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Similar effects of succinic acid dimethyl ester and glucose on islet calcium oscillations and insulin release

André Mukala-Nsengu[a], Sergio Fernández-Pascual[a], Francisco Martín[b], Rafael Martín-del-Río[c], Jorge Tamarit-Rodriguez[a,*]

[a]*Department of Biochemistry, Medical School, Complutense University, Madrid 28040, Spain*
[b]*Institute of Bioengineering, Miguel Hernandez University, San Juan 03550, Spain*
[c]*Research Department, Hospital "Ramón y Cajal", Madrid 28034, Spain*

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Abstract

The relative contribution of glycolysis vs. oxidative metabolism to the stimulus secretion coupling mechanism of β-cells was investigated in isolated islets. For that purpose, the secretory and intracellular calcium responses of islets to both glucose and succinic acid dimethyl ester (SAD) were compared. After 45 min of rat islet perifusion in the absence of substrates, the maximum secretory responses to glucose (20 mmol/L) and SAD (10 mmol/L) were qualitatively and quantitatively indistinguishable. Malonic acid dimethyl ester (a permeable citric acid cycle inhibitor) suppressed the insulin secretory response to both 20 mmol/L glucose and 10 mmol/L SAD (−70% on average). The inhibitor decreased within 70% the rate of $^{14}CO_2$-production from 10 mmol/L [2-^{14}C]pyruvate without affecting the rate of 20 mmol/L D-[5-^{3}H]glucose utilization. Both, 11.1 mmol/L glucose and 10 mmol/L SAD, elevated the intracellular calcium concentration and induced a similar pattern of oscillations that were rapidly ablated by 20 mmol/L malonic acid dimethyl ester. However, the intracellular concentration of calcium declined to basal values several minutes after the introduction of the inhibitor in the presence of SAD whereas it remained elevated in the case of glucose. In conclusion: (1) An exclusive increase of mitochondrial metabolism in pancreatic islets was sufficient to mimic the effects of glucose on intracellular calcium and insulin secretion. (2) Islet glycolysis and/or the re-oxidation of cytoplasmic NADH allowed the maintenance of an elevated, though non-oscillating, intracellular calcium concentration, but a reduced response to glucose.

Keywords: Insulin release; Islet metabolism; Glucose; Succinic and malonic acid; Cytosolic calcium

1. Introduction

The glucose signaling mechanism of β-cells is metabolic: an increase of the glucose concentration above a threshold value (3–5 mmol/L) is followed by an increased sugar metabolism which provides signals responsible for the stimulation of insulin secretion [1]. Cytosolic ATP has long been considered the unique messenger linking glucose metabolism with insulin secretion. An increase of its concentration closures ATP-dependent K^+-channels and the resulting membrane depolarization increases $[Ca^{2+}]_i$ through the opening of voltage-dependent, L-type Ca^{2+}-channels and hence insulin secretion [2].

There is, however, no full agreement concerning the relative contribution of the different metabolic pathways of glucose catabolism (aerobic glycolysis vs. mitochondrial oxidation) to the increase of the cytosolic concentration of ATP. There is partial experimental evidence supporting a privileged role of the shuttle systems for the reoxidation of cytosolic NADH in the stimulation of insulin secretion by glucose, as if the β-cell was able to discriminate which fraction of mitochondrial ATP production should be linked to the closure of ATP-dependent K^+-channels [3–5]. On the other hand, there is partial evidence indicating that mitochondrial oxidative metabolism might also be specifically implicated in the mechanism of metabolism-secretion coupling of the β-cell [6]. In this respect, it has been shown that the stimulation of insulin secretion in permeabilized INS-1 cells by succinic acid is accompanied by increases in both the mitochondrial membrane potential

* Corresponding author. Tel.: +34-913941449; fax: +34-913941691.
E-mail address: tamarit@med.ucm.es (J. Tamarit-Rodriguez).
Abbreviations: SAD, succinic acid dimethyl ester; MAD, malonic acid dimethyl ester.

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and the matrix concentration of Ca^{2+} [7]. Membrane permeable analogues of some Krebs cycle intermediates were originally used with the strategy of bypassing possible defects of glucose metabolism that have been associated with certain forms of diabetes [8,9]. The analogues more intensively investigated have been the methyl esters of succinic acid. They have been shown to induce a shift to the left of the relationship between insulin secretion and glucose concentration [10] and to exert a weak stimulation by themselves [11,12]. Succinic acid dimethyl ester (SAD) is hydrolyzed intracellularly to succinic acid and metabolized by islets as assessed by the production of $^{14}CO_2$ from either [1,4-^{14}C] or [2,3-^{14}C]SAD [13,14].

The general aim of our work was to investigate, in isolated islets, whether the elevation of intracellular calcium and the biphasic secretory response induced by glucose could be reproduced qualitatively and quantitatively with a mitochondrial substrate like SAD. Malonic acid dimethyl ester (MAD), a permeable citric acid cycle inhibitor [15], was used as a tool to evaluate the contribution of glycolysis to the stimulation of insulin secretion by glucose.

2. Materials and methods

Collagenase P was obtained from Roche Diagnostics S.L. Bovine serum albumin (BSA) and most of the organic compounds, including the dimethyl esters of succinic and malonic acid (Fluka Chemie GmbH), were obtained from Sigma-Aldrich Química S.A. Rat insulin was from Linco Research, Inc. Na[^{125}I] was obtained from Amersham Iberica S.A.; D-[U-^{14}C]glucose, D-[5-^{3}H]glucose, [2-^{14}C]pyruvate, $^{3}H_2O$, and NaH[$^{14}CO_3$] were from New England Nuclear or American Radiolabeled Chemicals. Fura-2 was from Molecular Probes Inc. Inorganic compounds and organic solvents were obtained from Merck Farma y Química S.A.

Islets were isolated from the pancreas of male Wistar–Albino rats (250 g BW) or of male Swiss OF1 mice (8- to 12-week-old) by collagenase digestion [16]. Insulin secretion was studied in perifused rat islets or in batch-type incubations of mouse islets. Two groups each of 40 rat islets were perifused in parallel and at a flow rate of 0.5 mL/min with Krebs-Ringer, buffered with 0.5 mmol/L $NaHCO_3$ and 20 mmol/L HEPES and supplemented with 0.5% BSA. Mouse islets (groups of five) were incubated for 60 min at 37° in 1 mL of Krebs medium supplemented with 1% BSA and continuously gassed with 95% O_2 + 5% CO_2. Immunorreactive insulin was measured in the perifusion effluent of rat islets and in the incubation medium of mouse islets. The insulin antiserum used for the measurement of rat insulin was kindly provided by Dr. Janove Sehlin from the Department of Medical Cell Biology at the University of Umea.

Rat islet metabolism was studied with adequate isotope tracers in batch-type incubations of 2 hr duration. Glucose utilization was measured as the production of $^{3}H_2O$ from D-[5-^{3}H]glucose [17]. Oxidation rates were measured as the production of $^{14}CO_2$ from either D-[U-^{14}C]glucose and [2-^{14}C]pyruvate [17]. Metabolic rates were correspondingly corrected according to the recovery of $^{3}H_2O$ or NaH[$^{14}CO_3$], measured in triplicate within each experiment. One or two groups of islets were taken from each preparation for the fluorometric determination of DNA [18].

The measurement of the intracellular calcium concentration ($[Ca^{2+}]_i$) was performed in mouse islets. They were loaded with Fura-2 by incubating them for 3 hr at room temperature with 5 μmol/L of the acetoxymethyl derivative of the fluorescent dye. Islets were then superfused at a rate of 0.5 mL/min with Krebs medium supplemented with 1% (w/v) BSA and constantly gassed with 95% O_2 + 5% CO_2. Fura-2 was alternately excited at 340 and 380 nm by a monochromator. The emitted fluorescence from the islets was reflected by a dicroic mirror, centered at 430 nm, to a CCD video camera (Hamamatsu Photonics KK) through a 510 nm filter. The fluorescence images were digitized using an image acquisition program (AquaCosmos 2.0, Hamamatsu Photonics KK). Four consecutive 100-ms frames were averaged at each wavelength before ratioing. The time interval between successive series of 340/380 images was 3 s. The resulting images were ratioed (340/380) pixel by pixel to produce ratio images. $[Ca^{2+}]_i$ was estimated from the ratio of the fluorescence at 340 and 380 nm by interpolation to an *in vitro* calibration curve adjusted to the equation of Grynkiewicz [19].

All the experimental data are presented as the mean values ± SE, and the numbers of separate experiments are given in parentheses. Statistical comparisons were usually performed with non-paired, two-tailed Student's *t* tests. Statistical differences in the insulin release dose–response curves of mouse islets were tested through the test of comparison of the multiple non-linear regression curves ($P < 0.01$ was taken as significant).

3. Results

3.1. Insulin secretion

SAD induced a sustained secretion of insulin in rat perifused islets already at 3 mmol/L. This secretory response was biphasic (Fig. 1) and augmented by increasing the concentration of SAD from 3 to 7 mmol/L (5.8 ± 0.6, N = 9 vs. 13.6 ± 1.5 ng/30 min × 40 islets, N = 19; $P < 0.001$) and from 7 to 10 mmol/L (13.6±1.5, N = 19 vs. 19.0 ± 1.4 ng/30 min × 40 islets, N = 21; $P < 0.02$). Higher concentrations of SAD (13 mmol/L, N = 12; 20 mmol/L, N = 10) did not exert any greater response. MAD is a membrane permeable analogue of malonic acid which is a known competitive inhibitor of the citric acid cycle enzyme succinic acid dehydrogenase [15]. As shown in Fig. 2, when 10 mmol/L MAD was simultaneously

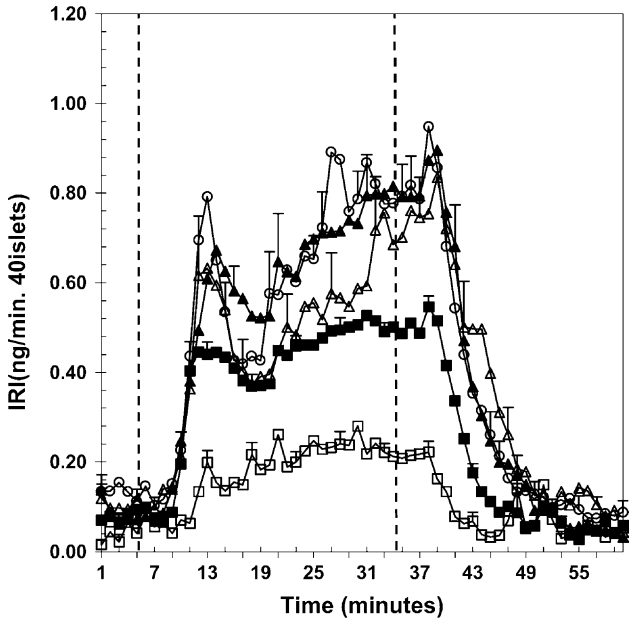

Fig. 1. Insulin secretion of rat perifused islets in response to variable concentrations of SAD. Groups of 40 islets each, pre-perifused without substrates for 45 min, were then stimulated for 30 min (between vertical broken lines) with variable concentrations of SAD (3, □; 7, ■; 10, ○; 13, ▲; 20 mmol/L, △). Pre-perifusion conditions were then re-established during the last 25 min. Symbols represent mean values ± SE and the number of observations is given in the text.

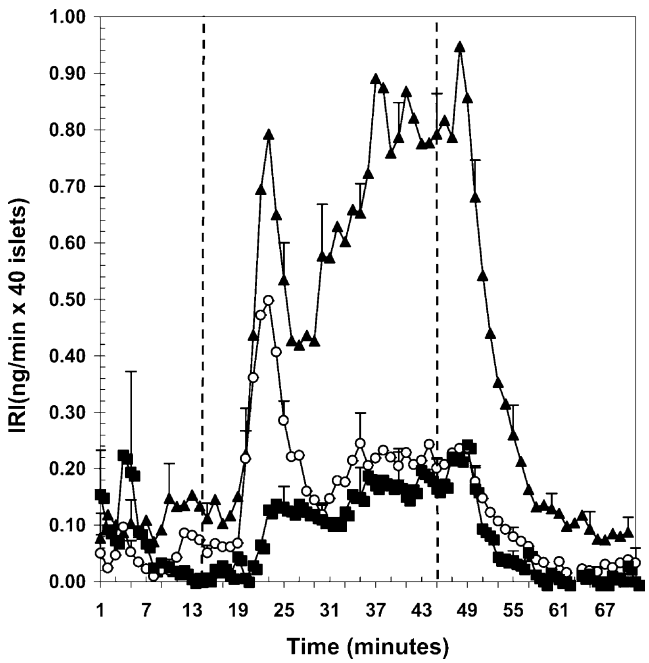

Fig. 2. Effect of 10 mmol/L MAD on the rate of insulin secretion of rat perifused islets stimulated by 10 mmol/L SAD. Groups of 40 islets each, pre-perifused without substrates for 45 min, were then stimulated for 30 min (between vertical broken lines) with SAD alone (▲, N = 21) or with the combination SAD + MAD (○, N = 12). One group of islets (■, N = 6) was pre-perifused for 30 min without substrates and then perifused with MAD alone for 15 min, before stimulating for 30 min with SAD + MAD. Pre-perifusion conditions were then re-established during the last 25 min. Symbols represent mean values ± SE and the number of observations (N) is given above, at the right of each symbol.

perifused with 10 mmol/L SAD the secretory response to the latter was decreased by 62% (7.2 ± 0.7, N = 12 vs. 19.0 ± 1.4 ng/30 min × 40 islets, N = 21; $P < 0.001$), mainly due to a reduction of the second phase of secretion. Pre-perifusion with 10 mmol/L MAD for 15 min decreased the subsequent response to 10 mmol/L SAD plus MAD by 77% (4.3 ± 1.1, N = 6 vs. 19.0 ± 1.4 ng/30 min × 40 islets, N = 21; $P < 0.001$) and almost completely suppressed the first phase of secretion (Fig. 2). The stimulation of insulin secretion by 20 mmol/L glucose was also diminished by 10 mmol/L (8.3 ± 0.9, N = 7 vs. 19.4 ± 1.6 ng/30 min × 40 islets, N = 33; $P < 0.001$) and 20 mmol/L MAD (3.3 ± 0.5, N = 7 vs. 19.4 ± 1.6 ng/30 min × 40 islets, N = 33; $P < 0.001$) (Fig. 3).

Succinic acid has to leave mitochondria as malate and to re-enter as pyruvate (after decarboxylation by malic enzyme) [13,14] in order to be completely oxidized to CO_2 in the Krebs cycle, feeding it with acetyl-CoA. As succinic acid conversion into pyruvate could be limiting both the overall rate of metabolism and its secretory capacity, so the effect of exogenous pyruvate on the secretory response of rat perifused islets to 10 mmol/L SAD was checked. Figure 4A shows that 10 mmol/L pyruvate alone did not stimulate insulin secretion but its combination with 10 mmol/L SAD led to an increase (+58%) of the secretory response to the latter (30.0 ± 2.1, N = 21 vs. 19.4 ± 1.4 ng/30 min × 40 islets, N = 21; $P < 0.001$) by an apparently proportionate enhancement of both phases of release. A similar enhancement was obtained with 20 mmol/L pyruvate (results not shown). The islet secretory response to 20 mmol/L glucose alone was almost identical, both qualitatively and quantitatively, to that triggered by 10 mmol/L SAD and it was also increased (+64%) by the simultaneous perifusion with 10 mmol/L pyruvate (31.7 ± 2.9, N = 8 vs. 19.4 ± 1.6 ng/30 min × 40 islets, N = 33; $P < 0.002$) (Fig. 4A). The combination of 10 mmol/L SAD together with 20 mmol/L glucose resulted in an enhancement (60–70%) of the insulin secretory response due to the separate perifusion of 10 mmol/L SAD (19.0 ± 1.4, N = 21 vs. 32.2 ± 2.3 ng/30 min × 40 islets, N = 28; $P < 0.001$) or 20 mmol/L glucose (19.4 ± 1.6, N = 33 vs. 32.2 ± 2.3 ng/30 min × 40 islets, N = 28; $P < 0.001$) (Fig. 4B).

In all previously described experiments, the secretory responses to glucose and SAD were studied after 45 min of perifusion without any added substrate, in order to avoid any possible interference of different pre-perifusion conditions on the subsequent stimulation of insulin secretion. Pre-perifusion of islets with 3 mmol/L glucose for this period of time improved the subsequent half an hour response to 20 mmol/L glucose by 2.4-fold (47.0 ± 4.8, N = 14 vs. 19.4 ± 1.6 ng/30 min × 40 islets, N = 33; $P < 0.001$) (Fig. 4B). Under this condition, the effects of glucose and SAD were not additive, with no statistically significant difference being found between the stimulation with 20 mmol/L glucose alone and the combination of

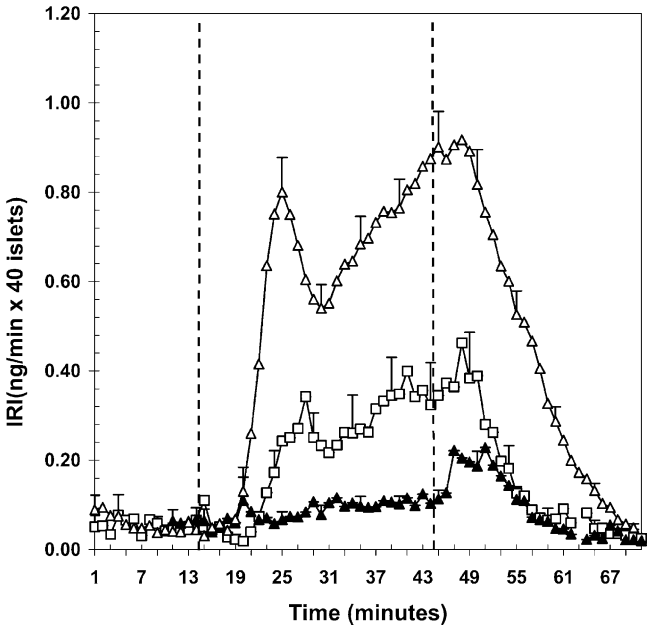

Fig. 3. Effect of 10 or 20 mmol/L malonic acid dimethyl ester (MAD10 or 20) on the rate of insulin secretion of rat perifused islets stimulated by 20 mmol/L glucose (G20). Groups of 40 islets each, pre-perifused without substrates for 45 min, were then stimulated for 30 min (between vertical broken lines) with either G20 (△, N = 33), G20 + MAD10 (□, N = 7) or G20 + MAD20 (▲, N = 7). MAD alone was already introduced in the perifusate during the last 15 min of pre-perifusion. Pre-perifusion conditions (in the absence of substrates) were re-established during the last 25 min. Symbols represent mean values ± SE and the number of observations (N) is given above, at the right of each symbol.

20 mmol/L glucose plus 10 mmol/L SAD (47.0 ± 4.8, N = 14 vs. 40.9 ± 3.5 ng/30 min × 40 islets, N = 11; N.S.) (Fig. 4B). Pre-perifusion with either 3 mmol/L glucose (Fig. 4B) or 1.5 mmol/L SAD (not shown) for 45 min significantly enhanced the first peak of the subsequent response to 10 mmol/L SAD (8.2 ± 0.6, N = 13, and 6.1 ± 0.6, N = 16, vs. 4.33 ± 0.4 ng/6 min × islet, N = 21; $P < 0.001$ and $P < 0.02$, respectively). However, the respective second-phase responses to 10 mmol/L SAD were not significantly modified by pre-treatment with either 3 mmol/L glucose (15.2 ± 1.7, N = 13 vs. 15.2± 1.2 ng/24 min × islet, N = 21; N.S.) or 1.5 mmol/L SAD (12.5 ± 1.5, N = 16 vs. 15.2 ± 1.2 ng/24 min × islet, N = 21; N.S.). The basal rates of insulin secretion recorded during pre-perifusion were significantly higher in the presence of 3 mmol/L glucose and 1.5 mmol/L SAD than in the absence of substrates (5.3 ± 0.8, N = 13, and 5.4 ± 0.8, N = 16 vs. 1.4 ± 0.3 ng/15 min × islet, N = 21; $P < 0.001$ in both cases).

3.2. Rat islet metabolism

As is shown in Fig. 5, the rate of D-[5-^{3}H]glucose utilization was increased 3-fold by changing the glucose concentration from 3 to 20 mmol/L and 10 mmol/L MAD (a citric acid cycle inhibitor) did not exert any effect at 20 mmol/L glucose.

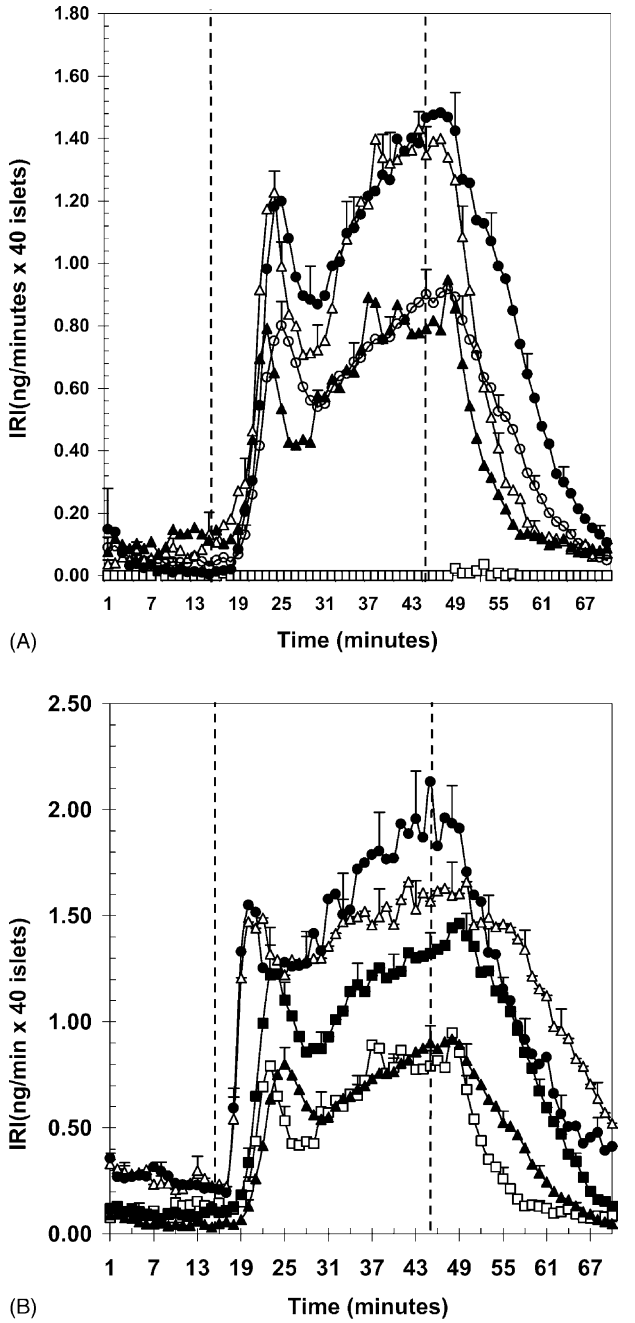

Fig. 4. (A) Effect of the combination of 10 mmol/L pyruvate (Pyr) with 10 mmol/L SAD or 20 mmol/L glucose (G20) on the rate of insulin secretion of rat perifused islets. Groups of 40 islets each, pre-perifused without substrates for 45 min, were then stimulated for 30 min (between vertical broken lines) with either Pyr10 alone (□, N = 8), SAD alone (▲, N = 21), SAD + Pyr (△, N = 21), G20 (○, N = 33), or G20 + Pyr (●, N = 8). Pre-perifusion conditions were then reestablished during the last 25 min. Symbols represent mean values ± SE and the number of observations (N) is given above, at the right of each symbol. (B) Insulin secretory responses of rat perifused islets to the combination of 20 mmol/L glucose (G20) and 10 mmol/L SAD. Influence of pre-perifusion with 3 mmol/L glucose (G3) on the subsequent responses. Groups of 40 islets each, pre-perifused with G3 (●, △) or without substrates (□, ▲, ■) for 45 min, were then stimulated for 30 min (between vertical broken lines) with either SAD alone (□, N = 21), G20 (▲, N = 33; ●, N = 14) and SAD + G20 (■, N = 28; △, N = 11). Pre-perifusion conditions were then re-established during the last 25 min. Symbols represent mean values ± SE and the number of observations (N) is given above, at the right of each symbol.

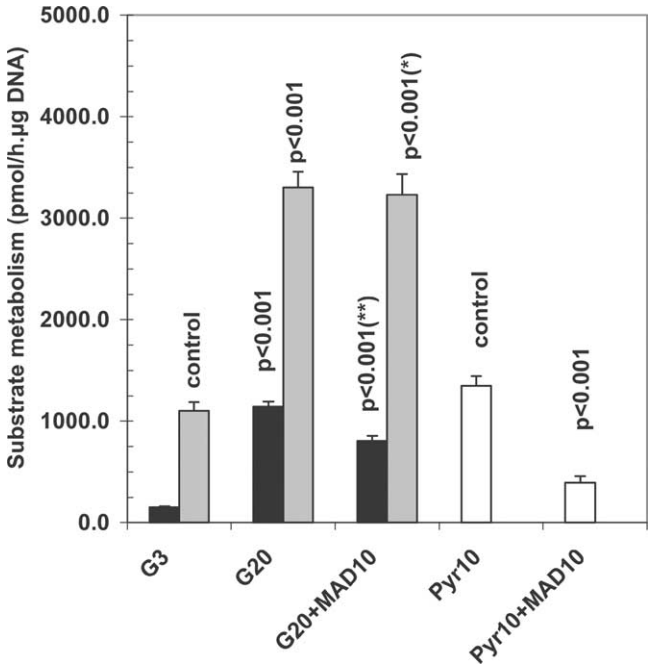

Fig. 5. Effect of 10 mmol/L MAD on rat islet glucose metabolism. Glucose utilization (□) was measured as the production of ^3H_2O from 3 mmol/L (N = 6) and 20 mmol/L (N = 18) D-[5-^{3}H]glucose during 2 hr of incubation, and the effect of MAD tested on 20 mmol/L glucose utilization (N = 6). Glucose oxidation (■) was measured as the amount of $^{14}CO_2$-produced from 3 mmol/L (N = 6) and 20 mmol/L (N = 23) D-[U-^{14}C]glucose during 2 hr of incubation, and the effect of MAD checked on 20 mmol/L glucose oxidation (N = 6). Pyruvate oxidation (□) was similarly measured as the rate of $^{14}CO_2$-production from 10 mmol/L [2-^{14}C]pyruvate during 2 hr of incubation in the absence (N = 9) and presence of MAD (N = 5). Columns represent mean values ± SE of the different metabolic rates. ((*): N.S. and (**): $P < 0.001$ compared with the corresponding value in the absence of MAD.)

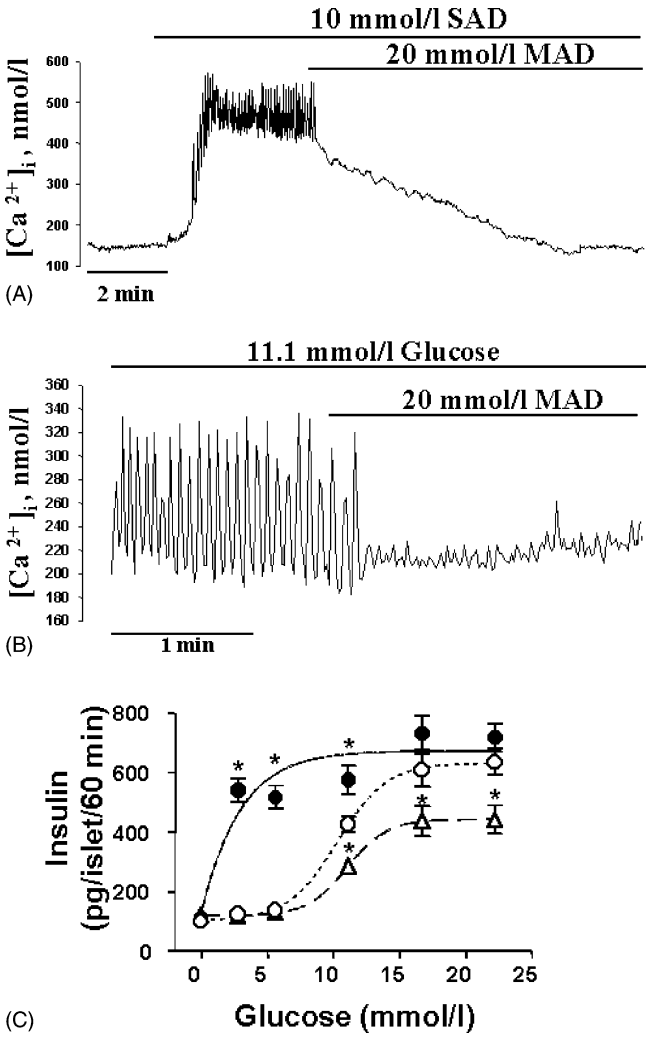

Fig. 6. Effects of SAD and MAD on glucose-induced stimulation in mouse islets. (A) Representative example of the effects on $[Ca^{2+}]_i$ of 10 mmol/L SAD and 10 mmol/L SAD plus 20 mmol/L MAD in islet perfused with 2.75 mmol/L glucose. (B) Representative example of the effects on $[Ca^{2+}]_i$ of 20 mmol/L MAD in islets perfused with 11.1 mmol/L glucose. (C) Insulin secretion experiments: islets were incubated in batches of five for 60 min at 37° in 1 mL of Krebs-Ringer bicarbonate buffer supplemented with 1% BSA plus different concentrations of glucose alone (○) or together with either 10 mmol/L SAD (●) or 20 mmol/L MAD (△). Values are expressed as mean ± SEM of four experiments. ((*): $P < 0.01$ when compared with glucose alone.)

The rate of $^{14}CO_2$-production from [U-^{14}C]glucose was increased 7- to 8-fold by changing the glucose concentration from 3 to 20 mmol/L. The rate of 20 mmol/L glucose oxidation was not modified by 5 mmol/L MAD (not shown) but 10 mmol/L MAD decreased it significantly by 30% ($P < 0.001$) (Fig. 5). [2-^{14}C]Pyruvate is a more specific isotope tracer to measure the rate of $^{14}CO_2$-production in citric acid cycle. The rate of 10 mmol/L [2-^{14}C]pyruvate decarboxylation was decreased within 71% by 10 mmol/L MAD (Fig. 5).

3.3. Intracellular calcium concentration ($[Ca^{2+}]_i$)

For technical reasons, the effects of nutrients on $[Ca^{2+}]_i$ were measured in mouse islets. The main difference between mouse and rat islets is that the latter scarcely show glucose-induced $[Ca^{2+}]_i$ oscillations; however, they share very important similarities [20,21] which allow the assumption of a common mechanism in the regulation of $[Ca^{2+}]_i$ and insulin release by nutrients in both species. Nevertheless, to be completely sure that the effects of SAD and MAD on mouse islet $[Ca^{2+}]_i$ might be extrapolated to rat islets, we also studied their effects on glucose-induced insulin release. Mouse islets showed a sigmoidal insulin response curve to glucose (2.8, 4.2, 5.6, 8.3, 11.1, 16.7, and 22.2 mmol/L) (Fig. 6C). SAD (10 mmol/L) was without effect by itself but it induced a significant ($P < 0.01$) increase of insulin secretion at both non-stimulatory (4.2 and 5.6 mmol/L) and stimulatory (11.1 mmol/L) glucose concentrations (Fig. 6C). MAD (20 mmol/L) significantly reduced ($P < 0.01$) glucose-induced insulin release at stimulatory concentrations (11.1, 16.7, and 22.2 mmol/L) (Fig. 6C).

Succinic acid dimethyl ester (10 mmol/L) increased basal $[Ca^{2+}]_i$ of mouse islets perifused at 2.75 mmol/L glucose. In eight out of nine islets, a clear increase in $[Ca^{2+}]_i$ was observed 30 s after SAD addition (Fig. 6A).

The sustained $[Ca^{2+}]_i$ increase was followed by regular $[Ca^{2+}]_i$ oscillations, similar to those induced by 11.1 mmol/L glucose, as seen in Fig. 6B and as previously reported in rodent perifused islets [22]. MAD (20 mmol/L) ablated SAD-induced $[Ca^{2+}]_i$ oscillations 10 s after its addition and decreased progressively $[Ca^{2+}]_i$ until it reached basal values after 12 min (Fig. 6A). On the other hand, addition of 20 mmol/L MAD resulted in the disappearance of the $[Ca^{2+}]_i$ oscillations induced by 11.1 mmol/L glucose but $[Ca^{2+}]_i$ remained elevated in the continuous presence of the inhibitor (Fig. 6B).

4. Discussion

In previously published reports, the mono or dimethyl ester of succinic acid stimulated in perifused rat islets a predominantly monophasic release of insulin [11,12] or a poor biphasic response in the absence of glucose [10] which became greater and clearly biphasic in the presence of 2.75–6 mmol/L glucose [10–12]. As far as is known, no detailed study of the dose–response stimulation of insulin secretion by succinic acid methyl esters, in islets perifused in the absence of glucose, had hitherto been performed.

The data here clearly demonstrate that SAD behaves as an initiator of secretion in rat islets. It stimulates "*per se*" a sustained and biphasic release of insulin which increases with its concentration in the range 3–10 mmol/L The maximum response to SAD (10 mmol/L) was undistinguishable, both qualitatively and quantitatively, from that elicited by 20 mmol/L glucose and they were partially additive when the two secretagogues were perifused together. This probably means that the metabolic flux in the citric acid cycle is not saturated by 20 mmol/L glucose under the prevailing condition of a pre-perifusion period (45 min) in the absence of substrates and can be increased by supplying mitochondrial substrates, like succinic acid or pyruvate. The latter has been shown to lack any secretagogue capacity by itself but to enhance glucose-induced insulin secretion in rat islets, only at submaximal sugar concentrations [23,24]. However, it is also known that long pre-incubations in the absence of glucose decrease more markedly the insulinotropic capacity of glucose than that of pyruvate, so that the enhancing action of exogenous pyruvate can then be documented at a high glucose concentration [23]. Indeed, after pre-perifusion for 45 min with 3 mmol/L glucose, the subsequent half an hour response to 20 mmol/L glucose was enhanced 2.4-fold. Under this more physiological circumstance, the secretory response to 20 mmol/L glucose has probably reached its maximum value and addition of SAD does not increase it further. Pre-perifusion with 3 mmol/L glucose or 1.5 mmol/L SAD enhanced only the magnitude of the first phase of the subsequent secretory response to 10 mmol/L SAD but not the second phase. This previous exposure to subthreshold substrate concentrations might keep intracellular ATP at permissive levels for priming secretory granules into the readily releasable pool [25], thus enhancing a subsequent secretory response. On average, the maximum islet response to glucose, obtained after pre-perifusing with 3 mmol/L glucose for 45 min, is only 30% higher than the maximum stimulation recorded, after pre-perifusion without substrates, by the combination of pyruvate and SAD. In summary, it is concluded that at least 70% of the maximum secretory response to glucose may be mimicked by the combination of mitochondrial substrates that are directly oxidized in the citric acid cycle without the participation of glycolysis. This conclusion is supported by previous work showing that α-ketoisocaproic acid, a pure mitochondrial substrate, elicits a biphasic, and concentration-dependent, secretory response in mouse islets [26].

Malonic acid is a known competitive inhibitor of succinic acid dehydrogenase [15] but it seems to be non-permeable through the plasma membrane as it does not alter either $^{14}CO_2$-production from 20 mmol/L D-[U-^{14}C]glucose, or islet levels of ATP or the ATP/ADP ratio (results not shown). However, its dimethyl ester (MAD, at 10 mmol/L) decreased the production of $^{14}CO_2$ from [2-^{14}C]pyruvate by approximately 70%, thus confirming its expected inhibitory action on the citric acid cycle dehydrogenase for succinic acid. As far as is known, this is the first report demonstrating an inhibitory effect of MAD on mitochondrial substrate oxidation by islets. In correspondence with this metabolic effect, 10 mmol/L MAD inhibited the secretory response to SAD by 62–77%. Similarly, glucose-induced insulin secretion (20 mmol/L glucose) was also suppressed within 54–82% by MAD (10–20 mmol/L, respectively). It has been previously shown [27] that the monomethyl ester of malonic acid (10 mmol/L) did not significantly modify the stimulation of insulin secretion induced in rat islets by glucose and monomethyl succinate. In this report, however, the effect of malonic acid monomethyl ester, at variance with the dimethyl ester (MAD) used in the present study, on islet metabolism was not characterized. Therefore, one cannot exclude the possibility that a lower permeability to the monomethyl ester, as compared with the dimethyl ester derivative, was responsible of its lower intracellular accumulation, hence of its failure to reach an effective inhibitory concentration.

The rate of glycolysis, measured as the detritiation of D-[5-^{3}H]glucose, was unaltered by 10 mmol/L MAD. Under these conditions, reoxidation of glycolysis-derived NADH by any of the shuttle systems is not expected to be affected, but the stimulation of insulin secretion by glucose is strongly suppressed. This evidence does not support the idea [3–5] that the stimulation of insulin secretion by glucose is exclusively linked to the re-oxidation of glycolysis-derived NADH. This conclusion is also strengthened by recently reported evidence in INS-1 cells expressing glycerol kinase, indicating that the magnitude of the redox potential of the NADH/NAD^+-couple does not determine the magnitude of the secretory response to glycerol or dihydroxyacetone [28].

As already commented in more detail in Section 3.3, mouse and rat islets seem to share a common mechanism for the regulation of $[Ca^{2+}]_i$ and insulin release in response to nutrient secretagogues. In mouse islets, the recording of islet $[Ca^{2+}]_i$ has allowed to demonstrate that SAD, at a non-stimulatory glucose concentration (2.75 mmol/L), is able of reproducing the elevation and regular oscillatory pattern induced by a stimulatory glucose concentration (11.1 mmol/L) in mouse islets. These effects are strictly dependent on the integrity of the citric acid cycle metabolism, as they are completely suppressed by MAD. They confirm a previous finding showing that mannoheptulose, an inhibitor of glucose metabolism at its very first step of phosphorylation, suppresses the glucose-induced, oscillatory pattern of $[Ca^{2+}]_i$ which returns to basal levels [29]. The fact that SAD alone does not increase insulin secretion in mouse islets confirms a recent report which associates this lack of stimulation to a species failure for the expression of malic enzyme activity particularly in islet tissue [30]. However, succinic acid can be partially metabolized in the citric acid cycle of mouse islets to oxaloacetate which might then be further metabolized after condensing with glucose-derived acetyl-CoA. This might be the reason why SAD stimulates both insulin secretion and the oscillating $[Ca^{2+}]_i$ in a glucose-dependent manner in mouse islets. In other words, SAD addition mimics the effects of an elevation of the glucose concentration from a non-stimulatory to a stimulatory value.

In the case of glucose, citric acid cycle inhibition by MAD ablated the regular oscillatory pattern of islet $[Ca^{2+}]_i$ which remained elevated and did not return to basal values, at variance to what occurred with SAD. The main metabolic difference between glucose and SAD in the presence of MAD being that glucose continues to be metabolised through an intact glycolysis, makes it tempting to propose that ATP produced by the latter and by re-oxidation of cytosolic NADH is sufficient to elevate $[Ca^{2+}]_i$ to a stimulatory level but not for a maximum stimulation of exocytosis. This might serve the purpose of priming the subsequent metabolism of pyruvate in the citric acid cycle through the activation of Ca^{2+}-sensitive dehydrogenases [31,32] and feeding forward the secretory response.

In summary, nutrient oxidation in the citric acid cycle seems to be necessary and sufficient for a close to maximum stimulation of exocytosis in the β-cells of isolated rat islets. An increased islet glycolysis and the re-oxidation of cytosolic NADH are sufficient for initiating exocytosis, as judged by the $[Ca^{2+}]_i$ elevation, but not for a full amplification of the insulin secretory response.

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