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ORIGINAL ARTICLE

Sonia Bolea · Jose A. G. Pertusa · Franz Martín
Juan V. Sanchez-Andrés · Bernat Soria

Regulation of pancreatic β-cell electrical activity and insulin release by physiological amino acid concentrations

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Abstract The mutual enhancement of insulin release by glucose and amino acids is not clearly understood. In this study, the effects on electrical activity and insulin release of a mixture of amino acids and glucose at concentrations found in fed (aa_{FD}) and fasted (aa_{FT}) animals were determined using freshly isolated mouse islets. Islets perifused with aa_{FD} mixture showed an oscillatory pattern of electrical activity at lower glucose concentrations (5 mmol/l) than in islets perifused with the aa_{FT} mixture and with glucose (G) alone (10 mmol/l). The concentration/response curve for the fraction of time spent by the membrane potential in the active phase in aa_{FD}-stimulated islets was found to be significantly shifted to the left and had a smaller slope than that for glucose-stimulated islets. Insulin release followed the same pattern. This resulted in a concentration/response curve for glucose that was closer to that recorded "in vivo". We have also found that four amino acids (leucine, isoleucine, alanine and arginine) are largely responsible for the observed effects and that there is a non-linear enhancement of insulin release as a consequence of the combined effect of amino acids and glucose. This effect was more pronounced in the second phase of insulin release and was dependent on intracellular Ca^{2+}. These findings indicate that amino acids account for most of the leftward shift in the concentration/response curve for glucose and that a reduction in the threshold for the glucose-induced oscillatory electrical activity response and in the generation of Ca^{2+} spikes accounts for the triggering of insulin release at lower glucose concentrations. Nevertheless, the effects on insulin release at high glucose concentrations cannot be explained solely by the increase in glucose-induced electrical activity.

Key words Amino acids · Electrical activity · Insulin release · Islet of Langerhans

S. Bolea · J.A.G. Pertusa · F. Martín · J.V. Sanchez-Andrés
B. Soria (✉)
Departamento de Fisiología e Instituto de Neurociencias,
Facultad de Medicina, Universidad de Alicante,
Campus de San Juan, Aptdo. 374, E-03080 Alicante, Spain

Introduction

Under physiological conditions the blood glucose concentration in fasted mammals, including humans, is maintained at around 5 mmol/l. It is broadly accepted that an increase in blood glucose concentration results in changes of metabolically induced intracellular mediators [2, 14] which, in turn, close ATP-regulated K^+ channels, depolarize the β-cell and activate voltage-dependent Ca^{2+} channels [13]. Subsequent cytosolic Ca^{2+} increases will activate the exocytotic machinery and promote insulin release [6, 8]. Insulin activates glucose uptake in peripheral tissues (muscle and adipose tissue), thus maintaining normal physiological glucose levels. A corollary of this is that the release of insulin in response to the glucose concentration should follow this pattern. But this is not the case. "In vitro" electrical activity and insulin release have a threshold at about 7 mmol/l and an EC_{50} value of around 12 mmol/l glucose, far outside the physiological range. On the other hand, we have recently shown that "in vivo" the EC_{50} (6.8 mmol/l) of the electrical response to glucose is close to the normal physiological level of glucose [16]. The difference observed between "in vitro" and "in vivo" conditions points to the contribution of other factors in the regulation of islet responsiveness.

Amino acids, like carbohydrates and lipids, are major components of food that control insulin secretion, and are therefore possible candidates for carrying out this regulatory role. Two early papers [3, 10] showed that some essential amino acids (L-leucine, L-isoleucine and L-arginine) stimulate insulin release. Moreover, it was demonstrated that L-leucine and L-isoleucine, at low concentrations (250 µmol/l each), doubled the insulin secretion rate [10]. Since then, amino acids have been thoroughly studied and are now well recognized as potent insulin secretagogues. However, in most of these studies amino acids were assayed at concentrations which are never reached under physiological conditions. In a recent study [8], it was shown that a mixture of amino acids and glucose at concentrations found in the plasma of fed animals induces slow intracellular Ca^{2+} oscillations. Since

cytosolic Ca^{2+} concentrations parallel oscillatory insulin release, these slow oscillations may constitute the framework for the pulsatile insulin release observed in vivo [8].

In an attempt to gain further insight into the physiological role of amino acids in insulin release and the possible mechanisms responsible for the difference observed between glucose sensitivity "in vivo" and "in vitro", we have studied the effects of amino acids, at concentrations found in the plasma of fasted and fed animals, on the electrical activity of, and insulin release from, mouse pancreatic islets.

Materials and methods

Albino mice (8–10 weeks old, 25–35 g mass) bred in our animal house were used. Except when otherwise stated, the animals were fed ad libitum. The experiments were carried out according to institutional animal care guidelines. For insulin release experiments, pancreatic islets were isolated by collagenase digestion [5] and hand-picked, using a glass loop pipette, with the aid of a stereomicroscope. Islets of similar medium size (100–200 μm diameter) were used for all the experiments. Once isolated, the islets were incubated for 1 h at 37°C in modified Krebs-Ringer bicarbonate (KRB) buffer containing (in mmol/l): 120 NaCl, 5 KCl, 25 $NaHCO_3$, 1.1 $MgCl_2$, 2.5 $CaCl_2$, 5 glucose and 3% bovine serum albumin. The medium was continuously bubbled with a mixture of O_2 (95%) and CO_2 (5%) for a final pH of 7.4. For electrophysiological experiments, islets were isolated by microdissection with the aid of a stereomicroscope [15], and perifused with modified KRB buffer without bovine serum albumin.

Solutions

Perifusion solutions consisted of the previoulsy described KRB buffer plus different mixtures of amino acids and glucose as follows. (1) Glucose, (G): at different mmol/l concentrations (2.75, 4.18, 5, 5.6, 7, 8.35, 10, 11.1, 15, 16.75, 20 and 22.2); (2) aa_{FD}: amino acids at concentrations found in the plasma of fed mice (in μmol/l) – 185 threonine, 155 serine, 119 glutamic acid, 388 glutamine, 80 proline, 293 glycine, 322 alanine, 182 valine, 52 cystine, 50 methionine, 88 isoleucine, 119 leucine, 91 tyrosine, 70 phenylalanine, 331 lysine, 108 histidine and 137 arginine; (3) aa_{FT}: amino acids at concentrations found in the plasma of 24-h-fasted mice (in μmol/l) – 151 threonine, 143 serine, 115 glutamic acid, 343 glutamine, 109 proline, 272 glycine, 349 alanine, 167 valine, 47 cystine, 52 methionine, 72 isoleucine, 90 leucine, 71 tyrosine, 53 phenylalanine, 316 lysine, 93 histidine and 112 arginine; (4) aa_{FD-4}: like aa_{FD} but alanine, leucine, isoleucine and arginine were included at concentrations found in the plasma of fasted mice; (5) aa_{FT+4}: like aa_{FT} but alanine, leucine, isoleucine and arginine were included at concentrations found in the plasma of fed mice [7].

Electrophysiology

The β-cell membrane potential was recorded as previously described [15]. Once isolated, islets were fixed with micropins to the bottom of a 50-μl chamber and perifused with modified KRB buffer at a rate of 0.8 ml/min. Different mixtures of glucose and amino acids were added to the superfusion medium. The test agents reached the chamber with a delay of 3 s. These delays have been corrected for in the figures. Bath temperature was maintained at 36 ± 1°C with a Peltier device. The temperature of the chamber was continuously monitored with a micro-thermistor. Recordings were made with an Axoclamp 2A amplifier (Axon Instruments, Foster City, Calif., USA). Data acquisition was performed with Axotape version 2.0 (Axon Instruments) and data analysis with MicroCal Origin version 3.7 (MicroCal Soft. Northampton, Mass., USA).

Insulin release experiments

For static incubations, islets were incubated in groups of three in 1 ml of fresh modified KRB buffer with 1% bovine serum albumin plus the different mixtures of amino acids and glucose for 30 min at 37°C. For the perifusion experiments, groups of ten islets were placed in a 50-μl chamber and perifused at a flow rate of 1 ml/min at 37°C. The islets were first perifused in 3 mmol/l glucose for 30 min to reach a state of stable insulin release. The solutions were prewarmed at 37°C and continuously gassed. Throughout the perifusion effluent was continuously collected at 2-min intervals. The dead time was 2 min and has been corrected for in the figures. Both static incubation and perifused samples were kept at –20ºC until insulin determination. Insulin was assayed by radioimmunoassay (RIA). The RIA included the following steps: addition of anti-porcine insulin guinea-pig serum, incubation for 36 h at 4°C, addition of freshly labelled [^{125}I]-(TYR A19)-human insulin, incubation for 12 h at 4°C and precipitation of bound insulin by ethanol. Rat insulin was used to prepare the standard curves. The minimum detectable amount of insulin was 10 pg islet^{-1} min^{-1} and 50% displacement of [^{125}I]-labelled insulin was achieved by 65 pg/tube. Standard curves and problems were run in triplicate, the intra-assay variation was 13% at 24 pg islet^{-1} min^{-1} and 9% at 205 pg islet^{-1} min^{-1}.

Reagents

The RIA kit was from Diagnostic Products (Los Angeles, Calif., USA). Collagenase was from Boehringer Mannheim (Mannheim, Germany). The rest of the reagents were purchased from Sigma (St. Louis, Mo., USA).

Statistics

Statistical significance was determined using the Student's *t*-test for unpaired data. Statistical differences in the dose/response curves were tested using the test of comparison of experimental curves [16]. A $P<0.001$ was taken as significant. Values presented in the figures and results represent means ± SE of at least five experiments.

Results

Leftward displacement of glucose-induced electrical activity by the effect of specific physiological amino acid mixtures

The action of different glucose concentrations on the electrical activity was as previously described [13] (Fig. 1A, left). A glucose concentration of 5 mmol/l kept the cell slightly depolarized, but with no oscillations in membrane potential, or action potentials. An increase in glucose concentrations from 3 to 5–7 mmol/l induced the onset of oscillatory activity. Membrane potential oscillated between silent (hyperpolarized) and active (depolarized) phases with fast spikes (Ca^{2+} action potentials) superimposed on them. Further increases in glucose concentrations up to 20 mmol/l increased the relative length of the active phase (Fig. 1A, left). At concentrations higher than 20 mmol/l the membrane remained steadily

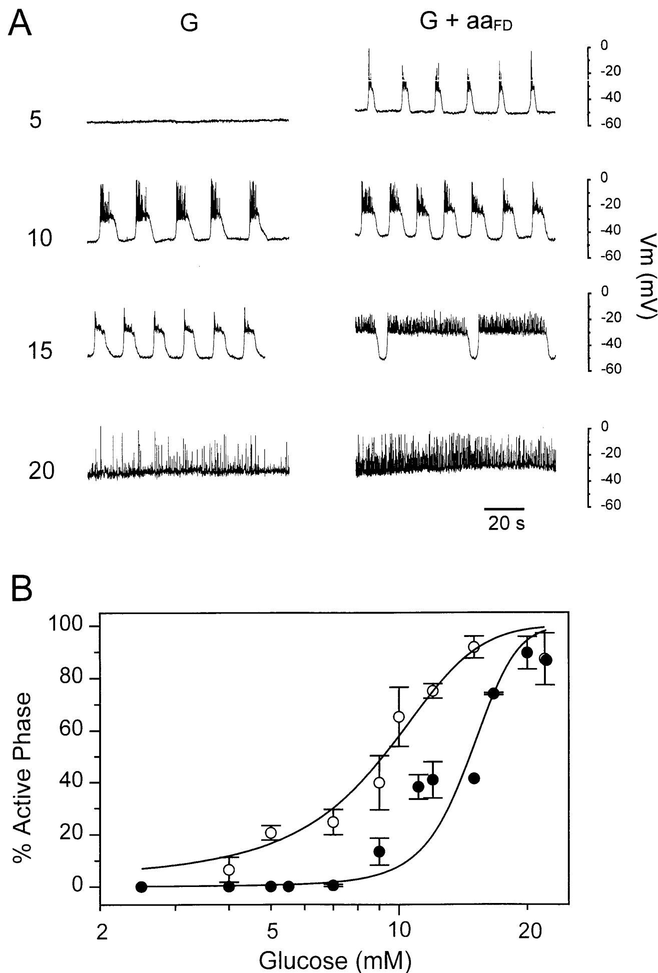

Fig. 1A, B Effect of a mixture of amino acids at concentrations found in the plasma of fed mice on glucose-induced electrical activity. **A** Representative examples of the effects of different glucose (*G*) concentrations (5, 10, 15 and 20 mmol/l) on intracellularly recorded membrane potential of a pancreatic β-cell in the intact islet perifused with Krebs-Ringer-bicarbonate (KRB) buffer (*left*) and KRB buffer plus aa_{FD} mixture (*right*). In both groups the same islet was perifused with the different glucose concentrations. **B** Dose/response curve showing the percentage of time spent in the active phase as a function of glucose concentration in the intact islet perifused with KRB buffer (●) and KRB buffer plus aa_{FD} mixture (○).Values are expressed as means ± SE of seven experiments. Glucose concentrations are expressed in semilogarithmic scale

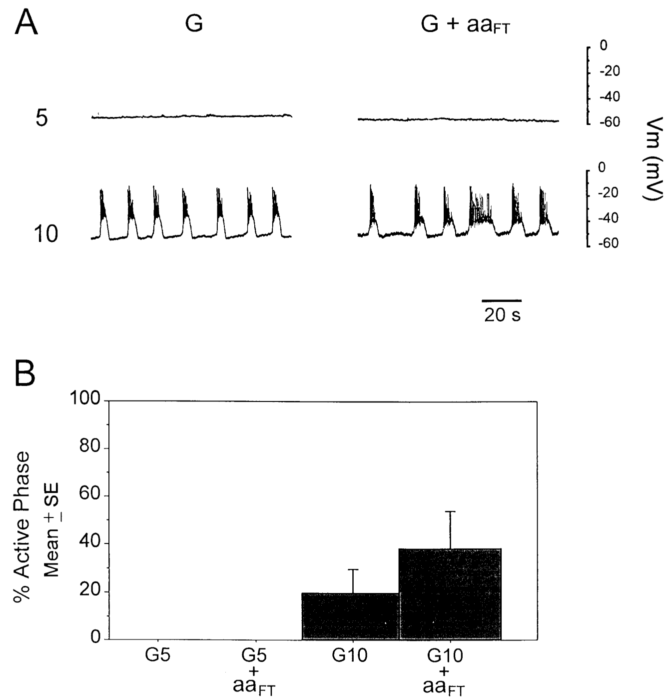

Fig. 2A, B Effect of a mixture of amino acids at concentrations found in the plasma of fasted mice on glucose-induced electrical activity. **A** Representative examples of the effects of different glucose (*G*) concentrations (5 and 10 mmol/l) on intracellularly recorded membrane potentials of a pancreatic β-cell in the intact islet perifused with KRB buffer (*left*) and KRB buffer plus aa_{FT} mixture (*right*). In both groups the same islet was perifused with both glucose concentrations. **B** *Bars* represent the percentage of time spent in the active phase for the different glucose concentrations (5 and 10 mmol/l) in the intact islet perifused with KRB buffer and KRB buffer plus aa_{FT} mixture.Values are expressed as means ± SE of six experiments. There was no significant difference between intact islets perifused with KRB buffer and those perifused with KRB buffer plus aa_{FT} mixture

depolarized at the plateau potential (Fig. 1A, left). Plotting the duration of the active phases versus the glucose concentration resulted in a sigmoidal dose/response curve (Fig. 1B) with an EC_{50} = 13.63 mmol/l (95% confidence interval 12.45–14.81 mmol/l). Islets perifused with aa_{FD} mixture elicited increased glucose-induced electrical activity when compared with islets perifused with G mixture (Fig. 1A, right), and showed an oscillatory pattern of electrical activity in response to glucose concentrations (5 mmol/l) that were unable by themselves to drive the cell into oscillations. Additionally, in the aa_{FD} group the plot of the duration of the active phases versus glucose concentration was found to be shifted to the left when compared with the G group plot (Fig. 1B). The EC_{50} of the aa_{FD} curve was 9.24 mmol/l (95% confidence interval 8.31–10.17 mmol/l). The G and aa_{FD} curves were significantly different ($F_{(2, 52)}$ = 37.57; P<0.001). The difference between curves was not based in an increased sensitivity, as would be expected if the shift was accompanied by a greater slope. Rather, the slope was lesser (2.27 for the aa_{FD} curve versus 2.90 for the G curve), and the change due to a broadening of the range of concentrations at which the cell responded with oscillatory electrical activity. This observation is supported by an inspection of the curve at low glucose levels (<7 mmol/l). This range corresponds to glucose concentrations that are unable to induce oscillatory electrical activity in the G group (Fig. 1B). In the case of islets perifused with aa_{FD} mixture, glucose concentrations as low as 4 mmol/l were able to drive the β-cells into an oscillatory pattern (Fig. 1B). Consequently, a reduction in the threshold for a glucose-induced oscillatory electrical activity response accounts for the observed effect. The same effect in islets perifused with aa_{FT+4} mixture was observed (data not shown).

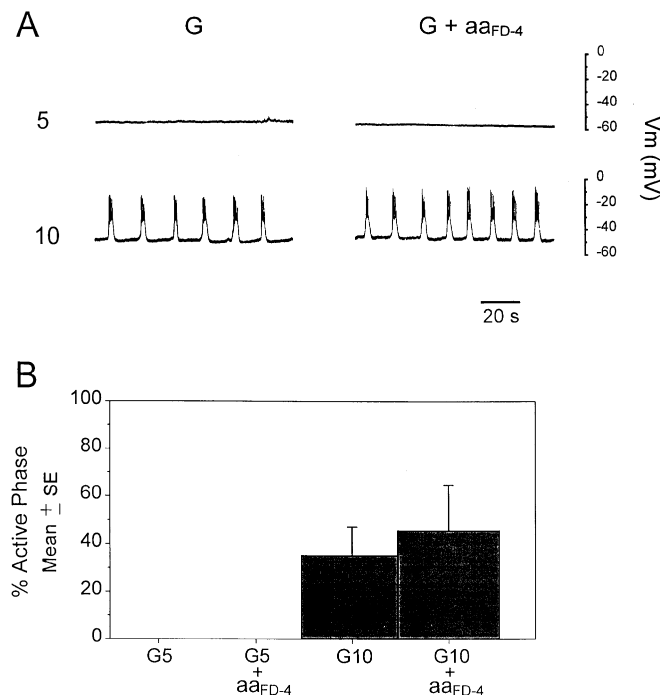

Fig. 3A, B Effect on glucose-induced electrical activity of a mixture of amino acids at concentrations found in the plasma of fed mice, but with alanine, leucine, isoleucine and arginine at concentrations found in the plasma of fasted mice. **A** Representative examples of the effects of different glucose (*G*) concentrations (5 and 10 mmol/l) on intracellularly recorded membrane potential of a pancreatic β-cell in the intact islet perifused with KRB buffer (*left*) and KRB buffer plus aa_{FD-4} mixture (*right*). In both groups the same islet was perifused with both glucose concentrations. **B** *Bars* represent the percentage of time spent in the active phase for the different glucose concentrations (5 and 10 mmol/l) in the intact islet perifused with KRB buffer and KRB buffer plus the aa_{FD-4} mixture. Values are expressed as means ± SE of seven experiments. There was no significant difference between intact islets perifused with KRB buffer and those perifused with KRB buffer plus aa_{FD-4} mixture

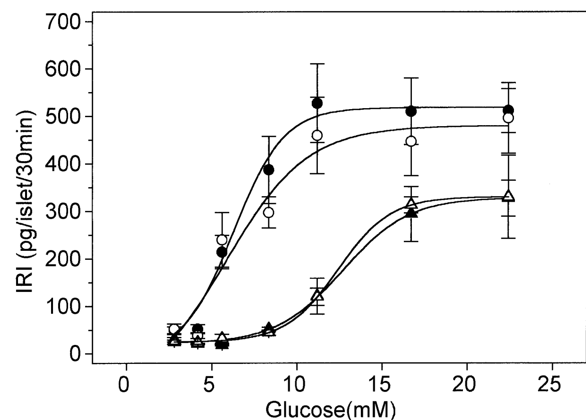

Fig. 4 Effect of specific physiological amino acids mixtures on glucose-induced insulin secretion. Islets were incubated in groups of 3 for 30 min at 37°C in 1 ml of fresh modified KRB buffer with 1% bovine serum albumin plus different glucose concentrations only (■), plus aa_{FT} mixture (□), plus aa_{FD} mixture (●) and plus aa_{FT+4} mixture (○). Insulin was assayed by radioimmunoassay and determinations were run in triplicate. Values are expressed as means ± SE of nine experiments. *P<0.001 when compared with glucose only and aa_{FT} mixture. (*IRI* Induced release of insulin)

On the other hand, the aa_{FT} perifusion mixture did not result in significant changes in the glucose-induced electrical response of pancreatic β-cells when compared with the G group. Figure 2A shows the results obtained at 5 and 10 mmol/l glucose. The threshold for induction of glucose-induced oscillatory electrical activity was not affected in islets perifused with aa_{FT} (Fig. 2A, right), in contrast with the displacement observed under the effect of aa_{FD} mixture. In the presence of higher glucose concentrations, i.e. those able to induce oscillatory electrical activity, the aa_{FT} mixture did not produce any significant changes in the electrical activity when compared with G group (Fig. 2B). Accordingly, the dose/response curve was not significantly affected (data not shown).

Four amino acids (leucine, isoleucine, alanine and arginine) are responsible for the leftward displacement of glucose-induced electrical activity

The above data strongly suggest that only an appropriately balanced mixture of amino acids is responsible for modulating the β-cell glucose response. This argument is based on the fact that aa_{FD} and aa_{FT} mixtures are composed of the same amino acids but at different concentrations, all of them being within the physiological range. To check this hypothesis we applied the aa_{FD-4} mixture. Figure 3A shows that this combination of amino acids was unable to modify the β-cell glucose-induced electrical activity. As with aa_{FT}, the aa_{FD-4} mixture showed a slight, but not significant, increase in the percentage of time in the active phase (Fig. 3B), and the threshold for the onset of oscillatory electrical activity was not different to that under G conditions (Fig. 3A, right).

Effects of physiological concentrations of amino acids on glucose-induced insulin secretion

The effects of different mixtures of amino acids on glucose-induced insulin release was tested. The relationship between the rate of release and the glucose concentration was sigmoidal and similar both for islets incubated with G and islets incubated with the aa_{FT} mixture (Fig. 4), the EC_{50} of both curves being similar (12.69 and 12.41 mmol/l respectively). However, when islets were incubated with the aa_{FD} and aa_{FT+4} mixtures, a significant displacement to the left in the dose/response curve was observed when compared with G group ($F_{(2, 107)} = 41.08$; $P<0.001$ and $F_{(2, 94)} = 27.08$; $P<0.001$ respectively) (Fig. 4). The fact that aa_{FT+4} mixture also showed a significant displacement to the left reinforces the hypothesis of an appropriately balanced mixture of amino acids required to modulate the β-cell glucose response. All these results were consistent with the previously described effect on electrical activity (Fig. 1B). Similarly, the effect consisted of a displacement and broadening of the glucose response range, through a reduction of the threshold of the glucose response. Islets incubated with aa_{FD} and

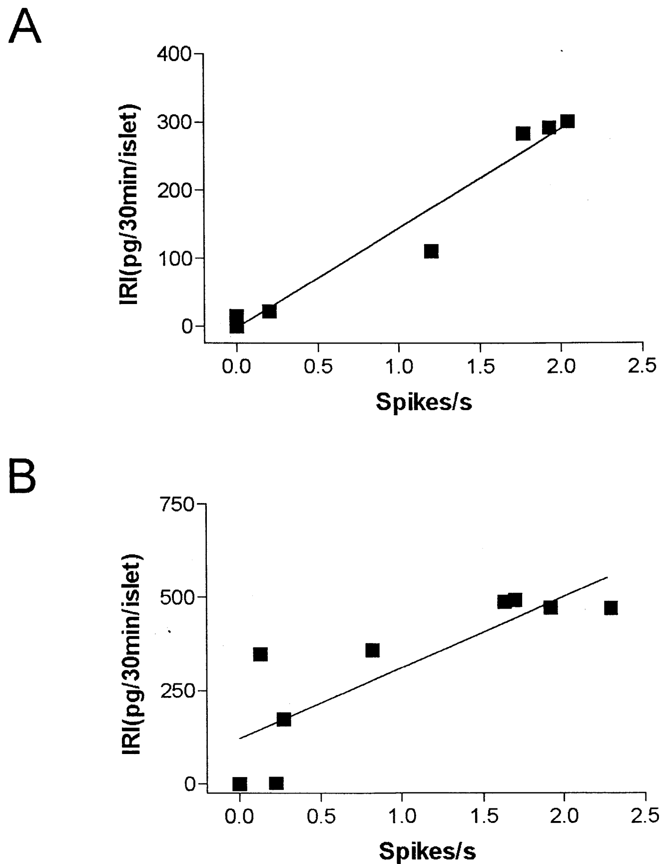

Fig. 5A, B Correlation between electrical activity and insulin release in islets perifused with glucose alone and with glucose plus a mixture of amino acids at concentrations found in the plasma of fed mice. **A** Islets were perifused with KRB buffer; $r = 0.98$. **B** Islets were perifused with KRB buffer plus aa_{FD} mixture; $r = 0.82$. In both conditions the values are calculated for the following glucose concentrations: 2.75, 4.18, 5, 7, 8.35, 11.1, 16.75, 20 and 22.2 mmol/l. Each *point* corresponds to the mean of 5 experiments carried out in parallel. Insulin release values are expressed following subtraction of basal values

aa_{FT+4} mixtures secreted insulin at glucose concentrations lower than 7 mmol/l (Fig. 4), which was the threshold level under G conditions. In addition, at glucose concentrations higher than 5 mmol/l, islets incubated with aa_{FD} and aa_{FT+4} mixtures (Fig. 4) showed a significant increase in insulin release ($P<0.001$) compared to islets incubated with G and with aa_{FT} mixture. A remarkable feature was the prominent increase in the amplitude of the glucose-induced response (Fig. 4). Both aa_{FD} and aa_{FT+4} mixtures produced a twofold increase in insulin release at glucose concentrations higher than 11.1 mmol/l. This increase did not correlate with the observed effect on the electrical activity, the duration of the active phase (Fig. 1B) or in the spike frequency (Fig. 5B). Figure 5B shows the lack of correlation between the increase in insulin release and the electrical activity when islets were incubated with aa_{FD} mixture ($r = 0.82$) in the presence of different glucose concentrations (2.75, 4.18, 5, 7, 8.35, 11.1, 16.75, 20 and 22.2 mmol/l). The correlation coeficient for data from islets perifused in the absence of amino acids was 0.98 (Fig. 5A).

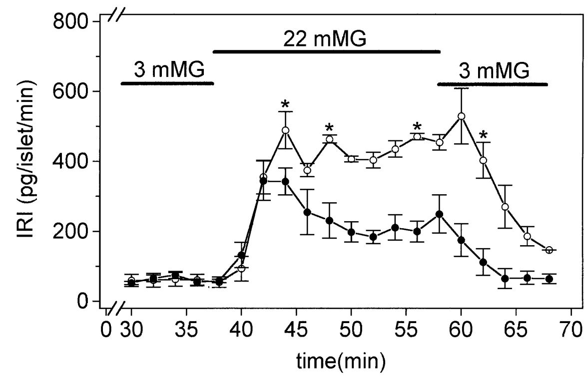

Fig. 6 Effect of a mixture of amino acids at concentrations found in the plasma of fed mice on glucose-induced insulin secretion. Groups of 10 islets were perifused at a flow rate of 1 ml/min and at 37°C. After a 30-min stabilization period with 3 mmol/l glucose, the islets were perifused for 10 min with 3 mmol/l glucose (*3 mMG*), then for 20 min with 22 mmol/l glucose (*22 mMG*) and finally for 10 min with 3 mmol/l glucose (*3 mMG*). The perifusion medium was fresh modified KRB buffer with 1% bovine serum albumin alone (●) and with aa_{FD} mixture added (○). Insulin was assayed by radioimmunoassay and determinations were run in triplicate. Values are expressed as means ± SE of six experiments. $^{*}P<0.001$ when compared with G

Perifused mouse islets responded to a stepwise increase in glucose concentration with a rapid and transient burst of insulin secretion (first phase) followed by a plateau (second phase) which lasted as long as glucose was present. Perifusion studies indicated that insulin release from islets stimulated with aa_{FD} mixture increased significantly ($^{*}P<0.001$, when compared with islets stimulated with G) mainly due to the second phase, although a small increase in the first phase was also evident (Fig. 6).

Discussion

The results described here show that a mixture of nutrients (amino acids + glucose) at the physiological concentrations found in mice plasma generate a concentration/response curve which is close to that recorded "in vivo" [16]. Consequently, the currently accepted model may explain the regulation of blood glucose concentration at values which are closer to the physiological range. Several features of this work which support this model should be highlighted: (1) there is a displacement to the left of the glucose concentration/response curve, together with a decrease in the glucose threshold; (2) there is an increase in the dynamic range of the system in terms of oscillatory electrical activity; (3) four amino acids (leucine, isoleucine, alanine and arginine) are largely responsible for the observed effects; and (4) there is a non-linear enhancement of insulin release as a consequence of the combined effect of amino acids and glucose.

It is broadly accepted that the sigmoidal curve which describes the rate of insulin release as a function of glucose concentration is mostly dominated by glucokinase activity [9] and the activity of the ATP-sensitive K^{+}

channel [1, 12]. It is particularly striking that a displacement to the left both for the dose/response curve for the percentage of time spent in the active phase and that for insulin secretion is not accompanied by an increase in slope, which would be expected from a change in the sensitivity of the system. Rather, the slope is decreased (as is the electrical activity, Fig. 1B) or unaffected (as is insulin secretion, Fig. 4). One explanation to account for these changes is a reduction in the glucose threshold potential. A reduction in the glucose threshold to approximately 4–5 mmol/l (in the range of the glucokinase K_m) would allow for an early initiation of insulin release and electrical oscillatory activity, implying the generation of Ca^{2+} action potentials at lower glucose concentrations. We propose that this mechanism could be responsible for triggering insulin release at these relatively low glucose concentrations.

It is likely that the role of amino acids can be seen as priming the mechanisms responsible for the onset of oscillatory electrical activity. As it is generally accepted that oscillations appear after a certain level of membrane depolarization is reached, it can be assumed that the observed effects on islets perifused with aa_{FD} and aa_{FT+4} mixtures (Figs. 1 and 4) come from an intrinsic action of electrically active amino acids (leucine, isoleucine, alanine and arginine) on membrane potential. In this regard, a recent study showed that under near-physiological conditions glucose, together with mitochondrially generated signals, is very effective at closing ATP-dependent K^+ channels [7]. In fact, leucine [4] and isoleucine [11] decrease the K^+ permeability of the β-cell membrane and there is general agreement that this decrease in K^+ conductance causes depolarization of the β-cell membrane. Moreover, the uptake of cationic amino acids (such as arginine) by transporters in the β-cell membrane [4] and of neutral amino acids (such as alanine) by a Na^+-amino acid co-transport mechanism [4] results in membrane depolarization. It is worth noting that in any case the effect of aa_{FD} mixture on electrical activity is dependent on the presence of substimulatory glucose concentrations (Fig. 1).

As seen in Fig. 1B, islets perifused with aa_{FD} mixture had a leftward displacement of the electrical activity dose/response curve similar to the recordings observed "in vivo" [16], with an EC_{50} (9.24 mmol/l; Fig. 1B) closer to that obtained "in vivo" (EC_{50} = 6.8 mmol/l) [16]. This leads us to propose that an appropriately balanced mixture of amino acids and glucose may be necessary to set the responsiveness of the pancreatic islets to nutrients. Nevertheless, a significant difference from the values obtained using the "in vivo" preparation, still exists, suggesting that other metabolic or neural factors contribute to the physiological response of the islets.

The facts that islets stimulated with aa_{FD} and aa_{FT+4} mixtures had a higher rate of insulin release (Fig. 4) and that there was poor correlation between electrical activity and insulin release in islets stimulated with aa_{FD} mixture at higher glucose concentrations (Fig. 5B) indicate that at physiological concentrations, in addition to their effect on electrical activity, amino acid metabolism may generate other messengers that act as distal signals for insulin release and exocytosis.

In conclusion, the present data suggest that at physiological concentrations amino acids (mainly the electrically active ones) induce a reduction in the glucose threshold potential due to depolarization of the β-cell membrane but the effects on insulin release cannot be explained alone by the increase in glucose-induced electrical activity.

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