Mechanisms for stimulation of rat anterior pituitary cells by arginine and other amino acids

Carlos Villalobos, Lucía Núñez and Javier García-Sancho*

Instituto de Biología y Genética Molecular (IBGM), Universidad de Valladolid y CSIC, Departamento de Bioquímica y Biología Molecular y Fisiología, Facultad de Medicina, 47005-Valladolid, Spain

1. Arginine and other amino acids are secretagogues for growth hormone and prolactin in the intact animal, but the mechanism of action is unclear. We have studied the effects of amino acids on cytosolic free calcium concentration ($[Ca^{2+}]_i$) in single rat anterior pituitary (AP) cells. Arginine elicited a large increase of $[Ca^{2+}]_i$ in about 40% of all the AP cells, suggesting that amino acids may modulate hormone secretion by acting directly on the pituitary.

2. Cell typing by immunofluorescence of the hormone the cells store showed that the arginine-sensitive cells are distributed uniformly within all the five AP cell types. The arginine-sensitive cells overlapped closely with the subpopulation of cells sensitive to thyrotrophin-releasing hormone.

3. Other cationic as well as several neutral (dipolar) amino acids had the same effect as arginine. The increase of $[Ca^{2+}]_i$ was dependent on extracellular Ca^{2+} and blocked by dihydropyridine, suggesting that it is due to Ca^{2+} influx through L-type voltage-gated Ca^{2+} channels. The $[Ca^{2+}]_i$ increase was also blocked by removal of extracellular Na^+ but not by tetrodotoxin. The substrate specificity for stimulation of AP cells resembled closely that of the amino acid transport system $B^{0,+}$. We propose that electrogenic amino acid influx through this pathway depolarizes the plasma membrane with the subsequent activation of voltage-gated Ca^{2+} channels and Ca^{2+} entry.

4. Amino acids also stimulated prolactin secretion *in vitro* with a similar substrate specificity to that found for the $[Ca^{2+}]_i$ increase. Existing data on the stimulation of secretion of other hormones by amino acids suggest that a similar mechanism could apply to other endocrine glands.

The anterior pituitary (AP) contains at least five different cell types which are able to secrete growth hormone (GH), prolactin, gonadotrophins (follicle-stimulating hormone (FSH) and luteinizing hormone (LH)), adrenocorticotrophin (ACTH) and thyrotrophin (thyroid-stimulating hormone (TSH), respectively. Hormone secretion is regulated mainly by the hypothalamus via releasing and inhibiting factors, which reach the pituitary through the hypophyseal portal system (Schally, Coy & Meyers, 1978; O'Leary & O'Connor, 1995). Releasing factors elicit in their cell targets an increase in the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$), which acts as a second messenger in pituitary stimulus–secretion coupling (Gershengorn, 1986; Kato, Hoyland, Sikdar & Mason, 1992; Prevarskaya *et al.* 1994; Kasahara *et al.* 1994).

Regulation of pituitary hormone secretion by plasma amino acids has been known for a long time and its possible role in postprandial stimulation of GH secretion has been considered. Arginine, the most studied amino acid, elicits a rise in the plasma level of growth hormone and prolactin. Arginine infusion as a measure of GH secretory reserve has become an important part of the diagnostic armamentarium of the clinical endocrinologist, particularly since, unlike hypoglycaemia, it is without toxic effects. Other amino acids reported to induce GH discharge include histidine, lysine, valine, phenylalanine, leucine, methionine and threonine (Reichlin, 1974). Arginine and other amino acids have been reported to induce, in addition, secretion of insulin, glucagon and somatostatin from pancreatic β-, α- and δ-cells, respectively (Malaisse, 1972). The stimulation of GH secretion is not secondary to the insulin-induced hypoglycaemia. Blood glucose levels fall only slightly during arginine infusion and the GH response to arginine is not blocked if plasma glucose levels are deliberately maintained above baseline (Reichlin, 1974). The site of action of arginine has not been fully established. Because glucose, which acts on the brain, blocks the arginine response, it has

* To whom correspondence should be sent at Departamento de Fisiología, Facultad de Medicina, 47005-Valladolid, Spain.

been assumed without adequate proof that this amino acid affects secretion of GH-releasing factor (Reichlin, 1974). The stimulatory effect of arginine on GH secretion has also been proposed to depend on a decrease in hypothalamic somatostatin tone (Alba-Roth, Muller, Schopol & Von Werder, 1988).

Here we address the question of whether arginine may act directly at the pituitary by studying its effects on $[Ca^{2+}]_i$ in single rat AP cells loaded with the Ca^{2+}-sensitive probe fura-2. We find that arginine and other amino acids are able to elicit an increase in $[Ca^{2+}]_i$ in some AP cells. The following points of this action have been investigated: (i) the substrate specificity, (ii) the mechanisms and (iii) the cell types that are sensitive to L-arginine. For (iii), immunocytochemical identification of the cells responding to L-arginine was performed in the same microscope fields as those used for the $[Ca^{2+}]_i$ measurements. Finally, the effects of amino acids on prolactin secretion have been studied.

METHODS

AP cells were obtained from 8- to 10-week-old male Wistar rats essentially as described by Dobson & Brown (1985). Rats were killed by decapitation and their anterior pituitary glands were quickly removed, transferred to culture medium (RPMI 1640 supplemented with 10% fetal calf serum, 100 i.u. ml^{-1} penicillin and 100 $\mu g\ ml^{-1}$ streptomycin) and handled under sterile conditions. The glands were washed three times with low Ca^{2+}–Mg^{2+} Krebs solution (solution W; composition (mM): NaCl, 118·5; KCl, 4·7; $CaCl_2$, 0·1; $MgSO_4$, 0·1, KH_2PO_4, 1·18; $NaHCO_3$, 25; 2% bovine serum albumin; 12 mg l^{-1} Phenol Red), warmed to 37 °C and equilibrated to pH 7·4. The glands were then chopped into little pieces (about 1 mm × 1 mm) with small dissecting scissors, washed again with solution W and incubated in solution W with 0·25% trypsin for 35–40 min at 37 °C under an atmosphere of 95% O_2–5% CO_2. After washing with solution W, the trypsin-treated pieces were incubated with 0·25% soy bean trypsin inhibitor for 5 min in solution W, washed again with Ca^{2+}–Mg^{2+}-free Krebs solution and dispersed by gentle drawing through a siliconized Pasteur pipette. The cells were sedimented by centrifugation at 200 *g* for 5 min and washed twice with Krebs solution (containing 1 mM of both $CaCl_2$ and $MgSO_4$). They were finally suspended in culture medium (RPMI 1640 supplemented with 10% fetal calf serum and antibiotics, as above) and allowed to attach to 11 mm diameter glass coverslips treated with poly-L-lysine (0·01 mg ml^{-1}, 5 min) at 2×10^4 to 4×10^4 cells per coverslip. The cells were then cultured at 37 °C under a 95% air–5% CO_2 atmosphere for 2–3 days, with daily exchanges of the culture medium.

Measurements of $[Ca^{2+}]_i$ were performed in cells loaded with fura-2 (Grynkiewicz, Poenie & Tsien, 1985) by incubation for about 60 min at room temperature (20–22 °C) with 5 μM fura-2 AM (the acetoxymethyl ester form of fura-2) in standard medium (composition (mM): NaCl, 145; KCl, 5; $MgCl_2$, 1; $CaCl_2$, 1; glucose, 10; Hepes-sodium, 10; pH 7·4). The coverslips were then mounted under the microscope (Nikon Diaphot) in a temperature-controlled chamber (36 °C) and epi-illuminated alternately at 340 and 380 nm. Light emitted above 520 nm was recorded by an extended ISIS-M camera (Photonic Science, Robertbridge, East Sussex, UK) and analysed using a Magical Image Processor (Applied Imaging, Newcastle, UK) with a 32 Mbyte video RAM. Four video frames of each wavelength were averaged by hardware with an overall time resolution of about 3 s for each pair of images at alternate wavelengths. Pixel-by-pixel ratios of consecutive frames obtained at 340 and 380 nm excitation were produced and $[Ca^{2+}]_i$ was estimated from these ratios by comparison with fura-2 standards. The incubation chamber was under continuous perfusion at 2–3 ml min^{-1} with either control or test solutions. This flow rate allowed > 95% exchange of the medium bathing the cells within 5–10 s. Further details of these procedures have been provided previously (Villalobos, Fonteriz, López, García & García-Sancho, 1992; López, García, Artalejo, Neher & García-Sancho, 1995; Villalobos & García-Sancho, 1995*a*).

For Mn^{2+} entry assays, coverslips were introduced at a fixed angle (45 deg) into a quartz cuvette placed into the sample compartment of a fluorescence spectrophotometer that allowed rapid (30–300 Hz) alternation of up to six different excitation wavelengths (Cairn Research Ltd, Newnham, Sittingbourne, Kent, UK). Temperature was 30 °C. Fluorescence emitted above 510 nm was measured and integrated at every 1 s period. Mn^{2+} entry was estimated from the quenching of the fura-2 fluorescence excited at 360 nm, a wavelength that is not sensitive to changes in Ca^{2+} concentration (Hallam & Rink, 1985). Simultaneous measurement of $[Ca^{2+}]_i$ can be performed from the ratio of the fluorescences excited at 340 and at 380 nm. This procedure has been described in detail elsewhere (Alonso, Sánchez & García-Sancho, 1989).

For identification of single cells according to the hormone they store, the coverslips were fixed with 4% paraformaldehyde at the end of the $[Ca^{2+}]_i$ measurements. Then indirect immunofluorescence using antibodies raised against one of the pituitary hormones was performed. The field of interest was located by positioning a cross engraved in the coverslip as in the $[Ca^{2+}]_i$ experiment and the fluorescence image was captured with the image processor. The image was digitalized, stored and later moved and rotated in the computer as required to match exactly the images obtained in the $[Ca^{2+}]_i$ experiment. This procedure has been described in detail elsewhere (Nuñez, De La Fuente, García & García-Sancho, 1995).

Reverse haemolytic plaque assays were performed to measure prolactin secretion according to the protocol described by Boockfor, Hoeffler & Frawley (1986). In brief, monodispersed AP cells were plated on plastic Petri dishes in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 100 i.u. ml^{-1} penicillin and 100 $\mu g\ ml^{-1}$ streptomycin. After 2 days in culture, cells were recovered by mild trypsinization and washed several times with standard medium. Then cells were mixed with protein A-coated goat erythrocytes and infused into Cunningham incubation chambers. After attachment of cells (about 1 h), chambers were flooded with 150 μl (5 × volume of chamber) of standard medium containing prolactin antiserum (1:80) alone or with amino acids or thyrotrophin-releasing hormone (TRH). After 1 h incubation at 37 °C, plaques were developed by adding standard medium containing guinea-pig complement (1:80) and incubating for 50 min. Then the cells were fixed with 2% glutaraldehyde and stained with Toluidine Blue. The percentage of plaque-forming cells was determined by examination under the light microscope. At least 250 cells were counted for each of the triplicate chambers for every treatment group.

Fura-2 AM was obtained from Molecular Probes. Antibodies against rat prolactin (rabbit, AFP425-10-91), β-TSH (rabbit, AFP1274789), GH (monkey, AFP4115), β-FSH (guinea-pig, AFP85GP9691BF-

Table 1. Effects of several amino acids and derivatives on $[Ca^{2+}]_i$ in single rat pituitary cells

Amino acid	Relative increase in $[Ca^{2+}]_i$ 50 μM	1000 μM
L-Arginine[a]	56 ± 6 (64)	100
D-Arginine	6 ± 4 (27)	71 ± 6 (16)
L-Lysine[b]	38 ± 5 (96)	n.d.
D-Lysine	7 ± 4 (27)	52 ± 7 (77)
L-Ornithine	2 ± 3 (20)	65 ± 15 (38)
Glycine[c]	46 ± 10 (32)	n.d.
L-Alanine	63 ± 7 (39)	84 ± 9 (31)
D-Alanine	32 ± 7 (10)	67 ± 13 (14)
N-Methyl-L-alanine	n.d.	4 ± 6 (14)
L-Proline	n.d.	35 ± 7 (38)
L-Serine	65 ± 9 (29)	n.d.
L-Homoserine	66 ± 14 (19)	80 ± 13 (16)
L-Threonine	23 ± 10 (19)	87 ± 14 (16)
L-Valine	56 ± 8 (30)	63 ± 12 (14)
D-Valine	n.d.	14 ± 8 (77)
L-Citrulline[d]	42 ± 10 (10)	n.d.
L-Leucine	25 ± 8 (54)	47 ± 10 (54)
L-Isoleucine	44 ± 6 (40)	n.d.
L-Tryptophan	29 ± 6 (20)	72 ± 15 (16)
D-Tryptophan	32 ± 13 (19)	90 ± 16 (16)
N-Methyl-L-tryptophan	n.d.	1 ± 7 (16)
Cycloleucine	5 ± 5 (40)	50 ± 14 (12)
AIB	n.d.	16 ± 10 (12)
N-Methyl-AIB	n.d.	5 ± 4 (38)
b(±)-BCH	0 ± 2 (10)	1 ± 7 (12)

Results were standardized by expressing them as a percentage of the increase in $[Ca^{2+}]_i$ produced by 1000 μM L-arginine. Values are means ± S.E.M. of all the cells that were sensitive to arginine. The number of cells is given in parentheses. n.d., not determined. [a] *N*-γ-Methyl-L-arginine produced 70 ± 7% stimulation ($n = 47$) at 1000 μM. *N*-γ-Nitro-L-arginine methyl ester (NAME) had no effect at 100 μM. The dipeptide L-arginine–L-lysine had no effect at 1000 μM. [b] L-Lysine methyl ester had no effect at 50 μM; L-lysinamide had no effect at 1000 μM. [c] Glycinamide had no effect at 1000 μM. [d] At neutral pH citrulline bears no net charge and can therefore be classified as a neutral amino acid.

SHB) and anti-human ACTH (rabbit, AFP39013082) were generous gifts from the National Hormone and Pituitary Program, the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), the National Institute of Child Health and Human Development, and the US Department of Agriculture, Rockville, MD, USA. Furnidipine was a gift from Laboratorios Alter S.A., Madrid, Spain. Fluorescein-labelled anti-rabbit, anti-guinea-pig or anti-monkey IgGs, the hypothalamic releasing hormones (growth hormone-releasing hormone (GHRH), TRH, gonadotrophin-releasing hormone (GnRH) and corticotrophin-releasing hormone (CRH)) and the amino acids and their derivatives were obtained from Sigma. RPMI 1640 was from Sigma (Ref. R 7880). DMEM (Ref. 12320) and the fetal calf serum were from Gibco. Other chemicals were either from Sigma or from Merck.

RESULTS

Arginine and other amino acids increase $[Ca^{2+}]_i$ in AP cells

Perfusion with 50 μM L-arginine for 30 s produced an increase in $[Ca^{2+}]_i$ in some AP cells. Figure 1*A* illustrates this response in a representative single cell. Some AP cells (10–40%, depending on the cell batch) showed spontaneous $[Ca^{2+}]_i$ oscillations. L-Arginine also increased $[Ca^{2+}]_i$ in some of these cells (Fig. 1*B*). In 2358 individual cells studied in thirty-two independent experiments we found that 37 ± 2% (mean ± S.E.M.) responded to L-arginine (100 μM). In these arginine-sensitive cells, $[Ca^{2+}]_i$ was increased from 102 ± 6 to 391 ± 13 nM (means ± S.E.M.). The increase in $[Ca^{2+}]_i$ induced by L-arginine was concentration dependent. The effects of different concentrations of the amino acid (5–100 μM) on a single cell are compared in Fig. 1*B*. The averaged response of twenty-three arginine-sensitive single cells from the same experiment is shown in Fig. 1*C*. On averaging, the contribution of spontaneous activity is filtered, as these spontaneous $[Ca^{2+}]_i$ peaks are generally out of phase in different cells. Figure 1*D* shows the dose–response curve, obtained by averaging the results obtained in three similar experiments. The half-maximal effect was reached at 36 μM L-arginine.

Both the increase in $[Ca^{2+}]_i$ on stimulation with L-arginine and the return to the pre-stimulatory levels (and often below) on removal of the amino acid from the perfusion medium were quick. A second pulse with the amino acid produced a similar $[Ca^{2+}]_i$ increase. When L-arginine was perfused for longer periods the increase in $[Ca^{2+}]_i$ was biphasic, composed of a transient peak followed by a sustained plateau, which persisted as long as the amino acid was present (Fig. 1*A*).

Many other amino acids, both cationic and neutral dipolar, were also able to increase $[Ca^{2+}]_i$ in AP cells. Figure 1*E* compares the effects of L-arginine and L-alanine, tested at concentrations giving maximal effects (1 mM). Results obtained with a long series of amino acids and amino acid derivatives are summarized in Table 1. Two different concentrations were tested, 50 and 1000 μM, in order to assess the relative affinities of the different compounds. Among cationic amino acids L-lysine was about as potent as L-arginine, and L-ornithine was less efficient (compare the effects at 50 μM). All the natural neutral L-amino acids tested, either with polar or non-polar side-chains, were able to increase $[Ca^{2+}]_i$ in AP cells with about the same efficiency as L-arginine (note significant effects at 50 μM, Table 1). Stereospecificity was not absolute. Whereas the D-isomers of arginine, lysine and valine were significantly less potent than the L-isomers, D- and L-isomers of alanine and tryptophan were about equipotent (Table 1).

The different amino acids acted on the same subpopulation of AP cells. Figure 2 shows that there was a good correlation between the $[Ca^{2+}]_i$ increases induced by L-arginine and

those induced by either L-alanine (*A*) or L-valine (*B*) in each individual single cell. The correlation coefficient (*r*) was 0·71 in *A* and 0·80 in *B*. When the effects of two successive pulses of L-arginine were compared in the same cells of Fig. 2*A*, *r* was 0·87. For other pairs of amino acids the values of *r* were similar (results not shown). The effects of different amino acids, when tested at concentrations giving maximal increase in $[Ca^{2+}]_i$, were not additive. Thus, the mixture of 1 mM L-alanine and 1 mM L-arginine had the same effect as 1 mM L-arginine alone (results not shown). The experiment shown in Fig. 1*E* reinforces the same conclusion using an even more sensitive experimental design. When 1 mM L-alanine was added to cells of which $[Ca^{2+}]_i$ had been increased by maximal stimulation by L-arginine (1 mM) no further increase in $[Ca^{2+}]_i$ was observed, and the same was true when 1 mM L-arginine was added on top of 1 mM L-alanine. Similar results were found with mixtures of 1 mM L-arginine and 1 mM L-tryptophan (results not shown).

Among non-metabolizable amino acids, cycloleucine was able to increase $[Ca^{2+}]_i$ at a concentration of 1 mM, α-amino-isobutyric acid (AIB) had little effect, and the model substrates for amino acid transport systems A and L, *N*-methyl-AIB (MeAIB; Christensen, Oxender, Liang & Vatz, 1965) and 2-aminobicyclo(2.2.1)heptane-2-carboxylic acid (BCH; Tager & Christensen, 1972), respectively, had no effect at 1 mM (Table 1). The presence of a free α-carboxyl group was required, as shown by the lack of effect of glycinamide, lysinamide and lysine methyl ester (see Table 1

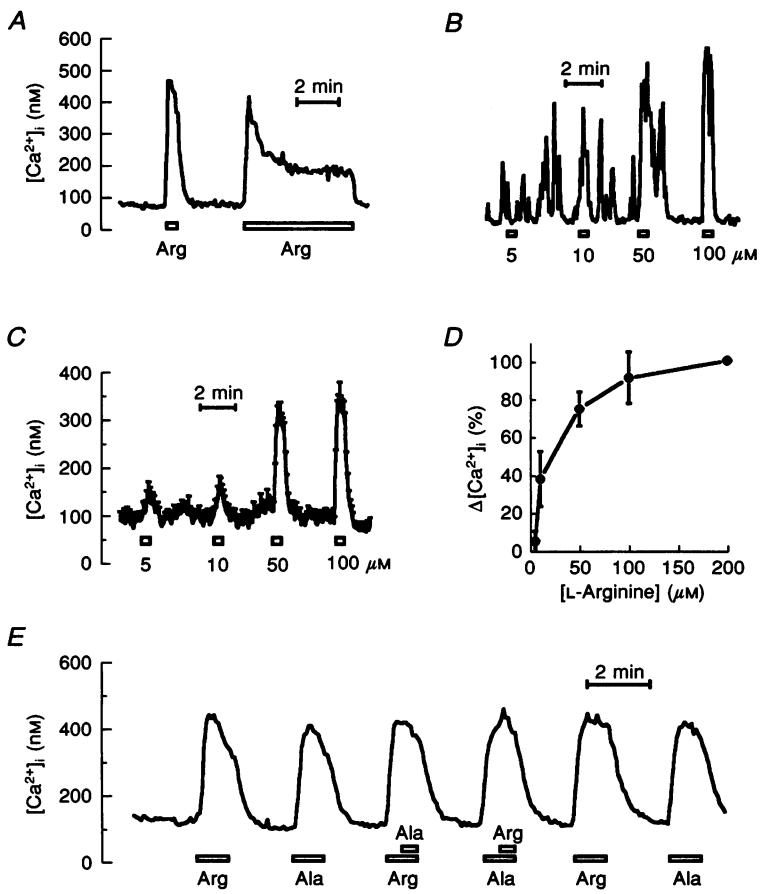

Figure 1. Effect of arginine on $[Ca^{2+}]_i$ in AP cells

A, comparison of the effects of a short (30 s) and a long (5 min) pulse with L-arginine (Arg, 50 μM) on $[Ca^{2+}]_i$ in a single pituitary cell. *B*, comparison of the effects of several concentrations of L-arginine (5–100 μM, as shown) in a single cell showing spontaneous $[Ca^{2+}]_i$ oscillations. *C*, average of the traces obtained in 23 arginine-sensitive single cells from the same experiment as in *B*; vertical bars represent S.E.M. *D*, dose–response curve for the increase in $[Ca^{2+}]_i$ induced by L-arginine; each point is the mean of the values obtained in 64 arginine-sensitive single cells from 3 different experiments. $\Delta[Ca^{2+}]_i$ values were computed for each cell as the difference between the mean $[Ca^{2+}]_i$ values obtained by integration of the 30 s periods before and after stimulation and standarized by expressing them as the percentage of the $\Delta[Ca^{2+}]_i$ value obtained with the largest concentration (200 μM); vertical bars represent S.E.M. *E*, comparison of the effects of L-arginine and L-alanine, both tested at 1 mM; the trace shown is the average of 26 arginine-sensitive single cells; the stimulation pulses always lasted for 1 min; In the third and fourth pulses perfusion was started with 1 mM of one of the amino acids and, after 30 s, switched to medium containing 1 mM of both amino acids for an additional 30 s period.

legend). An intact α-amino group was also required since *N*-methylation was not tolerated, as shown by the lack of effect of the *N*-methyl derivatives of alanine and tryptophan (Table 1). The dipeptide lysine–arginine had no effect. With regard to arginine derivatives known to inhibit NO synthase, *N*-γ-methyl-L-arginine was able to produce an increase in $[Ca^{2+}]_i$ whereas *N*-γ-nitro-L-arginine methyl ester (NAME) had no effect (Table 1). NAME (100 μM) did not modify the $[Ca^{2+}]_i$-increasing effect of 100 μM L-arginine (results not shown).

The increase in $[Ca^{2+}]_i$ is due to Ca^{2+} entry through L-type voltage-gated channels and depends on extracellular Na^+

Essentially all the cells present in the microscope field (91–98% in seven different experiments) possessed voltage-gated Ca^{2+} channels, as they showed a large increase in $[Ca^{2+}]_i$ when they were depolarized with high-K^+ (50 mM) solution. The increase in $[Ca^{2+}]_i$ induced by high K^+ was prevented by removal of external Ca^{2+} or by dihydropyridines (85–95% inhibition, with dihydropyridine blockers nimodipine (0·02 μM), nisoldipine (0·1 μM) or furnidipine (1 μM); results not shown).

Removal of extracellular Ca^{2+} prevented the effect of arginine (Fig. 3*A*), suggesting that the increase in $[Ca^{2+}]_i$ is due to Ca^{2+} entry. The addition of 1 mM Ni^{2+}, a generic blocker of Ca^{2+} entry, also abolished the effect of arginine (Fig. 3*B*). Finally, addition of 1 μM furnidipine, a dihydropyridine blocker of L-type voltage-gated channels (López *et al.* 1994), prevented most of the increase in $[Ca^{2+}]_i$ induced by L-arginine (84 ± 6% inhibition; mean ± S.E.M.; $n = 71$ cells), and this inhibitory effect was reversed on washout of furnidipine (Fig. 3*C*). Similar results were obtained using L-alanine or L-tryptophan as the stimulators of the $[Ca^{2+}]_i$ increase and using nisoldipine (0·1 μM) as the dihydropyridine blocker of Ca^{2+} channels.

L-type Ca^{2+} channels are also permeable to manganese (Villalobos *et al.* 1992). Figure 3*D* shows the results of an experiment in which Mn^{2+} was used as a surrogate of Ca^{2+} for entry. The entry of Mn^{2+} was measured by the quenching of the fura-2 fluorescence excited at 360 nm, a wavelength that is not sensitive to Ca^{2+} (Alonso *et al.* 1989). Arginine increased the rate of quenching indicating enhanced entry of Mn^{2+}. Again, the effect of arginine on Mn^{2+} entry was blocked by furnidipine.

Removal of the extracellular Na^+ (replaced by *N*-methyl-D-glucamine) fully prevented the effect of L-arginine on $[Ca^{2+}]_i$, which reappeared on readdition of Na^+ (Fig. 3*E*). The same results were obtained using L-alanine or L-tryptophan to increase $[Ca^{2+}]_i$ (results not shown). Tetrodotoxin (TTX), an inhibitor of voltage-gated Na^+ channels, had no effect on the increase in $[Ca^{2+}]_i$ induced by L-arginine (Fig. 3*F*).

Ca^{2+} release from the intracellular Ca^{2+} stores did not seem to contribute to the increase in $[Ca^{2+}]_i$, as previous emptying of the stores by treatment with the Ca^{2+} ionophore ionomycin (100 nM) or the endomembrane Ca^{2+}-

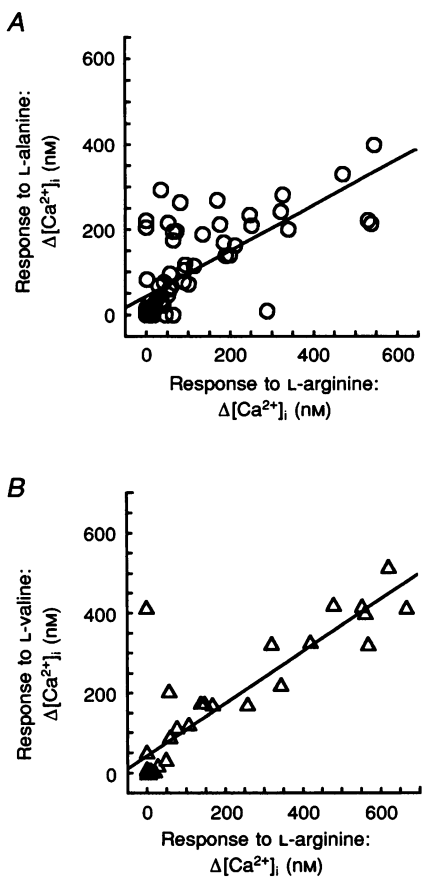

Figure 2. Cationic and neutral amino acids produce similar effects on the same subpopulation of AP cells

Correlation between the net increases in $[Ca^{2+}]_i$ ($\Delta[Ca^{2+}]_i$) elicited by L-arginine and either L-alanine (*A*) or L-valine (*B*). Stimulation was performed by giving a 1 min pulse with standard medium containing 100 μM of one of the amino acids and, 5 min later, a similar pulse with the other amino acid in the pair. Results are expressed as the net nanomolar increase in $[Ca^{2+}]_i$, computed as the difference between the mean $[Ca^{2+}]_i$ just before and just after application of the amino acid. The mean $[Ca^{2+}]_i$ values were computed as the average of all the $[Ca^{2+}]_i$ values within the integration period (1 min). Each point corresponds to a single cell. Lines were fitted by the least-squares procedure. Correlation coefficients (r) were 0·71 (*A*; $n = 71$) and 0·80 (*B*; $n = 34$). The results are representative of 3–6 similar experiments.

ATPase inhibitor thapsigargin (500 nM; Thastrup, 1990) did not modify the effect of L-arginine (results not shown; for effects of ionomycin and thapsigargin on the Ca^{2+} contents of the intracellular Ca^{2+} stores see Villalobos & García-Sancho, 1995*b*).

Identification of the cell types sensitive to L-arginine

The anterior pituitary contains five main cell types, each one secreting a different pituitary hormone. Since only 37% of the cells responded to arginine with an increase in $[Ca^{2+}]_i$ (see the first section in Results), experiments were designed to test whether arginine-sensitive cells are restricted to one or several cell types. For these purposes, immunocytochemical identification of the individual cells was performed in the same microscope field as that used for $[Ca^{2+}]_i$ measurements (see Methods for details). We have studied 1845 AP cells in twenty-two independent experiments in which either lactotrophs, somatotrophs, corticotrophs, gonadotrophs or thyrotrophs were identified using antibodies raised against their respective AP hormones. Results are summarized in Table 2. The arginine-sensitive cells were not restricted to a given cell type, but distributed rather

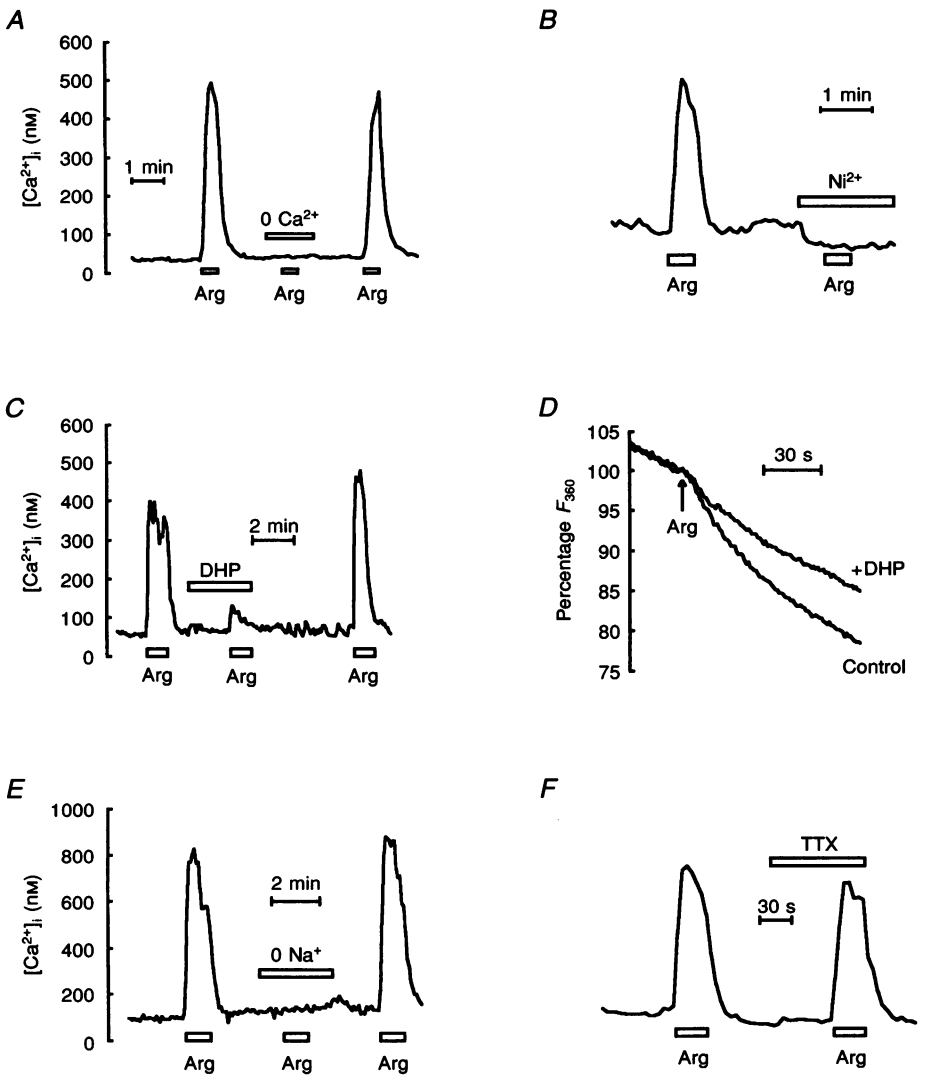

Figure 3. Inhibition of the effect of arginine on $[Ca^{2+}]_i$

A, effect of Ca^{2+} removal (0 Ca^{2+}) on the increase in $[Ca^{2+}]_i$ induced by L-arginine; the Ca^{2+}-free medium had the same composition as the standard incubation medium (see Methods), except that $CaCl_2$ was omitted and 0·1 mM EGTA was added. *B*, effect of $NiCl_2$ (1 mM). *C*, effect of the inhibitor dihydropyridine furnidipine (DHP, 1 μM). *D*, effect of furnidipine (DHP, 1 μM) on arginine-induced Mn^{2+} entry (expressed as a percentage of the fluorescence excited at 360 nm; see Methods for details); arginine was added at the time shown by the arrow; $MnCl_2$ (0·2 mM) was added 1 min before arginine; *E*, effect of Na^+ removal (0 Na^+); Na^+ was replaced by *N*-methyl-D-glucamine. *F*, effect of tetrodotoxin (TTX, 1 μM). All the traces, except for *B* and *D*, show the behaviour of a representative single cell. In *B* the average trace of 22 arginine-sensitive cells is shown. In *D* the measurement was done in a Cairn spectrofluorimeter and corresponds to the whole cell population. The concentration of L-arginine was allways 100 μM. In all cases similar results were obtained in at least two additional experiments.

homogeneously within all the five cell types, with a percentage of about 40% in each.

We have shown recently that an alternative classification of rat AP cells can be performed according to their response to hypothalamic releasing factors (Villalobos, Nuñez & García-Sancho, 1996; see also Kasahara *et al.* 1994). Using these criteria, a subpopulation of cells responding to several, and frequently to all, of the releasing factors can be identified. This subpopulation amounted to about one-third of the total and included cells within all the conventional cell types, defined by the hormone they store. All the cells in this subpopulation responded to TRH. Thus, the TRH-sensitive pool of AP cells includes not only thyrotrophs and lactotrophs, which are known to respond to TRH, but also this peculiar subpopulation (which in turn includes a large fraction of the lactotrophs) able to respond to several releasing factors (Villalobos *et al.* 1996). In the experiment shown in Fig. 4*A–C* the AP cells were stimulated first with L-arginine and later with TRH. Then, the traces coming from the TRH-sensitive (*A*) and the TRH-insensitive cells (*B*) were averaged. It seems clear that the arginine-sensitive cells are only within the TRH-sensitive group (compare the effects of arginine in Fig. 4*A* and *B*). In Fig. 4*C* this point is further supported by the good correlation existing between the $[Ca^{2+}]_i$ responses to L-arginine and to TRH in every single cell. Note particularly that there are no points located at the upper left corner in Fig. 4*C*. This means that cells which are sensitive to arginine but not to TRH do not exist. The experiment of Fig. 4*D–F* shows that when AP cells were grouped by the sensitivity to GHRH the outcome was a very different one. There was a response to arginine, both within the GHRH-sensitive (Fig. 4*D*) and the GHRH-

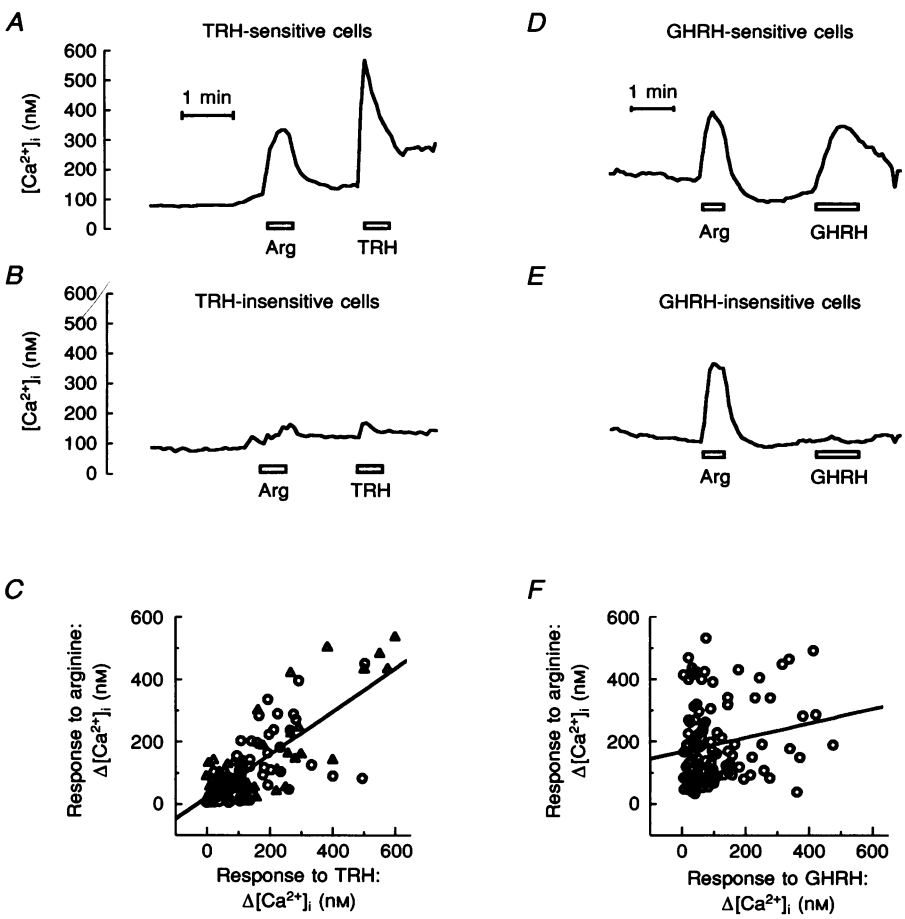

Figure 4. The response to arginine is restricted to cells sensitive to TRH

A–C, the cells were perfused first with L-arginine (100 μM) and then with TRH (100 nM), as indicated by the bars. After analysis of all the individual cell responses, the traces of either the TRH-sensitive (*A*, 50 cells) or the TRH-insensitive cells (*B*, 21 cells) were averaged. This experiment is representative of 5 similar ones. *C* shows the correlation between the responses to L-arginine and to TRH in two similar experiments, shown by different symbols. Each point corresponds to a single cell. Responses were quantified as the net increase in $[Ca^{2+}]_i$ (nM) computed as in Fig. 2. Lines were fitted by the least-squares procedure. The value for the correlation coefficient (r) was 0·87 ($n = 134$). *D–F*, the cells were stimulated first with 100 μM arginine and later with 10 nM GHRH. The average traces for 36 GHRH-sensitive and 75 GHRH-insensitive cells are shown in *D* and *E*, respectively. *F* shows the correlation between the responses to L-arginine and to GHRH; $r = 0{\cdot}19$ ($n = 111$). Other details as for *A–C*.

Table 2. Response to arginine in the five AP cell types defined by immunocytochemical identification of the hormone they store

Cell type (stored hormone)	Percentage of all the cells	Cells responding to arginine within this cell type (%)
Prolactin	58 ± 4	37 ± 5
GH	40 ± 7	49 ± 6
TSH	2 ± 1	40 ± 2
ACTH	18 ± 2	44 ± 4
FSH	8 ± 1	40 ± 2

In each experiment, after the $[Ca^{2+}]_i$ measurements, the cells were stained with the antibody against one of the AP hormones. Values are means ± S.E.M. of 3–6 independent experiments (264–492 cells) with each antibody. The concentration of L-arginine was 100 μM.

insensitive (Fig. 4*E*) groups. The correlation between both responses was very poor (Fig. 4*F*), with a large group of cells which were sensitive to arginine but not to GHRH, located at the upper left corner of Fig. 4*F*. This result is a good internal control to confirm that the excellent correlation between the responses to arginine and to TRH is not artefactual.

Amino acids induce prolactin secretion

The effects of amino acids on prolactin secretion were assessed by a reverse haemolytic plaque assay (Boockfor *et al.* 1986). The number of plaque-forming cells was measured after 1 h of incubation with or without amino acids. In control experiments, we determined that at least 2 h of incubation were required to reach the maximum percentage of plaque-forming cells (not shown). Therefore, after 1 h incubation, those cells that release more hormone per unit time will form plaque faster than those releasing fewer molecules (Boockfor *et al.* 1986) and the percentage of plaque-forming cells can be used as an index of the rate of prolactin secretion. Figure 5*A* shows the effects of several amino acids, tested at a 1 mM concentration, on prolactin secretion. L-Arginine, L-ornithine and L-alanine produced a significant stimulation of prolactin secretion that was

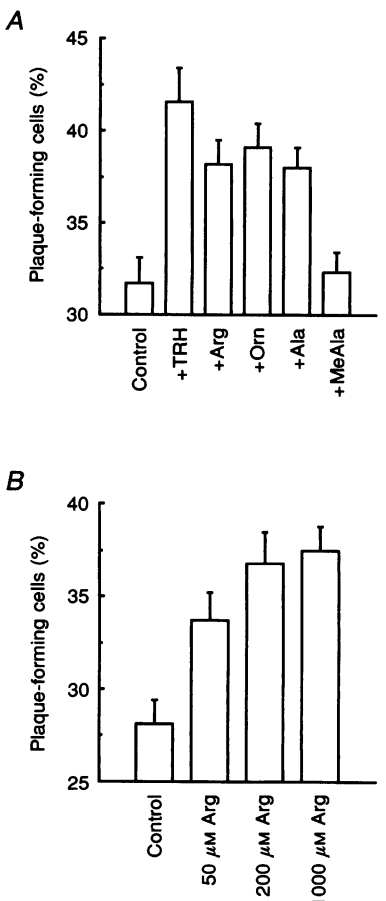

Figure 5. Effects of amino acids on prolactin secretion

A, cells were stimulated for 60 min with either 0·1 μM TRH or with a 1 mM concentration of the indicated L-amino acids. MeAla, *N*-methyl-L-alanine. The percentage of plaque-forming cells is shown. Each value is the mean of the values obtained in 3 different Cunningham chambers (see Methods). Vertical bars represent S.E.M. All the values except for that of *N*-methyl-L-alanine were significantly different from the control ($P < 0·05$, ANOVA). This experiment is representative of 3 similar ones. *B*, cells were stimulated with different concentrations of L-arginine (50–1000 μM). Other details as in *A*.

64–76% of the stimulation induced by TRH used in these experiments as a positive control. L-Lysine had a similar effect (not shown). *N*-Methyl-L-alanine, which did not have an effect on $[Ca^{2+}]_i$ (Table 1), did not stimulate prolactin secretion either (Fig. 5*A*). Figure 5*B* illustrates the dose–response curve for arginine stimulation of prolactin secretion. At 50 μM concentration the increase in plaque-forming cells was 60%, the maximal effect obtained with 1 mM L-arginine being taken as 100%. Thus the EC_{50} for secretion was comparable to the value estimated for the $[Ca^{2+}]_i$-increasing action (36 μM; see Fig. 1*D*).

DISCUSSION

We find that a wide series of both cationic and neutral dipolar amino acids, very similar to those reported to produce GH discharge *in vivo* (Reichlin, 1974), are able to increase $[Ca^{2+}]_i$ in AP cells. Since secretion of AP hormones is triggered by an increase in $[Ca^{2+}]_i$ (Gershengorn, 1986; Kato *et al.* 1992; Prevarskaya *et al.* 1994; Kasahara *et al.* 1994), it seems reasonable to conclude that amino acids may stimulate secretion acting directly at the AP cell level. This was confirmed here for prolactin secretion (Fig. 5). However, the subpopulation of AP cells sensitive to amino acids was not limited to any of the five AP cell types, classified by the hormone they store. Instead, it included about 40% of the cells within each cell type. Arginine-sensitive cells closely overlapped with the TRH-sensitive cells. These have been shown recently to constitute a cell subpopulation, amounting to about one-third of all the AP cells, which is able to respond, in addition, to other hypothalamic releasing factors and to glutamate (Villalobos *et al.* 1996). It is intriguing to find now that sensitivity to amino acids is also confined to this particular cell subpopulation, whose functional role remains unknown.

With regard to the mechanisms for stimulation, NO synthase has been proposed to be involved in the stimulation of insulin release induced by arginine (Schmidt, Warner, Ishii, Sheng & Murad, 1992), although this view is controversial (Drews & Krippeitdrews, 1995). In the present case, a role for NO synthase can be excluded since, apart from arginine, many others amino acids, including the NO synthase inhibitor, *N*-γ-methyl-L-arginine, were able to increase $[Ca^{2+}]_i$.

It is clear that the Ca^{2+} entry induced by amino acids in AP cells took place through dihydropyridine-sensitive Ca^{2+} channels (Fig. 3). The increase in $[Ca^{2+}]_i$ during phase 2 of TRH action (the delayed $[Ca^{2+}]_i$ plateau obtained on stimulation with TRH) is also due to Ca^{2+} entry through L-type Ca^{2+} channels, which results from increased electrical activity secondary to the membrane depolarization induced by TRH (Barros, Villalobos, García-Sancho, Camino & Peña, 1994). Membrane depolarization also seems the most likely mechanism to explain the increase in Ca^{2+} entry observed here. The depolarization induced by arginine and other amino acids in pancreatic β-cells (Blachier *et al.* 1989; Drews & Krippeitdrews, 1995; Aschroft, Coles, Gummerson, Sakura & Smith, 1995) and the increase in $[Ca^{2+}]_i$ induced by glutamate and other acidic amino acids in GH_3 pituitary cells (Villalobos & García-Sancho, 1995*a*) have both been attributed to electrogenic amino acid entry. We find that the $[Ca^{2+}]_i$-increasing effect of amino acids in AP cells is dependent on external Na^+, suggesting that it may require the operation of a Na^+-dependent transport system. Possible actions of Na^+ removal due to modifications of ion currents through voltage-gated Na^+ channels seem unlikely to interfere as tetrodotoxin did not prevent the $[Ca^{2+}]_i$-increasing action of the amino acids (Fig. 3*F*).

The broad substrate specificity found (Table 1) is puzzling. The close correlation for the activity of different amino acids at the single-cell level as well as the lack of additivity suggest that all the amino acids act through the same transport system. The two main systems for neutral amino acid transport, A and L (Oxender & Christensen, 1963), can be excluded because the model substrates, MeAIB and BCH, respectively, (Christensen *et al.* 1965; Tager & Christensen, 1972) had no effect on $[Ca^{2+}]_i$. The Na^+-dependent system, ASC (alanine, serine and cysteine), for neutral amino acids (Christensen, Liang & Archer, 1967; Christensen, 1975) does not fit either, as, in contrast to the present findings, it shows a high stereospecificity for D- and L-alanine, and cationic and large neutral amino acids are not transported through it.

Three transport systems able to accept both cationic and dipolar amino acids, y^+, $B^{0,+}$ and $b^{0,+}$, should be considered. System y^+ seems an unlikely candidate as arginine and lysine do not require Na^+ for transport through it (White, 1985), whereas the effect on $[Ca^{2+}]_i$ of AP cells is dependent on external Na^+ (Fig. 3*E*). Other details of the specificity profile (Table 1) do not fit to system y^+ either. For example, arginine and ornithine show a similar affinity for system y^+ (White, 1985) but arginine is much more efficient than ornithine in increasing $[Ca^{2+}]_i$ in AP cells (Table 1). Certain dipolar amino acids are able to react with system y^+ in the presence of Na^+, Na^+ taking the place of the terminal positively charged amino group (Christensen, Handlogten & Thomas, 1969; Christensen, 1975; Christensen & Greene, 1995). However, this mode of operation seems to produce only the exchange of intra- and extracellular amino acids (White, 1985), thus being unable to modify membrane potential and hence to produce the effects on $[Ca^{2+}]_i$ reported here. In any case, the dipolar amino acids have a much smaller affinity than arginine for system y^+ (White, 1985), and this seems not to be the case for the effects on $[Ca^{2+}]_i$ (Table 1).

The main difference between systems $B^{0,+}$ (Van Winkle, Christensen & Campione, 1985) and $b^{0,+}$ (Van Winkle, Campione & Gorman, 1988*a*) is that the former is Na^+ dependent. Since the effect on $[Ca^{2+}]_i$ reported here requires the presence of external Na^+ (Fig. 3*E*), system $B^{0,+}$ is the most likely candidate. The substrate specificity shown in Table 1 also favours this possibility. Although the substrate

specificities of systems $B^{0,+}$ and $b^{0,+}$ are very similar, the latter reacts poorly with amino acids branched at the β-carbon (Van Winkle *et al.* 1988*a*) and this does not fit with the good reactivity shown here for isoleucine and valine (Table 1). Other properties, which have been reported for system $B^{0,+}$, that fit to the substrate specificity shown in Table 1 are the poor stereospecificity for D- and L-alanine (Van Winkle *et al.* 1985), the relatively high affinity (Van Winkle *et al.* 1985) and the lack of tolerance to *N*-methylation (Van Winkle, Haghighat, Campione & Gorman, 1988*b*).

It seems pertinent to examine whether the results reported here may contribute to an explanation of the effects of amino acids on the secretion of AP hormones observed *in vivo* (see Introduction). Since the effects we found took place at rather low concentrations, it seems reasonable to ask whether the amino acid level in plasma would not be enough to produce near-maximal stimulation, even in resting conditions. For example, the normal plasma concentration of arginine, 50–150 μM, is well above the EC_{50} value found here for stimulation of AP cells, 36 μM. This would leave little room for further hormone release on increase of blood amino acid level, for example during postprandial amino acidaemia. In *in vivo* conditions hormone secretion at every moment, including 'resting' conditions, arises from the balance of multiple stimulating and inhibiting influences acting simultaneously. Amino acids might provide a tonic stimulation, leaving room for the inhibitory action of down-regulating factors. It is well known, for example, that when the AP is implanted far away from the hypothalamus prolactin secretion increases. This is attributed to the loss of the tonic downregulatory effect of dopamine, which reveals a previously hidden upregulatory tone (Lamberts & McLeod, 1990). Alternatively, other plasma components or down-regulating factors might counteract or make less efficient the stimulation by amino acids *in vivo*, thus allowing stimulatory action by increased amino acidaemia. Note that amino acids would not, in any case, replace the action of hypothalamic releasing factors, which continue to be the main regulators of AP hormone secretion. However, by changing the membrane potential of AP cells, amino acids may modify the threshold for the response to these main regulators thus contributing towards integration of neural and humoral factors in multifactorial control. Further research in the intact animal will be required to assess the physiological relevance of this regulatory mechanism.

The action of amino acids described here seems rather similar, both in substrate preferences and in mechanisms, to that reported previously in pancreatic β-cells (Malaisse, 1972; Blachier *et al.* 1989; Drews & Krippeitdrews, 1995; Ashcroft *et al.* 1995). Thus, electrogenic amino acid transport might act as a general mechanism for linking the nutritional and/or metabolic status with regulation of hormone release in secretory cells possessing voltage-gated Ca^{2+} channels.

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Acknowledgements

This paper has was supported by the Spanish Dirección General de Investigación Científica y Técnica (DGICYT), grant PB92-0268. We thank Drs H. N. Christensen, L. J. Van Winkle and M. Palacín for helpful suggestions on amino acid transport systems.

Author's email address

J. García-Sancho: jgsancho@cpd.uva.es

Received 13 January 1997; accepted 7 April 1997.