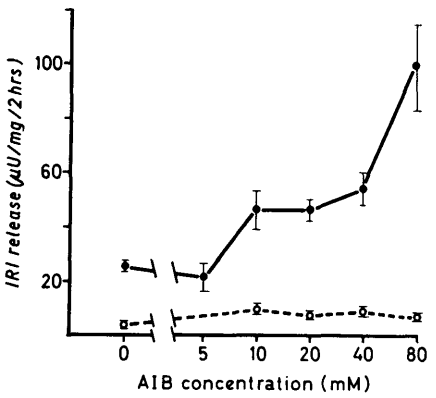

FIG. 1. Effect of AIB alone (O- -) or together with 10 m*M* caffeine (●) on IRI release from cultured fetal rat pancreas during a 2-hr incubation period. Each point represents the mean of 12 observations ± SEM.

and sonicated to complete tissue disruption in 1 ml of water. The radioactivity was measured in a 0.1-ml aliquot of this medium after mixing in 10 ml scintillation fluid (17). Protein was measured in another aliquot of the same medium (18). The results were expressed as disintegrations per minute per microgram of tissue protein and as distribution ratios (on a water basis), *i.e.*, the ratios (dpm/μl of tissue water):(dpm/μl of incubation medium). AIB and ^{3}H-AIB (sp act 1.29 Ci/mmole) were from Sigma, St. Louis, Missouri, and from the Radiochemical Centre, Amersham, England, respectively. One AIB sample was synthesized and purified by H.N.C. and results with it did not differ from those obtained with purchased AIB. BCH was an unresolved preparation containing a mixture of 2 isomers: 92% b-BCH and 8% a-BCH, each of them being a DL mixture.

Results. As shown in Fig. 1, AIB alone, at concentrations ranging from 10 to 80 m*M*, did not modify basal IRI release from cultured fetal explants. In the presence of 10 m*M* caffeine, an agent which favors cyclic AMP accumulation in several tissues by inhibiting phosphodiesterase (19), 5 m*M* AIB had similarly no effect, while at concentrations equal to or higher than 10 m*M*, it significantly enhanced caffeine-induced IRI output roughly in proportion to its concentration, and this in the absence of any exogenous metabolisable substrate. Moreover, the addition of 20 m*M* AIB to the incubation medium markedly enhanced glucose-induced IRI release as a function of glucose concentration from 2.75 to 22 m*M* (Fig. 2). This enhancing effect was inhibited approximately 50% in the presence of 1 μg/ml oligomycin, an agent interfering with oxidative phosphorylation (20), and was almost completely abolished when the incubation was performed at 4° (Fig. 2).

In a second group of experiments, AIB uptake was measured during a 2-hr incubation period. The results of these experiments are summarized in Table I. A significant uptake of ^{3}H-AIB by cultured fetal pancreas was observed in the presence of 20 m*M* AIB alone. This uptake was approximately doubled when the explants were incubated in a medium supplemented with 16.5 m*M* glucose. The enhancing effect of glucose was strongly inhibited by oligomycin (1 μg/ml). When expressed as distribution ratios, these data indicated that, at 37°, AIB was concentrated in fetal pancreatic cells by a factor of 2.5 to 5. The small amount of radioactivity found in these cells at a low temperature may be accounted for by passive diffusion. Autoradiographic studies combined with phase-

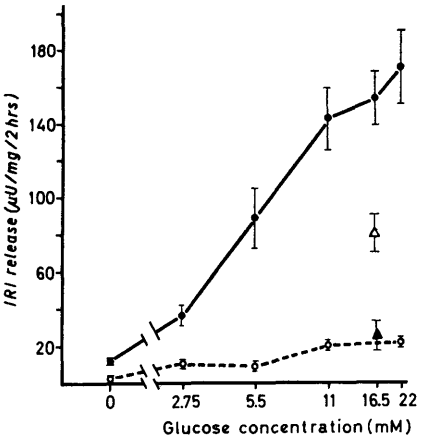

FIG. 2. Effect of glucose alone (O- -) or together with 20 m*M* AIB (●) on IRI release from cultured fetal rat pancreas during a 2-hr incubation period; (△) 16.5 m*M* glucose + 20 m*M* AIB + oligomycin (1 μg/ml); (▲) 16.5 m*M* glucose + 20 m*M* AIB, incubation performed at 4°. Each point represents the mean of 12 observations ± SEM.

TABLE I. Uptake and Distribution Ratios of ^{3}H-AIB in Cultured Fetal Explants Incubated for 2 hr in the Presence of 20 m*M* ^{3}H-AIB.[a]

Additions	Temp of incubation (°)	^{3}H-AIB uptake (dpm/μg of protein)	Distribution ratio (on a water basis)
None	37	1004 ± 84	2.66 ± 0.34
Glucose, 16.5 m*M*	37	1926 ± 140[b]	4.87 ± 0.20[b]
+ oligomycin, 1 μg/ml	37	1275 ± 93[c]	3.32 ± 0.30[c]
Glucose, 16.5 m*M*	4	178 ± 13[bd]	0.45 ± 0.10[bd]

[a] Results expressed as mean of six observations ± SEM.

[b] Significance of differences from the explants incubated with 20 m*M* ^{3}H-AIB at 37°, $p < 0.001$.

[c] Significance of differences from the explants incubated with 20 m*M* ^{3}H-AIB and 16.5 m*M* glucose at 37°: $p < 0.01$; [d] $p < 0.001$.

contrast microscopy performed during these experiments have provided morphological evidence for the accumulation of radioactivity in the endocrine pancreas of explants incubated at 37° with ^{3}H-AIB and 16.5 m*M* glucose. Such accumulation was not observed at 4° (data not shown).

At a concentration of 5 m*M*, BCH did not significantly stimulate IRI release from cultured explants (Fig. 3) nor did it enhance the effect of 11 m*M* glucose (16.6 ± 4.0 versus 13.1 ± 2.3 μU/mg/2 hr for glucose alone; $n = 6$). By contrast, 5 m*M* BCH significantly enhanced the IRI release induced by caffeine (Fig. 3). In the presence of caffeine, 10 and 20 m*M* BCH were not more effective than 5 m*M*.

Discussion. The present results, confirming and extending previous studies from this and other laboratories (12–14) indicate that two nonmetabolized amino acid analogues stimulated IRI release from fetal explants *in vitro*. It would seem, therefore, that the metabolism of natural amino acids is not the only process by which they may stimulate insulin release. The presence of caffeine markedly enhanced the insulin-releasing capacity of AIB and BCH, being necessary for the latter, in a manner similar to that observed with glucose and most of the natural amino acids (8, 21). These data are in agreement with the hypothesis that cyclic AMP is involved in insulin release mechanisms from cultured fetal pancreas. Of particular interest was the observation that AIB stimulated IRI output not only in the presence of caffeine, but also in its absence, provided that glucose was added to the incubation medium. Insulin release is an energy-requiring process. This energy might be supplied by an exogenous substrate, such as glucose or by endogenous energy stores, when caffeine is used, possibly as a result of enhanced glycogenolysis.

These experiments have demonstrated that fetal B-cells accumulate AIB, supporting the observation that AIB accumulates in whole islets of adult obese hyperglycemic mice (22). This accumulation was enhanced in cultured explants by glucose and inhibited by agents or conditions interfering with cell metabolism, suggesting the existence of energy-dependent transport mechanisms for amino acids in the B-cell membrane. The fact that BCH and AIB stimulated IRI release under different conditions might be related to differences in transport mechanisms by which these amino acids enter the cells. Indeed, BCH is known to be transported in Ehrlich cells by the Na^+-independent *L* system (23), whereas AIB is transported in the same cells by the Na^+-dependent *A* system (24).

When considering the effect of natural amino acids, two major possibilities for the initiation of insulin release are present, namely the effects of these agents on cell membranes, presumably related to their mode of transport, and their metabolic activity as substrates. With AIB and BCH, only the former should be involved. That AIB but not BCH enhances glucose-mediated IRI release sug-

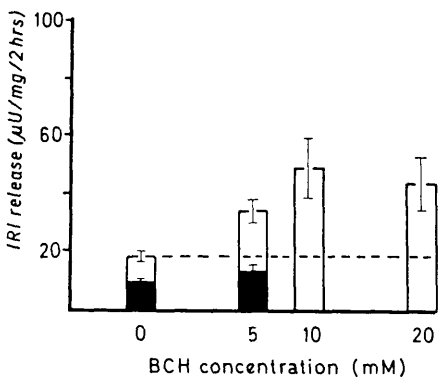

FIG. 3. Effect of BCH in the presence of 10 m*M* caffeine but in the absence of glucose (open columns) on IRI release from cultured fetal rat pancreas during a 2-hr incubation period; (black columns) incubation performed in the absence of caffeine and glucose. Each column represents the mean of 12 observations ± SEM.

gests that the transport system for AIB, but not that for BCH, may be linked to other processes, possibly changes in ionic fluxes (25), which, in turn, may stimulate IRI release (26). The existence on the B-cell membrane of several selective transport mechanisms for amino acids similar to those described in the Ehrlich cell (24) might explain the differences in the ability of various natural amino acids to promote insulin release. Indeed, it was found *in vivo* (1) and *in vitro* (8) that the most potent amino acids were basic amino acids, such as lysine and arginine. Neutral amino acids transported by the *A* system, such as alanine and threonine, were somewhat less effective; whereas the least effective were neutral amino acids transported by the *L* system (leucine, phenylalanine, and histidine). These transport mechanisms might also explain the synergistic effect of amino acid pairs (2) transported by different mechanisms. However, the activity of such transport sites does not appear sufficient in itself, at least in our preparation, to promote IRI release, since a significant AIB uptake was found in the presence of AIB alone even though IRI release was not stimulated.

In conclusion, the results of the present studies suggest that the stimulation of insulin release by amino acids in our preparation depends on the combined action of at least two components: (i) the metabolic activity of the B cell supplied either exogenously (by glucose or amino acids) or endogenously; and (ii) the activity of receptor sites which may well prove to be transport sites.

Summary. Two nonmetabolized amino acid analogues, α-aminoisobutyric acid (AIB) and 2-aminobicyclo[2,2,1]heptane-2-carboxylic acid (BCH) stimulated immunoreactive insulin (IRI) release from cultured fetal rat pancreas incubated *in vitro*. AIB was effective in the presence of either caffeine or glucose; whereas BCH was only active in the presence of the methylxanthine. AIB-induced IRI release together with glucose was inhibited by oligomycin or incubation at a low temperature. The uptake of ^{3}H-AIB was demonstrated in this preparation. With changing experimental conditions, IRI release induced by AIB paralleled changes in AIB uptake (*i.e.*, its active transport). The results suggest the existence of a relationship between the two phenomena.

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