Glutamate increases cytosolic calcium in GH_3 pituitary cells acting via a high-affinity glutamate transporter

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ABSTRACT **Hormone secretion by GH_3 pituitary cells is regulated by oscillations of the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$), which are driven by electrical activity and modulated by hypothalamic releasing factors. We find that micromolar concentrations of L-glutamate and other acidic amino acids, but not selective excitatory amino acid receptor agonists, increase $[Ca^{2+}]_i$ in GH_3 cells. Activation by glutamate is blocked by dihydropyridines or removal of extracellular Ca^{2+} or Na^+, but not by tetrodotoxin or excitatory amino acid receptor antagonists. Glutamate also accelerated the entry of Mn^{2+} used as a Ca^{2+} surrogate for Ca^{2+} channels. L-Glutamate and other acidic amino acids were taken up into GH_3 cells by an Na^+-dependent high-affinity transporter. The half-maximal effect of glutamate on $[Ca^{2+}]_i$ was reached at concentrations similar to the K_m for the glutamate transporter. Moreover, only those amino acids taken up through this transporter were able to increase $[Ca^{2+}]_i$. We propose that electrogenic entry of Na^+-glutamate depolarizes the plasma membrane, thus causing an increase of action potentials firing and Ca^{2+} entry through voltage-gated channels. Our results suggest that glutamate may cooperate to the modulation of pituitary hormone secretion by an unconventional mechanism involving a high-affinity glutamate transporter rather than excitatory amino acid receptors.—Villalobos, C., García-Sancho, J. Glutamate increases cytosolic calcium in GH_3 pituitary cells acting via a high-affinity glutamate transporter. *FASEB J.* 9, 815-819 (1995)**

Key Words: glutamate receptors • Ca^{2+} channels • GH_3 pituitary cells • prolactin secretion

GH_3 pituitary cells are a useful model to study the stimulus-secretion coupling of pituitary hormones. They secrete prolactin and growth hormone in response to several physiological secretagogues, such as thyrotropin-releasing hormone (TRH)[2] or vasoactive intestinal peptide (VIP) (1). The effects on secretion are often mediated by changes in the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$), which can be due to Ca^{2+} release from the intracellular Ca^{2+} stores and/or Ca^{2+} entry from the external medium (2). Ca^{2+} entry is often observed as $[Ca^{2+}]_i$ oscillations, which are driven by electrical activity and modulated by hypothalamic releasing factors (3, 4).

Parenteral administration of glutamate is able to stimulate pituitary hormone secretion. This effect was hypothesized to depend on action at a central site in the brain (5) but, alternatively, it could also result from a direct action on the pituitary. In fact, it has been reported that addition of glutamate to rat pituitary cells in primary culture is able to induce secretion of prolactin (6). Here we have studied the effects of glutamate and other acidic amino acids on $[Ca^{2+}]_i$ in fura-2 loaded GH_3 pituitary cells. We find that glutamate stimulates Ca^{2+} entry through L-type Ca^{2+} channels and that this effect is secondary to membrane depolarization induced by electrogenic uptake through a NA^+-dependent high-affinity glutamate transporter present in GH3 cells.

EXPERIMENTAL PROCEDURES

GH_3 pituitary cells were grown in RPMI 1640 medium supplemented with 15% horse serum and 2.5% fetal calf serum at 37°C and under air-5% CO_2 atmosphere. For fluorescence measurements (7), GH_3 cells were allowed to attach to poly-L-lysine-coated (0.01 mg/ml, 5 min) glass coverslips and grown for 2–3 days. The cell-coated coverslips were washed with standard medium containing (in mM) NaCl,145; KCl,5; $MgCl_2$,1; $CaCl_2$,1; glucose, 10; sodium-Hepes, 10; pH, 7.4, and loaded with fura-2 by incubation with 5 μM fura-2/AM at room temperature for about 1 h. $[Ca^{2+}]_i$ measurements were performed either on cell populations or at the single-cell level, as described below.

For measurements of $[Ca^{2+}]_i$ and Mn^{2+} entry on cell populations, glass coverslips were introduced at a fixed angle (45°) 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. Newnhan, Sittingbourne, Kent, U.K.). Temperature was 30°C. Emitted fluorescence was measured at 520 nm and integrated at every 1 s period. $[Ca^{2+}]_i$ was estimated from the ratio of the fluorescence values excited at 340 and at

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[2]Abbreviations: TRH, thyrotropin-releasing hormone; VIP, vasoactive intestinal peptide; $[Ca^{2+}]_i$, cytosolic free calcium concentration; EC_{50}, agonist concentration that produces 50% of maximal effect; IC_{50}, inhibitor concentration that produces 50% inhibition; NMDA, N-methyl-D-aspartate; EARs, excitatory amino acid receptors; AP-7, 2-amino-7-phosphonoheptanoic acid; TPDC, L-trans-pyrrolidine-2,4-dicarboxylic acid.

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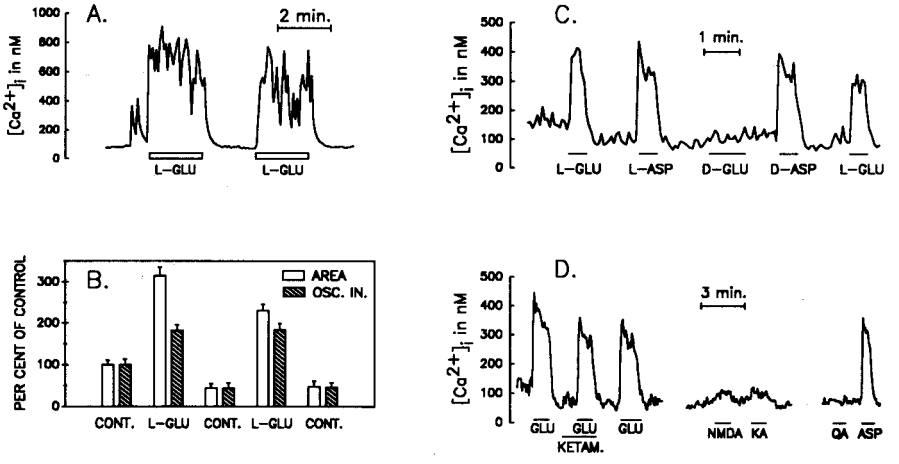

Figure 1. Increase of $[Ca^{2+}]_i$ induced by L-glutamate and other acidic amino acids in GH_3 cells. The effects of two successive pulses with 10 μM L-glutamate on $[Ca^{2+}]i$ in a representative single cell (A) and average effects on 42 cells (B), expressed in terms of mean $[Ca^{2+}]i$ increase (area) and oscillation index (see Experimental Procedures), are shown. Integration periods of 90 s were chosen in B in order to avoid transients from one condition to the next. Values are expressed as percent of the control before the first L-glutamate pulse. This experiment is representative of 20 similar ones. C, D) Comparison of the effects of several acidic amino acids on $[Ca^{2+}]i$. Concentrations of L-glutamate, L-aspartate, and D-aspartate were 10 μM whereas D-glutamate, N-methyl-D-aspartate (NMDA), kainate (KA), and quisqualate (QA) were used at 100 μM. The lack of effect of ketamine (KETAM, 40 μM) on the L-glutamate action is also illustrated (D). These experiments are representative of 4–7 similar ones.

380 nm (8). Mn^{2+} entry was evidenced by the quenching of the fura-2 fluorescence excited at 360 nm, a wavelength that is not sensitive to changes in Ca^{2+} concentration (9).

For single-cell measurements, the coverslips coated with fura-2-loaded cells were mounted under the microscope (Nikon Diaphot) in a chamber thermostated at 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, U.K.) and analyzed using an Applied Imaging Magical image processor (Sunderland, Tyne & Wear, U.K.). 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. Consecutive frames obtained at 340 and 380 nm excitation were ratioed pixel by pixel and $[Ca^{2+}]_i$ was estimated by comparison with fura-2 standards (7). To quantify the behavior of many individual cells in terms of $[Ca^{2+}]_i$ oscillations, two parameters were used: the mean $[Ca^{2+}]_i$ increase and the oscillation index. The mean $[Ca^{2+}]_i$ increase was computed as the average of all the $[Ca^{2+}]_i$ values during the integration period minus the "resting" $[Ca^{2+}]_i$, arbitrarily chosen as the lowest $[Ca^{2+}]_i$ value of the whole trace. It is equivalent to the mean area under the $[Ca^{2+}]_i$ peaks and is sensitive to any $[Ca^{2+}]_i$ increase, either oscillatory or nonoscillatory. The oscillation index was computed as the sum of all the differences (in absolute value) between every $[Ca^{2+}]_i$ value and the next, and is sensitive only to oscillatory $[Ca^{2+}]_i$ increases[3] (10).

For measurements of glutamate uptake, cell-coated coverslips were incubated at 37°C in 0.3 ml of medium containing either different concentrations (1–500 μM) of [^{14}C]L-glutamate (0.3 μCi/ml) or 1 μM [^{14}C]L-glutamate and 10 to 100 μM of the competing amino acid as indicated (see Results). At the end of the incubation period the coverslips were washed with ice-cold medium and radioactivity and protein content was determined (11). Uptake was computed in nmoles of [^{14}C]glutamate/mg of protein. In preliminary experiments the time course of [^{14}C]L-glutamate uptake was determined. The rate of uptake was the same in 0.5 and 1 min incubation periods and decreased somewhat after 2 and 5 min. A 1 min incubation period was used for all experiments shown here.

Fura-2/AM was obtained from Molecular Probes, Eugene, Oreg. Nisoldipine was a generous gift from Bayer A. G., Germany. Thapsigargin was obtained from Alomon Laboratories, Jerusalem, Israel. L-Transpyrrolidine-2,4-dicarboxylic acid was obtained from RBI, Natick, Mass. [^{14}C]L-Glutamate was from Amersham Ibérica, Madrid, Spain. MK-801 was a generous gift from Merck Sharp & Dohme de España, Madrid, Spain. Other chemicals were obtained either from Sigma, Madrid, Spain, or from E. Merck, Darmstandt, Germany.

RESULTS AND DISCUSSION

Figure 1*A* shows that L-glutamate (10 μM) reversibly enhanced $[Ca^{2+}]i$ in a single representative GH_3 pituitary cell. Figure 1B summarizes the average behavior of 42 single cells present in the same microscope field. Both the mean $[Ca^{2+}]i$ increase and the oscillation index were increased by L-glutamate (see Experimental Procedures and Fig. 1 legend for details). This indicates that the increase of $[Ca^{2+}]i$ was due, at least in part, to an increase of the $[Ca^{2+}]i$ oscillations. Upon removal of glutamate, the $[Ca^{2+}]i$ activity returned to or often decreased below basal levels. The effect of L-glutamate on $[Ca^{2+}]i$ was concentration-dependent, 50% stimulation (EC_{50}) being reached at about 6 μM (results not shown).

Figure 1*C* and Fig. 1*D* show a representative selection of the effects of other amino acids. Several acidic amino acids (L-aspartate, EC_{50}, 4 μM; D-aspartate, EC_{50}, 6 μM; L-cysteate) reproduced the effects of L-glutamate, whereas others (D-glutamate, α-methyl-DL-aspartate, L-homocysteate, the excitatory amino acid receptor (EARs) agonists (12, 13) N-methyl-D-aspartate -NMDA-, kainate, quisqualate, trans-(1S, 3R)-1-amino-1,3-cyclopentane dicarboxylic acid,[4] and the dipeptide L-asp-L-asp, all tested at 100 μM) had no effect. None of the neutral amino acids tested (L-

[3]The noise of the measurements had a minor contribution to the value of the oscillation index. For example, addition of nisoldipine (0.5 μM), which is known to block $[Ca^{2+}]i$ oscillations in GH_3 cells (3; see also Fig. 3A) to control cells, reduced the oscillation index by 81 ± 3% (mean ± SEM; n = 33).

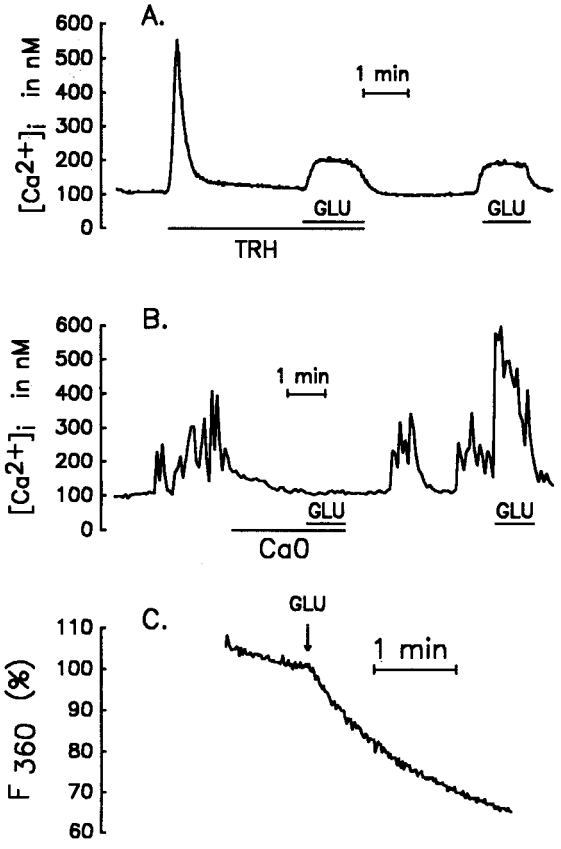

Figure 2. The increase of $[Ca^{2+}]_i$ induced by L-glutamate is due to Ca^{2+} entry. *A)* After emptying the intracellular Ca^{2+} stores by addition of TRH (0.1 μM), L-glutamate (10 μM) was still able to increase $[Ca^{2+}]_i$. A second glutamate addition after removal of TRH had the same effect. The trace shown is the average of all the cell population. The experiment is representative of three similar ones. *B)* L-Glutamate (10 μM) has no effect in Ca^{2+}-free medium. Trace in a representative single cell is shown. The Ca^{2+}-free medium (Ca0) contained 0.5 mM EGTA. Readdition of external Ca^{2+} restored both $[Ca^{2+}]_i$ oscillations and the $[Ca^{2+}]_i$ increase induced by glutamate. *C)* Effect of L-glutamate on the entry of Mn^{2+}. 100 μM $MnCl_2$ was added 1 min before L-glutamate (10 μM, added at the arrow); quenching of the fura-2 fluorescence excited at 360 nm, which is insensitive to Ca^{2+}, was monitored (see Experimental Procedures). Measurements were performed in the entire cell population. The experiment shown is representative of three similar ones.

glutamine, L-asparagine, L-aspartic acid amide, L-alanine, L-serine, L-cysteine, L-homocysteine, α-aminobutyric, and α-aminoisobutyric acids; all at 100 μM) reproduced the effects of L-glutamate (results not shown). Neither the inhibitors of the NMDA receptor (13, 14) ketamine (Fig. 1D), MK-801 (1 μM) and 2-amino-7-phosponoheptanoic acid (100 μM, AP-7) nor the broad spectrum EARs antagonists kynurenic acid (1 mM; 13) and D-glutamylglycine (1 mM; 15) inhibited the effects of L-glutamate on $[Ca^{2+}]$i (results not shown)[4]. These results suggest that the effects of glutamate on $[Ca^{2+}]$i of GH3 cells are not mediated by EARs.

L-Glutamate applied after TRH still increased $[Ca^{2+}]_i$ (**Fig. 2*A***). Because TRH is known to release Ca^{2+} from the intracellular Ca^{2+} stores (2)[5], these results suggest that mobilization of stored Ca^{2+}, which should have been emptied by TRH treatment, is not involved in the $[Ca^{2+}]_i$ increase induced by glutamate. Emptying the intracellular Ca^{2+} stores by treatment with the Ca^{2+} ionophore ionomycin (16, 17) or the endomembrane Ca^{2+}-ATPase inhibitor thapsigargin (19) did not prevent the $[Ca^{2+}]_i$ increase induced by glutamate either (results not shown)[5]. These results also show that TRH receptor occupancy does not avoid the effects mediated by glutamate, indicating that glutamate does not interact with GH_3 cells at the level of the TRH receptor. Removal of external Ca^{2+} prevented the effects of glutamate (Fig. 2*B*), suggesting that the increase of $[Ca^{2+}]_i$ is caused by Ca^{2+} entry. Figure 2*C* shows that L-glutamate accelerated the entry of Mn^{2+}, a Ca^{2+} surrogate for Ca^{2+} channels in GH_3 cells (7), as assayed from the quenching of fura-2 fluorescence. This result provides direct evidence of the stimulation of a plasma membrane divalent cation entry pathway by L-glutamate.

The effects of L-glutamate on $[Ca^{2+}]_i$ were completely prevented by dihydropyridines (**Fig. 3*A***), indicating that voltage-dependent L-type Ca^{2+} channels (20) are essential for the glutamate-induced Ca^{2+} entry. Mn^{2+} is known to permeate voltage-dependent Ca^{2+} channels of GH_3 cells (7), and the glutamate-induced Mn^{2+} entry (see Fig. 2*C*) was also blocked by dihydropyridines (results not shown). Dihydropyridines also prevented the spontaneous $[Ca^{2+}]_i$ oscillations (see Fig. 3*A*, before glutamate addition). Therefore, the action mechanism of glutamate may be to increase the spontaneous electrical activity, most likely by moderately depolarizing the plasma membrane, thus increasing excitability and the frequency of action potentials firing. Figure 3*B* shows that removal of extracellular Na^+ also prevented the effects of glutamate. This suggests that glutamate depolarizes the membrane by increasing Na^+ entry. The Na^+ channel poison tetrodotoxin (1 μM) did not prevent the effects of glutamate (results not shown). This suggests that the Na^+ entry pathway does not involve voltage-dependent Na^+ channels. On the other hand, voltage-dependent Na^+ channels are scarce in GH_3 cells and are not essential for spontaneous firing, which is dominated by Ca^{2+} channels (21, 22).

An electrogenic high-affinity transport system for glutamate with substrate specificity similar to the one reported here for the increase of $[Ca^{2+}]_i$ has been described in several excitable and nonexcitable cells (23–27). This system cotransports 2 Na^+ + 1 glutamate in exchange for 1 K^+ + 1 OH^-, thus producing net Na^+ entry and membrane depolarization as well as cell acidification (24, 25). We find that

[4]The agonists and antagonists of the EARs were also tested using the same protocol for their effects on $[Ca^{2+}]_i$ in cerebellar granule neurons, which possess EARs. In these experiments the effects were as expected from the known pharmacology of these compounds (13, 14).

[5]Ionomycin (100 nM) is known to mobilize the whole stored Ca^{2+} pool in GH_3 cells (17). The extent of depletion of the Ca^{2+} stores by TRH (100 nM for 5 min) or thapsigargin (500 nM for 5 min) was estimated by comparing the size of the $[Ca^{2+}]_i$ peaks induced by ionomycin (100 nM) added in Ca^{2+}-free medium before and after treatment (18). The stores were depleted by 89 ± 3% and 95 ± 4% by TRH and thapsigargin, respectively (mean ± SEM; 5 independent experiments).

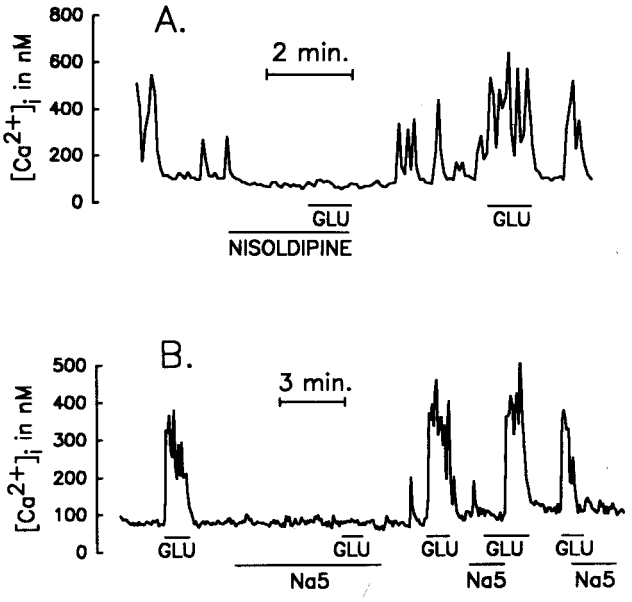

Figure 3. Effects of nisoldipine (0.5 μM, *A*) and low sodium (5 mM, *B*) on the increase of $[Ca^{2+}]_i$ produced by 10 μM L-glutamate. Effects on representative single cells are shown. For low sodium experiments most of the sodium chloride (140 mEq/l) was replaced by N-methyl-D-glucamine chloride (labeled Na5). Full Na^+ removal was avoided because it often produced a slow $[Ca^{2+}]_i$ increase. Replacement of Na^+ by choline instead of N-methyl-D-glucamine produced the same results. Results are representative of 5 similar experiments.

the addition of 10 μM L-glutamate produces a small (0.05–0.1 pH units) decrease of the intracellular pH of GH_3 cells, which reverts upon removal of glutamate (measurements performed in cells loaded with bis-carboxyethyl-carboxyfluorescein, ref 28; results not shown). The transport of [^{14}C]L-glutamate by GH_3 cells was then studied. We find that GH_3 cells take up glutamate by a saturable transport system with Vmax = 7.4 nmol/mg of protein/min and Km = 21 μM (Fig. 4A). The values for these kinetics parameters are comparable to the ones reported previously for other high-affinity glutamate transporters present in neural cells and glia (23–27). The uptake of [^{14}C]glutamate by GH_3 cells was completely prevented by removal of extracellular Na^+ (results not shown).

Other acidic amino acids are carried by the high-affinity glutamate transporter with the ensuing membrane depolarization (24, 25). We then tested the effects of several selected amino acids (10–100 μM) on the uptake of [^{14}C]L-glutamate (1 μM) by GH_3 cells. Percent inhibition by the competing amino acid would be an index of the relative affinity for the glutamate transporter. We find that L-cysteate, L- and D-aspartate, and L-glutamate are efficient inhibitors of the uptake of [^{14}C]glutamate (68–42% inhibition at 10 μM) whereas D-glutamate, L-homocysteate, NMDA, and all the neutral amino acids tested are not (<16% inhibition at 100 μM). Only those amino acids that inhibited the transport of [^{14}C]L-glutamate (and presumably were carried by the glutamate transporter) were able to increase $[Ca^{2+}]$i. The correlation between the increase of $[Ca^{2+}]$i and the inhibition of [^{14}C]L-glutamate uptake achieved by several amino acids is shown in Fig. 4*B*. The correlation coefficient (r) was 0.98. These results suggest that the effects on $[Ca^{2+}]$i are due to the entry of the amino acid through the glutamate transporter. This view was confirmed using the selective glutmate transport inhibitor L-trans-pyrrolidine-2,4-dicarboxylic acid (TPDC). TPDC competes with glutamate for transport through the glutamate carrier (27). TPDC at 20 μM produced essentially the same $[Ca^{2+}]$i increase as 20 mM L-glutamate (84 ± 4%; mean ± SEM, n = 185 cells from three independent experiments). On the other hand, TPDC added on top of L-glutamate did not produce an additional effect on $[Ca^{2+}]$i, and vice versa (results not shown).

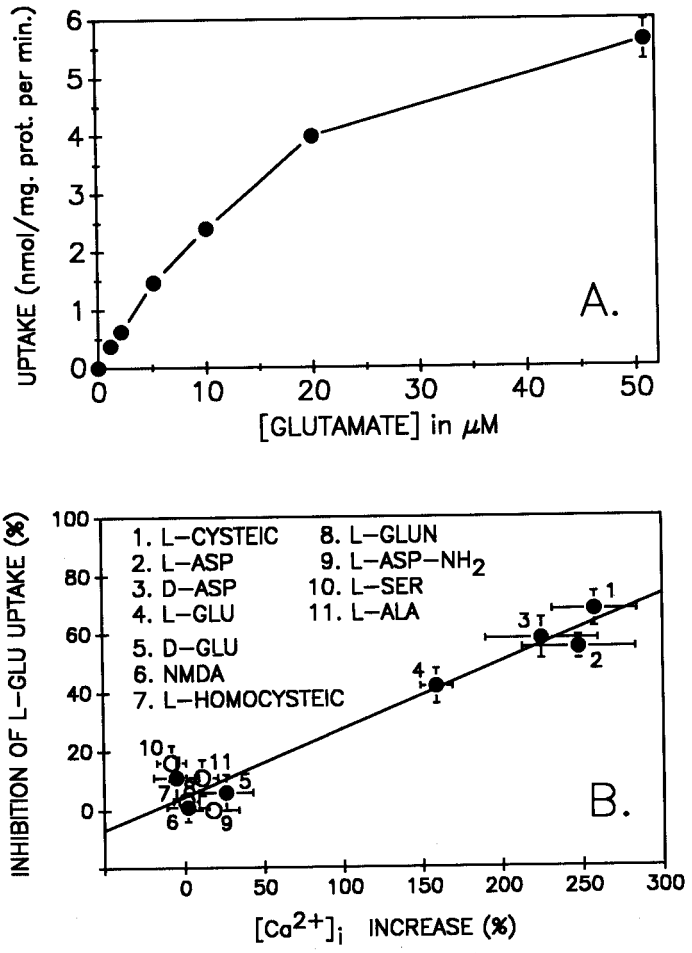

Figure 4. Saturation kinetics of the uptake of [^{14}C]L-glutamate by GH_3 cells (*A*) and correlation between the inhibition of glutamate uptake and the increase of $[Ca^{2+}]_i$ produced by several amino acids (*B*). In *A* each value is the mean of 4 determinations. The nonsaturable uptake (min^{-1}) amounted 0.04 × [glutamate] and was subtracted from the raw data. Straight-line fitting of the data in a double-reciprocal plot gave a correlation coefficient (*r*) of 0.98. Values for V_{max} and K_m were 7.4 and 21, respectively. In *B* inhibition was tested at 1 μM [^{14}C]L-glutamate and 10 (#1 to #4) or 100 μM (#5 to #11) of the competing amino acid. The increase of $[Ca^{2+}]_i$ was estimated as the mean $[Ca^{2+}]_i$ increase (see Fig. 1 legend). Each value is the mean of 4–7 (inhibition) or 21–304 (increase of $[Ca^{2+}]_i$) determinations. Bars represent standard errors. Straight lines were fitted by the least squares procedure.

The effects of glutamate on $[Ca^{2+}]i$ of GH_3 cells are very similar to those observed during phase 2 of TRH action. Prolactin release during this phase is associated with dihydropyridine-sensitive oscillations of $[Ca^{2+}]_i$, which are secondary to an increase of action potential firing (1–4). This increase of electrical activity ultimately arises from depolarization of the plasma membrane by inhibition of an inwardly rectifying K^+ current (10). We propose that entry of L-glutamate through the Na^+-dependent electrogenic transporter depolarizes the plasma membrane of GH_3 cells. This produces an increase of the rate of firing of Ca^{2+} action potentials and the ensuing $[Ca^{2+}]_i$ increase. This conclusion provides experimental support for a possible novel action of glutamate as an extracellular messenger that fits the known properties of the glutamate carrier (24–26). Note that the range of glutamate concentrations at which effects are observed is comparable to that required to activate the receptors for excitatory amino acids. Negative results with EAR agonists (NMDA, quisqualate, and kainate) and antagonists (ketamine, MK-801, and AP-7) suggest that GH_3 cells do not possess functional EARs. This may not necessarily be true for normal pituitary cells because it has been reported that some membrane receptors present in these cells, for example dopamine receptors, are missing in GH_3 cells (29). Evidence for the existence of EARs in normal pituitary cells is contradictory. A recent work reported the presence of non-NMDA EARs in the pituitary gland (30), but this was at variance with other results (31).

Our results also open the question of whether glutamate may act as a nonpeptidic prolactin-releasing factor under physiological conditions. Glutamate could reach the adenohypophysis from the systemic blood, from the hypothalamus via the hypophyseal portal system, and from the trigeminal ganglia, or it could even be produced by the hypophyseal cells (30). An increase of prolactin secretion, both in vivo after parenteral administration of glutamate (5) and in vitro by addition of glutamate to rat anterior pituitary cells in primary culture (6), has been reported. Several neurotransmitters such as dopamine and GABA (32) act as nonsynaptic inhibitors of prolactin secretion. Stimulation by glutamate could be the first example of nonsynaptic regulation for this neurotransmitter (see also ref 30). FJ

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