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Ionic Dependence of Amino-Acid Transport in the Exocrine Pancreatic Epithelium: Calcium Dependence of Insulin Action

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Summary. Rapid unidirectional transport (15 sec) of L-serine and 2-methylaminoisobutyric acid (MeAIB) was studied in the isolated perfused rat pancreas using a dual-tracer dilution technique. Time-course experiments in the presence of normal cation gradients revealed a time-dependent transstimulation of L-serine influx and transinhibition of MeAIB influx. Transport of the model nonmetabolized System A analog MeAIB was Na^+ dependent and significantly inhibited during perfusion with 1 mM ouabain. Although transport of L-serine was largely Na^+ independent, ouabain caused a time-dependent inhibition of transport. Influx of both amino acids appeared to be inhibited by the ionophore monensin but unaffected by a lowered extracellular potassium concentration. Removal of extracellular calcium had no effect on influx of the natural substrate L-serine, whereas stimulation of transport by exogenous insulin (100 μU/ml) was entirely dependent upon extracellular calcium and unaffected by ouabain. Paradoxically, exogenous insulin had no effect on the time-course of MeAIB influx. The characteristics of L-serine influx described in earlier studies together with our present findings suggest that insulin may modulate the activity of System asc in the exocrine pancreatic epithelium by a calcium-dependent mechanism.

Key Words amino-acid transport · insulin · diabetes · calcium dependency · ouabain · 2-methylaminoisobutyric acid · exocrine pancreas

Introduction

The postulated existence of a pancreatic portal circulation conveying islet hormones directly to the exocrine epithelium provides a mechanism for local endocrine control of acinar cell nutrient transport, metabolism and secretion. Recent morphological [16, 32] and functional [18, 36, 42] evidence supports this hypothesis. Insulin receptors have been identified on pancreatic acinar cells [20], and both exogenous and endogenously released insulin potentiates cholecystokinin-induced amylase release in the isolated perfused pancreas [36]. Moreover, exogenous insulin has been shown to stimulate neutral amino-acid transport in the perfused exocrine pancreas and to reverse the paradoxically high transport rate observed in pancreata isolated from streptozotocin-diabetic rats [26]. Earlier studies of pancreatic amino-acid transport established no regulatory role for islet hormones [4, 5, 8, 20] or alloxan-induced diabetes [4], albeit glucose transport was stimulated by insulin in pancreatic acini [20]. In rat hepatocytes Na^+-dependent neutral amino-acid transport via the hormone-sensitive System A [14, 38] is elevated by insulin, glucagon and streptozotocin-induced diabetes, and activation of transport by diabetes may occur *in vivo* as a result of the hyperglucagonemia associated with the disease [2].

The important role of cation gradients in energizing amino-acid transport [9, 12, 14, 21, 30, 33, 38, 41, 43, 45, 47] together with the modulation of ionic events by insulin and its ability to induce a membrane hyperpolarization [*see* 1, 18, 30, 39, 47] implies a close relationship between hormonal regulation of transport [*see* reviews 14, 38] and cation gradients. Previous studies with exocrine pancreatic fragments, acini or vesicles have identified a number of parallel amino-acid carriers differing in their substrate specificity, ionic dependency and ouabain sensitivity [4, 23, 29, 33, 43]. Electrogenic Na^+-alanine cotransport has been described in exocrine pancreatic membrane vesicles [43], and moreover many neutral amino acids evoke a membrane depolarization in mouse pancreatic acinar cells [*see* review 33]. An increase in intracellular Na^+ would activate the Na^+-K^+ pump thereby sustaining the Na^+ gradient [6, 9, 21, 30, 33, 38, 43]. Accumulated K^+ could exit via Ca^{2+}-activated monovalent cation channels identified in rat pancreatic acinar cells [34]. Our recent studies in the perfused pancreas have confirmed that influx of the nonmetabolized System A analog MeAIB is critically dependent upon extracellular sodium [29, 31], whereas influx of L-serine [29] is largely mediated by a novel Na^+-independent System asc preferring alanine, serine and cysteine [11, 44].

In the present study we have examined the time-course of MeAIB and L-serine transport in the intact pancreas during perfusion with different concentrations of potassium and calcium, as well as ouabain and the Na^+-selective ionophore monensin. In the presence of extracellular sodium 1 mM ouabain inhibited both methylaminoisobutyric acid and L-serine influx in a time-dependent manner. The selective stimulation of L-serine transport by insulin was dependent upon extracellular calcium and unaffected by ouabain. Preliminary abstracts of part of this work have been communicated [27, 28].

Materials and Methods

Isolation and Perfusion of the Pancreas

Male Sprague-Dawley rats weighing between 180 and 250 g were fasted for 24 hr but allowed water *ad libitum*. Animals were anesthetized with an intraperitoneal injection of sodium pentobarbitone (60 mg/kg 'Sagatal') and supplementary anesthetic was administered as required via a cannula in the external jugular vein. Pancreata were surgically isolated from the spleen, stomach and small intestine except for a small duodenal segment attached to the head of the pancreas, and then perfused with a Krebs-Henseleit bicarbonate medium via the superior mesenteric and celiac arteries [31, 36]. The fully isolated preparation was placed in a temperature-regulated Perspex® perfusion bath containing colloid-free perfusate gassed with 95% O_2, 5% CO_2. The pancreatic effluent was collected from the cannulated portal vein and the outflow rate was continuously monitored using a photoelectric drop recorder. At a constant flow of 1.75 ml/min perfusion pressures ranged between 20 and 35 mm Hg. The isolated preparation and all perfusates were maintained at 37°C in a temperature-regulated cabinet.

Perfusates

The composition of the perfusate was (mM): NaCl 131, KCl 5.6, $CaCl_2$ 2.5, $MgCl_2$ 1.0, $NaHCO_3$ 25.0, NaH_2PO_4 1.0, D-glucose 2.5, dextran T70 (5% wt/vol, Pharmacia, Sweden), bovine serum albumin (0.25% wt/vol, Cohn Fraction V, Sigma Chemical Co., U.K.). When insulin was included in the perfusates, it was diluted just prior to the experiment from a stock bovine insulin solution (40 IU/ml, Wellcome Laboratories, U.K.). Some perfusates also contained either 10 mM L-serine or 50 μM 2-methylaminoisobutyric acid, and all solutions were continuously gassed with 95% O_2, 5% CO_2 at 37°C to a pH between 7.3 and 7.4.

In ionic replacement experiments with potassium and calcium no osmotic adjustments were made since the total change was always less than 5 mM. Monensin (10^{-5} and 10^{-7} M, sodium salt, Calbiochem-Boehringer Corp., USA) and ouabain (10^{-3} M, Strophanthin G octahydrate, Sigma Chemical Co., U.K.) were added directly to select perfusates. The concentration of sodium and potassium in each perfusate and in the pancreatic venous effluent was measured using a Type 343 flame photometer (Instrumentation Laboratory Ltd., U.K.).

Measurement of Unidirectional Amino-Acid Influx

Pancreatic amino-acid transport was quantitated using a rapid dual tracer dilution technique [46] previously applied to the perfused rat pancreas [26–29, 31], in which small metabolites rapidly diffuse across the fenestrated capillary endothelium [16]. As the endocrine pancreas comprises only 2 to 4% of the total pancreatic weight and preferentially utilizes D-glucose as an energy substrate [8], we believe that our measured transport rates reflect amino-acid uptake at the basolateral membrane of the exocrine epithelium. Moreover, neutral amino-acid transport rates measured in isolated rat islets during 2 to 15-min incubation in vitro [35] are at least two orders of magnitude lower than our influx values measured in 15 sec.

Unidirectional amino-acid uptake was quantitated by directly comparing portal vein tracer concentration-time curves obtained for a labeled amino-acid and D-mannitol (extracellular reference marker) following an intra-arterial bolus injection (100 μl in 1 to 2 sec) of perfusate containing both tracer molecules (Fig. 1). Usually 20 to 30 successive 100-μl venous samples were collected during 45 to 60 sec, and a final venous sample was accumulated for a further 4 min to maximize tracer recoveries. The time-course of either L-(3-^{3}H)serine or (^{14}C)MeAIB uptake was assessed relative to D-mannitol in each of the successive venous samples using the expression: uptake = 1 − (test amino acid/D-mannitol). Depending on the label associated with the amino acid, either D-(^{14}C or ^{3}H)mannitol was used as the extracellular reference tracer. Unidirectional amino-acid influx was quantified from the maximal fractional tracer uptake (U_{max}), the perfusion rate (ml/min per g wet weight) and the perfusate concentration of the unlabeled amino acid (C_a, mM): influx = $-F \cdot \ln (1 - U_{max}) \cdot C_a$. As previously reviewed [46], the logarithmic transformation corrects for an exponential tracer profile along the length of the microvascular exchange site.

Time-Dependent Transport Changes

In control experiments pancreata were initially perfused with an amino-acid-free solution for 10 min and then with one containing either 10 mM L-serine or 50 μM MeAIB. In order to assess time-dependent changes in amino-acid transport, repeated measurements of influx were made 5, 15, 25, 35 and 45 min after switching to an amino-acid-containing solution. This experimental design enabled us to also investigate transstimulation or transinhibition of influx during prolonged perfusion with a fixed extracellular amino-acid concentration. The same protocol was adopted in ionic replacement experiments or during perfusion with ouabain or monensin. When the effects of insulin were tested, pancreata were perfused for 25 min with an amino-acid-free solution containing 100 μU/ml bovine insulin. This preliminary perfusion interval was found to be adequate for detecting stimulation of L-serine transport by insulin in the rat exocrine pancreas [26]. After 25 min the perfusate was switched to one containing 100 μU/ml insulin and either 10 mM L-serine or 50 μM MeAIB and repeated influx measurements were made. The dependency of insulin's action on extracellular calcium was tested by perfusing pancreata for 25 min with 100 μU/ml insulin in absence or presence of 2.5 mM calcium, and then switching to the appropriate amino-acid perfusate containing insulin and either zero or 2.5 mM calcium.

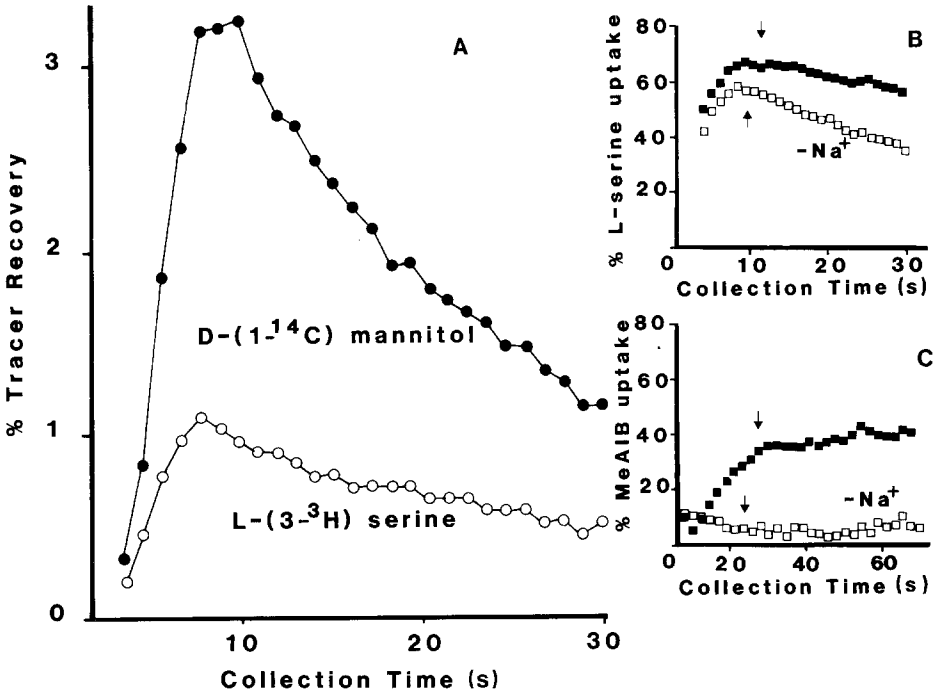

Fig. 1. Analysis of pancreatic amino-acid transport by rapid dual tracer dilution. (*A*) Simultaneous portal vein dilution curves obtained for L-(^{3}H)serine and D-(^{14}C)mannitol (extracellular reference) following a bolus injection (100 μl in 1 to 2 sec) of both tracers into the perfusate supplying the isolated rat pancreas. Tracer recoveries in successive venous samples have been expressed as percentages of the respective radioactive doses injected intra-arterially and are plotted against the venous sampling time. (*B*) Time-course of L-(^{3}H) serine uptake relative to D-(^{14}C)mannitol (uptake % = 1 − L-(^{3}H)serine/D-(^{14}C)mannitol × 100) in a pancreas perfused in the presence (■—■, dilution data shown in panel *A*) and then absence (□—□, dilution data not shown) of 156 mM sodium. Small arrows denote the time of maximum venous recovery recovery for D-(^{14}C)mannitol, (*C*) Time-course of 2-(^{14}C)methylaminoisobutyric acid uptake obtained in a pancreas perfused in the presence (■—■) and then absence (□—□) of 156 mM sodium. The inhibitory effects of sodium removal were fully reversible upon reperfusion with normal sodium

Insulin and Glucagon Radioimmunoassays

We assessed the effects of experimental interventions on the endocrine pancreas by assaying immunoreactive insulin and glucagon levels in the portal vein effluent. Samples of pancreatic effluent were collected at 2- or 4-min intervals in chilled tubes containing 30 μl aprotonin (Trasylol, Bayer, Belgium) and frozen at −20°C until time of assay. Insulin and glucagon were assayed in duplicate using 100-μl aliquots of the venous samples and release were quantified as ng/min per gram wet weight of pancreas. The tracers were [^{125}I] iodinated rat insulin and [^{125}I] iodinated pig/bovine glucagon and standards were purified rat insulin (Lot R170, Novo, Denmark) and pig/bovine glucagon (Novo, Denmark). The limit of detection for these assays was 0.08 ng/ml for insulin and 78 pg/ml for glucagon; details have previously been published [17].

Radioactive Molecules and Counting Procedures

The radioactive molecules L-[3-^{3}H]serine (20 Ci/mmol), D-[1-^{14}C] mannitol (53.4 mCi/mmol), D-[1-^{3}H]mannitol (19 Ci/mmol), 2-[1-^{14}C]methylaminoisobutyric acid (48.4 mCi/mmol) were obtained from New England Nuclear Chemicals, Dreieich, F.R.G. or Amersham International, U.K. Paired tracer injectates contained a radioactive amino acid and D-mannitol and were made up to volume with a given perfusate. Each isotope injectate normally contained 2.1 μCi of [^{14}C] and 7 μCi of [^{3}H]. Portal vein effluent samples, aliquots of the paired tracer injectates and [^{3}H] and [^{14}C] channel standards were mixed with 2 ml of liquid scintillant (Ready-Solv HP, Beckman, U.K.) and counted concurrently on a Beckman LS-250 liquid scintillation counter interfaced to a CBM microcomputer.

Results

Epithelial Uptake of L-Serine and 2-Methylaminoisobutyric Acid

Figure 1 illustrates the analysis of paired venous dilution profiles obtained for labeled L-serine and D-mannitol (extracellular reference) following a bolus injection of both tracers into the arterial circulation of the isolated perfused rat pancreas. The lower concentration-time profile for L-[^{3}H]serine relative

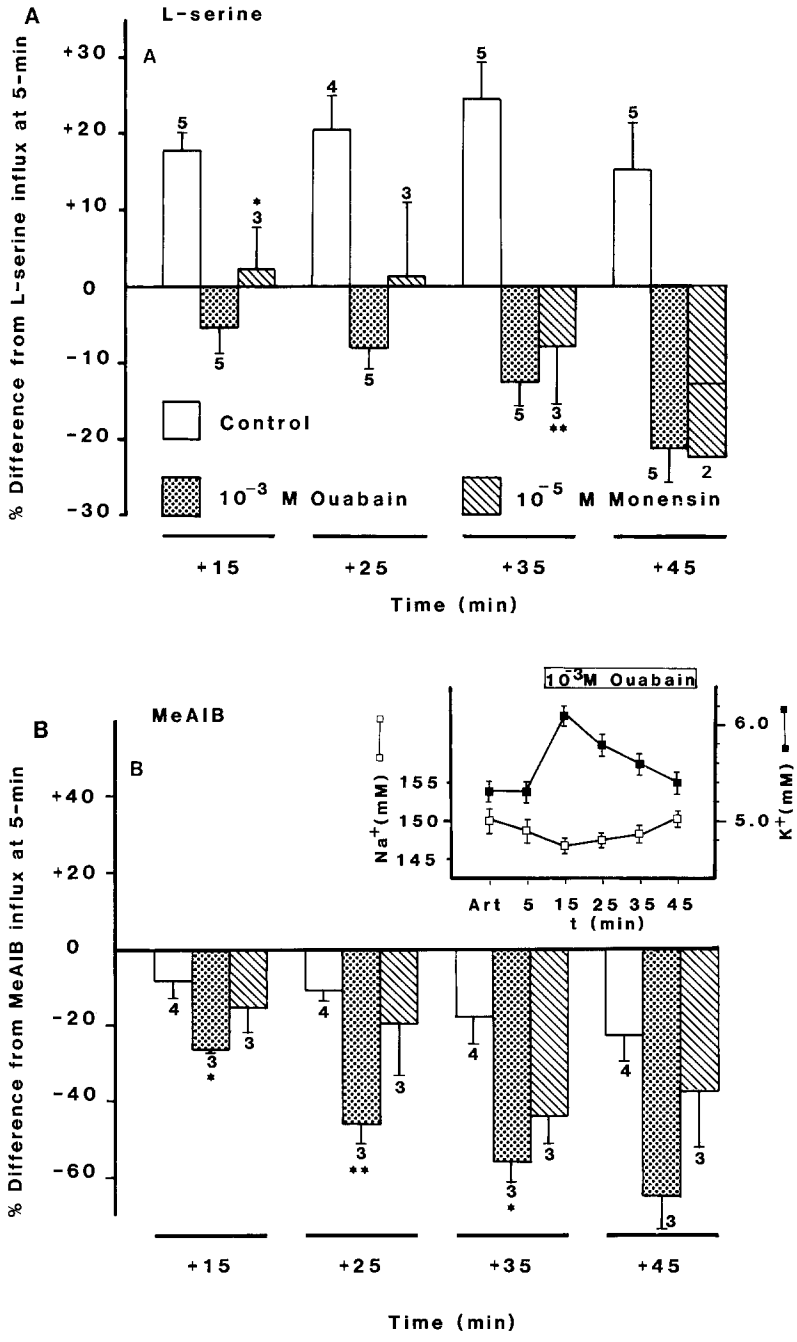

Fig. 2. Effects of ouabain and monensin on the time-course of amino-acid influx. Pancreata were preperfused for 10 min with an amino-acid free solution and then at zero time the perfusate was switched to one containing either 10 mM L-serine (panel *A*) or 50 μM 2-methylaminoisobutyric acid (panel *B*). After 5-min perfusion with either unlabeled amino acid an initial 5-min influx measurement was made and thereafter repeated influx measurements were made at 10-min intervals. When 1 mM ouabain or 10^{-5} M monensin was included in the amino-acid-containing perfusates, pancreata were perfused with these solutions 10 min after switching to either 10 mM L-serine or 50 μM 2-methylaminoisobutyric acid. In each experiment influx data at each time interval has been expressed as a percentage difference from the 5-min influx determination made in the same pancreas, and the mean ± SE for *n* animals is shown. The inset illustrates the effect of 1 mM ouabain on sodium and potassium concentrations in the pancreatic venous effluent. An unpaired Student's *t*-test was used to assess the significance of mean differences between the unhatched control columns and the hatched columns for ouabain or monensin at time intervals of 15, 25, 35 and 45 min. The ouabain-induced inhibition in L-serine influx was significant ($P \leq 0.001$) at all time intervals and other significance levels were: $^{*}P = 0.02$, $^{**}P = 0.008$

to D-[^{14}C]mannitol indicates uptake of the amino acid at the basolateral membrane of the pancreatic epithelium. Quantitative analysis of the tracer dilution profiles (Fig. 1*A*) revealed that the time-course of L-[^{3}H]serine uptake in the presence of normal sodium reached a maximum (U_{max}) of 64 ± 2% within 6 to 8 sec after injection of the tracers (Fig. 1*B*). This maximal unidirectional uptake was often followed by a declining uptake profile indicating efflux of transported serine from the epithelium [26, 29]. As shown in Fig. 1*C*, a much lower uptake was measured for the nonmetabolized System A analog MeAIB. When sodium dependence of transport was investigated, NaCl and $NaHCO_3$ in the Krebs-Henseleit perfusate were replaced by buffered Trizma HCl (Sigma) and unidirectional amino-acid uptake was measured 5 min equilibration with the sodium-free solution. Transport of L-serine (Fig. 1*B*) was

largely Na^+-independent [29], whereas transport of MeAIB (Fig. 1*C*) was dependent upon extracellular sodium [31].

Time-Course of Amino-Acid Influx

When time-dependent changes in amino-acid influx via Systems asc and A were studied, pancreata were equilibrated with an amino-acid free solution (2.5 mM D-glucose, 156 mM Na) for 10 min, and then at zero time the perfusate was switched to one containing either 10 mM L-serine or 50 μM MeAIB. Measurements of unidirectional amino-acid influx were obtained 5, 15, 25, 35 and 45 min after switching to either 10 mM L-serine (Fig. 2*A*) or 50 μM MeAIB (Fig. 2*B*). As illustrated in Fig. 2*A*, a time-dependent increase in unidirectional L-serine transport was observed when sequential influx measurements were compared to the value determined at 5 min (influx data shown in Fig. 5*B*). During continued perfusion (15 to 45 min) with unlabeled L-serine, influx increased by 19 ± 2% (n = 19 observations in 5 pancreata), suggesting that transport of L-serine may have been transstimulated by elevated intracellular concentrations. When in parallel experiments MeAIB influx was assessed in the presence of 50 μM MeAIB, a time-dependent inhibition in influx was observed (Fig. 2*B*) with a mean decrease of 14 ± 3% (n = 16 observations in 4 pancreata) occurring over the 15 to 45-min perfusion interval. A comparison of transport rates measured for L-serine and MeAIB after 5-min perfusion with 10 mM unlabeled substrate revealed that MeAIB influx (0.41 ± 0.02 μmol/min · g, n = 3) was significantly lower than that measured for L-serine (9.8 ± 1 μmol/min · g, n = 5).

Time-Dependent Effects of Ouabain and Monensin

To characterize the dependency of amino-acid transport on existing cation gradients we evaluated the effects of ouabain, an inhibitor of Na^+, K^+-ATPase, and monensin, an ionophore mediating electroneutral Na^+/H^+ exchange. In experiments with ouabain pancreata were perfused with either 10 mM L-serine or 50 μM MeAIB and an initial influx measurement was obtained in the absence of ouabain. The perfusate was then switched to one containing the respective unlabeled amino acid and 1 mM ouabain, and repeated measurements of influx were obtained at 10-min intervals. Ouabain inhibited transport of both amino acids in a time-dependent manner, although the maximum inhibition of L-serine influx relative to the influx measured at 5 min was only 25 ± 4% (n = 5) compared to 65 ± 10% (n = 5) for MeAIB (Fig. 2). When unpaired data for L-serine influx measured in the absence or presence of ouabain were compared, it was apparent that ouabain not only inhibited influx but also abolished the transstimulation of influx at each time interval (Table). In these experiments we also measured the sodium and potassium concentrations in the venous effluent by flame photometry. Ouabain caused a measurable release of K^+ into and loss of Na^+ from the perfusion medium (Fig. 2, inset). In experiments using the same protocol 10^{-7} M monensin had a negligible effect on influx (*data not shown*), whereas 10^{-5} M monensin generally decreased influx compared to the transport rate measured at 5 min in the absence of the ionophore. We were unable to detect monensin-induced changes in the venous concentrations of sodium or potassium.

Effects of Low Extracellular K^+ on Amino-Acid Transport

In a similar series of experiments we investigated the effects of a low K^+ perfusate (0.3 mM) on the time-course of 10 mM L-serine and 50 μM MeAIB influx. Five min after switching to a perfusate containing either unlabeled amino acid, an initial influx measurement was made in the presence of 5.1 mM K^+. The perfusate was then switched to one containing the same amino-acid concentration but only 0.3 mM K^+; however, the portal vein effluent K^+ concentration only approached 0.7 mM after 15-min perfusion. Both the time-dependent stimulation of L-serine transport and the inhibition of MeAIB transport were unaffected by this reduction in extracellular K^+ (*data not shown*).

Immunoreactive Insulin and Glucagon Release

Previous studies [16, 18, 32, 36, 42] have concluded that an insulo-acinar portal axis conveys islet hormones to the exocrine epithelium. Since we had demonstrated that L-serine transport was activated by insulin [26], we considered it important to monitor immunoreactive insulin (IRI) and glucagon (IRG) release during perfusion of isolated pancreata with initially an amino-acid-free solution (2.5 mM D-glucose), and then 10 mM L-serine or 50 μM MeAIB in the absence or presence of ouabain, monensin or low K^+. Figure 3 compares the IRI and IRG responses of the isolated pancreas to perfusion with 10 mM L-serine and 16.8 mM D-glucose. As shown

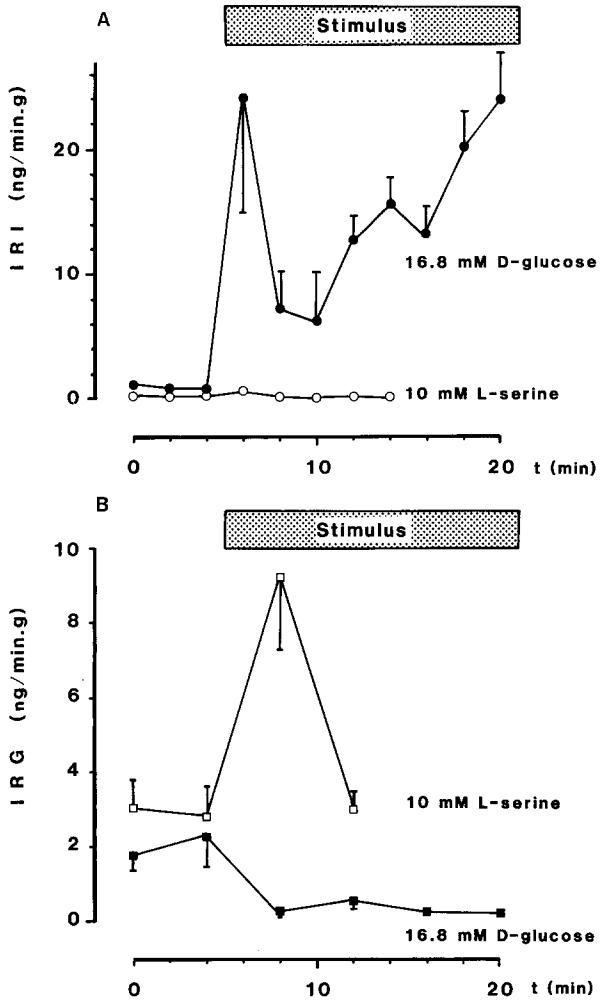

Fig. 3. Effects of L-serine and D-glucose on immunoreactive insulin (IRI) and glucagon (IRG) release from the isolated perfused pancreas. Control measurements of IRI and IRG release were obtained during perfusion of pancreata with an amino-acid-free medium containing 2.5 mM D-glucose. (*A*) Immunoreactive insulin (IRI) release in response to 16.8 mM D-glucose ($n = 4$) and 10 mM L-serine (2.5 mM D-glucose, $n = 5$). (*B*) Immunoreactive glucagon (IRG) release in response to 16.8 mM D-glucose ($n = 3$) and 10 mM L-serine ($n = 4$). Values denote the mean ± SE of duplicate measurements in three to five animals

in Fig. 3*A*, perfusion with L-serine for 1 min transiently elevated insulin release from 0.2 ± 0.06 to 0.7 ± 0.3 ng/min · g ($n = 5$, $P = 0.005$) but subsequent IRI levels were not significantly different from levels measured during amino-acid-free perfusion ($n = 53$ observations in five animals). Although 10 mM L-serine evoked a significant release of glucagon, this level was not maintained and returned to the amino-acid-free level after 8 min. In similar experiments 50 μM MeAIB had no effect on immunoreactive insulin or glucagon release (*data not shown*). Perfusion of pancreata with 16.8 mM D-glucose evoked the characteristic and well-reported biphasic insulin release profile (Fig. 3*A*) and an inhibition of IRG release (Fig. 3*B*).

In the presence of 2.5 mM D-glucose and 10 mM L-serine, 1 mM ouabain caused an immediate release of IRI insulin which continued to rise linearly with time (Fig. 4*A*). The maximum ouabain-induced insulin release was similar to the level measured in the presence of 16.8 mM D-glucose (Fig. 3*A*). In two of the experiments with 0.3 mM K^+ we monitored the time-course of immunoreactive insulin release and observed that it rose markedly once the effluent K^+ concentration approached 0.7 mM (Fig. 4*B*). In three experiments 10^{-7} M monensin had a negligible effect on insulin release, and when determined in one experiment 10^{-5} M monensin stimulated glucagon release.

Effect of Exogenous Insulin on L-Serine and MeAIB Transport

In these experiments pancreata were initially perfused for 25 min with an amino-acid-free solution containing 100 μU/ml bovine insulin and then with either 10 mM L-serine or 50 μM MeAIB in the continued presence of 100 μU/ml insulin. Repeated measurements of influx were made 5, 15, 25, 35 and 45 min after switching to the respective amino acid containing perfusates. In the presence of 2.5 mM extracellular calcium, insulin stimulated L-serine influx (Fig. 5*A*) compared to values measured in the absence of insulin (Fig. 5*B*). A closer analysis of the time-course of L-serine transport in the presence and absence of insulin revealed that transport was only significantly elevated over the 5 to 15-min perfusion interval. In the presence of insulin transstimulation of L-serine influx (Fig. 2*A*) was absent, and moreover the elevated transport rate remained constant throughout the time-course of the experiment (Fig. 5*A*). The transstimulation of transport observed in the absence of insulin may partially explain the lack of a significant difference between influx measurements in the presence or absence of insulin at later perfusion intervals. However, the full insulin-stimulated transport rate was never attained simply by transstimulation. Contrary to the inhibitory effect of 1 mM ouabain on L-serine transport in the absence of insulin (Fig. 2*A*), no inhibition of insulin-stimulated L-serine transport was observed (Table).

In similar experiments 100 μU/ml insulin had no effect on the time-course of 50 μM MeAIB influx. After 25-min perfusion with or without insulin unidirectional influx of MeAIB was, respectively, 39 ± 7 nmol/min · g ($n = 3$) and 36 ± 8 nmol/min · g

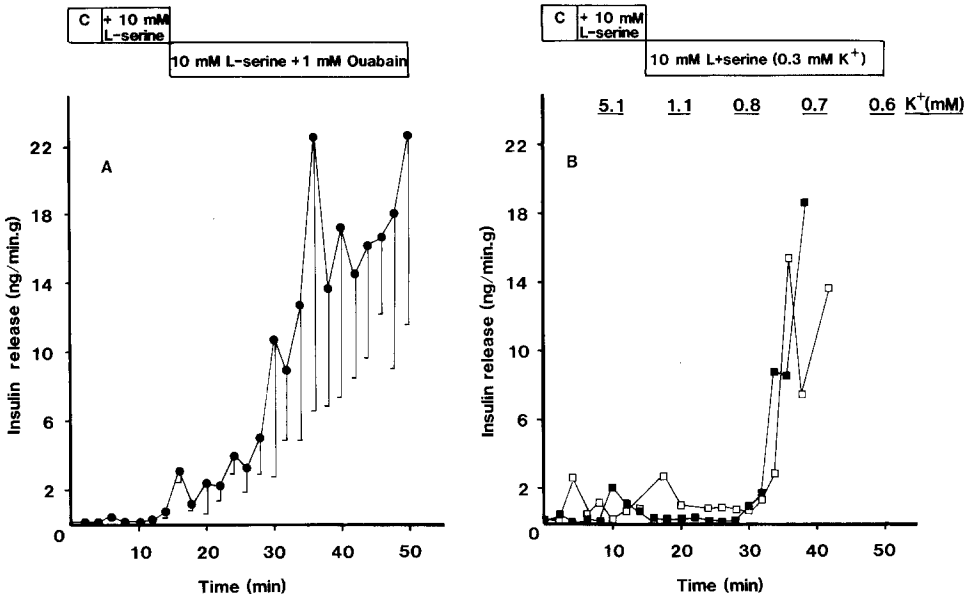

Fig. 4. Immunoreactive insulin release during perfusion of the pancreas with ouabain or low potassium. (*A*) In the presence of 10 mM L-serine 1 mM ouabain caused an immediate release of insulin which rose linearly over the 35-min experimental period. Amino-acid influx (Fig. 2*A*) and the Na^+ and K^+ concentrations in the pancreatic venous effluent were measured simultaneously (inset in Fig. 2). Values denote the mean ± SE of duplicate 2-min measurements in three pancreata. (*B*) release of IRI was quantified in two experiments during perfusion with 10 mM L-serine and initially 5.1 mM K^+ and then 0.3 mM K^+. The mean portal vein effluent K^+ concentrations (mM) are shown at the top of the Figure

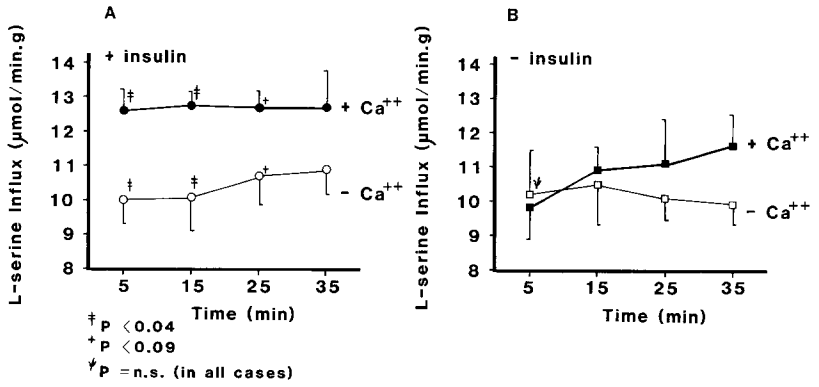

Fig. 5. Calcium dependence of insulin's stimulatory effect on L-serine transport. The effect of extracellular calcium removal on L-serine influx was tested in pancreata perfused in the presence (*A*) or absence (*B*) of 100 µU/ml bovine insulin. Pancreata were preperfused for 25 min with an amino-acid-free medium (± insulin and/or ± 2.5 mM calcium) and the time-course of L-serine influx was then measured repeatedly at 10-min intervals after switching to an identical perfusate but containing 10 mM L-serine. Values denote the mean ± SE of measurements in five to six perfused pancreata

($n = 3$). Furthermore, the time-dependent reduction in MeAIB influx observed previously (Fig. 2*B*) was unaffected by insulin.

Is Insulin Stimulation of L-Serine Transport Ca^{2+} Dependent?

In order to test whether L-serine transport and/or its stimulation by insulin were dependent upon extracellular calcium, pancreata were continually perfused with a calcium-free solution both in the absence or presence of 100 µU/ml insulin. In these experiments calcium was also omitted from the solution bathing the pancreas. In the absence of exogenous insulin, removal of calcium had no significant effect on the time-course of L-serine influx, although a time-dependent increase in L-serine influx (Fig. 5*B*) was not observed. The insulin-induced

Table. Influence of insulin on ouabain-induced inhibition of L-serine transport[a]

	Perfusion time (min)	% Inhibition in L-serine influx (mean difference)	*n*	*P*
Insulin-free	15	23.5	10	0.0006
	25	28.3	9	0.001
	35	36.9	10	0.0004
	45	37.1	10	0.0002
+ 100 μU/ml insulin	15	9.2	9	0.14
	25	1.5	9	0.67
	35	5.0	9	0.32
	45	11.7	9	0.14

[a] Pancreata were preperfused with an amino-acid-free solution containing either no insulin or 100 μU/ml insulin, and then perfused with 10 mM L-serine in the continued absence or presence of insulin. 1 mM ouabain was added to the respective perfusate 10 min after switching to 10 mM L-serine. The time-dependent inhibition of L-serine influx induced by ouabain was quantified as the mean difference between the unhatched (control) and hatched (ouabain) columns at each time interval shown in Fig. 2*A*. The data for ouabain effects in pancreata perfused with insulin is not shown graphically. An unpaired Student's *t*-test was used to assess significance and *n* denotes the total number of animals.

stimulation of L-serine transport was completely inhibited following 25-min preliminary perfusion with insulin and zero calcium (Fig. 5*A*).

Discussion

It is generally accepted that influx of Na^+ and the energy from the Na^+ gradient can drive uphill amino-acid transport [9], although some studies have concluded that active amino-acid transport also occurs in the absence of cation gradients [15, 23]. In the pancreas many amino acids evoke a similar depolarization of the acinar cell membrane [33], but it is difficult based on these electrical measurements to quantify what fraction of amino-acid uptake was actually Na^+ dependent. Our previous studies in the perfused rat exocrine pancreas demonstrated that influx of L-serine was largely mediated by a Na^+ independent System asc for small neutral amino acids, whereas influx of MeAIB occurred via the classically Na^+-dependent System A [29, 31]. We have now exploited this differential Na^+ sensitivity in characterizing the effects of ouabain, monensin, low K^+, calcium removal and insulin on the transport activity of L-serine and MeAIB.

Effects of Ouabain, Monensin, Low K^+ and Calcium Removal

Ouabain and monensin are known to inhibit amino-acid transport in exocrine pancreatic fragments [4, 23] and vesicles [43] but, to our knowledge, the time-course of this inhibition and their effects of L-serine and MeAIB influx in the perfused pancreas have not been reported. The time-dependent transstimulation of L-serine influx (Fig. 2) by accumulated intracellular substrate is characteristic of System asc [11, 44] and, as in other tissues [7, 14, 38], MeAIB transport via System A would be inhibited by elevated intracellular MeAIB.

Ouabain inhibited unidirectional influx of MeAIB to a greater extent than that of L-serine, but the significance of the effect on MeAIB was reduced as a consequence of the time-dependent transinhibition of influx (Fig. 2). Although inward transport of L-serine was predominantly Na^+ independent (Fig. 1), it is possible that exchange diffusion of L-serine with intracellular amino acids may have been modified by altered ionic gradients and membrane potential. The Na^+/H^+ exchanger monensin was a less effective inhibitor of pancreatic amino-acid transport (Fig. 2). Dissipation of the Na^+ gradient by monensin has been shown to inhibit Na^+-dependent aminoisobutyric acid (AIB) transport in rat parotid membrane vesicles [41]. In pancreatic acinar cells extrusion of cytosolic free calcium by Na^+/Ca^{2+} countertransport is also decreased in the presence of monensin [3]. In cultured 3T3 mouse fibroblasts monensin apparently stimulated AIB transport by elevating intracellular Na^+ and activating the Na^+-K^+ pump [40]. Moreover, in this study ouabain abolished stimulation of AIB uptake by monensin but had no effect on valinomysin-stimulated AIB uptake.

Following incubation of Chinese hamster ovary cells in 0.3 mM K^+, both K^+ influx and AIB transport via Na^+-dependent and Na^+-independent pathways was found to be stimulated [12]. When we examined the time-course of MeAIB and L-serine transport in pancreata perfused with 0.3 mM K^+, no significant changes in unidirectional influx were observed. The delayed washout of extracellular K^+ during 30-min perfusion with 0.3 mM K^+ may partially explain the lack of an effect on amino-acid transport (Fig. 4*B*). In other experiments omission of calcium from the perfusate had no effect on the time-course of L-serine transport (Fig. 5*B*), albeit removal of extracellular calcium evokes an acinar cell membrane hyperpolarization in the perfused rat pancreas [18].

When immunoreactive insulin and glucagon release was monitored under different experimental conditions, 1 mM ouabain and 0.3 mM K^+ (in the presence of 2.5 mM D-glucose) caused a similar delayed release of insulin (Fig. 4). Earlier studies in the perfused rat pancreas revealed that in the presence of 2.8 mM D-glucose 1 mM ouabain only transiently stimulated glucagon release, whereas omission of K^+ evoked a rapid and sustained inhibition of glucagon release but had not effect upon insulin release [22].

Regulation of Amino-Acid Transport by Insulin

Stimulation of L-serine transport by insulin was calcium dependent (Fig. 5*A*) and insensitive to ouabain (Table). As in the pancreas, MeAIB transport in the perfused rat liver is insulin-insensitive but stimulated by glucagon [19]. Although insulin hyperpolarizes pancreatic acinar cells [18, 39], it seems unlikely that stimulation of L-serine transport by insulin was dependent upon activation of the sodium pump. It remains controversial whether or not the hyperpolarization induced by insulin occurs by a ouabain-inhibitable mechanism [30, 47].

In perfused rat skeletal muscle ouabain was recently shown to have no effect on 200 μU/ml insulin-stimulated AIB transport, although it significantly inhibited the Na^+-K^+ pump and the basal rate of AIB uptake [48]. It was suggested in that paper that activation of amino-acid transport by insulin was not dependent upon the Na^+ electrochemical potential difference but rather the consequence of an increased activity or number of membrane transporters. Experiments in vitro with frog skeletal muscle have shown that resting AIB uptake was paradoxically Na^+ independent, whereas stimulation of AIB transport by 250 mU/ml insulin was dependent upon extracellular sodium [13]. In this study ouabain at 10^{-5} M had no inhibitory effect on either resting or insulin-stimulated AIB uptake. Moreover, the action of insulin appeared to be independent of protein synthesis since cycloheximide did not diminish AIB uptake. In diced mammary gland tissue 0.4 μU/ml insulin has been reported to increase AIB transport and the rate of intracellular amino-acid utilization [24]. It was proposed that a decrease in the intracellular amino-acid pool due to hormonal activation of metabolism or protein synthesis could stimulate the transport activity of preexisting membrane carriers.

Insulin also modulates cyclic nucleotide levels, and in barnacle muscle 7 to 50 μM insulin increases the cyclic GMP content, decreases the cyclic AMP content and lowers cytosolic ionized calcium [1]. Changes in cyclic nucleotide levels preceded stimulation of sugar transport induced by insulin, and both calcium and cGMP appeared to mimic the action of insulin. By contrast, in superfused mouse pancreatic acinar cells 1 μM insulin evoked an overall increase in cyclic AMP levels and only a transient increase in cyclic GMP concentrations [39]. Whether these differential effects of insulin reflect tissue and/or species differences or actual variations in intracellular regulatory mechanisms remains to be clarified.

Activation of Na^+/H^+ exchange by insulin has been shown to elevate intracellular pH and to increase System A amino-acid transport and incorporation into protein [30]. Moreover, an inhibition of Ca^{2+}-activated ATPase and a stimulation of endoplasmic reticulum calcium uptake by insulin could also lead to an increase in cytosolic pH [25, 30]. These mechanisms together with the Na^+ pump may play important roles in mediating the action of insulin in the pancreas. In addition to its effects on ions, insulin has recently been shown to enhance phosphatidylinositol breakdown and amylase secretion in the pancreas [10]. Incubation of adipocytes with insulin has been reported to remove a 90 kD protein from the plasma membrane [37]. These authors speculated that this protein might be a glucose transport-limiting factor. Their hypothesis is attractive, insofar as insulin may stimulate glucose transport by removal of this protein (or prevention of its accumulation) from the plasma membrane [37].

The characteristics of L-serine transport in the pancreas closely resemble those of the Na^+-independent System asc identified in erythrocytes [11, 44], and it is conceivable this membrane carrier may be regulated by insulin. Since L-serine entry is mediated by several parallel carriers [29], we cannot exclude the possibility that the stimulatory action of insulin was restricted to a fraction of L-serine influx mediated by Systems A or ASC. If insulin modulated the intracellular concentration of other natural amino acids, its effects on L-serine transport may be the consequence of transstimulation associated with System asc. In recent experiments somatostatin-14 (another islet hormone) completely inhibited the stimulation of L-serine transport induced by insulin (G.E. Mann and P.S.R. Norman, *unpublished data*). Our present findings provide a basis for evaluating the cellular mechanisms by which islet and other gastrointestinal hormones interact to

modulate basolateral amino-acid transporters in the exocrine pancreatic epithelium.

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