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Regulation of L-alanine transport systems A and ASC by cyclic AMP and calcium in a reptilian duodenal model

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Summary

The regulation of neutral amino acid transport by cyclic AMP (cAMP) and calcium across the isolated duodenum of the lizard *Gallotia galloti* has been studied under short-circuit conditions. Active L-alanine transport was stimulated by forskolin, theophylline and dibutyryl cyclic AMP (db-cAMP). All these agents increased transmural potential difference (PD) and short-circuit current (I_{sc}) in a manner consistent with the activation of a chloride secretory pathway. Both forskolin and theophylline increased intracellular cAMP levels in the lizard duodenal mucosa. Addition of calcium ionophore A23187 rapidly reduced mucosa-to-serosa L-alanine fluxes and diminished net L-alanine transport. Despite the reduction of alanine fluxes by A23187, transepithelial PD and I_{sc} values were increased by the ionophore. Analyses of the responses of isolated transport pathways indicated that the Na^+-independent L-alanine transport system was unaffected by db-cAMP or calcium ionophore. By contrast, Na^+-dependent transport activities were profoundly modified by these agents. Thus, while system A [α-methylamino-isobutiric acid (MeAIB)-transporting pathway] was stimulated by increased calcium, system ASC activity was nearly abolished. Calcium ionophore also potentiated the electrogenic response of system A. Forskolin strongly stimulated system ASC activity but left system A activity unchanged. Activation of system ASC by forskolin was clearly electroneutral, as pre-incubation of the tissues with the chloride channel blocker diphenylamine-2-carboxilic acid (DPC) completely prevented forskolin-induced transepithelial electrical responses. It is concluded that intracellular messengers cAMP and calcium oppositely modulate active Na^+-dependent L-alanine transport in the lizard intestine. The different sensitivity exhibited by individual transport pathways may well account for the changes observed in overall alanine transport.

Key words: system A, system ASC, neutral amino acid transport, L-alanine transport, intracellular transducers, cyclic AMP, calcium, *Gallotia galloti*.

Introduction

Over the past two decades, many laboratories have demonstrated that, besides adaptive regulation, neutral amino acid transport is also subject to regulation by hormonal and environmental stimuli (Kilberg, 1986; McGivan and Pastor-Anglada, 1994; Moule and McGivan, 1990; Pastor-Anglada et al., 1996). In particular, L-alanine transport through hepatocyte and skeletal muscle is clearly regulated by the hormones EGF (epidermal growth factor), cortisol and, especially, glucagon and insulin in a manner that is consistent with the gluconeogenic properties of L-alanine.

Pioneering work by Edmonson and Lumeng (1980) on isolated rat hepatocytes showed that glucagon stimulation of neutral amino acid transport was biphasic, with short- and long-term effects. Although the first phase was totally insensitive to inhibitors of protein and RNA synthesis, the second phase was completely prevented by cycloheximide. Later studies have demonstrated that the rapid stimulation of L-alanine transport by glucagon is caused by the rapid generation of cyclic AMP (cAMP), and it has been suggested that the short-term stimulation of alanine transport is secondarily due to a cAMP-mediated membrane hyperpolarization (Moule et al., 1987). Although this model of regulation has been generally accepted for hepatocytes, it has not been validated for other preparations expressing similar transport systems. In fact, studies performed in vascular endothelial cells (Escobales et al., 1994), kidney cortex (Goldstone et al., 1983), lymphocytes (Woodlock et al., 1989), glioblastoma cells (Zafra et al., 1994) and placental slices (Karl et al., 1988; Ramamoorthy et al., 1992) have pointed out that alanine transport is acutely regulated by intracellular calcium or diacylglycerol but not by cAMP. Interestingly, despite the existence of multiple distinguishable transport systems for neutral amino acids within each cell type, the regulation of transport activity appears to be

restricted to individual systems. For instance, neutral amino acid transport systems in the plasma membrane of hepatocytes is carried out by systems A, ASC and L, but only system A appears to be subjected to adaptive regulation and pancreatic hormones (Gazzola et al., 1981; Handlogten and Kilberg, 1984; Shotwell et al., 1983).

In the small intestine of vertebrates, neutral amino acid transport is catalyzed by several Na^+-dependent and Na^+-independent systems (for reviews, see Kilberg et al., 1993; Munck and Munck, 1994; Stevens, 1992). Nonetheless, despite extensive description of amino acid transport pathways, kinetic properties, energy requirements and membrane localization in isolated epithelia, enterocytes and brush-border membranes from different animal models, studies describing short-term regulation of intestinal neutral amino acid transport across epithelial cells are scant.

The purpose of this study was to investigate the regulation of L-alanine transport across the lizard duodenum by increased intracellular cAMP and calcium levels. This epithelium has been studied over the past two decades, and the different pathways for neutral amino acid transport have been recently identified and characterised physiologically (Díaz et al., 2000; Medina et al., 2001).

Materials and methods

Chemicals

MeAIB (α-methylamino-isobutiric acid), L-alanine, L-cysteine, L-serine, diphenylamine-2-carboxilic acid (DPC) and theophylline were obtained from Sigma-Aldrich S.A. (Madrid, Spain). Forskolin, calcium ionophore A23187 and dibutyryl cyclic AMP were purchased form Calbiochem (Madrid, Spain). [^{2,3}H]L-alanine and [^{14}C]MeAIB were purchased from Amersham Ibérica (Madrid, Spain). All reagents used were analytical grade.

Animals and solutions

Adult male lizards (*Gallotia galloti* sauria, lacertidae) weighing 25–40 g were sacrificed by spinal transection and the duodenum was removed and rinsed in ice-cold bathing solution. The standard Ringer solution contained 107 mmol l^{-1} NaCl, 4.5 mmol l^{-1} KCl, 25 mmol l^{-1} $NaHCO_3$, 1.8 mmol l^{-1} Na_2HPO_4, 0.2 mmol l^{-1} NaH_2PO_4, 1.25 mmol l^{-1} $CaCl_2$ and 1.0 mmol l^{-1} $MgCl_2$ and had a final pH of 7.3. Duodenal segments were mounted in water-jacketed Ussing chambers with an exposed area of 0.21 cm^2 and bathed on both sides with 4 ml of Ringer solution. Chambers were continuously gassed with 5% CO_2 and 95% O_2 and the temperature was maintained at 27°C. In some experiments, choline was used to replace sodium ions in the bathing solutions.

Electrical measurements

The electrical measurements were made as described previously (Gómez et al., 1986) using calomel (for voltage sensing) and Ag/AgCl electrodes (for current passage) connected to the bathing solutions through 4% (v/w) agar bridges. Electrical measurements were continuously monitored with an automatic computer-controlled voltage-clamp device (AC-microclamp) and hardcopied. The tissues were first incubated under open-circuit conditions for 20 min and then under short-circuit conditions; i.e. the potential difference (PD) and the short-circuit current (I_{sc}) were determined every minute. Every 5 s, the tissues were pulsed with 1 s ± 10 μA pulses, and, from the displacement of the PD, the tissue conductance (G_t) was derived. Corrections for electrode offset potential and solution resistance were determined throughout the experiments and stored in the computer-controlled voltage-clamp device.

Transepithelial fluxes

Unidirectional amino acid fluxes were measured under short-circuit conditions using the procedure described in detail by Diaz et al. (2000). Briefly, 20 min after the tissue was properly mounted in the chamber, 185 kBq of the appropriate labelled substrate ([^{2,3}H]L-alanine or [^{14}C]MeAIB) was added to the serosal or mucosal side of the tissue. After an additional 20 min period, by which isotope fluxes had reached steady state, duplicate 200 μl aliquot samples were taken from the unlabelled side at regular 20 min intervals for 1 h and replaced by an equal volume of Ringer solution. Inhibition experiments were carried out by adding small volumes (100 μl) of concentrated stock solution containing the amino acid or analogue to the mucosal and/or serosal compartments. The unidirectional and net fluxes were determined using a computer program written in our laboratory (Díaz and Cozzi, 1991), which also provided the statistical tools required for data analysis.

Cyclic AMP determination

Tissue cAMP accumulation in response to forskolin and theophylline was determined on freshly isolated tissues. Once removed, tissues were washed several times in cold saline, weighed, minced into small pieces and immediately transferred to the standard bathing solution. Vehicles and drugs were added at time zero to control and test tubes, respectively, and allowed to preincubate for different times. Samples were then homogenized in a Tris/EDTA buffer (0.5 ml per 100 mg tissue mass) at pH 7.5. Samples were then deproteinized by heat in boiling oil for 3 min and centrifuged at 1000 ***g*** for 5 min. Duplicated 50 μl samples from each supernatant were taken for cAMP determination using a standard cAMP assay kit (Amersham Ibérica).

Statistical analysis

Results are expressed as means ± S.E.M. Statistical comparisons of mean values were made using two-tailed Student's *t*-test or one-way analysis of variance (ANOVA) followed by Tukey's test, where appropriate. In some experiments, PD and I_{sc} values were normalized by transformation to percentage of maximal change within each experiment.

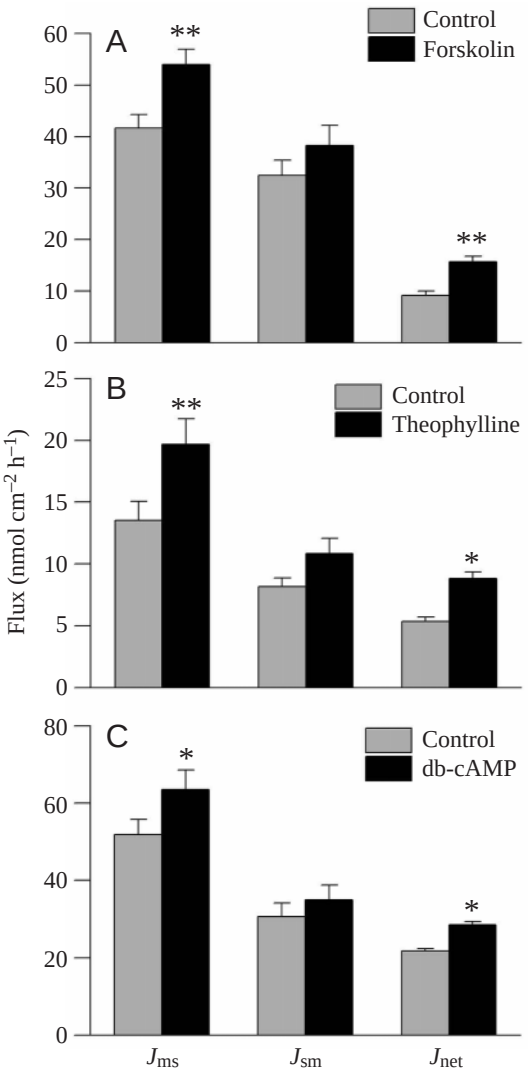

Fig. 1. Effects of (A) forskolin (10 μmol l^{-1}), (B) theophylline (3 mmol l^{-1}) and (C) dibutyryl cyclic AMP (db-cAMP; 500 μmol l^{-1}) on unidirectional and net L-alanine fluxes across the duodenum of *Gallotia galloti*. Experiments were carried out under short-circuit conditions in the presence of sodium. The concentration of L-alanine was 1 mmol l^{-1} throughout the experiments and was added to both serosal and mucosal bathing solutions 20 min before the control values were obtained. Drugs were added in small volumes (<50 μl) to both sides of the tissue immediately after the last control values were obtained and were left for an additional 10 min before test samples were taken (see Materials and methods). Results are means ± S.E.M. for 16, 12 and 8 different duodenal segments, respectively. J_{ms}, mucosa-to-serosa flux; J_{sm}, serosa-to-mucosa flux; J_{net}, net flux. *P<0.05; **P<0.01 compared with control.

Results

Effects of forskolin, theophylline and db-cAMP on total L-alanine transport

The effects of different pharmacological agents affecting cAMP metabolism on unidirectional and net fluxes of L-alanine (1 mmol l^{-1}) across the isolated lizard duodenum have been determined under short-circuit conditions (Fig. 1). Using Na^+-containing Ringer solutions, mean control net L-alanine transport (J_{net}) was 14.81±1.05 nmol cm^{-2} h^{-1} (significantly different from zero at P<0.01), which was in the range reported previously for this same preparation (Medina et al., 2001). Addition of forskolin (10 μmol l^{-1}), theophylline (3 mmol l^{-1}) or the membrane-permeable analogue dibutyryl cyclic AMP (db-cAMP; 500 μmol l^{-1}) significantly increased J_{net}, these effects being due to a rise in the mucosal-to-serosal flux (J_{ms}) without altering the flux in the opposite direction (J_{sm}). The elevation of J_{net} induced by the three compounds was, on average, 47% above the control value, which closely reflected the increase observed for J_{sm}.

Table 1. *Effects of forskolin, theophylline and db-cAMP on bioelectrical parameters across the duodenal mucosa of* Gallotia galloti

	PD (mV)	I_{sc} (μequiv. cm^{-2} h^{-1})	G_t (mS cm^{-2})
Control	2.01±0.2	1.01±0.15	7.95±1.1
Forskolin (10 μmol l^{-1}) (16)	2.80±0.2**	1.50±0.2*	8.95±1.2
Control	2.3±0.2	0.70±0.15	8.01±0.6
Theophylline (3 mmol l^{-1}) (12)	2.8±0.2*	1.15±0.18*	8.05±0.5
Control	2.60±0.2	0.60±0.1	8.01±0.8
db-cAMP (500 μmol l^{-1}) (8)	3.05±0.1*	0.95±0.1*	8.01±0.5

Number of experiments for each experimental condition indicated in parentheses. *P<0.05; **P<0.01 compared with control tissues.

db-cAMP, dibutyryl cAMP; PD, potential difference; I_{sc}, short-circuit current; G_t, tissue conductance.

Bioelectrical parameters were measured under short-circuit conditions for the different compounds and the results are illustrated in Table 1. The tissues were maintained for 20–40 min under open-circuit conditions and were then short-circuited. As has been reported several times for this same preparation (Gómez et al., 1986; Lorenzo et al., 1989b), no differences were observed between the transmural potential difference (PD), short-circuit current (I_{sc}) and tissue conductance (G_t) between stable open-circuit and short-circuit measurements. As can be seen in Table 1, under control conditions, transepithelial PD was approximately 2.0 mV and the mean I_{sc} was 0.77 μequiv. cm^{-2} h^{-1}, the serosal side of the tissue being electrically positive compared with the luminal side, which parallels our previous observations (Gómez et al., 1986; Medina et al., 2001). Both PD and I_{sc} were significantly increased by forskolin, theophylline and db-cAMP, in agreement with most studies performed in different animal models, including lizard intestine (Bridges et al., 1983; Díaz and Lorenzo, 1991; Lorenzo et al., 1989a). The tissue

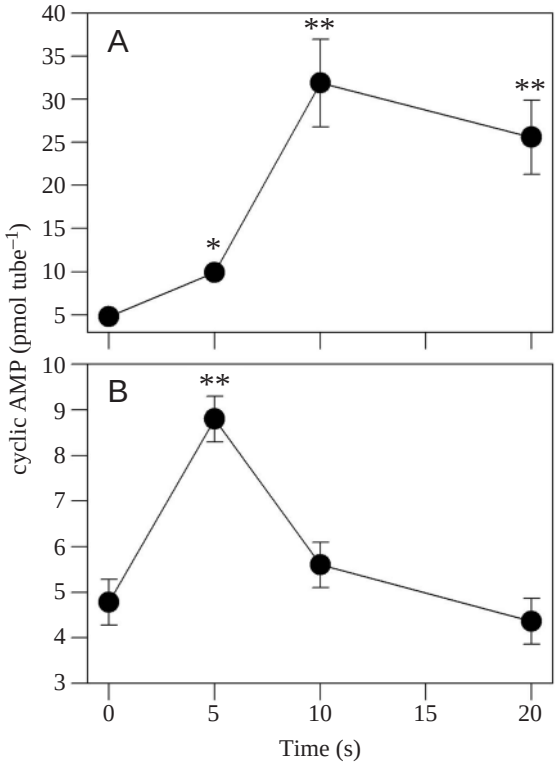

Fig. 2. Time-course for cyclic AMP production in response to (A) forskolin (10 μmol l^{-1}) and (B) theophylline (3 mmol l^{-1}) in the duodenal mucosa of *Gallotia galloti*. Forskolin and theophylline were added at time zero. Each point represents the mean ± S.E.M. for six separate determinations. *P<0.05; **P<0.01 compared with time 0.

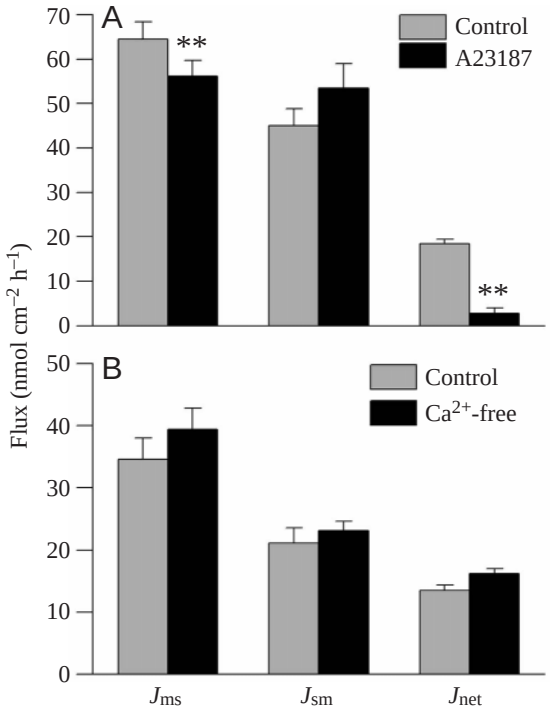

Fig. 3. Effects of (A) calcium ionophore A23187 and (B) extracellular calcium replacement on duodenal unidirectional and net L-alanine fluxes. Experiments were carried out under short-circuit conditions in the presence of sodium. The concentration of L-alanine was 1 mmol l^{-1} throughout the experiments and was added to both serosal and mucosal bathing solutions 20 min before the control values were obtained. A23187 (0.5 μmol l^{-1}) was added to both sides of the tissue immediately after the last control values were obtained. Calcium replacement was achieved by replacing all the standard solution with a Ca^{2+}-free solution containing 0.5 mmol l^{-1} EGTA. Tissues were left for an additional 10 min before test samples were taken (see Materials and methods). Results are means ± S.E.M. for 16 and 10 duodenal segments, respectively. J_{ms}, mucosa-to-serosa flux; J_{sm}, serosa-to-mucosa flux; J_{net}, net flux. **Statistically different from its corresponding control value at P<0.01.

conductance remained unaltered throughout the experiments at approximately 8 mS cm^{-2}.

Effects of forskolin and theophylline on intracellular cAMP concentration

In order to test whether the effects of forskolin and theophylline on L-alanine transport across lizard duodenum could be attributed to changes in the intracellular levels of cAMP, we performed radioimmunoassays to determine the time-course of cAMP concentration in isolated mucosal preparations. The results shown in Fig. 2 revealed that both forskolin (10 μmol l^{-1}) and theophylline (3 mmol l^{-1}) significantly increased cAMP levels, although the time-course and the magnitude of change were clearly different for the two drugs. Thus, cAMP concentration reached a maximal value of 31.87±5.1 pmol tube^{-1} 10 min after the exposure of mucosal tissues to 10 μmol l^{-1} forskolin. On the other hand, theophylline induced a transient increase of cAMP level that was much lower (8.8±0.5 pmol tube^{-1}) than for forskolin, this value being reached 5 min after exposure to the secretagogue. In addition, the effect of forskolin was maintained for the rest of the experiment whereas the effect of theophylline rapidly declined to control values 20 min after drug exposure.

Effects of calcium ionophore A23187 and calcium-free solutions on total L-alanine transport

In the next series of experiments, manoeuvres known to alter cellular calcium homeostasis were tested for their ability to affect L-alanine transport across the lizard duodenum. Initially, the effects of calcium ionophore A23187 (0.5 μmol l^{-1}) on unidirectional and net L-alanine fluxes were analysed. As can be observed in Fig. 3A, addition of calcium ionophore to both sides of the tissue brought about a considerable decrease in J_{net}, this effect being entirely attributable to the reduction of J_{ms}. The remaining J_{net} (2.76±1.19 nmol cm^{-2} h^{-1}) was still significantly different from zero (P<0.05, N=16), suggesting that a fraction of L-alanine transport was unaffected by the ionophore. Despite the dramatic reduction of L-alanine transport by A23187, PD and I_{sc} (but not G_t) were increased by the ionophore (Table 2), indicating that increased calcium levels had stimulated a conductive process.

Table 2. *Effects of calcium ionophore on bioelectrical parameters across the duodenal mucosa of* Gallotia galloti

	PD (mV)	I_{sc} (μequiv. cm^{-2} h^{-1})	G_t (mS cm^{-2})
Control	1.90±0.19	0.64±0.05	8.90±0.11
A23187 (0.5 μmol l^{-1}) (8)	2.65±0.19*	0.78±0.04*	9.10±0.15
Control	2.03±0.13	0.91±0.11	8.95±1.1
Ca^{2+}-free (6)	2.00±0.20	0.90±0.12	8.25±1.2

Number of experiments for each experimental condition indicated in parentheses. *P<0.05 compared with control tissues.

PD, potential difference; I_{sc}, short-circuit current; G_t, tissue conductance.

In another set of experiments, the effects of extracellular calcium replacement on L-alanine fluxes and biolectrical parameters were determined. The results shown in Fig. 3B and Table 2 indicate that none of the parameters under study were modified by calcium replacement.

Effects of db-cAMP and A23187 on individual L-alanine transport systems

We have previously demonstrated that, under short-circuit conditions, the overall L-alanine transport in the lizard duodenum could be completely explained by the simultaneous participation of two Na^+-dependent transport systems, endowed with properties of systems ASC and A, plus one Na^+-independent electrogenic carrier (Díaz et al., 2000; Medina et al., 2001). These three different transport systems could be individually dissected by means of a strategy based on the differential substrate affinities and sodium dependence. In order to determine whether individual L-alanine transport systems were equally affected by cAMP and calcium, we performed experiments aimed at assessing the effects of these intracellular transducers on each individual transport system.

First, we measured L-alanine fluxes under short-circuit conditions using Na^+-free solutions. Under these conditions, active L-alanine transport was carried by an electrogenic cycloleucine- and 2-aminobicyclo-(2,2,1)-heptane-2-carboxilic acid (BCH)-sensitive pathway (Díaz et al., 2000). The results shown in Figs 4A and 5A indicate that neither db-cAMP nor A23187 affected unidirectional or net L-alanine fluxes, which strongly suggests that changes in the activity of the Na^+-independent carrier were not responsible for the effects on the overall L-alanine transport observed above, but rather these resulted from the activities of the Na^+-dependent systems.

Therefore, we measured the effects of cAMP and calcium on the activity of the two Na^+-dependent transport pathways identified in the lizard duodenum, i.e. system A and system ASC. Initially, system A activity was determined by using the specific *N*-methylated derivative α-methylamino-isobutiric acid (MeAIB) in the presence of sodium. Our results showed that neither unidirectional nor net MeAIB fluxes were altered

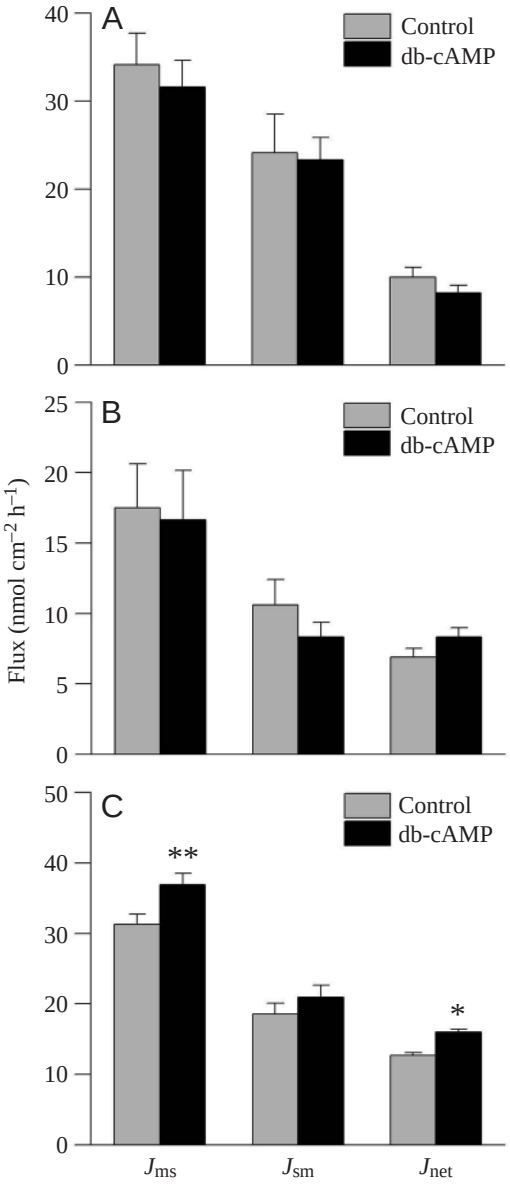

Fig. 4. Effects of dibutyryl cyclic AMP (db-cAMP; 500 μmol l^{-1}) on isolated L-alanine transport systems across the duodenal mucosa of *Gallotia galloti*. (A) Effects on Na^+-independent L-alanine transport. (B) Effects on system A activity as determined by measuring the unidirectional and net MeAIB (α-methylamino-isobutiric acid) fluxes (10 mmol l^{-1}) in Na^+-containing solutions. (C) Effects on system ASC. System ASC transport activity was obtained by determining L-alanine unidirectional and net fluxes in the presence of a saturating concentration of MeAIB (20 mmol l^{-1}) in Na^+-containing solutions. Effects of db-cAMP were measured 20 min after its addition to the bathing solutions. The concentration of L-alanine in the bathing solutions was 1 mmol l^{-1}. All experiments were performed under short-circuit conditions. Results are means ± S.E.M. for 16 duodenal segments under each condition. *P=0.05; **P=0.01 compared with control.

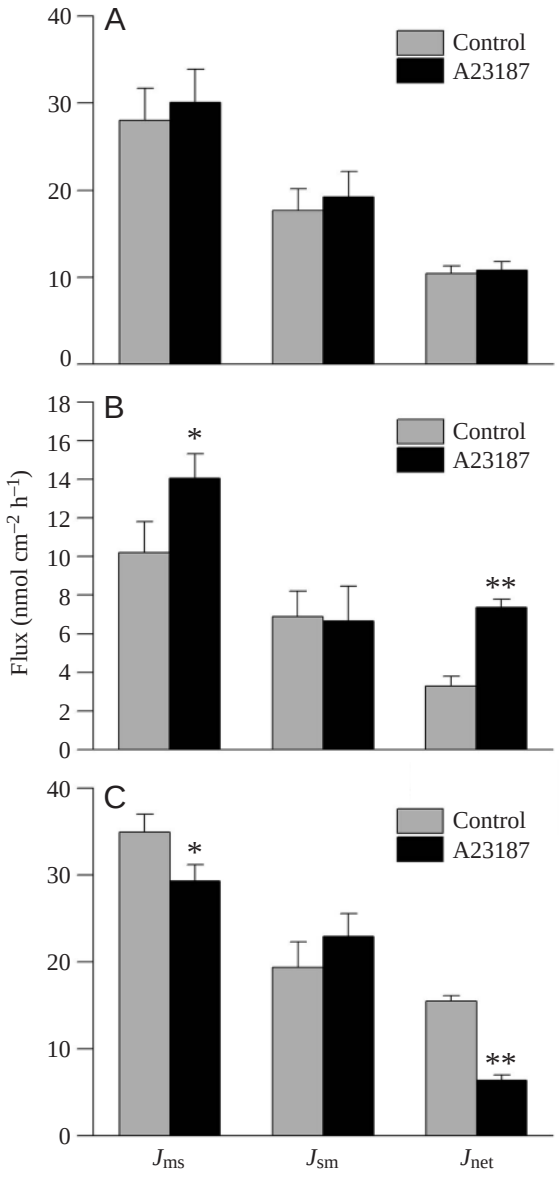

Fig. 5. Effects of calcium ionophore A23187 (0.5 μmol l^{-1}) on isolated L-alanine transport systems across the duodenal mucosa of *Gallotia galloti*. (A) Effects on Na^+-independent L-alanine transport. (B) Effects on system A activity as determined by measuring the unidirectional and net MeAIB (α-methylamino-isobutiric acid) fluxes (10 mmol l^{-1}) in Na^+-containing solutions. (C) Effects on system ASC. System ASC transport activity was obtained by determining L-alanine unidirectional and net fluxes in the presence of a saturating concentration of MeAIB (20 mmol l^{-1}) in Na^+-containing solutions. A23187 effects were determined 20 min after its addition to the bathing solutions. The concentration of L-alanine in the bathing solutions was 1 mmol l^{-1}. All experiments were performed under short-circuit conditions. Results are means ± S.E.M. for 16 duodenal segments under each condition. *P<0.05; **P<0.01 compared with control.

by db-cAMP at concentrations known to increase duodenal L-alanine transport (Fig. 4B). Conversely, addition of A23187 significantly increased J_{ms} (but not J_{sm}), which consequently augmented J_{net} (Fig. 5B; P<0.01). These observations were striking because the ionophore effectively stimulated MeAIB transport (therefore system A activity) but, as shown before, dramatically reduced net L-alanine fluxes.

Finally, in order to assess the effects of intracellular messengers on the activity of system ASC, we measured L-alanine fluxes in the presence of a saturating concentration of MeAIB (20 mmol l^{-1}) under short-circuit conditions and in the presence of sodium. This same procedure has been used to isolate the ASC transport pathway in different preparations, including lizard duodenum (Medina et al., 2001). Interestingly, under these conditions, both intracellular messengers affected L-alanine transport but in a completely opposite direction (Figs 4C, 5C). Thus, while db-cAMP significantly increased J_{ms} and J_{net}, addition of A23187 reduced both J_{ms} and J_{net}. Neither compound affected J_{sm}, ruling out a possible change on membrane permeability and indicating that system ASC activity is stimulated by cAMP and depressed by increased intracellular free calcium.

Effects of db-cAMP and A23187 on L-alanine- and MeAIB-induced bioelectrical parameters

To further explore the effects of cAMP and calcium on the activity of the different L-alanine transport pathways, we designed experiments to ascertain the electrical responses of isolated duodenum to the addition of transported substrates before and after addition of the drugs. As can be seen in Fig. 6A, addition of L-alanine (4 mmol l^{-1}) to the bath readily elevated PD and I_{sc}, consistent with our previous demonstration of the activation of Na^+- and L-alanine-dependent conductive pathways (Medina et al., 2001). Following washout, application of forskolin to the bath induced an increase in PD and I_{sc} that could be readily reversed by addition of the chloride channel blocker diphenylamine-2-carboxilic acid (DPC; 1 mmol l^{-1}) but not by addition of 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS; 0.5 mmol l^{-1}; data not shown), suggesting that forskolin had activated the Cl^- conductance typically involved in the secretory response to cAMP-elevating agents (Anderson and Welsh, 1991; Anderson et al., 1992; Liedtke, 1989). Under these conditions, subsequent addition of L-alanine elicited changes on the electrical parameters that were similar in magnitude to those observed in the control period (Fig. 6A, inset). These results indicate that, although forskolin increases unidirectional and net L-alanine fluxes, the pathway(s) activated by increased cAMP levels do not provide any additional electrogenicity but, rather, behave in an electroneutral way. Similar effects were observed using MeAIB instead of L-alanine. These observations suggest the involvement of an Na^+-dependent, MeAIB-excluding, electroneutral pathway, which may well be ascribed to the electrical operation of system ASC (Bussolati et al., 1992; Medina et al., 2001).

The effects of calcium ionophore on L-alanine-induced electrical responses suggested that increased intracellular calcium potentiated the response to L-alanine. Indeed, results

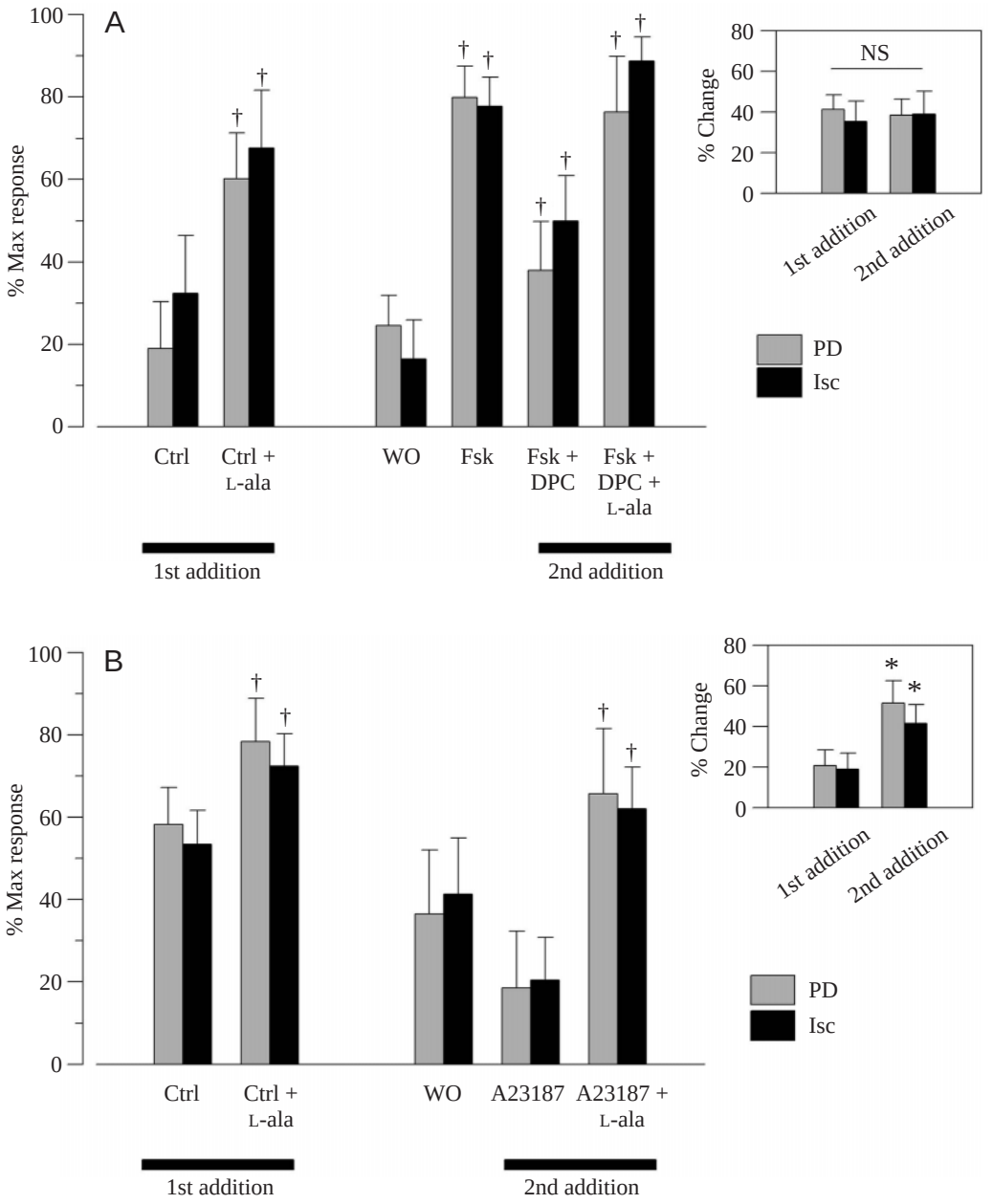

Fig. 6. Effects of (A) forskolin (Fsk; 10 μmol l^{-1}) and (B) calcium ionophore A23187 (0.5 μmol l^{-1}) on L-alanine-induced bioelectrical changes. Each bar represents the steady-state value for the indicated manoeuvre within the experiment. The effects of drugs, alone or in combination with inhibitors, were assessed by comparing the magnitude of potential difference (PD) or short-circuit current (I_{sc}) changes upon consecutive additions of L-alanine (L-ala). Values are expressed as a percentage of maximal change (% max response) within each experiment. The insets show comparisons between percentage PD and I_{sc} changes in response to consecutive L-alanine additions. Further details are provided in the text. Results are means ± S.E.M. of 10 different duodenal segments. *P<0.05 compared with control; †P<0.05 compared with the previous experimental condition. Ctrl, control; WO, washout; NS, non-significant.

illustrated in Fig. 6B show that exposure of the tissues to A23187 (0.5 μmol l^{-1}) did not significantly affect either PD or I_{sc}. This observation was interesting because it ruled out the possible activation of rheogenic calcium-dependent Cl^- or K^+ conductances in response to the ionophore (Anderson and Welsh, 1991; Anderson et al., 1992; Liedtke, 1989). However, subsequent addition of L-alanine to A23187-treated tissues brought about a 2–3-fold increase in both PD and I_{sc}, the changes being statistically significant compared with the first amino acid challenge (Fig. 6B, inset). Identical results were obtained using the system A-specific substrate MeAIB. Taken together, these results indicate that, although increased intracellular calcium reduces total L-alanine transport (see above), it stimulates an electrogenic pathway endowed with properties of system A.

Discussion

The data presented in this study clearly indicate that L-alanine transport across the lizard duodenal epithelia is stimulated by dibutyryl cAMP as well as by factors increasing cAMP levels, namely forskolin and theophylline. The fact that unidirectional mucosa-to-serosa and net L-alanine fluxes were significantly increased under short-circuit conditions by these agents strongly indicates that L-alanine is actively transported across the epithelia and that this vectorial movement is stimulated by cAMP. Although this is the first demonstration of cAMP effects on L-alanine transport in a reptile intestine, several studies have been published showing that intestinal neutral amino acid transport may also be affected by stimulating adenylate cyclase and/or cAMP-phosphodiesterase inhibition. For instance, a proabsorptive effect of forskolin has

been reported for lysine, leucine and glycine transport in the rat jejunum (Reymann et al., 1986). Similarly, previous studies from our laboratory have shown that theophylline enhances phenylalanine absorption and intracellular accumulation in the lizard intestine (Lorenzo et al., 1989a). Apparently, the effects of forskolin and theophylline on L-alanine transport are a consequence of an increase in amino acid uptake at the site of the brush-border carriers and not the result of a reduction in permeability across the basolateral border. This conclusion is supported by our data of unchanged unilateral fluxes from the serosal compartment.

Using electrophysiological and radiotracer techniques, we have recently demonstrated that active L-alanine transport across the lizard duodenum could be explained by the simultaneous operation of different carriers (Díaz et al., 2000; Medina et al., 2001). These distinct transport pathways could be resolved on the basis of their different Na^+ dependence, substrate affinity, kinetic features and electrical properties. These approaches led us to ascertain that the lizard duodenum displayed two Na^+-dependent pathways, endowed with properties of system A (MeAIB-transporting electrogenic system) and system ASC (MeAIB-insensitive L-cysteine- and L-serine-transporting electroneutral system), which coexist with a unique Na^+-independent, K^+-dependent, electrogenic L-alanine transport pathway.

Under this scenario, we thought it worthwhile to investigate the effects of increased cAMP and intracellular calcium levels on L-alanine transport pathways in order to assess whether individual systems were affected equally by intracellular signal transducers. The data shown in Fig. 4 suggest that not all transport pathways exhibited similar responses to db-cAMP; instead, only system ASC appeared to be stimulated by the nucleotide. The finding that MeAIB transport was not affected by db-cAMP was striking, since most literature data on the short-term regulation of neutral amino acid transport by cAMP (or glucagon) in hepatocytes and adipocytes indicate that system A represents the main target for cAMP stimulation (Guma et al., 1993; McGivan et al., 1981; Miller and Bhandari, 1986; Moule et al., 1987; Moule and McGivan, 1990).

Our results also demonstrate that, aside from stimulating net L-alanine transport, db-cAMP, as well as forskolin and theophylline, increased transepithelial PD and I_{sc} (see Table 1). These observations indicate that the rise in cAMP in duodenal tissues had eventually triggered an electrogenic process. It has been suggested that rapid stimulation of L-alanine transport by cAMP in rat hepatocytes was due to membrane hyperpolarization (Moule et al., 1987). These authors have suggested that cAMP hyperpolarizes hepatocytes by stimulation of the Na^+/H^+-exchange and Na^+/K^+-ATPase activities, therefore reducing intracellular Na^+, which would enhance Na^+–alanine symport activity in the plasma membrane of hepatocytes (Moule et al., 1987). In addition, numerous studies have demonstrated that these agents stimulate an electrogenic chloride secretion by activation of apical Cl^- channels in the intestinal epithelia (see Anderson et al., 1992; Liedtke, 1989; Merlin et al., 1998). Hence, evaluation of electrical changes associated with stimulation of L-alanine transport by increasing cAMP is hampered by the possible induction of an electrogenic chloride secretion. In order to circumvent this inconvenience, we designed the sequential protocol shown in Fig. 6A. Firstly, the effects of L-alanine on PD and I_{sc} were determined and the percentage of change was measured (1st addition). Following washout, addition of forskolin to the bath brought about a considerable increase in PD and I_{sc}, which was reduced to control levels by DPC, a well-known inhibitor of cAMP-activated apical Cl^- channels (Anderson et al., 1992; Becq et al., 1993). Then, still in the presence of forskolin and DPC, addition of L-alanine (2nd addition) rapidly increased PD and I_{sc}, but no appreciable differences were observed among the two consecutive L-alanine additions, suggesting that, although L-alanine fluxes were considerably stimulated by forskolin, the system responsible for this change was not conductive but rather behaved in an electroneutral manner, ruling out the activation of system A. In the case of lizard duodenum, this feature defined the electrical operation of system ASC (Medina et al., 2001). Moreover, the magnitude of system ASC activity stimulation was sufficient to explain the increase in overall L-alanine transport observed following forskolin. Consistent with our results, studies performed in skeletal muscle and placental membranes have demonstrated that neither forskolin nor cholera toxin, both cAMP-elevating factors, affect system A activity (Guma et al., 1992; Pastor-Anglada et al., 1996). Likewise, results from trout hepatocytes have revealed that system ASC, but not system A, was rapidly stimulated by glucagon (Gallardo et al., 1996). Although speculative, our results, together with those from fish hepatocytes, suggest that rapid regulation of L-alanine transport could have been subjected to phylogenetic variations.

The experiments aimed at assessing a possible role of calcium on the regulation of L-alanine transport across the lizard duodenum suggest that L-alanine transport pathways appear to be oppositely modulated by intracellular messengers. Indeed, permeabilizing duodenal cells to calcium using the ionophore A23187 dramatically reduced J_{ms} and J_{net}, without altering J_{sm}, revealing that an important fraction of total L-alanine transport was depressed by a rise in cytosolic calcium. Although the literature on the regulation of amino acid transport by calcium is scarce, the inhibition of L-alanine and glucose uptake across brush-border vesicles from rabbit small intestine by intravesicular calcium loading has been reported by Miyamoto et al. (1990). Similar findings have been observed for several Na^+-dependent carriers in rat small intestine vesicles preloaded with a high calcium concentration (Fondacaro and Madden, 1984).

Strikingly, in spite of the considerable reduction of duodenal L-alanine transport by A23187, PD and I_{sc} were augmented by the ionophore, reflecting the activation of some conductive mechanism (see Table 2). It is well known that cytosolic calcium may regulate electrolyte transport in airway and intestinal epithelia (Brown, 1987; Liedtke, 1989) by different mechanisms, often involving activation of Ca^{2+}-dependent Cl^-

channels and/or calcium-dependent K^+ channels (Anderson and Welsh, 1991; McCabe and Smith, 1985; Schultheiss and Diener, 1998). However, this does not seem to be the case for the lizard duodenum, as addition of A23187 to control tissues (in the absence of L-alanine) failed to produce any significant change in either PD or I_{sc} (Fig. 6B), therefore ruling out the possibility of an electrogenic Cl^- secretion activated by calcium. The first evidence for the identification of the putative electrogenic pathway activated by rising intracellular calcium emerged from the comparison of the effects of L-alanine addition to the lizard duodenum before and after the ionophore challenge. As can be seen in Fig. 6B, application of the calcium ionophore potentiated the responses of PD and I_{sc} to L-alanine, suggesting that the rise in cytoplasmic calcium had activated an L-alanine-dependent conductive pathway. Hence, we determined the effects of calcium ionophore on individual transport pathways using the same dissection protocol mentioned before for forskolin (Fig. 5). The results showed that calcium ionophore dramatically reduced system ASC and augmented system A, while the Na^+-independent pathway remained unaffected.

These observations provided a clue to reconcile the apparent contradiction that the ionophore reduced total L-alanine transport but increased L-alanine-induced electrogenicity. Thus, as system ASC represents the most important contribution to total duodenal L-alanine transport (serving >70%), its inhibition would account for the dramatic reduction observed on the overall net L-alanine transfer. On the other hand, as system A catalyses an electrogenic Na^+–L-alanine cotransport, stimulation of system A activity by calcium would lead to enhanced sodium transfer to the serosal side (most likely involving Na^+/K^+-ATPase activity), thereby generating the concomitant increase in transepithelial PD and I_{sc}. In agreement with our observations, human T cells demonstrate rapid enhancement of system A uptake when treated with ionomycin, an effect that was blocked by extracellular EGTA (Woodlock et al., 1989). Similar stimulatory actions of calcium on system A activity have also been observed in endothelial cells (Escobales et al., 1994) and glioblastoma cells (Zafra et al., 1994).

In summary, our data provide evidence that L-alanine transport in the lizard intestine is differently modulated by intracellular signal transducers, namely cAMP and calcium. The extent to which each transport pathway is affected by intracellular messengers varies depending on the individual system considered. Thus, while system A appears to be stimulated by elevated intracellular calcium, system ASC activity is clearly reduced. However, system ASC, which represents the largest transport pathway in unstimulated tissues, is clearly stimulated by increased cAMP levels. Although the precise mechanisms of regulation remain elusive, the magnitude of the effects observed here suggests that amino acid transport across the duodenum may be precisely controlled by circulating hormones and paracrine factors reaching the submucosa and the epithelia. Current research is being undertaken to assess the significance of these findings in the context of the physiological responses to endogenous agonists triggering acute changes in the levels of these intracellular messengers.

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