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Mechanisms of arginine-induced increase in cytosolic calcium concentration in the beta-cell line NIT-1

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Summary The effects of L-arginine and its analogues N^G-nitro-L-arginine, N^G-methyl-L-arginine, L-homoarginine and D-arginine on cytosolic calcium concentration were investigated to characterise the mechanisms of arginine-induced stimulation and to determine if nitric oxide production played a role in this stimulation. NIT-1 cells, a transgenic beta-cell line, were used for this purpose since they release insulin in response to typical beta-cell stimuli. Our data demonstrate that the arginine-induced increase in cytosolic calcium concentration was completely dependent on the influx of extracellular Ca^{2+} via verapamil-sensitive voltage-activated Ca^{2+} channels and that arginine stimulation requires the presence of a nutrient in order to cause an increase in cytosolic calcium concentration. The nutrient likely acted by closing the K^+_{ATP} channels, since its effect could be inhibited by activation of these channels with diazoxide. L-arginine, as well as nitro-arginine and methyl-arginine which are not substrates for the production of nitric oxide, caused similar increases in cytosolic calcium concentration. Non-metabolisable arginine analogues homoarginine and D-arginine also caused increases in the cytosolic calcium concentration although not to the same extent. Insulin secretion was enhanced to the same extent by all analogues of arginine. It can be concluded that the arginine-induced increase in cytosolic calcium concentration in NIT-1 cells is attributable to an electrogenic effect following the transport of arginine leading to depolarisation of the plasma membrane potential, although metabolism of the amino acid itself may also partially contribute to the response. [Diabetologia (1997) 40: 374–382]

Keywords Arginine, N^G-nitro-L-arginine, N^G-methyl-L-arginine, L-homoarginine, fura-2, intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), NIT-1 cells.

Changes in beta-cell cytosolic calcium concentration ($[Ca^{2+}]_i$), whether by an influx of extracellular Ca^{2+} or by release of Ca^{2+} from intracellular stores, are thought to be the primary trigger for the initiation of insulin secretion [1–4]. Glucose, the major insulin secretagogue, stimulates insulin release by the production of ATP during its metabolism thus increasing the cytosolic ATP/ADP ratio. This in turn closes the K^+_{ATP} channels in the plasma membrane [5, 6] leading to the depolarisation of the membrane potential [7, 8]. This depolarisation causes the opening of the voltage-activated Ca^{2+} channels, also present in the plasma membrane [9–12], resulting in the influx of Ca^{2+} into the cell and the subsequent elevation of $[Ca^{2+}]_i$.

The amino acid arginine causes insulin release from the perfused pancreas, pancreatic explants and perifused islets of the rat as well as from glucose-insensitive human [13] and rat [14, 15] fetal beta cells. Arginine also causes an increase in $[Ca^{2+}]_i$ [11, 16–18] in beta cells; however, the cellular mechanisms involved in this response are not well understood.

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Abbreviations: $[Ca^{2+}]_i$, Intracellular Ca^{2+} concentration; Nit-arg, N^5-[Nitroaminidino]-L-2,5-diaminopentanoic acid; Me-arg, N^G-methyl-L-arginine (N^5-[Methylamidino]-L-ornithine; $NMDG^+$, N-methyl-D-glucamine; Sin-1, 3-morpholino-syndonimine-hydrochloride; SNP, sodium nitroprusside.

Several mechanisms for arginine-induced beta-cell stimulation have been proposed. These include the metabolism of L-arginine leading to the formation of ATP [19, 20], the generation of nitric oxide [21, 22], and the direct depolarisation of the plasma membrane potential, due to the accumulation of the cationic amino acid [18, 23, 24].

We performed experiments on the NIT-1 insulinoma cell line established from a transgenic mouse [25]. We first characterised the responses of this cell line to arginine and other stimulants of insulin secretion to confirm that its properties were similar to those of other beta cells. We then used the ratiometric fluorescent probe of Ca^{2+}, fura-2, to study the increase in $[Ca^{2+}]_i$ in response to arginine and arginine analogues to determine the role of nitric oxide production in arginine-induced stimulation of beta cells.

Materials and methods

Materials. L-arginine and its analogues N^G-nitro-L-arginine (N^5-[Nitroaminidino]-L-2,5-diaminopentanoic acid)(nit-arg), N^G-methyl-L-arginine (N^5-[Methylamidino]-L-ornithine)(me-arg), L-homoarginine (2-amino-6-guanidinohexanoic acid), D-arginine as well as D,L-glyceraldehyde, N-methyl-D-glucamine ($NMDG^+$), verapamil hydrochloride and the disodium salt (grade I) of adenosine 5′-triphosphate (ATP) were purchased from Sigma Chemical Co. (St. Louis, Mo., USA). 3-morpholino-syndonimine-hydrochloride (sin-1) was a generous gift from Pharmaforschung (Frankfurt, Germany). Diazoxide was obtained from Research Biochemicals Incorporated (RBI) (Natick, Mass., USA) and ionomycin from Calbiochem (San Diego, Calif., USA). All chemicals were analytical reagent grade.

NIT-1 cells (passage 90–110) were maintained in culture in antibiotic free, low-glucose (5.6 mmol/l) DMEM medium (Gibco, Grand Island, N. Y., USA) supplemented with 10 % fetal calf serum at 37 °C in 5 % CO_2 and air. For microfluorometric experiments cells were grown on glass coverslips for 2 days. Coverslips covered with cells were incubated for 35 min at 37 °C with 12 μmol/l of the fluorescent intracellular dye fura-2 AM (Molecular Probes, Eugene, Ore., USA) plus 12 μl/ml of a 20 % (w/v) stock Pluronic F-127 solution (Molecular Probes) in a shaking water bath. Following incubation, the cells were washed three times, a piece of the coverslip cut off with a diamond knife, transferred to a closed perfusion chamber of approximately 0.3 ml volume and perfused at a flow rate of 1 ml/min by a peristaltic pump (Minipuls, Gilson, Middleton, Wis., USA). All experiments were performed at room temperature (20–22 °C).

The solution used for loading, washing and perfusion consisted of the following (in mmol/l): 145 NaCl, 5 KCl, 1 $MgCl_2$, 10 H-HEPES, 1 $CaCl_2$, 1 D-glucose, pH adjusted to pH 7.35 with NaOH at room temperature. In the experiments performed in the absence of glucose, this sugar was replaced with equimolar amounts of sucrose, glyceraldehyde or leucine. In experiments performed in the absence of Ca^{2+}, a perfusion solution was used containing no added $CaCl_2$ and 1.0 mmol/l EGTA. In experiments performed in Na^+-free solution, Na^+ was replaced by equimolar substitution of N-methyl-D-glucamine ($NMDG^+$).

Measurements of $[Ca^{2+}]_i$. Microfluorometric experiments were conducted using a Nikon Diaphot inverted microscope (Nikon Corporation, Tokyo, Japan) equipped with × 40 Fluor objective. Fura-2 loaded cells were irradiated alternately with light at 340 and 380 nm using a filter wheel. The emitted light was passed through a 505 ± 10 nm bandpass filter, detected by a photomultiplier and the resultant signal recorded on a 4-channel MacLab (ADInstruments Pty Ltd., Sydney, Australia). Fura-2 ratios (R) were calculated as 340/380 ratio and converted into $[Ca^{2+}]_i$ according to the equation derived by Grynkiewicz et al. [26]. The dissociation constant (K_d) for Fura-2 at room temperature was taken to be 135 nmol/l [26]. Calibrations of the fura-2 signal were performed at the end of each study day. For calibration, the cells were exposed to a 150 mmol/l KCl solution plus 2 μmol/l ionomycin (pH 8.0) [27], containing either 10 mmol/l EGTA (R_{min}) or 10 mmol/l $CaCl_2$ (R_{max}). The resting $[Ca^{2+}]_i$ measured in this study (30 nmol/l) is at the lower end of the reported range (see [28, 29]). This may be because of the present experiments being carried out at room temperature.

Measurements of nitric oxide. Nitric oxide production from NIT-1 cells was assayed by measuring the production of nitrate and nitrite with the Griess reagent [30–32]. NIT-1 cells grown in 35 mm tissue culture Petri dishes were washed in perfusion solution before being incubated for 10 min at room temperature in the presence of either perfusion solution alone (as a control), or perfusion solution supplemented with one of the following: 20 mmol/l L-arginine, 20 mmol/l nitro-arginine, 20 mmol/l homoarginine or 100 μmol/l sin-1. Immediately following the stimulation, the samples were collected and stored at –20 °C until assayed. The cells were trypsinised off the dish and total cell numbers were counted with the use of a haemocytometer. The Griess reagent was added to the samples before being transferred to 96 multiwell plates and the absorbances read at 540 nm (Titertek Multiscan, Pathtech, Victoria, Australia). Measurements for the determination of nitrate required the reduction of this compound to nitrite by the addition of 0.6 mmol/l NADPH and 5 units/ml nitrate reductase before the addition of the Griess reagent. Results were read from standard curves for reduced $NaNO_3$ and $NaNO_2$ (4 to 1000 nmol/l). Nitric oxide production was expressed as the concentration of nitrite plus nitrate ($NO_2 + NO_3$) and expressed as nmol · 10^6 cells^{-1} · 10 min^{-1}.

Measurements of insulin release. NIT-1 cells grown in 35-mm Petri dishes were washed in perfusion solution and then exposed for 10 min at room temperature to either perfusion solution alone (as a control), or to perfusion solution supplemented with one of the following: 20 mmol/l L-arginine, 20 mmol/l nitro-arginine, 20 mmol/l homoarginine, or 100 μmol/l sin-1. Following the stimulation, samples were collected and stored at –20 °C until assayed. The cells were trypsinised off the dish and total cell numbers were counted with the use of a haemocytometer. The levels of insulin secretion were measured by radioimmunoassay (RIA) using a rat standard (Novo Laboratories, Bagsvaerd, Denmark). Insulin release was expressed as ng · 10^6 cells^{-1} · 10 min^{-1}.

Statistical analysis. Results are expressed as mean ± SEM (number of observations). Statistical significance was assessed by using the Student's unpaired *t*-test, or analysis of variance (ANOVA) when variances were equal; and the Mann-Whitney non-parametric test when variances were unequal.

Results

$[Ca^{2+}]_i$ measurements

Response to insulin secretagogues. NIT-1 cells were exposed to glucose, glyceraldehyde and leucine, agents which are metabolised to form ATP, thereby increasing the cytosolic ATP/ADP ratio and closing K^+_{ATP} channels [17, 33–38]. The resting $[Ca^{2+}]_i$ in NIT-1 cells was 30 ± 1 nmol/l ($n = 199$). Exposure of NIT-1 cells to 20 mmol/l glucose elicited an increase in $[Ca^{2+}]_i$ of 21 ± 3 nmol/l ($n = 6$, $p < 0.01$)(Fig. 1A) above resting levels. NIT-1 cells also responded to 20 mmol/l glyceraldehyde and 20 mmol/l leucine with increases in $[Ca^{2+}]_i$ of 76 ± 21 nmol/l ($n = 7$, $p < 0.02$)(Fig. 1B) and 88 ± 8 nmol/l ($n = 13$, $p < 0.01$)(Fig. 1C), respectively. Membrane depolarisation following exposure to 20 mmol/l KCl [10, 11, 34] produced an increase in $[Ca^{2+}]_i$ of 96 ± 13 nmol/l ($n = 7$, $p < 0.01$)(Fig. 1D) showing that depolarisation of the membrane potential leads to opening of voltage-activated Ca^{2+} channels in NIT-1 cells. These results demonstrate that the NIT-1 cell possesses the complete mechanism to translate stimulation by known secretagogues to an increase in $[Ca^{2+}]_i$.

Characterisation of arginine-induced beta-cell stimulation. Exposure of the NIT-1 cells to a 5-min pulse of arginine (20 mmol/l) caused an increase in $[Ca^{2+}]_i$ of 72 ± 4 nmol/l ($n = 76$, $p < 0.01$) above resting levels. The increase in $[Ca^{2+}]_i$ occurred within 60 s of exposure reaching a peak within 152 ± 31 s ($n = 10$) (Fig. 2A). The magnitude of the increase in $[Ca^{2+}]_i$ in response to a second stimulus to 20 mmol/l arginine (57 ± 6 nmol/l, $n = 14$) was not significantly different from that observed in the first exposure ($p = 0.474$, $n = 14$) (Fig. 2A), indicating that NIT-1 cells were not desensitized following repeated stimulation with arginine.

The increase in $[Ca^{2+}]_i$ in response to 20 mmol/l arginine was not dependent on extracellular Na^+. When the Na^+ in the perfusion solution was replaced by $NMDG^+$, exposure to 20 mmol/l arginine caused an increase in $[Ca^{2+}]_i$ of 62 ± 8 nmol/l ($n = 5$, $p = 0.150$ compared to arginine in the presence of Na^+) (Fig. 2B), consistent with previous reports that arginine transport across the cell membrane occurs by a Na^+-independent mechanism [39]. The removal of extracellular Na^+ caused a slight increase in $[Ca^{2+}]_i$ of 20 ± 4 nmol/l ($p < 0.001$, $n = 11$) likely due to the inhibition of Na^+/Ca^{2+} exchange which is present in these cells [40–42].

It is well established that arginine-induced insulin secretion from beta cells is nutrient dependent [18, 24, 43, 44]. The arginine-induced increase in $[Ca^{2+}]_i$ in NIT-1 cells was also found to be nutrient dependent since removal of glucose from the extracellular solution inhibited the response to 20 mmol/l arginine by $82 \pm 5\%$ ($n = 10$) compared to control ($p < 0.001$, $n = 10$) (Fig. 2C). When glucose was replaced by other nutrients, the arginine-induced increase in $[Ca^{2+}]_i$ was also observed. Exposure of the cells to 20 mmol/l arginine in the perfusion solutions in which the glucose was replaced by either 1 mmol/l glyceraldehyde or 1 mmol/l leucine, caused increases in $[Ca^{2+}]_i$ of 66 ± 4 nmol/l ($n = 3$), and 55 ± 14 nmol/l ($n = 3$), respectively, not significantly different from the responses observed in the presence of glucose. In contrast, the response to direct membrane depolarisation by 20 mmol/l KCl was not significantly inhibited by the removal of glucose compared to controls ($15 \pm 8\%$ inhibition, $n = 3$). Diazoxide, a specific activator of K^+_{ATP} channels [35, 45], is able to counter

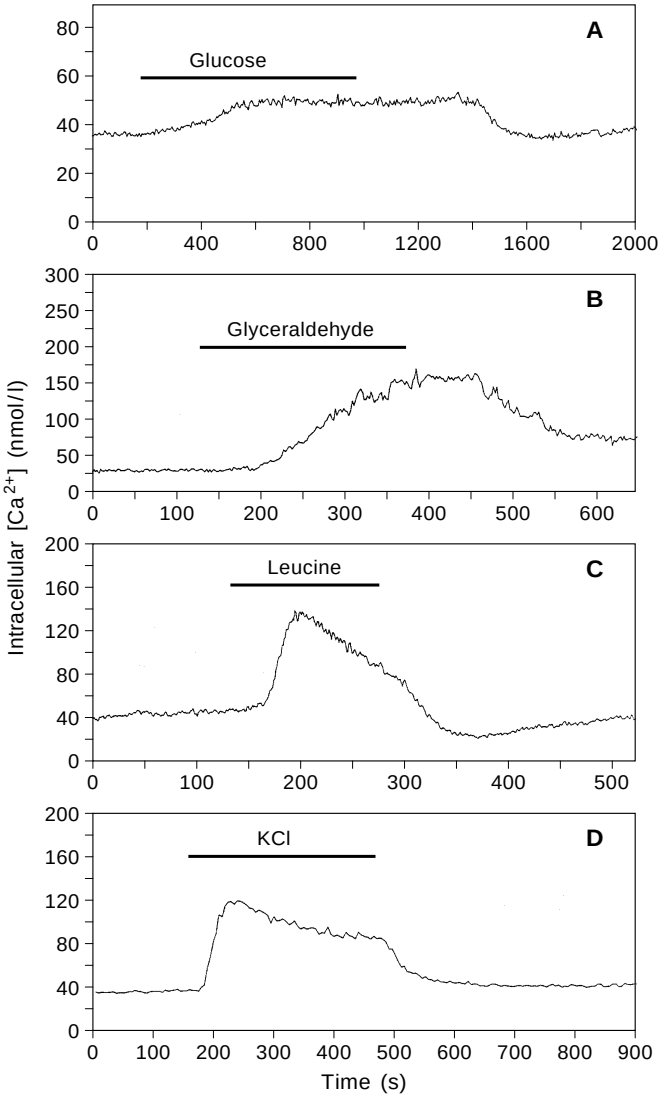

Fig. 1A–D. The effects of glucose (**A**), glyceraldehyde (**B**), leucine (**C**) and KCl (**D**) exposure on NIT-1 cells. The increase in glucose concentration (1 to 20 mmol/l) and the addition of glyceraldehyde (20 mmol/l), leucine (20 mmol/l) and KCl (20 mmol/l) to the perifusion solution are indicated by the black bars

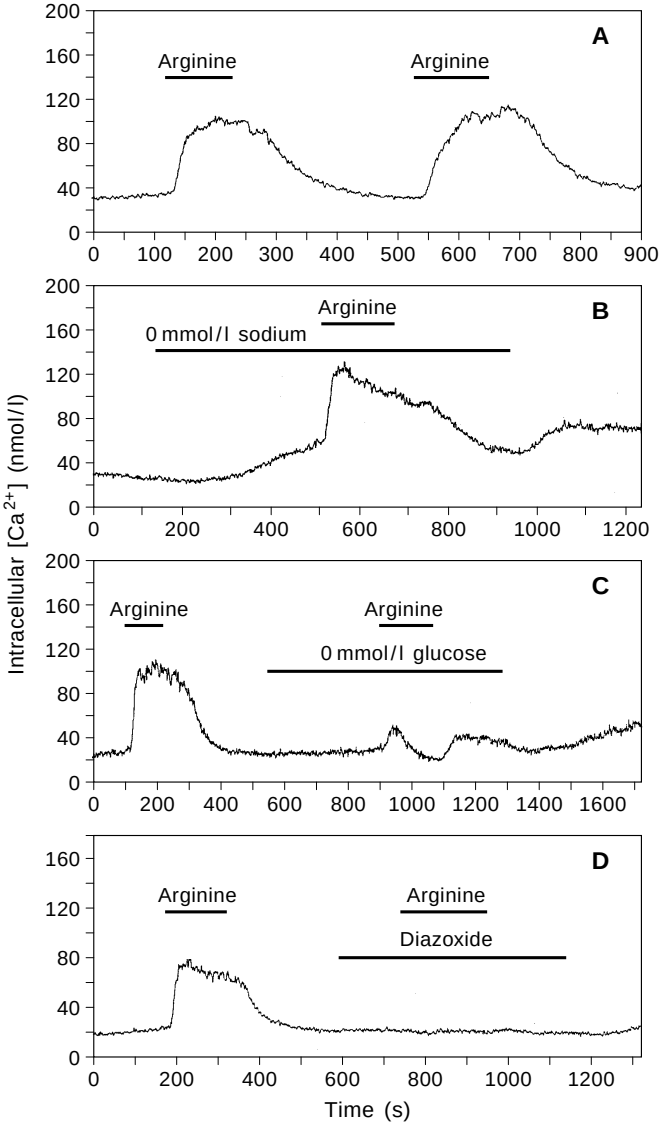

Fig. 2A–D. The effect of iterative stimulation (**A**), the removal of extracellular sodium (**B**), the removal of extracellular glucose (**C**) and the exposure of diazoxide (**D**) on arginine-induced increase in $[Ca^{2+}]_i$ in NIT-1 cells. The addition of arginine (20 mmol/l) and diazoxide (100 μmol/l); as well as the decrease in glucose concentration (1 to 0 mmol/l) and the removal of sodium from the perifusion solution are indicated by the black bars

membrane depolarisation in beta cells [9, 45–48]. Exposure of NIT-1 cells to diazoxide (100 μmol/l) did not change the $[Ca^{2+}]_i$ during a 5-min exposure (2 ± 1 nmol/l, $n = 9$), but inhibited the response to 20 mmol/l arginine by $97.5 \pm 1.4\%$ ($p < 0.001$, $n = 10$) (Fig. 2D), suggesting that arginine-induced depolarisation of the membrane potential can be prevented by activation of the K^+_{ATP} channels.

Experiments were conducted to determine if the arginine-induced increase in $[Ca^{2+}]_i$ was due to Ca^{2+} flux from the extracellular solution. Removal of Ca^{2+} from the extracellular solution caused a decrease in $[Ca^{2+}]_i$ of 25 ± 4 nmol/l from resting levels ($p < 0.01$, $n = 11$). In addition, the removal of extracellular Ca^{2+} completely inhibited the response to 20 mmol/l arginine ($p < 0.001$, $n = 11$) (Fig. 3A). Exposure of NIT-1 cells to ATP, which binds to cell surface receptors and causes release of Ca^{2+} from intracellular stores in beta cells [29, 49], caused an increase in $[Ca^{2+}]_i$ of 110 ± 41 nmol/l (100 μmol/l ATP, $n = 3$) and 50 ± 8 nmol/l (10 μmol/l ATP, $n = 2$), respectively, in the presence of 1 mmol/l extracellular Ca^{2+}. In the absence of extracellular Ca^{2+}, there was no inhibition of the response to either 100 μmol/l ATP or 10 μmol/l ATP ($17 \pm 2\%$, $n = 3$ and $0 \pm 16\%$, $n = 3$, respectively), indicating that the response to ATP was attributable solely to intracellular Ca^{2+} stores. These results show that the arginine-induced increase in $[Ca^{2+}]_i$ is dependent upon Ca^{2+} influx from the extracellular solution and does not involve release from intracellular stores.

The addition of 20 μmol/l verapamil, a blocker of voltage-activated Ca^{2+} channels in beta cells [1, 50–52], had no effect on resting $[Ca^{2+}]_i$ in NIT-1 cells ($n = 9$). It did, however, significantly inhibit the response to 20 mmol/l arginine by $86.0 \pm 4.6\%$ ($p < 0.001$, $n = 9$) (Fig. 3B). This demonstrates that the arginine-induced increase in $[Ca^{2+}]_i$ in NIT-1 cells occurs due to the influx of Ca^{2+} from the extracellular solution via voltage-activated Ca^{2+} channels.

The effect of nitric oxide producing agents. The production of nitric oxide has been implicated as the mechanism involved in arginine-induced stimulation of beta cells [21], and has been shown to cause an increase in $[Ca^{2+}]_i$ in endothelial cells [53, 54]. Experiments were performed to investigate the effects of increasing the intracellular nitric oxide concentration on $[Ca^{2+}]_i$ in the NIT-1 cell.

Sin-1 was used for this purpose because of previous reports showing that it causes an increase in the cytosolic concentration of nitric oxide in beta cells [21]. Exposure of the NIT-1 cells to 100 μmol/l sin-1 caused an increase in $[Ca^{2+}]_i$ of 89 ± 11 nmol/l ($n = 11$) (Fig. 4A). The magnitude of this increase was not significantly different from that caused by 20 mmol/l L-arginine ($n = 11$). Removal of extracellular glucose inhibited the response to 100 μmol/l sin-1 by $54.8 \pm 15.4\%$ ($p = 0.024$, $n = 3$), an effect similar to that of the removal of glucose on the arginine-induced increase in $[Ca^{2+}]_i$.

NIT-1 stimulation by arginine analogues. In order to further investigate the role of nitric oxide production in the arginine-induced increase in $[Ca^{2+}]_i$, we repeated the experiments with the arginine analogues nit-arginine and me-arginine which are not substrates for nitric oxide synthase and therefore do not generate nitric oxide in beta cells [22, 55–57]. Exposure of the NIT-1 cells to 20 mmol/l nit-arg and 20 mmol/l

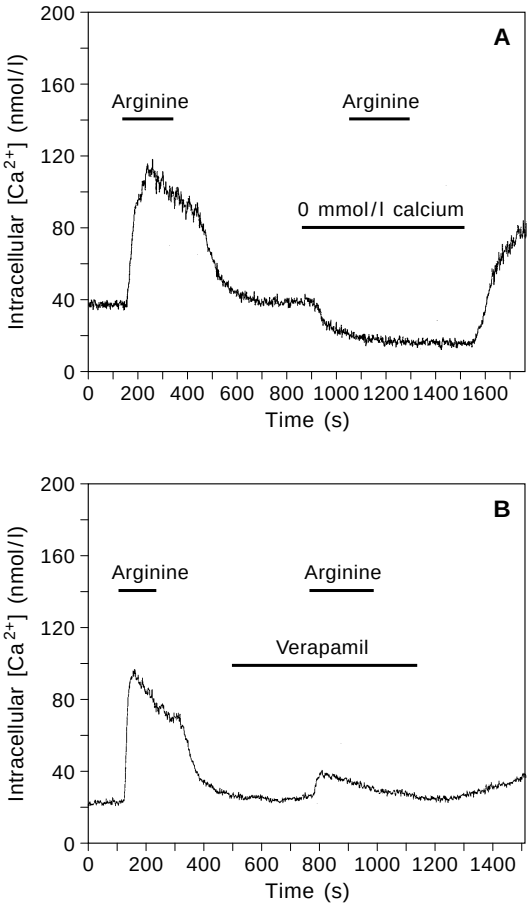

Fig. 3A, B. The effect of the removal of extracellular Ca^{2+} (**A**) and of the addition of the Ca^{2+} channel blocker verapamil (**B**) on the arginine-induced increase in $[Ca^{2+}]_i$ by NIT-1 cells. The removal of calcium; and the addition of arginine (20 mmol/l) and verapamil (20 μmol/l) to the perifusion solution are indicated by the black bars

me-arg caused increases in $[Ca^{2+}]_i$ of 83 ± 7 nmol/l ($n = 13$) and 74 ± 10 nmol/l, ($n = 5$) (Fig. 5). At all concentrations examined (0.01–20 mmol/l) (Fig. 5), the responses to nit-arginine and me-arginine were not significantly different from those to L-arginine. Removal of extracellular Ca^{2+} inhibited the response to nit-arginine by 83 ± 3 % ($n = 3$), just as it did for L-arginine.

The arginine-analogue homoarginine [23, 24, 58], and the arginine enantiomer D-arginine [57, 59], are non-metabolisable and are not substrates for nitric oxide synthase for the production of nitric oxide in the beta cell. Exposure of the NIT-1 cells to 20 mmol/l homoarginine and 20 mmol/l D-arginine caused increases in $[Ca^{2+}]_i$ of 39 ± 7 nmol/l ($n = 7$) and 26 ± 3 nmol/l ($n = 9$) (Fig. 5). These responses were not significantly different from each other at any concentration (0.01–20 mmol/l), but were significantly less than those to L-arginine at concentrations above 10 mmol/l ($p = 0.030$, $n = 7$) and below 1 mmol/l ($p = 0.004$, $n = 3$).

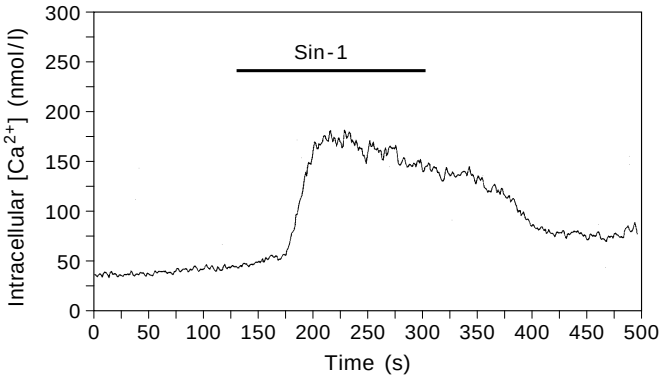

Fig. 4. The effect of the nitric oxide producer sin-1 on $[Ca^{2+}]_i$ in NIT-1 cells. The addition of sin-1 (100 μmol/l) to the perifusion solution is indicated by the black bar

Since these results suggested that the production of nitric oxide was not involved in the arginine-induced increase in $[Ca^{2+}]_i$, we tested this hypothesis by using a different agent, sodium nitroprusside (SNP), to elevate nitric oxide levels. Exposure of the cells to 1 mmol/l SNP had no effect on the levels of $[Ca^{2+}]_i$ (change in $[Ca^{2+}]_i$ -1 ± 1 nmol/l, $n = 5$). This result is further evidence that nitric oxide is not involved in the arginine-induced increase in $[Ca^{2+}]_i$.

Nitric oxide measurements

Exposure of NIT-1 cells for 10 min to 20 mmol/l L-arginine and 100 μmol/l sin-1 caused the production of 29.8 ± 4.8 and 36.7 ± 3.0 nmol/l nitric oxide $(NO_2 + NO_3) \cdot 10^6$ cells$^{-1} \cdot 10$ min^{-1} ($n = 3$ preparations each in quintuplicate) (Fig. 6A). Exposure of the cells to the perfusion solution (control), 20 mmol/l nit-arg and 20 mmol/l homoarginine failed to stimulate the production of nitric oxide above detectable levels (4 nmol/l $\cdot 10^6$ cells$^{-1} \cdot 10$ min^{-1}) ($n = 3$).

Insulin secretion measurements

Insulin release measurements are summarised in Figure 6B ($n = 3$ for all experiments). The NIT-1 cells released 2.7 ± 0.4 ng insulin $\cdot 10^6 \cdot$ cells in 10 min during incubation in perfusion solution alone (control). Exposure to 100 μmol/l sin-1, 20 mmol/l L-arginine, 20 mmol/l nitro-arginine and 20 mmol/l homoarginine caused the release of insulin at 4.7 ± 0.5, 6.0 ± 1.3, 9.9 ± 1.3 and 6.9 ± 1.9 ng $\cdot 10^6 \cdot$ cells$^{-1} \cdot$ 10 min^{-1}, respectively. These agents all released insulin in amounts significantly greater than the amount released by the control ($p = 0.017$, 0.028, 0.003, and 0.016), but did not differ significantly from one another.

Discussion

Arginine is a well-known secretagogue for the stimulation of insulin release from the pancreatic beta

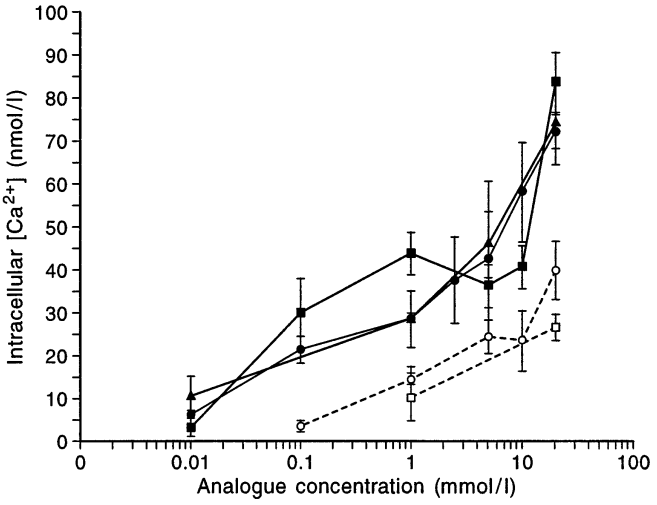

Fig. 5. Dose response curves (0.01–20 mmol/l) for the increases in $[Ca^{2+}]_i$ in response to L-arginine (●), and the arginine analogues nit-arg (■), met-arg (▲), homoarginine (○) and D-arginine (□)

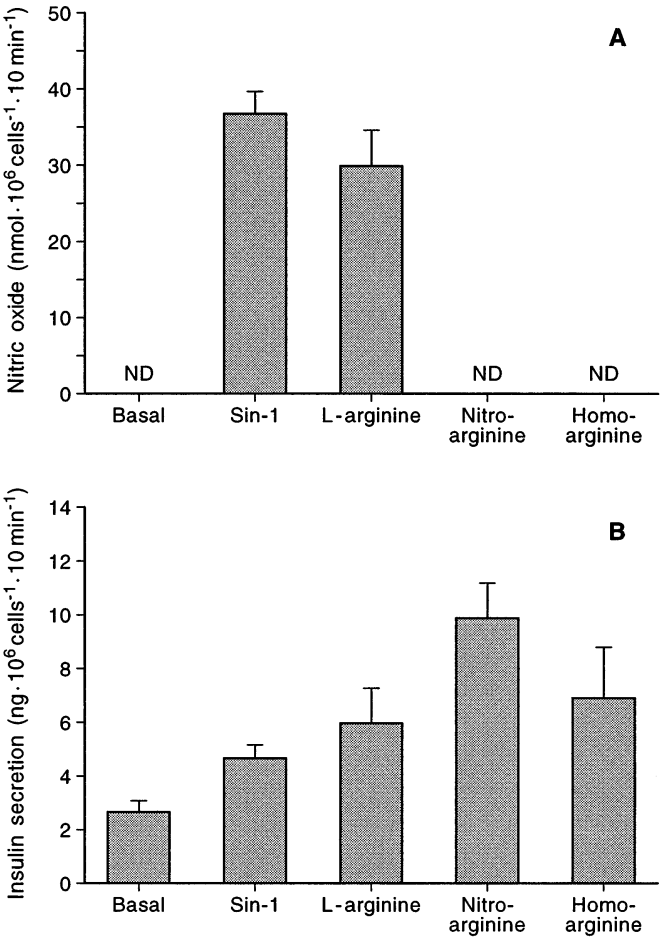

Fig. 6. Nitric oxide production (**A**) and insulin secretion (**B**) from NIT-1 cells in response to sin-1 (100 μmol/l), L-arginine (20 mmol/l), nitro-arginine (20 mmol/l) and homoarginine (20 mmol/l) ($n = 3$ preparations measured in quintuplicate in all experiments)

cell. The mechanism by which arginine-induced stimulation occurs is, however, not well characterised. Our data are consistent with a mechanism in which arginine enters NIT-1 cells via a Na^+-independent transport system, as previously described [39, 60]. Interestingly, the concentration-response curve for arginine (Fig. 5) suggests the presence of two distinct transporter systems for arginine and its analogues, one with an affinity for arginine of approximately 100 μmol/l, the other with an affinity in the millimolar range. These correspond with the known affinities of the amino acid transporters MCAT-1 and MCAT-2a which are present in islet cells [60]. Since the plasma level of arginine in fasted animals is approximately 100 μmol/l [61], the higher affinity component is likely to be physiologically relevant.

The arginine-induced increase in $[Ca^{2+}]_i$ is dependent upon the influx of extracellular Ca^{2+} and occurs via verapamil-sensitive and voltage-activated Ca^{2+} channels. This arginine-induced increase in $[Ca^{2+}]_i$ requires the presence of a nutrient and is inhibited by the activation of K^+_{ATP} channels with diazoxide. These observations suggest that the K^+_{ATP} channels, when fully open, act to prevent membrane depolarisation caused by arginine. The presence of a nutrient, such as glucose, produces sufficient closure of K^+_{ATP} channels to allow arginine-induced membrane depolarisation and activation of the voltage-activated Ca^{2+} channels. Our observations are consistent with previous reports in which arginine-induced stimulation of insulin was abolished by the complete removal of ATP from permeabilised islets [62], patch-clamp experiments that demonstrated that arginine alone did not cause the closure of K^+_{ATP} channels [35] and radioisotope studies which demonstrated that arginine exposure did not cause a reduction in $^{86}Rb^+$ efflux [18, 63].

There are considerable inconsistencies in the literature about whether arginine-induced insulin secretion is due to nitric oxide production. A number of studies have suggested that arginine-induced insulin release was caused by an increase in the cytosolic nitric oxide concentration. For example, nit-arginine and me-arginine inhibited insulin secretion in rat beta cells, HIT-T15 insulinoma cells [21, 57] and from perfused rat pancreas [22]. In contrast, other studies have shown that nit-arginine and me-arginine [22, 64], as well as homoarginine and D-arginine [24, 57, 65] all caused insulin release from rat islets. Studies using electropermeablised rat islets demonstrated that the addition of either nit-arginine or me-arginine with L-arginine inhibited arginine-derived nitric oxide production without affecting insulin release [56]. In addition, nit-arginine alone did not increase insulin release from isolated mouse islets, but when combined with L-arginine markedly increased D-arginine-induced insulin release [66].

The present results demonstrate by fluorimetric measurements that the arginine analogues, nit-arginine and met-arginine, which do not produce nitric oxide, cause increases in $[Ca^{2+}]_i$ in beta cells similar to those caused by L-arginine. We also show that the arginine analogues which are completely non-metabolisable, homoarginine and D-arginine, cause an increase in $[Ca^{2+}]_i$ although to a smaller extent than those caused by L-arginine. From the present data it is not possible to state whether the lesser effects on $[Ca^{2+}]_i$ of these non-metabolisable analogues are attributable entirely to them having lower affinities for the arginine transport system, or to metabolism of L-arginine, nit-arginine and met-arginine playing some role in producing the $[Ca^{2+}]_i$ response. The production of ATP from the metabolism of L-arginine alone is not likely to be this hypothetical metabolism-dependent mechanism because L-arginine stimulation does not lead to the closure of K^+_{ATP} channels in patch-clamp studies [35], nor does it increase $[Ca^{2+}]_i$ in the absence of glucose in the present study. Alternatives to the production of ATP by the metabolism of arginine include the formation of polyamines [20, 67].

In addition, because the nitric oxide producer nitroprusside did not cause an increase in $[Ca^{2+}]_i$, the increased $[Ca^{2+}]_i$ observed in the presence of sin-1 was presumably attributable to the transport of this zwitterionic compound into the cell, rather than to nitric oxide release. These findings indicate that nitric oxide production is not the mechanism leading to the arginine-induced increase $[Ca^{2+}]_i$ in NIT-1 cells, rather it is due to a process involving electrogenic transport of arginine and its analogues leading to depolarisation of the plasma membrane.

The fluorimetric data are supported by insulin release data which demonstrate that the agents which were able to cause an increase in $[Ca^{2+}]_i$ such as sin-1, L-arginine, nit-arg and homoarginine were also able to stimulate the release of insulin. The analogues of arginine caused similar amounts of insulin release suggesting that arginine-induced stimulation in beta cells has a threshold [23, 24] such that an increase in $[Ca^{2+}]_i$, even to the extent caused by homoarginine, is sufficient in itself to cause insulin secretion. Beyond this threshold a further increase in $[Ca^{2+}]_i$ does not further enhance insulin release.

In conclusion, these results suggest that in NIT-1 cells, L-arginine does not act via the nitric oxide pathway since nit-arg and met-arg which do not produce nitric oxide, caused increases in $[Ca^{2+}]_i$ and insulin release to the same extent as L-arginine. The data suggest that the arginine-induced increase in $[Ca^{2+}]_i$ occurs as a result of membrane depolarisation caused by the cationic amino acid, although metabolism of the amino acid itself may also partially contribute to the response.

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