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Stimulus-Secretion Coupling of Arginine-Induced Insulin Release

Comparison Between the Cationic Amino Acid and its Methyl Ester

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The role currently ascribed to the accumulation of L-arginine in the pancreatic islet B-cell as a determinant of its insulinotropic action was reevaluated by comparing the uptake and the metabolic, ionic, electric, and secretory effects of the cationic amino acid with those of its more positively charged methyl ester in rat pancreatic islets. The response to L-arginine methyl ester differed from that evoked by the unesterified amino acid by a lower uptake and oxidation, lack of inhibitory action on D-glucose metabolism, more severe inhibition of the catabolism of endogenous L-glutamine, inhibition of ^{45}Ca net uptake, decrease in both ^{86}Rb outflow from prelabeled islets perifused at normal extracellular Ca^{2+} concentration and ^{45}Ca efflux from prelabeled islets perifused in the absence of extracellular Ca^{2+}, and delayed and lesser insulinotropic action. These findings reinforce the view that the carrier-mediated entry of L-arginine into the islet B-cells, with resulting depolarization of the plasma membrane, represents the essential mechanism for stimulation of insulin release by this cationic amino acid.

Key Words: Arginine; L-arginine methyl ester; pancreatic islets; insulin release.

Introduction

The stimulation of insulin release by L-arginine is currently ascribed to the transporter-mediated accumulation of this cationic amino acid inside the B-cell with resulting depolarization of the plasma membrane *(1–10)*. To evaluate further such a hypothesis, we compared the uptake, cationic and metabolic effects, and insulinotropic action of L-arginine with those of its more positively charged methyl ester.

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Results

Uptake of L-[U-^{14}C]Arginine and Its Methyl Ester

In a first series of experiments, the distribution space of ^{3}HOH was not significantly different after either 5 or 20 min of incubation, averaging, respectively, 3.99 ± 0.14 nL/islet (n = 58) and 3.78 ± 0.16 nL/islet (n = 47). After 5 min of incubation, the distribution space of L-[1-^{14}C]glucose (2.0 m*M*) averaged 1.25 ± 0.08 nL/islet (n = 12), representing 35.4 ± 0.9% of the paired ^{3}HOH space.

After both 5 and 20 min of incubation and in both the absence and presence of D-glucose (7.0 m*M*), the apparent distribution space of L-[U-^{14}C]arginine (10.0 m*M*) exceeded (p <0.005 or less) the paired ^{3}HOH space (Table 1), as if the intracellular concentration of the cationic amino acid were to exceed its extracellular concentration. An opposite situation prevailed in the case of the methyl ester of L-[U-^{14}C]arginine (also 10.0 m*M*), whose apparent distribution space was lower (p <0.03 or less) than the paired ^{3}HOH space. The distribution space of the cationic amino acid and its ester was twofold higher after 20 than 5 min of incubation and, in both cases, not significantly affected by the presence of D-glucose in the incubation medium.

The time course for the net uptake of L-[U-^{14}C]arginine and its ester over 20–60 min of incubation was investigated in a second set of experiments. In these experiments, the distribution space of ^{3}HOH after 5–20 min of incubation averaged 4.05 ± 0.20 nL/islet (n = 33), a value comparable with that found in the preceding set of investigations. The ^{3}HOH space was not significantly different after 20, 40, or 60 min of incubation (data not shown).

After 5 min of incubation, the distribution space of L-[1-^{14}C]glucose averaged 1.21 ± 0.15 nL/islet (n = 12), representing 31.7 ± 2.2% of the paired ^{3}HOH space. The latter two mean values were not significantly different from those found in the first series of experiments. After 20 min of incubation, the apparent distribution spaces of L-[U-^{14}C]arginine and its ester, expressed relative to the paired ^{3}HOH space, were also close to those measured in the preceding experiments (Table 1).

As illustrated in Fig. 1, the time course for the net uptake of both L-[U-^{14}C]arginine and its methyl ester suggested a

Table 1
Apparent Distribution Space of L-[U-^{14}C]Arginine and Its Methyl Ester Expressed as Percentage of Paired ^{3}HOH Space

Experiment Number	D-Glucose (m*M*)	L-[U-^{14}C]Arginine (m*M*)	L-[U-^{14}C]Arginine methyl ester (m*M*)	Incubation time (min) 5	20	40	60
1	Nil	10.0	Nil	125.5 ± 6.9 (11)	256.7 ± 9.5 (12)	—	—
	7.0	10.0	Nil	132.9 ± 7.0 (11)	266.6 ± 11.7 (12)	—	—
	Nil	Nil	10.0	49.0 ± 4.0 (12)	81.8 ± 2.9 (12)	—	—
	7.0	Nil	10.0	44.4 ± 2.1 (12)	90.3 ± 3.7 (11)	—	—
2	Nil	10.0	Nil	—	288.7 ± 9.6 (11)	401.5 ± 11.8 (10)	464.4 ± 16.6 (10)
	Nil	Nil	10.0	—	95.3 ± 4.0 (10)	129.7 ± 4.7 (11)	155.8 ± 8.0 (10)

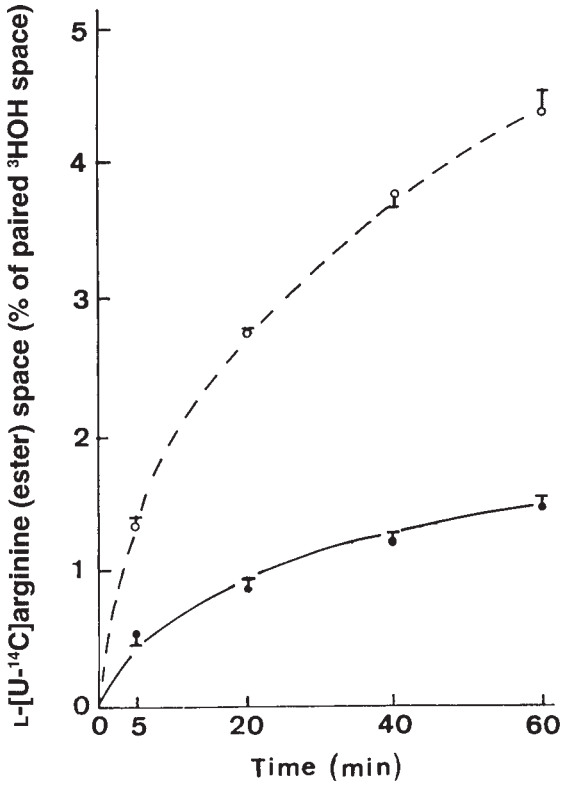

Fig. 1. Time course for the uptake of L-[U-^{14}C]arginine (– s –) and its methyl ester (— d —), both tested at 10.0 m*M*, by islets incubated in the absence of D-glucose. The paired ratio between the apparent distribution space of the amino acid or its ester and that of ^{3}HOH is shown relative to the mean value found within the same experiments after 20 min of incubation in the presence of L-[U-^{14}C]arginine. Mean values (±SEM) refer to 10–23 individual measurements.

progressive increase tending toward a saturation value. After 60 min of incubation, the apparent intracellular concentration of radioactive material (expressed as L-[U-^{14}C]arginine equivalent) represented, after correction for extracellular contamination, 6.33 ± 0.30 and 1.81 ± 0.13 times the extracellular concentration of L-[U-^{14}C]arginine and its ester, respectively. However, note that the radioactive content of the islets refers not solely to L-[U-^{14}C]arginine or its ester but also to their ^{14}C-labeled metabolites.

Oxidation of L-[U-14C]arginine and its Methyl Ester

At a 10.0 m*M* concentration, the oxidation of L-[U-^{14}C]-arginine and its methyl ester averaged, respectively, 8.79 ± 0.44 and 4.08 ± 0.42 pmol/(islet/120 min) (n = 16 in both cases). In the presence of 7.0 m*M* D-glucose, the oxidation of L-[U-^{14}C]arginine was decreased (p <0.04) to 82.0 ± 7.0% (n = 16) of the mean corresponding value found, within the same experiments, in the absence of the hexose (100.0 ± 4.7%; n = 16). By contrast, the presence of D-glucose (7.0 m*M*) increased (p <0.03) the oxidation of L-[U-^{14}C]arginine methyl ester to 131.5 ± 8.9% (n = 16) of the value recorded in the absence of D-glucose (100.0 ± 10.3%; n = 16). Never-

Table 2
Effect of D-Glucose, L-Arginine, and Its Methyl Ester on $^{14}CO_2$ Output from Islets Prelabeled with L-[U-^{14}C]Glutamine

D-Glucose (m*M*)	L-Arginine (m*M*)	L-Arginine methyl ester (m*M*)	$^{14}CO_2$ output		
			fmol/(30 min · islet)	% of paired ^{14}C content	Normalized values[a]
Nil	Nil	Nil	953 ± 102 (15)	18.87 ± 1.75 (15)	100.0 ± 10.8 (15)
7.0	Nil	Nil	1085 ± 82 (16)	21.34 ± 1.19 (16)	118.0 ± 9.1 (16)
7.0	10.0	Nil	1030 ± 88 (15)	20.95 ± 1.40 (15)	113.0 ± 7.8 (15)
7.0	Nil	10.0	1185 ± 123 (16)	22.60 ± 1.27 (16)	125.3 ± 9.9 (16)
16.7	Nil	Nil	1575 ± 153 (16)	27.54 ± 2.94 (16)	161.9 ± 17.2 (16)
16.7	10.0	Nil	1432 ± 125 (16)	21.23 ± 1.94 (16)	122.6 ± 16.1 (16)
16.7	Nil	10.0	1099 ± 122 (16)	15.12 ± 1.88 (16)	84.5 ± 16.0 (16)
Nil[b]	Nil	Nil	271 ± 41 (16)	3.44 ± 0.51 (16)	0.0 ± 3.0 (16)

[a]After correction for the measurements made within the same experiments in the presence of metabolic poisons.
[b]Islets incubated in the presence of 5 m*M* KCN, 0.01 m*M* antimycin A, and 0.01 m*M* rotenone.

Table 3
Effect of L-Arginine and Its Methyl Ester on Metabolism of D-Glucose

D-Glucose (m*M*)	L-Arginine (m*M*)	L-Arginine methyl ester (m*M*)	D-[U-^{14}C]Glucose oxidation	D-[5-^{3}H]Glucose utilization	$^{14}CO_2/^{3}HOH$ paired ratio (%)
			(pmol D-glucose equivalent/[islet · 120 min])		
7.0	Nil	Nil	22.4 ± 1.0 (20)	60.8 ± 3.7 (20)	38.0 ± 1.6 (20)
7.0	10.0	Nil	17.9 ± 1.0 (20)	50.7 ± 2.8 (20)	35.8 ± 1.2 (20)
7.0	Nil	10.0	20.1 ± 0.9 (20)	56.7 ± 3.0 (20)	36.0 ± 0.7 (20)
16.7	Nil	Nil	91.3 ± 2.8 (20)	189.4 ± 6.0 (20)	48.3 ± 0.7 (20)
16.7	10.0	Nil	83.6 ± 3.1 (20)	184.3 ± 6.9 (19)	45.2 ± 1.0 (19)
16.7	Nil	10.0	89.4 ± 4.8 (20)	192.7 ± 11.9 (19)	47.2 ± 1.8 (19)

theless, in the presence of D-glucose, the oxidation of L-[U-^{14}C]arginine (7.21 ± 0.64 pmol/[islet/120 min]) remained significantly higher (p <0.02) than that of its methyl ester (5.36 ± 0.36 pmol [islet/120 min]).

Production of Nitric Oxide

Over 180 min of incubation in the presence of 7.0 m*M* D-glucose, the production of nitric oxide (NO) averaged 0.36 ± 0.04 pmol/islet (n = 5). It failed to be significantly affected by either 10.0 m*M* L-arginine (0.46 ± 0.08 pmol/islet; n = 5) or 10.0 m*M* L-arginine methyl ester (0.30 ± 0.08 pmol/islet; n = 5).

Metabolism of Endogenous L-Glutamine

After 30 min of preincubation in the presence of L-[U-^{14}C]-glutamine (1.0 m*M*), the radioactive content of islets averaged 7.32 ± 0.25 pmol of L-[U-^{14}C]glutamine equivalent/islet (n = 128). Relative to such a content, the paired production of $^{14}CO_2$ over 30 min of incubation in the absence or presence of 5 m*M* KCN, 0.01 m*M* antimycin A, and 0.01 m*M* rotenone averaged 18.9 ± 1.8% (n = 15) and 3.4 ± 0.5% (n = 16). After correction for the latter value, the $^{14}CO_2$ output (relative to the paired radioactive content of the islets) during incubation in the presence of 7.0 and 16.7 m*M* D-glucose was increased to 118.0 ± 9.1% (n = 16; p <0.25) and 161.9 ± 17.2% (n = 16; p <0.01) of the mean corresponding control value found within the same experiments in the absence of D-glucose (100.0 ± 10.8%; n = 15). At the low concentration of D-glucose (7.0 m*M*), both L-arginine and its methyl ester (10.0 m*M*) each failed to affect significantly the output of $^{14}CO_2$ (Table 2). However, in islets exposed during incubation to 16.7 m*M* D-glucose, L-arginine and its ester decreased $^{14}CO_2$ output (in excess of the value found in the presence of metabolic poisons) to 72.8 ± 7.6% (n = 16; p <0.06) and 46.5 ± 6.9% (n = 16; p <0.001) of the mean corresponding value found within the same experiments and at the same concentration of D-glucose in the sole presence of hexose.

D-Glucose Metabolism

In islets exposed to 7.0 m*M* D-glucose, L-arginine (10.0 m*M*) slightly decreased the oxidation of D-[U-^{14}C]glucose and utilization of D-[5-^{3}H]glucose (p <0.05 or less), but failed to affect significantly the paired ratio between these two variables (Table 3). At a higher concentration of D-glucose (16.7 m*M*), L-arginine failed to affect significantly the catabolism of the sugar, except for a minor decrease (p <0.02) in the paired ratio between D-[U-^{14}C]glucose oxidation and

Table 4
Effect of L-Arginine and Its Methyl Ester on Net Generation of ^{14}C-Labeled Amino Acids and Acidic Metabolites from D-[U-^{14}C]Glucose

D-Glucose (m*M*)	L-Arginine (m*M*)	L-Arginine methyl ester (m*M*)	D-[U-^{14}C]Glucose conversion to radioactive	
			Amino acids[a]	Acidic metabolites[a]
16.7	Nil	Nil	15.9 ± 0.9 (20)	36.4 ± 1.8 (20)
16.7	10.0	Nil	27.0 ± 0.8 (20)	28.0 ± 1.9 (20)
16.7	Nil	10.0	25.9 ± 1.1 (20)	29.9 ± 2.6 (19)

[a]Results are expressed as picomoles of D-glucose equivalent/(islet · 120 min).

Table 5
$^{45}Ca^{2+}$ Net Uptake (pmol/islet at min 120) by Islets Incubated at Increasing Concentrations of D-Glucose in the Absence or Presence of L-Arginine and Its Methyl Ester

D-Glucose (m*M*)	Nil	Other secretagogues	
		L-Arginine (10 m*M*)	L-Arginine methyl ester (10 m*M*)
Nil	4.60 ± 0.36 (18)	7.50 ± 0.70 (19)	3.52 ± 0.27 (20)
7.0	6.78 ± 0.22 (50)	8.38 ± 0.24 (49)	5.08 ± 0.15 (50)
16.7	7.82 ± 0.20 (30)	8.27 ± 0.30 (30)	7.04 ± 0.33 (30)

D-[5-^{3}H]glucose utilization. The metabolism of hexose was not significantly affected by the ester of L-arginine (also 10.0 m*M*), whether in islets exposed to 7.0 or 16.7 m*M* D-glucose. In these experiments, the rise in D-glucose concentration from 7.0 to 16.7 m*M* augmented significantly ($p < 0.001$) all metabolic variables, including the paired ratio between D-[U-^{14}C]-glucose oxidation and D-[5-^{3}H]glucose utilization.

Indirect evidence that L-arginine as well as its methyl ester were metabolized in the sequence of reactions catalyzed by arginase and glutamate-ornithine transaminase was provided by the finding that in islets exposed to 16.7 m*M* D-glucose, both the amino acid and its ester augmented ($p < 0.001$) the net generation of ^{14}C-labeled amino acids from D-[U-^{14}C]glucose, coinciding with a lesser production ($p < 0.05$ or less) of radioactive acidic metabolites from ^{14}C-labeled hexose (Table 4).

Cellular pH

In four of four cells tested, neither L-arginine nor its methyl ester (both 20 m*M*) had any significant effect on intracellular pH, as assessed from 2',7'-*bis*(carboxyethyl)5'(6')-carboxyfluorescein fluorescence. In these experiments, application of 10.0 m*M* NH_4Cl raised intracellular pH by 0.5–0.7 units (positive control).

$^{45}Ca^{2+}$ Net Uptake

After 120 min of incubation, the net uptake of $^{45}Ca^{2+}$ increased above basal value as the concentration of D-glucose was raised to 7.0 m*M* ($p < 0.001$) and 16.7 m*M* ($p < 0.005$). L-Arginine (10.0 m*M*) also increased $^{45}Ca^{2+}$ net uptake (Table 5). In relative terms, this effect was most marked in glucose-deprived islets ($p < 0.001$), less pronounced at an intermediate D-glucose concentration ($p < 0.001$), and failed to achieve statistical significance at the highest concentration of hexose (16.7 m*M*) tested in these experiments. By contrast, the methyl ester of L-arginine decreased $^{45}Ca^{2+}$ net uptake ($p < 0.05$ or less) whether in the absence or presence of D-glucose.

^{86}Rb and ^{45}Ca Efflux

In prelabeled islets perifused in the presence of D-glucose (7.0 m*M*) at normal extracellular Ca^{2+} concentration (1.0 m*M*), L-arginine and its methyl ester (10.0 m*M* each) exerted opposite effects upon ^{86}Rb outflow (Fig. 2, top). During the 25-min exposure to the cationic amino acid, the integrated ^{86}Rb FOR was 35.8 ± 3.0% higher ($n = 4$; $p < 0.005$) than the paired theoretical value calculated by exponential extrapolation of the measurements made between min 31 and 45 (i.e., before introduction of L-arginine). On the contrary, the methyl ester of L-arginine decreased such an integrated ^{86}Rb FOR by 23.1 ± 2.3% ($n = 4$; $p < 0.005$).

Nevertheless, both L-arginine and its ester augmented, in a rapid, sustained, and rapidly reversible manner, ^{45}Ca efflux. Between min 46 and 70, the increment in ^{45}Ca FOR above theoretical value averaged, for L-arginine and its ester, 31.6 ± 3.2% ($n = 4$; $p < 0.005$) and 32.1 ± 9.1% ($n = 4$; $p < 0.05$), respectively (Fig. 2, middle). This increase in ^{45}Ca efflux was suppressed in the absence of $CaCl_2$ but presence of EGTA (0.5 m*M*), with the paired difference between the experimental and theoretical value for ^{45}Ca FOR over the 25 min of administration of L-arginine and its ester representing relative changes of +0.6 ± 4.2% ($n = 4$; $p > 0.85$) and –15.2 ± 2.7% ($n = 4$; $p < 0.02$), respectively. Thus, in the absence of extracellular Ca^{2+}, L-arginine methyl ester, but not the unesterified amino acid, decreased significantly ^{45}Ca outflow (Fig. 3, top).

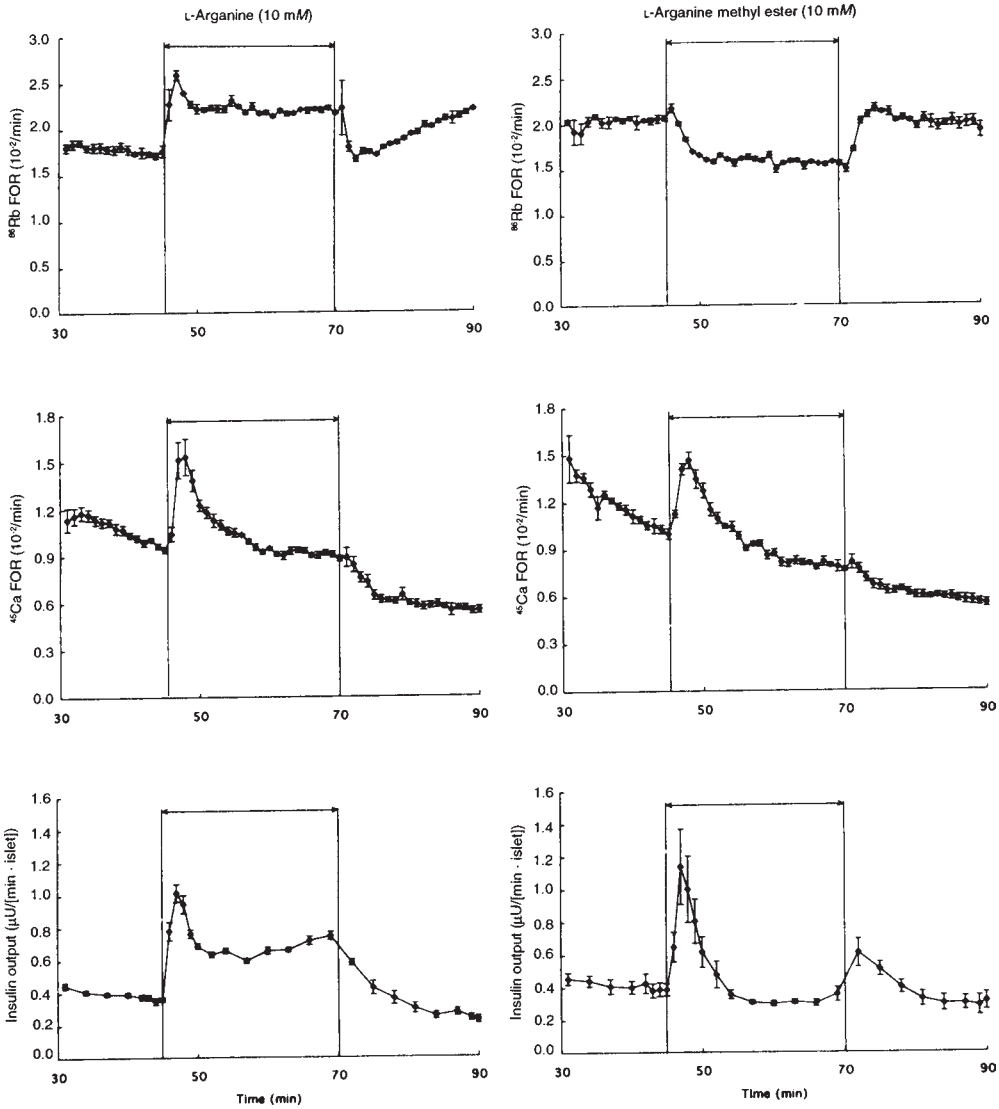

Fig. 2. Time course for the changes in ^{86}Rb FOR (**top**), ^{45}Ca FOR (**middle**), and insulin release (**bottom**) evoked by L-arginine (**left**) or its ester (**right**) in islets perifused in the presence of 7.0 m*M* D-glucose at normal extracellular Ca^{2+} concentration (1.0 m*M*). Mean values (±SEM) refer to four (top and middle) or eight (bottom) individual experiments.

The secretory response to L-arginine and its ester also differed significantly from one another (Fig. 2, bottom). Thus, L-arginine caused a biphasic, sustained, and rapidly reversible increase in insulin output. After 2 and 24 min of exposure to the amino acid, the release of insulin was, respectively, 0.69 ± 0.07 and 0.49 ± 0.04 μU/(min·islet) higher than the mean theoretical value ($n = 8$ and $p < 0.001$ in both cases). By contrast, L-arginine methyl ester only caused, in six out of eight individual experiments, an initial short-lived increase in insulin output. Between min 52 and 69, however, the output of insulin was no more significantly different from that calculated by extrapolation of the data recorded from min 31 to 45.

When the same experiments were repeated in the absence of extracellular Ca^{2+}, the output of insulin during exposure to L-arginine or its ester (min 46–70) was 43.6 ± 10.6 and 71.6 ± 22% lower ($n = 4$ in both cases) than the paired theoretical value (Fig. 3, bottom).

Cytosolic Ca^{2+} Concentration

In the presence of 7.0 m*M* D-glucose, L-arginine (10.0 m*M*), but not its methyl ester (also 10.0 m*M*), caused an immediate, sustained, and rapidly reversible increase in cytosolic Ca^{2+} concentration in a cluster of B-cells (Fig. 4). After 5 min of exposure to L-arginine the Ca^{2}-responsive fluorescence signal averaged 112.7 ± 3.8% ($n = 3$) of the paired reference value found in the absence of the cationic amino acid, distinct ($p < 0.025$) from only 99.3 ± 0.3% ($n = 3$) after 5 min of exposure to L-arginine methyl ester. As shown in Fig. 4, in these experiments, L-arginine and its ester were administered for 10–25 min, with an interval of 10–15 min between these two successive administrations. When the B-cells were stimulated with KCl (25 m*M*) at the end of the experiments, the Ca^{2+} signal increased to 180.7 ± 16.0% ($n = 3$; $p < 0.05$) of the paired reference value.

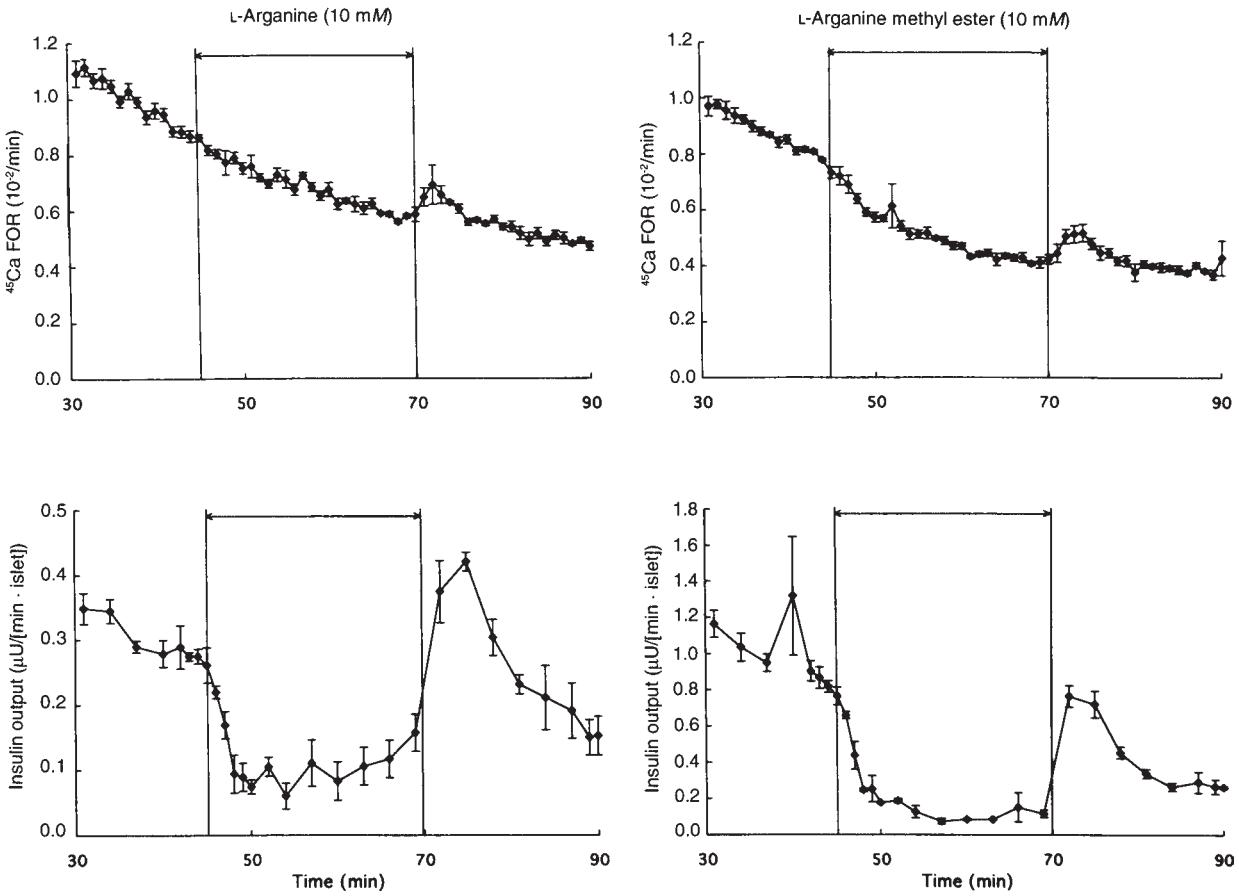

Fig. 3. Time course for the changes in ^{45}Ca FOR (**top**) and insulin release (**bottom**) evoked by L-arginine (**left**) or its ester (**right**) in islets perifused in the presence of 7.0 m*M* D-glucose in the absence of extracellular Ca^{2+} (no $CaCl_2$, 0.5 m*M* EGTA). Mean values (±SEM) refer to four individual experiments in all cases.

The results illustrated in Fig. 4 are in fair agreement with the time course of the secretory response to L-arginine and its ester in perifused (Fig. 2) or incubated islets (*see* p. 337). Thus, in such islets, a sustained stimulation of insulin release by L-arginine methyl ester is only observed after about 30 min of exposure to this secretagogue. In further experiments, therefore, the cluster of B-cells was exposed for 20–30 min to L-arginine or its ester (Figs. 5 and 6). The rapid response to the unesterified amino acid was, as a rule, followed by a late and progressive increase in the fluorescent signal (340/380 nm ratio). When L-arginine was removed from the perifusion medium, a further increase in this signal was also usually observed (Fig. 5). In the case of L-arginine methyl ester, the progressive rise in the fluorescent signal, during prolonged exposure of the islet cells to this agent, was much less pronounced than in the case of unesterified L-arginine (Fig. 6). The paired ratio between the readings made after 30 min and just before exposure to the ester averaged 113.1 ± 5.2% ($n = 8$), distinct ($p < 0.02$) from 155.3 ± 10.5% ($n = 5$) in the case of unesterified L-arginine. These findings are compatible with the late occurrence of a sustained secretory response to L-arginine methyl ester, when $[Ca^{2+}]_i$ eventually reaches the threshold value for stimulation of insulin release.

Electrophysiologic Data

Figures 7 and 8 illustrate membrane potential recordings from isolated rat pancreatic islet B-cells that were cultured for 1–5 d and then superfused in the presence of 7.0 m*M* D-glucose. Electrical activity was induced by 10 m*M* L-arginine in half of the cells (four of eight) tested. The proportion of responsive cells increased to three of four when the concentration of L-arginine was raised to 20 m*M*. Figure 7 (top) illustrates a cell (cell 5) that did not respond to 10 m*M* L-arginine, but later displayed electrical activity in response to a rise in D-glucose concentration from 7.0 to 12.0 (or 19.0) m*M*. Figure 7 (middle) refers to a cell that responded on two successive occasions to 10.0 m*M* L-arginine. Likewise, the bottom of Fig. 7 refers to a cell that responded on two successive incubations to 20.0 m*M* L-arginine. Electrical activity evoked by L-arginine was usually transient, with the membrane potential returning to the resting level within about 10 min (*see* Fig. 7, middle).

Several cells were found that failed to respond to 10.0 m*M* L-arginine but responded well to 10.0 m*M* L-arginine methyl ester. A comparable situation is illustrated in Fig. 8 (top), which refers to a cell that failed to respond to 20.0 m*M* L-arginine, but later displayed electrical activity in response

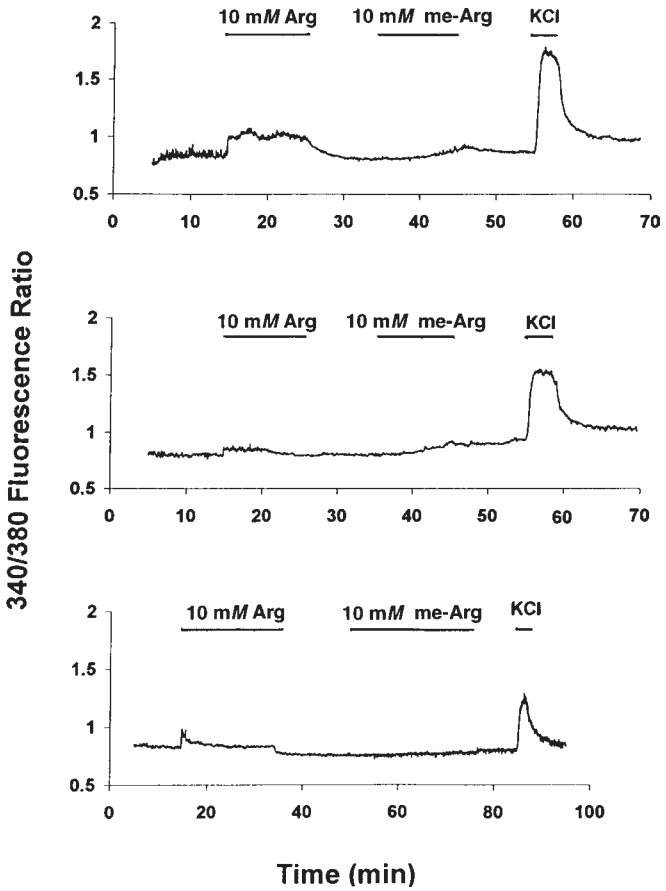

Fig. 4. Time course for the early changes in cytosolic Ca^{2+} concentration, as judged from the 340/380 nm fluorescence ratio in fura-2-loaded B-cells in response to the administration of 10.0 m*M* L-arginine and its methyl ester in the presence of 7.0 m*M* D-glucose. At the end of the experiments, the cells were stimulated with 25 m*M* KCl.

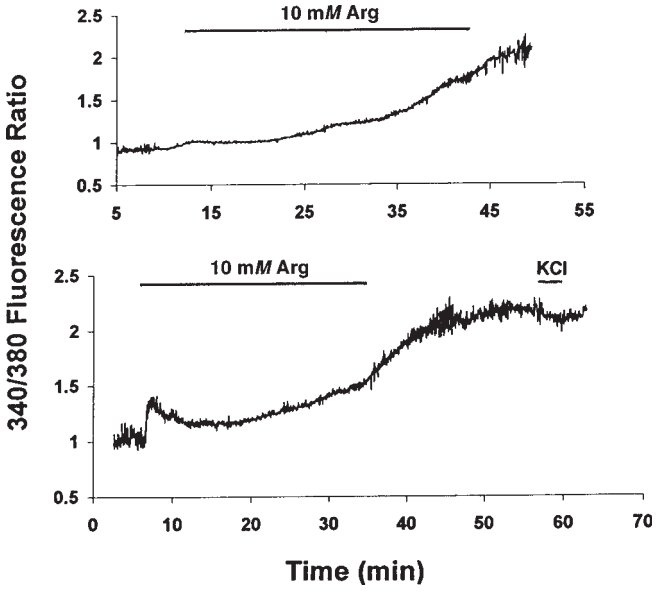

Fig. 5. Changes in cytosolic Ca^{2+} concentration during prolonged exposure to 10.0 m*M* L-arginine in the presence of 7.0 m*M* D-glucose. Presentation is the same as in Fig. 4.

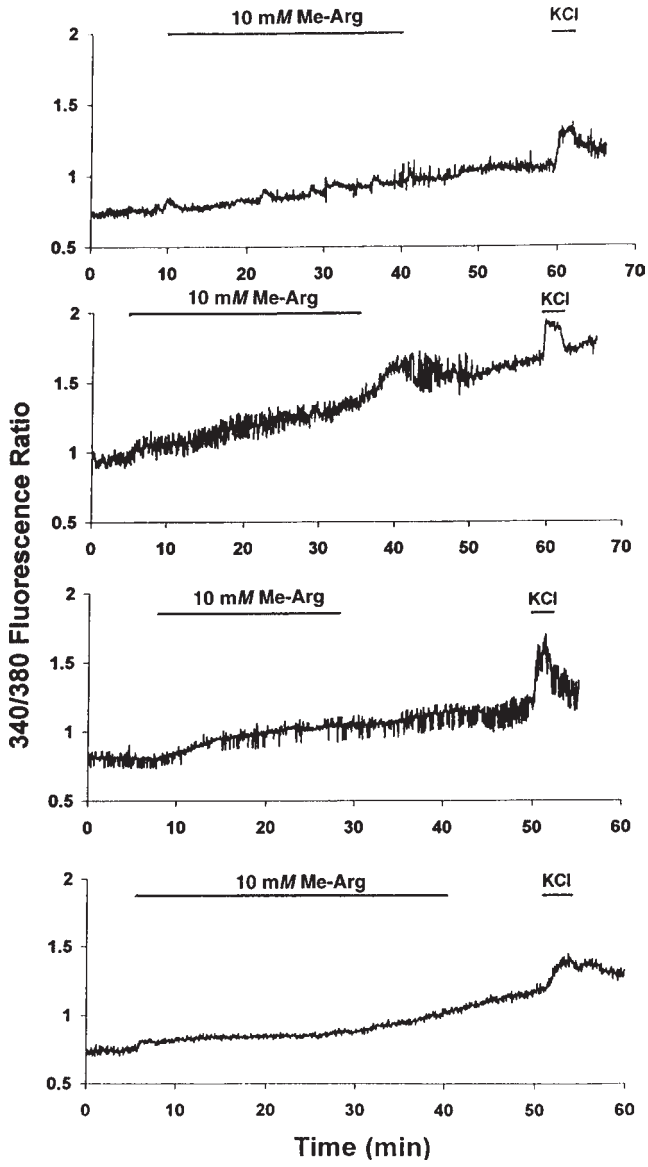

Fig. 6. Changes in cytosolic Ca^{2+} concentration during prolonged exposure to 10.0 m*M* L-arginine methyl ester in the presence of 7.0 m*M* D-glucose. Presentation is the same as in Fig. 4.

to 20.0 m*M* L-arginine methyl ester. As shown in the bottom of Fig. 8, the ester induced a prolonged depolarization, lasting for at least 30 min (data not shown). The proportion of cells that responded to the ester amounted to six of eight and three of three at L-arginine methyl ester concentrations of 10.0 and 20.0 m*M*, respectively.

Figure 9 shows measurements of B-cell input conductance, used to assess whole-cell K_{ATP} channel activity. In cells equilibrated in 7 m*M* D-glucose, G_{input} was 0.75 ± 0.06 nS (n = 16). Exposure of the cells to 20 m*M* L-arginine had no significant effect on G_{input}, whose value was 0.75 ± 0.04 nS (n = 8) 2–5 min following application of L-arginine. By contrast, the addition of 20 m*M* L-arginine methyl ester reversibly reduced G_{input} to the significantly (p <0.005) lower value of 0.41 ± 0.03 nS (n = 8).

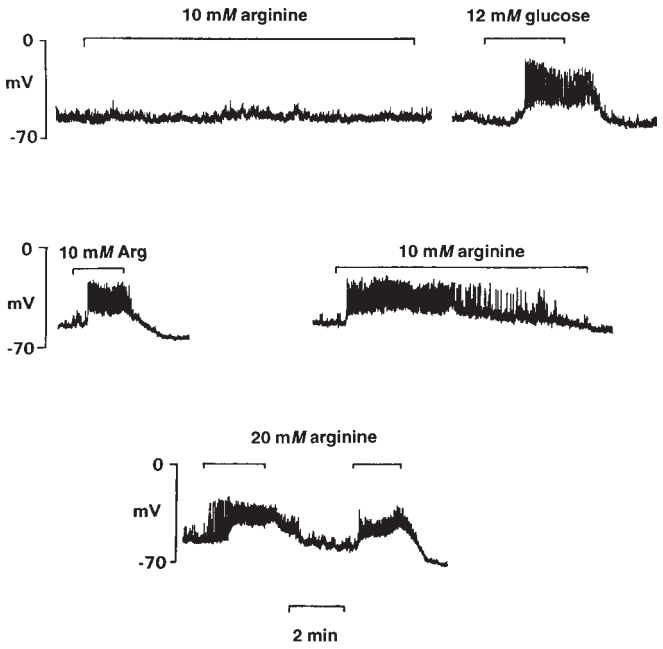

Fig. 7. Effects of 10.0 or 20.0 m*M* L-arginine on membrane potential in isolated rat pancreatic B-cells exposed to 7.0 m*M* D-glucose. (**Top**) The concentration of D-glucose was raised from 7.0 to 12.0 m*M* approx 10 min after the removal of L-arginine. (**Middle**) An interval of approx 7 min separated the two administrations of L-arginine.

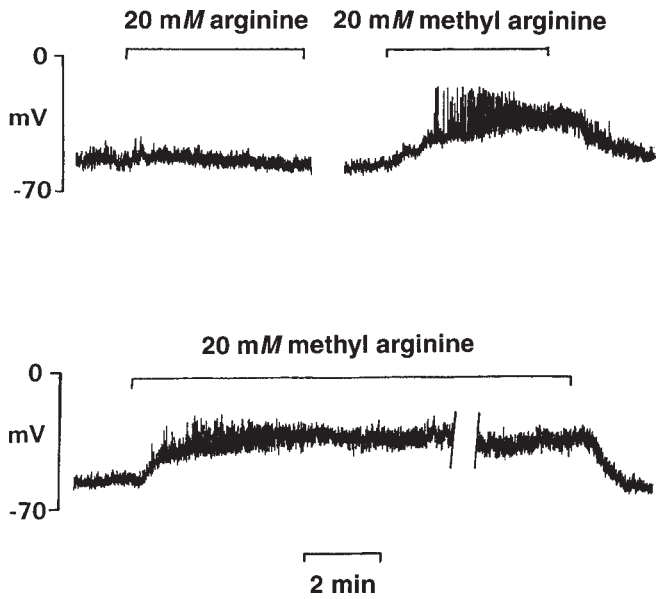

Fig. 8. Effects of L-arginine and its methyl ester on membrane potential in isolated rat pancreatic B-cells exposed to 7.0 m*M* D-glucose. (**Top**) An interval of approx 10 min separated the administration of L-arginine and that of its ester. (**Bottom**) The gap represents a period of about 5 min during which electrical activity was continuous.

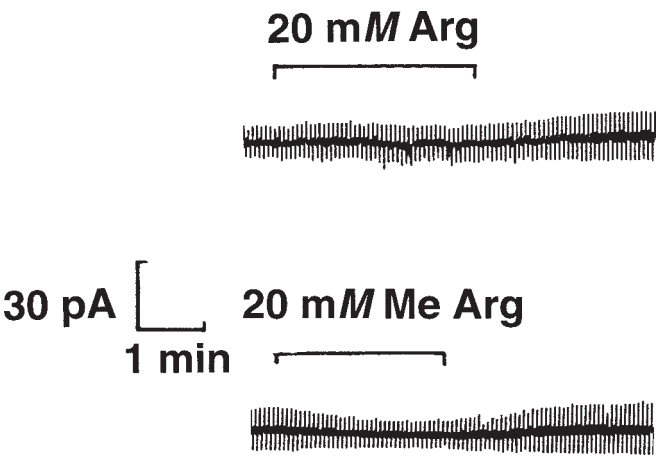

Fig. 9. Effects of L-arginine and its methyl ester (both 20.0 m*M*) on input conductance in B-cells exposed to 7.0 m*M* D-glucose.

Concentration and Glucose Dependency of Insulinotropic Action of L-Arginine and Its Ester

The concentration of 10.0 m*M* L-arginine and its ester used in all experiments was close to that required to evoke a maximal secretory response. For instance, over 120 min of incubation in the presence of 8.3 m*M* D-glucose, no significant further increase in insulin output was recorded when the concentration of the cationic amino acid or its ester was raised from 10.0 to 20.0 m*M* (data not shown).

As shown in Table 6, L-arginine (10.0 m*M*) failed to affect significantly insulin output in the absence of D-glucose or at a 4.0 m*M* concentration of the hexose, increased insulin release ($p < 0.001$) in the range of 6.0 to 11.1 m*M* D-glucose, and again failed to increase significantly the release of insulin at 16.7 m*M* D-glucose. The methyl ester of L-arginine (also 10.0 m*M*) also increased insulin output ($p < 0.01$ or less) at D-glucose concentrations ranging from 6.0 to 8.3 m*M*. Its insulinotropic action was less pronounced, however, than that of L-arginine ($p < 0.001$ in all cases). Moreover, over 120 min of incubation, the ester failed to enhance insulin release at 11.1 m*M* D-glucose and even decreased insulin output ($p < 0.001$) at 16.7 m*M* D-glucose.

Effect of Ca2+ on Arginine-Stimulated Insulin Release

In the nominal absence of Ca^{2+} (no $CaCl_2$), the release of insulin evoked by 7.0 m*M* D-glucose averaged 10.8 ± 0.8 and 14.0 ± 1.7 μU/(islet · 120 min) ($n = 22$ in both cases) in the absence and presence of 10 m*M* L-arginine, respectively. Thus, the cationic amino acid provoked a modest but not significant increment (+3.3 ± 1.9 μU/[islet · 120 min]) in insulin release under these experimental conditions. At increasing Ca^{2+} concentrations (0.5–4.0 m*M*), the increments in insulin output found in the sole presence of D-glucose above the corresponding value measured in the absence of Ca^{2+} and those caused by L-arginine at each Ca^{2+} concentration both displayed a comparable sensitivity to Ca^{2+}, with an apparent K_m close to 1.45 m*M* Ca^{2+} (Fig. 10).

Table 6
Insulin Output (μU/[islet · 120 min]) Evoked by Increasing Concentrations of D-Glucose from Islets Incubated in Absence or Presence of L-Arginine and Its Methyl Ester

D-Glucose (m*M*)	Nil	Other secretagogues	
		L-Arginine (10 m*M*)	L-Arginine methyl ester (10 m*M*)
Nil	11.5 ± 1.7 (19)	11.9 ± 3.4 (15)	13.0 ± 3.0 (16)
4.0	15.2 ± 2.4 (16)	14.6 ± 1.7 (15)	12.3 ± 1.7 (16)
6.0	21.9 ± 1.2 (74)	44.8 ± 2.0 (50)	27.6 ± 1.1 (50)
7.0	44.9 ± 2.6 (65)	78.8 ± 3.2 (65)	62.9 ± 2.9 (41)
8.3	67.3 ± 3.2 (102)	119.4 ± 5.7 (78)	79.8 ± 3.8 (78)
11.1	119.8 ± 5.8 (42)	163.9 ± 6.6 (42)	119.3 ± 4.4 (42)
16.7	184.2 ± 8.7 (38)	196.4 ± 5.7 (38)	161.0 ± 3.5 (38)

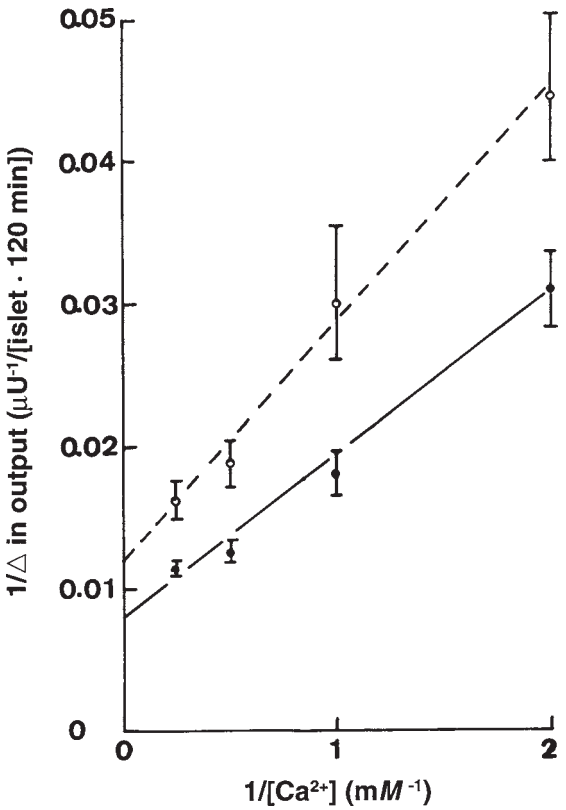

Fig. 10. Double reciprocal plot for the increment (Δ) in insulin release evoked by 7.0 m*M* D-glucose (— d —) or 10.0 m*M* L-arginine (– s –) from islets incubated at increasing concentrations of extracellular Ca^{2+}. Mean values (±SEM) refer to 23 individual observations in all cases.

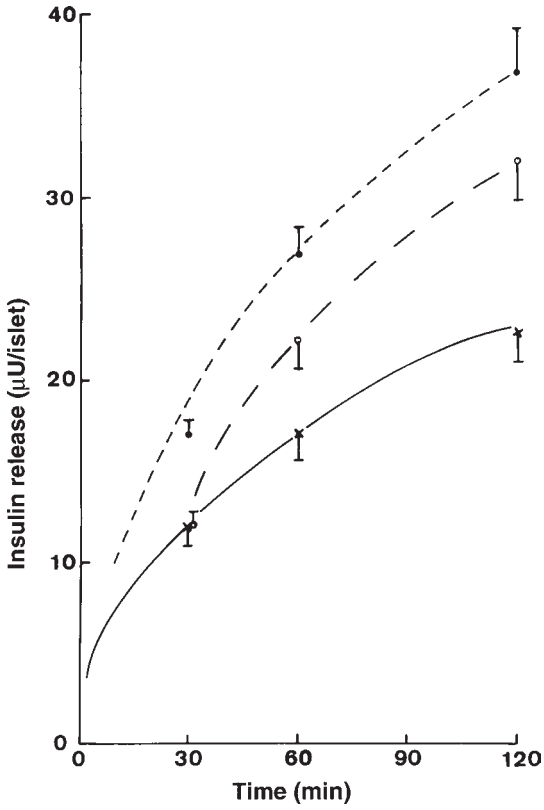

Fig. 11. Time course for insulin release evoked by 7.0 m*M* D glucose in the absence (— × —) or presence of either L-arginine (- - d - -) or its methyl ester (– s –) both tested at 10.0 m*M*. Mean values (±SEM) refer to 20 individual observations in all cases.

Time Course of Secretory Response

Over only 30 min of incubation, L-arginine (10.0 m*M*), but not its ester (also 10.0 m*M*), significantly augmented (p <0.005) insulin output evoked by 7.0 m*M* D-glucose (Fig. 11). Beyond the first 30 min of incubation, however, the time course of insulin release was virtually identical in the case of L-arginine and its ester, the increment in insulin release averaging, respectively, 10.1 ± 1.6 and 10.3 ± 1.7 μU/islet between min 31 and 60 and 9.9 ± 2.6 and 9.8 ± 2.3 μU/islet between min 61 and 120 of incubation (df = 36 and p <0.001 in all cases). In other words, whereas L-arginine augmented insulin secretion significantly at all time points (p <0.005 or less), its ester only did so after 60 and 120 min of incubation (p <0.01 or less).

These findings were confirmed in a further series of experiments in which the islets were preincubated for 30 min in the absence of any exogenous nutrient or in the sole presence of either L-arginine or its methyl ester (10.0 m*M* each). In the former case, the functional integrity of the islets was assessed by the fact that over 120 min of incubation, the output of insulin was increased (p <0.001) by

Table 7
Effect of L-arginine and Its Methyl Ester on Glucose-Stimulated Insulin Release in Islets from GK Rats

Experiment Number	D-Glucose (m*M*)	L-Arginine (m*M*)	L-Arginine methyl ester (m*M*)	Insulin output (μU/[islet · 120 min])
1	Nil	Nil	Nil	28.5 ± 5.1 (24)
	7.0	Nil	Nil	52.5 ± 6.8 (24)
	7.0	10.0	Nil	96.8 ± 5.7 (24)
	7.0	Nil	10.0	105.4 ± 5.9 (24)
2	Nil	Nil	Nil	49.6 ± 11.5 (24)
	16.7	Nil	Nil	190.4 ± 16.7 (24)
	16.7	10.0	Nil	252.7 ± 17.4 (24)
	16.7	Nil	10.0	145.2 ± 11.1 (24)

D-glucose (7.0 m*M*) from a basal value of 10.8 ± 1.7 to 28.6 ± 2.4 μU/islet (n = 39-40) and, in the presence of the hexose, further increased by 18.5 ± 2.7 μU/islet (df = 68; p <0.001) by L-arginine (10.0 m*M*). After preincubation in the presence of L-arginine, the output of insulin evoked by D-glucose (7.0 m*M*) and the cationic amino acid (10.0 m*M*) averaged 34.9 ± 2.4 [μU/(islet · 120 min) (n = 39), a value virtually identical to that found after preincubation with the ester of L-arginine and during incubation in the presence of both such an ester (10.0 m*M*) and D-glucose (7.0 m*M*), i.e., 35.2 ± 2.3 μU/(islet · 120 min) (n = 39). Both values were significantly higher (p <0.02 or less) than those found after preincubation in the absence of exogenous nutrient and incubation in the sole presence of 7.0 m*M* D-glucose, with mean increments of 6.3 ± 2.4 and 6.5 ± 2.2 μU/(islet · 120 min) (df = 70 in both cases). Moreover, and as expected from the data illustrated in Fig. 11, the output of insulin evoked by L-arginine and D-glucose was lower after preincubation in the presence, rather than absence, of the cationic amino acid (paired ratio: 75.5 ± 6.4%, df = 68; p <0.01), whereas the opposite situation prevailed in the case of the methyl ester of L-arginine (paired ratio: 133.7 ± 10.8%; df = 69; p <0.005).

Experiments in GK Rats

In the last set of experiments in this series, the secretory response to L-arginine and its methyl ester was investigated in islets from female GK rats. These animals were 96 ± 7 d old, weighed 212 ± 9 g, and displayed mean plasma D-glucose and insulin concentrations of 14.89 ± 0.68 m*M* and 35.2 ± 2.7 μU/mL, respectively. The protein and insulin content of their islets averaged, respectively, 1.37 ± 0.09 μg/islet and 1.29 ± 0.11 mU/islet (n = 11 in both cases).

As shown in Table 7, in a first set of experiments, D-glucose (7.0 m*M*) augmented (p <0.01) insulin output above basal value to 52.5 ± 6.8 μU/(islet · 120 min). Both L-arginine and its ester (10.0 m*M*) augmented significantly (p <0.001) the secretory response to hexose. In this respect, there was no significant difference (p > 0.3) between the amino acid and its ester. At a higher concentration of D-glucose (16.7 m*M*), L-arginine further increased insulin output (p <0.02), whereas its methyl ester exerted an opposite effect (p <0.05). In these experiments, the basal insulin output, which was not significantly different (p > 0.1) in the first and second set of investigations, averaged 39.0 ± 6.4 μU/(islet · 120 min) (n = 48) and, as such, exceeded (p <0.01) the value otherwise found in normal rats (*see* Table 6).

Discussion

As indicated in the Introduction, the primary aim of this study was to compare the effect of L-arginine and its methyl ester on several variables of B-cell function, with the naive idea that the more cationic ester may prove more efficient than unesterified L-arginine as an insulinotropic agent.

The present data indicate that both L-arginine and its ester are taken up by intact islets, the latter much less efficiently, however, than the unesterified amino acid. In this respect, it is quite conceivable that only L-arginine is transported by the carrier system mediating its passage across the plasma membrane *(9–11)*.

Both L-arginine and its ester are metabolized in islet cells. Thus, both L-[U-^{14}C]arginine and L-[U-^{14}C]arginine methyl ester were converted to $^{14}CO_2$. Like its uptake by islet cells, the production of $^{14}CO_2$ from L-[U-^{14}C]arginine was higher, however, than that from L-[U-^{14}C]arginine methyl ester. Moreover, both unesterified and esterified L-arginine increased the net generation of amino acids from D-[U-^{14}C]glucose and inhibited the production of $^{14}CO_2$ by islets prelabeled with L-[U-^{14}C]glutamine and incubated at a high concentration of D-glucose (16.7 m*M*).

As expected from its role as a source of pyruvic acid, considered a transamination partner, D-glucose (7.0 and 16.7 m*M*) indeed caused a concentration-related increase in the output of $^{14}CO_2$ from islets prelabeled with L-[U-^{14}C]-glutamine *(12)*. At a low hexose concentration (7.0 m*M*), which failed to increase significantly $^{14}CO_2$ output, neither L-arginine nor its methyl ester affected significantly the production of $^{14}CO_2$. At a higher concentration of D-glucose (16.7 m*M*), however, when the output of $^{14}CO_2$ was indeed increased above basal value, both L-arginine and its methyl ester opposed the effect of hexose. This is probably attrib-

utable, at least in part, to competition between L-[U-^{14}C]glutamate generated from L-[U-^{14}C]glutamine and unlabeled L-ornithine generated from L-arginine or its ester as a transamination partner for D-glucose-derived pyruvic acid (and its further acidic metabolites, e.g., α-ketoglutaric acid and oxaloacetic acid). Incidentally, under the present experimental conditions, the generation of methanol by hydrolysis of L-arginine methyl ester is too low to affect the metabolic and secretory response to D-glucose *(13)*.

L-Arginine, but not its ester, also inhibited D-glucose catabolism, at least at a low concentration of D-glucose (7.0 m*M*), in fair agreement with a prior observation *(7)*. Likewise, D-glucose inhibited the oxidation of L-[U-^{14}C]arginine, whereas it increased that of L-[U-^{14}C]arginine methyl ester.

In good agreement with prior observations *(8)*, the cationic, electrophysiologic, and secretory effects of L-arginine were all compatible with the view that the accumulation of this cationic amino acid in the B-cell leads to depolarization of the plasma membrane and subsequent gating of voltage-sensitive Ca^{2+} channels. This would indeed account for the increase in ^{86}Rb outflow caused by L-arginine, probably as a result of the gating of voltage- and/or Ca^{2+}-responsive K^+ channels, and the stimulation of Ca^{2+} influx, as documented by both the comparison of ^{45}Ca efflux from prelabeled islets perifused in the presence or absence of extracellular Ca^{2+} and the amino acid–induced increase in ^{45}Ca net uptake. L-Arginine failed to affect whole-cell K_{ATP} channel activity in B-cells voltage clamped at –70 mV, but did cause depolarization of the plasma membrane and induced electrical activity in single B-cells. This coincided with a rapid, sustained, and rapidly reversible increase in cytosolic Ca^{2+} concentration and stimulation of insulin release, which was abolished in the absence of extracellular Ca^{2+} and modulated by the concentration of extracellular Ca^{2+} in a manner similar to that found for D-glucose-stimulated insulin secretion.

Most of the results obtained with the methyl ester of L-arginine, however, suggested that, at variance with unesterified arginine, its ester acted mainly as a nutrient secretagogue. It decreased ^{86}Rb outflow from prelabeled islets, as well as ^{45}Ca efflux from islets perifused in the absence of extracellular Ca^{2+}. The reduction in G_{input} evoked by the ester is consistent with inhibition of ^{86}Rb fractional outflow rate. Like nutrient secretagogues, L-arginine methyl ester also caused depolarization of the B-cell plasma membrane, induced electrical activity, and stimulated Ca^{2+} influx, as judged from the comparison between ^{45}Ca influx in the absence and presence of extracellular Ca^{2+}. In islet cells that were first cultured for 1–5 d and in islets that were first incubated at high D-glucose concentration (16.7 m*M*) or preincubated in the presence of L-arginine methyl ester, the cationic, electrical, and secretory response to the ester was rapid. This was not the case, however, in islets that were exposed to the ester immediately after isolation in a nutrient-free medium. In such a case, the delay preceding the detection of a significant increase in insulin output may reflect the time required to achieve a sufficient uptake of the ester and sufficient rate for its intracellular hydrolysis and further catabolism, as to increase the rate of adenosine triphosphate generation above the threshold value for stimulation of insulin release *(14)*. In this respect, it should be underlined that, at variance with L-arginine, its ester failed to affect adversely the catabolism of exogenous D-glucose.

The sole finding apparently arguing against such a proposal consisted in the inhibition of ^{45}Ca net uptake by L-arginine methyl ester. It is conceivable, however, that the subcellular site of L-arginine generation from its ester (e.g., in mitochondria) opposed the expected sequestration of $^{45}Ca^{2+}$ by intracellular organelles. Such a difference in the subcellular location of catabolism of a given nutrient and its methyl ester was already previously documented in islets exposed to pyruvate and methyl pyruvate *(15)*. Moreover, the present data do not rule out a late untoward effect of L-arginine methyl ester on ^{45}Ca handling, which could account for the inhibition of insulin release evoked over 120 min of incubation by a high concentration of D-glucose.

In summary, the present findings are consistent with the hypothesis that the transport of L-arginine by a specific carrier system represents an essential determinant of its insulinotropic action.

Materials and Methods

L-[U-^{14}C]Arginine was purchased from NEN (Boston, MA). Its methyl ester was synthesized as follows. An aliquot (0.5 mL) of a solution of L-[U-^{14}C]arginine (313 Ci/mol; 0.1 mCi/mL) in ethanol:H_2O (2:98 v/v) was introduced in a pre-weighed quickfit glass tube provided with a magnetic stirrer. The solvent was dried under N_2 flow. Fifty micromoles of unlabeled L-arginine, 0.13 mL of methanol, and 0.1 mL of thionyl chloride were then added, and the tube was kept at –10°C for 8 h and at 20°C for 16 h. After heating under reflux for 2 h, the solvent methanol in excess was evaporated under N_2 flow, and the final product was eventually dried under vacuum in a desiccator over KOH/P_2O_5. About 44.4 μmol of the white crystalline ester was obtained. Thin-layer chromatographs and ^{1}H nuclear magnetic resonance data were identical to those of the unlabeled ester.

All experiments were conducted in isolated rat pancreatic islets *(16)* or isolated rat B-cells *(13)* obtained from either fed female normal Wistar rats or GK rats. The methods used to measure the distribution space of ^{3}HOH and ^{14}C-labeled molecules *(17)*, the metabolism of endogenous L-[U-^{14}C]glutamine *(18)*, the catabolism of D-[5-^{3}H]-glucose and D-[U-^{14}C]glucose *(19)*, the oxidation of L-[U-^{14}C]arginine and its methyl ester *(7)*, the intracellular pH *(20)*, the net uptake of ^{45}Ca *(21)*, the outflow of ^{86}Rb *(22)* and ^{45}Ca *(23)* and release of insulin *(24)* from prelabeled perifused islets, the membrane potential of single B-cells *(13)*, and the secretion of insulin by incubated islets *(16)*

were as previously described. In addition, the plasma D-glucose *(25)* and insulin *(26)* concentration and the protein *(27)* and insulin *(16)* content of pancreatic islets were measured by methods previously reported.

For measurement of NO production, groups of 250–300 pancreatic islets were cultured in a microwell plate (Nunc, Roskilde, Denmark) for 180 min at 37°C in 120 µL of HEPES- and bicarbonate-buffered medium *(28)* equilibrated against a mixture of O_2-CO_2 (95:5 [v/v]) and containing bovine serum albumin (5 mg/mL), 7 m*M* D-glucose, and, as required, 10 m*M* L-arginine or 10 m*M* L-arginine methyl ester. The nitrite content of the culture medium was measured by a microplate assay method *(29)*. An aliquot (0.1 mL) of the culture medium was mixed with 20 µL of Griess reagent. After 10 min of incubation at 20°C, the absorbance was read at 540 nm in a microplate reader (Titertek Multiscan MCC/340 MKII Elab, Finland). Nitrite concentration was calculated using $NaNO_2$ as the standard.

The method used for the measurement of intracellular Ca^{2+} concentration was similar to that described previously *(13)*, except that the experiments were conducted on groups of B-cells (approx 5–20) that had been first cultured for 2 d and then loaded with 5 µ*M* fura-2 acetomethoxy ester for 40–50 min at 20°C in the dark. The perifusion medium consisted of a HEPES (25.0 m*M*) buffer containing 128.0 m*M* NaCl, 5.0 m*M* KCl, 1.2 m*M* $CaCl_2$, 1.0 m*M* $MgCl_2$, and 1.0 m*M* NaH_2PO_4 and was maintained at 28–30°C.

Whole-cell K_{ATP} channel activity in single B-cells was assessed from input conductance (G_{input}), which was measured under perforated patch conditions using a method described previously *(30)*. Briefly, cells were held at –70 mV and subjected to 50-ms pulses of ±10 mV at 2-s intervals. In all cases, access resistance was <25 MΩ, and whole-cell capacitance was within the range of 12–15 pF.

All results are presented as mean values (±SEM) together with the corresponding number of individual observations (*n*) or degrees of freedom (df). The statistical significance of differences between mean values was assessed by use of Student's *t*-test.

Acknowledgments

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