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Genistein inhibits Ca^{2+} influx and glutamate release from hippocampal synaptosomes: putative non-specific effects

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Abstract

The role of protein tyrosine kinases on glutamate release was investigated by determining the effect of broad range inhibitors of tyrosine kinases on the release of glutamate from rat hippocampal synaptosomes. We found that lavendustin A and herbimycin A did not inhibit glutamate release stimulated by 15 mM KCl, but genistein, also a broad range inhibitor of tyrosine kinases did inhibit the intracellular Ca^{2+} concentration response to KCl and, concomitantly, decreased glutamate release evoked by the same stimulus, in a dose-dependent manner. These effects were not observed with the inactive analogue genistin. Therefore, we investigated the mechanism whereby genistein modulates Ca^{2+} influx and glutamate release. Studies with voltage-gated Ca^{2+} channel inhibitors showed that ω-conotoxin GVIA did not further inhibit glutamate release or the Ca^{2+} influx stimulated by KCl in the presence of genistein. This tyrosine kinase inhibitor and ω-agatoxin IVA had a partially additive effect on those events. Nitrendipine did not reduce significantly the KCl-induced responses. Genistein further reduced Ca^{2+} influx in response to KCl in the presence of nitrendipine, ω-conotoxin GVIA and ω-agatoxin IVA, simultaneously. The effect of tyrosine phosphatase inhibitors was also tested on the influx of Ca^{2+} and on glutamate release stimulated by KCl-depolarization. We found that the broad range inhibitors sodium orthovanadate and dephostatin did not significantly affect these KCl-evoked events.

Our results suggest that genistein inhibits glutamate release and Ca^{2+} influx in response to KCl independently of tyrosine kinase inhibition, and that tyrosine kinases and phosphatases are not key regulators of glutamate release in hippocampal nerve terminals.

Keywords: Ca^{2+} influx; Glutamate release; Hippocampal synaptosomes; Tyrosine phosphorylation

1. Introduction

Protein tyrosine kinases are highly expressed in several brain regions, including the hippocampus, being particularly enriched in synaptic membrane and synaptic vesicle fractions (Hirano et al., 1988; Lev et al., 1995; Ellis et al., 2000). This suggests that tyrosine phosphorylation may play a role in hippocampal synaptic plasticity. In fact, tyrosine kinase activity is involved in the induction of long-term potentiation (LTP) in hippocampus (O'Dell et al., 1991). The level of tyrosine phosphorylated proteins does not depend solely on the activity of tyrosine kinases but are the result of a tight balance between tyrosine kinase and tyrosine phosphatase activity. Protein-tyrosine phosphatases, also expressed in adult hippocampus (Van Vactor, 1998), can positively or negatively regulate tyrosine kinase activity which, on the other hand, may also modulate tyrosine phosphatase activity (reviewed in Petrone and Sap, 2000; Ostman and Bohmer, 2001).

Both tyrosine kinases and phosphatases can exist as integral membrane proteins, known as receptor tyrosine kinases (RTK) and receptor protein-tyrosine phosphatases (RPTP), or as cytosolic proteins. Brain derived neurotrophic factor (BDNF), a neurotrophin that binds to TrkB, a RTK, has been extensively described to act on hippocampal synaptic plasticity (Levine et al., 1995; Patterson et al., 1996; Leßmann and Heumann, 1998; Jovanovic et al., 2000). Cytosolic tyrosine kinases like c-src, proline-rich tyrosine kinase 2 (PYK2), c-fyn and $pp125^{FAK}$ may also be involved in synaptic plasticity (for review see Gurd, 1997). On the other hand, the activity of these cytosolic proteins can be regulated by RPTPs like CD45 and RPTPα (Petrone and Sap, 2000).

Although, the modulation of ion channel activity by phosphorylation is usually associated to serine/threonine kinases and phosphatases, an equally important role for tyrosine phosphorylation has been emerging (reviewed in Siegelbaum, 1994; Gurd, 1997). Tyrosine kinase activity increases voltage-gated Ca^{2+} channel (VGCC) activity

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(Cataldi et al., 1996; Potier and Rovira, 1999), reduces K^+ channel currents (Huang et al., 1993; Lev et al., 1995; Holmes et al., 1996), and enhances *N*-methyl-D-aspartate (NMDA) channel currents (Wang and Salter, 1994; Gurd, 1997). This suggests that tyrosine kinases may contribute to an overall increase in neuronal excitability. However, a recent report showed that tyrosine phosphorylation is a negative regulator of Na^+ channel activity (Ratcliffe et al., 2000).

Synaptic vesicle proteins and other proteins involved in exocytosis may also be targets for tyrosine phosphorylation, suggesting new mechanisms for the modulation of synaptic plasticity by tyrosine kinases and phosphatases (Pang et al., 1988; Jovanovic et al., 1996; Janz et al., 1999). The synaptic vesicle proteins synaptophysin and synaptotagmin seem to have an important function in hippocampal short- and long-term plasticity, although the physiological relevance of tyrosine phosphorylation of these proteins is still unclear (Janz et al., 1999). Synapsin I, a protein associated to synaptic vesicles, is involved in the modulation of glutamate release by BDNF, probably through phosphorylation of the protein by the mitogen activated protein kinase (MAPK), one of the effectors of TrkB signaling (Jovanovic et al., 2000).

Since depolarization of nerve endings increases tyrosine phosphorylation (Woodrow et al., 1992; Siciliano et al., 1996; Barrie et al., 1996), and taking into account that tyrosine kinases and phosphatases may modulate ion channel function and the activity of the exocytotic machinery, we investigated a role for tyrosine kinase activity in the modulation of glutamate release from hippocampal synaptosomes.

2. Experimental procedures

2.1. Materials

The acetoxymethyl ester of Indo-1 (Indo-1/AM), the broad range protein molecular weight standards, and the sypro orange protein gel stain were purchased from Molecular Probes Europe BV (Leiden, The Netherlands). Ionomycin, fatty acid-free bovine serum albumin (BSA), lavendustin A and dephostatin were obtained from Calbiochem–Novabiochem International (La Jolla, CA). Genistein and genistin were from Sigma and herbimycin A was from Biomol Research Labs Inc. (Plymouth Meeting, PA). The VGCC inhibitors ω-agatoxin IVA and ω-conotoxin GVIA were obtained from Peptide Institute Inc. (Osaka, Japan). Nitrendipine was from Research Biochemicals International (Natick, MA). Reagents used in immunoblotting experiments were purchased from Bio-Rad (Hercules, CA). The polyvinylidene difluoride (PVDF) membranes, the alkaline phosphatase-linked anti-mouse secondary antibody, the enhanced chemifluorescence (ECF) reagent and Percoll were obtained from Amersham Pharmacia Biotech (Buckinghamshire, England). The anti-phosphotyrosine antibody was a kind offer from New England Biolabs (Beverly, MA). All other reagents were from Sigma or from Merck.

2.2. Isolation of crude (P_2) and purified hippocampal synaptosomal fractions

The crude synaptosomal P_2 fraction from male Wistar rat hippocampi was isolated as described elsewhere (McMahon et al., 1992) with minor modifications. Briefly, 6–8 week old animals were sacrificed by cervical dislocation and their hippocampi were dissected and homogenized (5%, w/v) in 0.32 M sucrose, 10 mM HEPES, pH 7.4 (sucrose buffer), using a Thomas B homogenizer. The suspension was centrifuged at $3000 \times g_{max}$, for 2 min, and the supernatants were spun at $14{,}600 \times g_{max}$, for 12 min. The upper white layer of the pellet (P_2), containing the majority of the synaptosomes, was removed and resuspended in sucrose buffer at a protein concentration of about 8–12 mg/ml, as determined by the Biuret method (Layne, 1957). Alternatively, a purified hippocampal synaptosomal suspension was isolated using the Percoll method described elsewhere (Dunkley et al., 1988) with some modifications. The crude synaptosomal P_2 fraction was resuspended in sucrose buffer and layered on top of Percoll discontinuous gradients consisting of layers of sucrose buffer containing 23, 10 and 3% Percoll. After centrifugation at $23{,}200 \times g_{max}$, for 5 min, the purified synaptosomal fraction was removed from the interface between the 23 and 10% Percoll solutions, and resuspended in 15 volumes of sucrose buffer. The synaptosomal suspension was spun twice at $20{,}000 \times g_{max}$, for 20 min, and resuspended in sucrose buffer at a protein concentration of 4.0–5.5 mg/ml, as determined by the bicinchoninic acid (BCA) method (Smith et al., 1985). The synaptosomes (purified or crude fraction) were stored on ice as drained pellets and used within 4 h. The whole procedure was performed at 0–4 °C.

2.3. Glutamate release

The release of endogenous glutamate was measured using a continuous fluorimetric assay, based on the reduction of $NADP^+$ catalyzed by glutamate dehydrogenase (GDH, EC 1.4.1.3) in the presence of glutamate (Nicholls et al., 1987). In short, synaptosomes (1 mg protein/ml) were incubated for 20 min, at 30 °C, in incubation medium (1 ml of 132 mM NaCl, 1 mM KCl, 1 mM $MgCl_2$, 1.2 mM H_3PO_4, 0.1 mM $CaCl_2$, 10 mM glucose, 10 mM HEPES-Na^+, pH 7.4) supplemented with 0.1 mg/ml fatty-acid-free BSA. After centrifugation at $15{,}800 \times g_{max}$, for 20 s, the synaptosomes were resuspended in reaction medium (similar to the incubation medium but without BSA, and containing either 1 mM $CaCl_2$ or 200 nM Ca^{2+} [50 μM EGTA; 38 μM $CaCl_2$] for Ca^{2+}-independent glutamate release measurements). $NADP^+$ (1 mM, final concentration) and GDH (40 U) were added, and fluorescence was measured using excitation and emission wavelengths of 340 and 460 nm, with 5 and 10 nm slits, respectively. The experiments were

conducted at 37 °C, under continuous stirring, and the calibration was performed at the end of each experiment with 2.5 nM of glutamate. When tyrosine kinase/phosphatase inhibitors, ω-agatoxin IVA and ω-conotoxin GVIA were tested, they were present both in incubation and in reaction medium. Nitrendipine, a lipophylic drug, binds rapidly to L-type Ca^{2+} channels and therefore was added only in the reaction medium.

2.4. Intracellular $[Ca^{2+}]_i$ measurements

The $[Ca^{2+}]_i$ was determined by measuring the ratio of Indo-1/Ca^{2+} complex fluorescence at two distinct emission wavelengths, essentially as described previously (Duarte et al., 1996). Briefly, synaptosomes (1.5 mg protein/ml) were loaded with Indo-1 at 25 °C for the first 20 min, followed by 10 min at 35 °C, in incubation medium containing 3 μM Indo-1/AM. The synaptosomes were spun and resuspended at 0.375 mg protein/ml in reaction medium containing 1 mM $CaCl_2$. Incubation with tyrosine kinase/phosphatase inhibitors and/or VGCC inhibitors was performed as described for glutamate release experiments. When dephostatin was tested, Fura-2 was used instead of Indo-1 since this drug interfered with Indo-1 fluorescence. Fluorescence was monitored at 30 °C, under continuous stirring, at the excitation wavelength of 335 nm and emission wavelengths of 410 and 485 nm, for Indo-1, and excitation wavelengths of 340 and 380 nm and emission wavelength of 505 nm, for Fura-2. Five nm slits were used. Calibration was performed at the end of each experiment with 2.5 μM ionomycin followed by 20 mM Tris/4 mM EGTA (final concentrations), to determine the maximal fluorescence and the autofluorescence, respectively. Fluorescence intensities were converted into $[Ca^{2+}]_i$ using the calibration equation for double emission wavelength measurements (Grynkiewicz et al., 1985).

2.5. Immunoblotting

Purified synaptosomes (3.5 mg protein/ml) were preincubated, for 20 min at 30 °C, in incubation medium, followed by incubation in reaction medium (containing either 1 mM $CaCl_2$ or 200 nM free Ca^{2+}), at 37 °C, for 5 min, or for 4 min followed by 1 min stimulation with 15 mM KCl. The reaction was stopped by adding 2 × concentrated sample buffer [100 mM Tris, 100 mM glycine, 4% sodium dodecyl sulfate (SDS), 8% β-mercaptoethanol, 8 M urea and 3 mM sodium orthovanadate] and heating the samples for 5 min at 95 °C. Equal amounts of protein (25 μg) were separated by electrophoresis on 10% SDS-poliacrylamide gels (SDS-PAGE), and transferred electrophoretically to PVDF membranes. The membranes were then blocked for 1 h at room temperature, in Tris-buffered saline [137 mM NaCl, 20 mM Tris–HCl, pH 7.6] containing 0.1% Tween-20 (TBS-T) and 5% low fat milk. The membranes were incubated overnight at 4 °C, with a mouse monoclonal anti-phosphotyrosine antibody diluted 1:2000 in TBS-T with 5% BSA. Alternatively, the antibody was incubated with phosphothreonine, phosphoserine or phosphotyrosine prior to membrane probing, to assess for antibody specificity. After extensive washing, membranes were incubated with alkaline phosphatase-linked anti-mouse serum (1:20,000 in TBS-T with 5% low fat milk) for 1 h at room temperature. Protein immunoreactive bands were visualized by ECF on a Storm 860 Gel and Blot Imaging System (Amersham Pharmacia Biotech), following incubation of the membranes with ECF reagent for 5 min.

2.6. Statistical analysis

Results are presented as means ± S.E.M. of the indicated number of experiments. Statistical significance was determined using one-way ANOVA followed by Newman–Keuls or Dunnett post tests, as indicated.

3. Results

3.1. KCl-depolarization induces tyrosine phosphorylation of synaptosomal proteins in a Ca^{2+}-dependent manner

In order to determine the content in phosphotyrosine residues in synaptosomal proteins and tyrosine phosphorylation changes in response to depolarization, immunoblotting assays were performed using a monoclonal anti-phosphotyrosine antibody. As depicted in Fig. 1A, there are several tyrosine phosphorylated proteins in control conditions, in purified hippocampal nerve terminals. The specificity of the phosphotyrosine antibody was tested by previously incubating the antibody with phosphotyrosine, phosphothreonine or phosphoserine (Fig 1B). Depolarization of the nerve terminals with 15 mM KCl, for 1 min, increased the tyrosine phosphorylation of two proteins of approximately 107 and 44 kDa (Fig. 1, arrowheads). K^+-depolarization of nerve terminals in a low Ca^{2+} reaction medium (200 nM free Ca^{2+}) did not induce tyrosine phosphorylation of synaptosomal proteins (Fig. 1A), suggesting Ca^{2+} influx as a key factor for tyrosine kinase activation following depolarization.

3.2. Protein tyrosine kinase inhibitors have contradictory effects on glutamate release and $[Ca^{2+}]_i$ increase in response to KCl-depolarization of hippocampal nerve terminals

Taking into account that several synaptic proteins are tyrosine phosphorylated and that this phosphorylation increases following K^+-depolarization, we investigated a role for tyrosine kinases on the release of glutamate from hippocampal synaptosomes. Therefore, we studied the effect of genistein, lavendustin A and herbimycin A, three broad spectrum inhibitors of tyrosine kinases (Akiwama et al., 1987; Onoda et al., 1989; Akiwama and Ogawara,

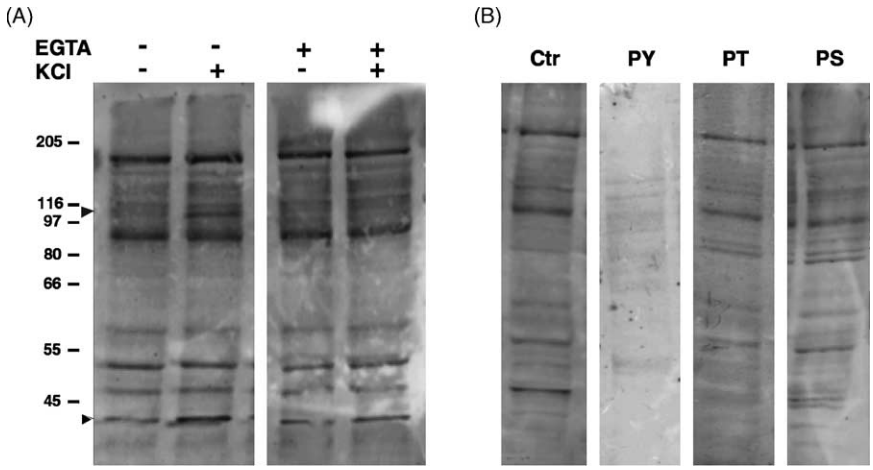

Fig. 1. Effect of KCl-depolarization on the phosphotyrosine content of synaptosomal proteins. (A) Representative membranes showing phosphotyrosine immunoreactivity of protein extracts from synaptosomal suspensions in control conditions, or following stimulation with 15 mM KCl for 1 min (KCl), as described in Section 2. Comparison between control and KCl-stimulated phosphotyrosine signals were done in reaction medium containing either 1 mM $CaCl_2$ or 200 nM free Ca^{2+} (EGTA). Arrowheads depict differences in intensity between bands in lanes 1 and 2, corresponding to proteins of approximately 107 and 44 kDa. (B) Immunoreactivity against anti-phosphotyrosine antibody incubated or not (Ctr) with phosphotyrosine (PY), phosphothreonine (PT) or phosphoserine (PS) peptides prior to membrane probing.

1991; Uehara and Fukazawa, 1991), on the release of glutamate evoked by KCl-depolarization. Synaptosomes were pre-incubated with the tyrosine kinase inhibitors for 20 min and the depolarization-evoked glutamate release was then monitored also in the presence of the inhibitors. Basal glutamate release was measured for 4 min and KCl-stimulated release was followed for the next 5 min. As depicted in Fig. 2A, the different tyrosine kinase inhibitors had contrasting effects on glutamate release. Genistein showed a concentration-dependent inhibitory effect on the release of glutamate in response to KCl-depolarization. At a concentration of 100 μM, genistein decreased glutamate release to 38.9 ± 3.2% of the control. Genistin, an inactive analogue of genistein (Akiwama and Ogawara, 1991), did not affect the release of glutamate evoked by K^+-depolarization, suggesting that the effect of genistein on glutamate release was specific. However, lavendustin A and herbimycin A had no significant effect on glutamate release when used at concentrations known to inhibit tyrosine kinase activity (Onoda et al., 1989; Uehara and Fukazawa, 1991; Satoh et al., 1992) (Fig. 2A).

The Ca^{2+}-independent release of glutamate, which is attributed to the reversal of the glutamate transporter (Nicholls, 1989), was measured by depolarizing the synaptosomes with 15 mM KCl in a medium containing 200 nM free Ca^{2+}. The Ca^{2+}-independent release under these experimental conditions was 40.1 ± 0.9% of the control. Genistein, at 100 μM, had no significant effect on the Ca^{2+}-independent glutamate release (36.2 ± 0.3% of the control, $P > 0.05$, Dunnett comparison test). These results suggest that genistein inhibits the Ca^{2+}-dependent release of glutamate. The other tyrosine kinase inhibitors had also no significant effect on the Ca^{2+}-independent release of glutamate (lavendustin A, 10 μM, 41.3 ± 2.2%, $P > 0.05$; herbimycin A, 10 μM, 38.3 ± 2.6%, $P > 0.05$, Dunnett test).

Since genistein reduced the Ca^{2+}-dependent glutamate release, we investigated whether this effect could be attributed to an inhibition of the $[Ca^{2+}]_i$ increase evoked by plasma membrane depolarization. Incubation of the nerve terminals with genistein decreased both the basal $[Ca^{2+}]_i$ and the initial $[Ca^{2+}]_i$ increase in response to KCl, in a dose-dependent manner (Fig. 2B and C). At 100 μM, genistein significantly reduced the basal $[Ca^{2+}]_i$ to 68.6 ± 2.0% of the control (Fig. 2B), and inhibited the $[Ca^{2+}]_i$ response to 15 mM KCl to 32.9 ± 2.6% of the control (Fig. 2C). Genistin, at 100 μM, did not affect significantly the basal $[Ca^{2+}]_i$ nor the $[Ca^{2+}]_i$ rise evoked by KCl (Fig. 2B and C). The other tyrosine kinase inhibitors were also tested and although lavendustin A was without effect on the basal $[Ca^{2+}]_i$ and on the $[Ca^{2+}]_i$ response to depolarization (Fig. 2B and C), herbimycin A slightly reduced the $[Ca^{2+}]_i$ increase stimulated by KCl (Fig. 2C). At the concentration of 10 μM, herbimycin A reduced the $[Ca^{2+}]_i$ response to K^+-depolarization to 76.0 ± 3.3% of the control (Fig. 2C). Incubation of the nerve terminals with higher concentrations of herbimycin A did not further inhibit KCl-induced $[Ca^{2+}]_i$ increase (data not shown). Interestingly, this extent of $\Delta[Ca^{2+}]_i$ inhibition by herbimycin A is quite similar to the inhibition caused by 10 μM genistein, which also had no significant effect on glutamate release at that concentration.

3.3. Genistein and ω-agatoxin IVA show partially additive inhibitory effects on glutamate release and on the $[Ca^{2+}]_i$ response to KCl

In order to further understand the effect of genistein on Ca^{2+} influx, a comparative study using VGCC inhibitors and

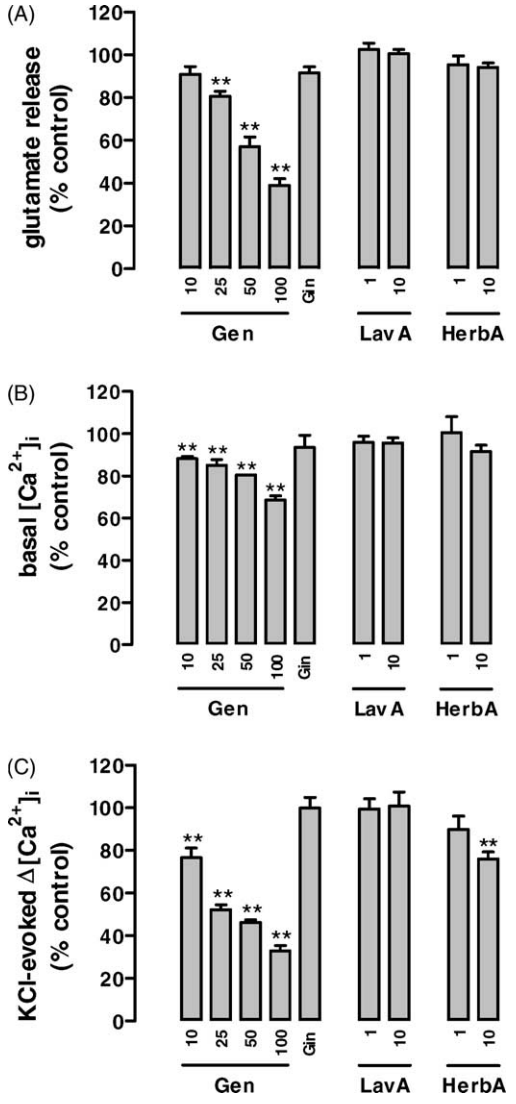

Fig. 2. Effect of protein tyrosine kinase inhibitors on glutamate release and $[Ca^{2+}]_i$ increase evoked by KCl. (A) Effect of genistein (Gen), genistin (Gin), lavendustin A (LavA) and herbimycin A (HerbA) on total glutamate release evoked by 15 mM KCl. The concentrations used for each inhibitor are expressed in μM, under the correspondent bar, except for genistin that was used at 100 μM. Results are expressed as a percentage of the control response ± S.E.M., of 3–4 experiments performed in different preparations. Glutamate release in control conditions was 5.02 ± 0.23 nM/mg protein/5 min ($n = 19$). (B) Effect of the protein tyrosine kinase inhibitors mentioned earlier on the basal $[Ca^{2+}]_i$ of hippocampal synaptosomes. Results are expressed as mean % of the control ± S.E.M. for $n = 3$–5. In control conditions, the basal $[Ca^{2+}]_i$ was 258.2 ± 9.1 nM ($n = 28$). C, Effect of protein tyrosine kinase inhibitors on the initial $[Ca^{2+}]_i$ response to 15 mM KCl. The changes in $[Ca^{2+}]_i$ ($\Delta[Ca^{2+}]_i$) were calculated as the difference between the peak values following depolarization and the basal values acquired just prior to the stimulus. In control conditions, the $\Delta[Ca^{2+}]_i$ induced by 15 mM KCl was 285.8 ± 10.9 nM ($n = 28$). The results are expressed as mean % control ± S.E.M. Statistical significance was determined by one-way ANOVA followed by Dunnett comparison test performed for each condition as compared to the correspondent control response in the absence of all drugs (**$P < 0.01$).

genistein was performed, both on the release of glutamate and on the $[Ca^{2+}]_i$ increase stimulated by KCl.

The L-type VGCC inhibitor nitrendipine (Duarte et al., 1996), at a concentration of 1 μM, had no significant effect on the KCl-evoked glutamate release (Fig. 3A), and caused only a minor decrease in the correspondent $[Ca^{2+}]_i$ rise, to 92.0 ± 1.9% of the control ($n = 6$, $P < 0.05$, Dunnett comparison test; Fig. 3B). Nitrendipine had also no effect on the basal $[Ca^{2+}]_i$ (96.2 ± 2.0% of the control, $n = 6$, $P > 0.05$, Dunnett test). Co-application of 50 μM genistein and 1 μM nitrendipine did not further inhibit glutamate release and the $[Ca^{2+}]_i$ response to 15 mM KCl, as compared to the effect of genistein on its own.

The contribution of the N-type VGCC in the events studied was assessed using the N-type VGCC inhibitor ω-conotoxin GVIA (ω-CgTx GVIA) (Duarte et al., 1996). Pre-incubation of the nerve terminals with 500 nM ω-CgTx GVIA lead to a reduction of the $[Ca^{2+}]_i$ response to KCl-depolarization, to 77.2 ± 2.6% of the control ($n = 4$, $P < 0.01$, Dunnett test), and a concomitant decrease in glutamate release, to 89.4 ± 2.4% of the control ($n = 6$, $P < 0.05$, Dunnett test) (Fig. 3A and B), without affecting the resting $[Ca^{2+}]_i$ (91.5 ± 4.5%, $n = 4$, $P > 0.05$, Dunnett test). When ω-CgTx GVIA and genistein were applied together, the inhibition of glutamate release and the $[Ca^{2+}]_i$ rise following K^+-depolarization was not significantly different from the effect of genistein alone. The N-type VGCC inhibitor had no significant effect on the resting $[Ca^{2+}]_i$ (91.5 ± 4.5%, $n = 4$, $P > 0.05$, Dunnett test).

The P-/Q-type VGCC inhibitor ω-agatoxin IVA (ω-Aga IVA) (Duarte et al., 1996), at a concentration of 100 nM, inhibited the $[Ca^{2+}]_i$ response to KCl, to 50.9 ± 3.2% ($n = 6$, $P < 0.01$, Dunnett test), and caused a reduction in glutamate release, to 42.0 ± 3.6% of the control ($n = 7$, $P < 0.05$, Dunnett test) (Fig. 3A and B). The basal $[Ca^{2+}]_i$ was also inhibited by ω-Aga IVA to 86.2 ± 2.6% of the control ($n = 6$, $P < 0.01$, Dunnett test). The P-/Q-type VGCC inhibitor further reduced glutamate release and the $[Ca^{2+}]_i$ rise following KCl-depolarization in the presence of genistein, but the effect of both drugs was only partially additive (Fig. 3A and B). To determine whether the ability of ω-Aga IVA to further reduce these events in the presence of genistein was due to the use of a submaximal concentration of this drug (see Figs. 2 and 3), the same study was performed using 100 μM genistein. The toxin ω-Aga IVA was again able to further inhibit the $[Ca^{2+}]_i$ response to KCl under these conditions (data not shown).

The simultaneous inhibition of L-, N- and P-/Q-type of VGCC, using all of the earlier mentioned VGCC inhibitors, caused a decrease in the $[Ca^{2+}]_i$ response to KCl to 33.1 ± 2.2% of the control ($n = 5$) (Fig. 3B), and a concomitant reduction of the glutamate release to 30.3 ± 2.0% of the control ($n = 5$) (Fig. 3A). Genistein further inhibited the $[Ca^{2+}]_i$ response to KCl in the presence of all VGCC inhibitors, simultaneously.

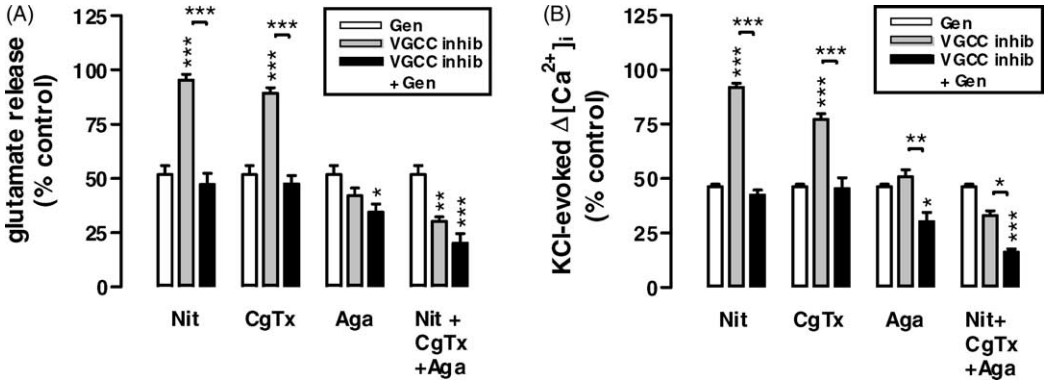

Fig. 3. Comparative effect of genistein and VGCC inhibitors on glutamate release and $[Ca^{2+}]_i$ increase in response to KCl. (A) Inhibitory effect of genistein (Gen) and VGCC inhibitors (VGCC inhib) on glutamate release stimulated by 15 mM KCl. When tested, these drugs were present both in the pre-incubation and in the reaction medium at the following concentrations: 50 μM genistein, 500 nM ω-conotoxin GVIA (CgTx) and/or 100 nM ω-agatoxin IVA (Aga). Nitrendipine (Nit, 1 μM) was applied only in the reaction medium. Results are shown as mean % of control ± S.E.M., for $n = 4$–7. (B) Comparison of the inhibitory effects of genistein and VGCC inhibitors on the $[Ca^{2+}]_i$ response to 15 mM KCl. The $[Ca^{2+}]_i$ measurements were performed as described in Section 2, in the presence or absence of genistein, nitrendipine, ω-CgTx GVIA and ω-Aga IVA, at the concentrations described previously for glutamate release experiments. Data is presented as mean % control ± S.E.M. for $n = 4$–9. Statistical significance was determined by one-way ANOVA followed by Newman–Keuls multiple comparison test ($^{*}P < 0.05$; $^{**}P < 0.01$ and $^{***}P < 0.001$). Unless otherwise indicated, the comparison is made between the indicated bar and the bar corresponding to the effect of genistein on its own.

The effects of nitrendipine, ω-CgTx GVIA and ω-Aga IVA on the release of glutamate were due to a direct action on Ca^{2+} influx, since glutamate release evoked by 5 μM ionomycin was not affected by these drugs (data not shown). This rules out a non-specific effect of the Ca^{2+} channel antagonists at a step downstream of Ca^{2+} entry.

These results confirm previous reports indicating P-/Q-type VGCC as the main channels responsible for the Ca^{2+} influx leading to glutamate release in the hippocampus, and N-type VGCC and unidentified types of VGCC as minor contributors (Malva et al., 1995; Ambrósio et al., 1997). Furthermore, our results suggest that genistein may reduce Ca^{2+} influx and glutamate release following depolarization, mainly by partial inhibition of P-/Q-type of VGCC, but also by inhibiting N-type and unidentified types of VGCC.

3.4. Protein tyrosine phosphatase inhibitors have no effect on glutamate release and $[Ca^{2+}]_i$ increase in response to KCl-depolarization of hippocampal nerve terminals

Since the effects of tyrosine kinase inhibitors on glutamate release and on the $[Ca^{2+}]_i$ response to KCl-depolarization were contradictory, we determined the effect of tyrosine

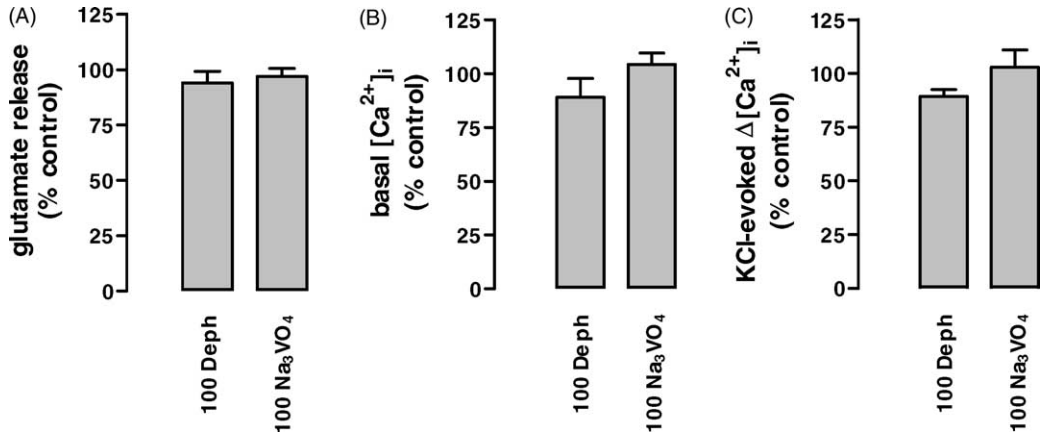

Fig. 4. Effect of protein tyrosine phosphatase inhibitors on glutamate release and $[Ca^{2+}]_i$ increase evoked by KCl. (A) Effect of dephostatin (Deph) and sodium orthovanadate (Na_3VO_4), at the concentration of 100 μM, on total glutamate release evoked by 15 mM KCl. These phosphatase inhibitors were present both in the incubation and the reaction medium. Results are expressed as mean % of control ± S.E.M. for $n = 3$. (B) Effect of the protein tyrosine phosphatase inhibitors mentioned earlier in the basal $[Ca^{2+}]_i$ of isolated hippocampal nerve terminals. (C) Effect of protein tyrosine phosphatase inhibitors on the initial $[Ca^{2+}]_i$ response to 15 mM KCl. The changes in $[Ca^{2+}]_i$ ($\Delta[Ca^{2+}]_i$) were calculated as described in the caption of Fig. 2C. The results are expressed as mean % control ± S.E.M. for $n = 3$–4. None of the tyrosine phosphatase inhibitors showed statistical significant effects as determined by one-way ANOVA followed by Dunnett comparison test.

phosphatase inhibitors on these KCl-evoked events, in order to further investigate the involvement of tyrosine phosphorylation on glutamate release from hippocampal nerve endings. The tyrosine phosphatase inhibitors dephostatin and sodium orthovanadate were used at concentrations known to inhibit protein tyrosine phosphatase activity (Swarup et al., 1982; Imoto et al., 1993). Dephostatin, at a concentration of 100 μM, had no significant effect on the glutamate release stimulated by 15 mM KCl (Fig. 4A). The resting $[Ca^{2+}]_i$ and the $[Ca^{2+}]_i$ response to 15 mM KCl were also not significantly affected by 100 μM dephostatin (Fig. 4B and C). Furthermore, sodium orthovanadate was without effect on the basal $[Ca^{2+}]_i$, on the $[Ca^{2+}]_i$ increase evoked by KCl and on the concomitant release of glutamate (Fig. 4A–C). These results, together with the general lack of effect of tyrosine kinase inhibitors on glutamate release and $[Ca^{2+}]_i$ response to KCl, suggest that tyrosine phosphorylation of synaptosomal proteins is not essential for exocytosis of glutamate-containing vesicles in the hippocampus.

4. Discussion

In the present work, we demonstrated that KCl-depolarization evoked a Ca^{2+}-dependent increase in the phosphotyrosine content of hippocampal synaptosomal proteins. Two proteins of approximate molecular weights of 107 and 44 kDa showed a Ca^{2+}-dependent increase in tyrosine phosphorylation in response to KCl. A Ca^{2+}-dependent rise in the tyrosine phosphorylation of several proteins following depolarization was previously described in rat forebrain synaptosomes and hippocampal slices (Woodrow et al., 1992; Barrie et al., 1996; Siciliano et al., 1996). In spite of the increase in tyrosine phosphorylation following KCl stimulation, with the exception of genistein, all the tyrosine kinase and tyrosine phosphatase inhibitors had no effect on glutamate release and Ca^{2+} influx stimulated by KCl-depolarization, in hippocampal synaptosomes. Therefore, our results strongly suggest that tyrosine phosphorylation does not affect glutamate release in hippocampal nerve terminals.

Although, we have not determined the identity of the tyrosine phosphorylated proteins, previous reports suggest PYK2 and $pp125^{FAK}$ as strong candidates for the 107 kDa protein that showed an increase in tyrosine phosphorylation after KCl-depolarization (Siciliano et al., 1996). The Kv2.1 K^+ channel α-subunit (105–115 kDa), that can be phosphorylated on tyrosine residues, is also a possible candidate for the 107 kDa protein (Peretz et al., 1999). As for the 44 kDa protein that showed an increase in its phosphotyrosine content in response to KCl in our system, it is most likely one of the MAPK isoforms, p42 or p44. In fact, using an antibody against the dually phosphorylated (activated) form of MAPK we found that KCl-depolarization activates this kinase in hippocampal synaptosomes, in a Ca^{2+}-dependent manner (Pereira et al., 2000).

In contrast to the other tyrosine kinase inhibitors, genistein inhibited the Ca^{2+}-dependent glutamate release from hippocampal nerve terminals, possibly by acting on Ca^{2+} influx through partial inhibition of P-/Q-type VGCC, and by inhibiting N-type and unidentified VGCC. Genistin had no effect on glutamate release and $[Ca^{2+}]_i$ response to KCl. Nevertheless, the lack of effect of the other tyrosine kinase inhibitors and tyrosine phosphatase inhibitors on the same events strongly argue against the specificity of the inhibitory effects of genistein. This isoflavone was initially reported as being a specific tyrosine kinase inhibitor, showing in vitro IC_{50} of 18–30 μM, depending on the kinase inhibited (Akiwama et al., 1987; O'Dell et al., 1991; Akiwama and Ogawara, 1991). The IC_{50} values for in vivo inhibition of tyrosine kinase activity by genistein are quite higher, being 111 μM for EGF receptor autophosphorylation (Akiwama and Ogawara, 1991). At this concentration, genistein already had a nearly maximal effect in glutamate release and Ca^{2+} influx inhibition in our system (Fig. 2), supporting the lack of specificity of the effect of genistein in these KCl-stimulated events.

Genistein was reported to inhibit the activity of other enzymes besides tyrosine kinases, such as β-galactosidase, DNA topoisomerase I and II, S6 kinase and cAMP phosphodiesterase 4, at the same range of concentration used to inhibit tyrosine kinase activity (Akiwama and Ogawara, 1991; Nichols and Morimoto, 1999). Recently, the list of genistein non-specific effects has been increasing and it now includes direct inhibition of voltage-sensitive Na^+ channels (Paillart et al., 1997), cardiac L-type Ca^{2+} channels (Chiang et al., 1996), $GABA_A$ and glycine receptors (Dunne et al., 1998; Huang and Dillon, 2000), inhibition of myocyte delayed-rectifier K^+ currents (Washizuka et al., 1998) and direct activation of cystic fibrosis transmembrane conductance regulator Cl^- channels (French et al., 1997).

In our study, genistein inhibited depolarization-induced Ca^{2+} influx and glutamate release, most likely through direct or indirect inhibition of VGCC. This is suggested by the differential effect of genistein on $[Ca^{2+}]_i$ rise mediated through different types of VGCC (Fig. 3). In fact, genistein directly inhibits L-type Ca^{2+} channels in guinea pig ventricular myocytes (Chiang et al., 1996). The non-specific inhibition of K^+ channels by genistein (Washizuka et al., 1998) is another possibility, since depolarization with high extracellular $[K^+]$ is driven by inward K^+ currents. Therefore, by inhibiting K^+ channels, genistein would reduce Ca^{2+} influx and the concomitant glutamate release. The direct inhibition of voltage-activated Na^+ channels, also reported for genistein (Paillart et al., 1997), is not a suitable mechanism for the effects described in this report, since KCl-stimulated glutamate release and Ca^{2+} influx are not dependent on Na^+ channel activity (Tibbs et al., 1989). However, although the effect of genistein in these KCl-evoked events is very likely non-specific, a specific effect through a tyrosine kinase that is not inhibited by lavendustin A or herbimycin A can not be entirely ruled out.

Our present results suggesting that the phosphotyrosine content of nerve terminal proteins does not influence neurotransmitter release in the hippocampus were not expected based on previous reports. Genistein was shown to inhibit glutamate release from hippocampal synaptosomes in a previous study (Mullany et al., 1996), although neither the specificity of the effect nor the mechanisms involved were further characterized. The present results suggest that the effect of genistein reported by Mullany et al. is independent of tyrosine kinase inhibition. However, in bovine adrenal chromaffin A cells, tyrosine kinase inhibitors suppress [^{3}H]noradrenaline ([^{3}H]NA) release evoked by nicotine, KCl or a Ca^{2+} ionophore (Cox et al., 1996). Also, in pheochromocytoma PC12 cells, ionomycin- and KCl-stimulated [^{3}H]NA release is enhanced by tyrosine phosphatase inhibition (Kitamura et al., 2000). These reports are not necessarily contrary to our results since the involvement of tyrosine kinases and phosphatases on exocytosis is likely to depend on cellular context.

On the other hand, a role for tyrosine kinases and phosphatases in the regulation of voltage-gated K^+ channel and VGCC is extensively described, suggesting that tyrosine phophorylation may contribute to an overall increase in synaptic excitability currents (Lev et al., 1995; Cataldi et al., 1996; Gurd, 1997). This contrasts with recent reports proposing Na^+ channels as novel targets for tyrosine kinases and phosphatases, being negatively regulated by tyrosine phosphorylation (Ratcliffe et al., 2000), or by the outcome of RTK signaling (Hilborn et al., 1998).

Several studies on the effect of tyrosine kinase and tyrosine phosphatase inhibitors on L-, N- and T-type VGCC, using different neuronal and non-neuronal cell types, suggested that tyrosine kinases and phosphatases act as positive and negative modulators of VGCC activity, respectively (Cataldi et al., 1996; Strauss et al., 1997; Morikawa et al., 1998; Wijetunge et al., 1998). In our study, genistein was the only tyrosine kinase inhibitor that showed a notorious inhibitory effect on Ca^{2+} influx, probably through a non-specific mechanism as discussed above. Herbimycin A also had a small inhibitory effect on the $[Ca^{2+}]_i$ increase stimulated by KCl that did not influence glutamate release (Fig. 2). Therefore, our results do not rule out a small contribution of tyrosine kinase activity on the $[Ca^{2+}]_i$ response to depolarization in hippocampal nerve terminals. The tyrosine phosphatase inhibitors, however, had no significant effect on Ca^{2+} influx in our system (Fig. 4). A recent report showed a 30–40% reduction of P-/Q-type Ca^{2+} currents, but not L- or N-type Ca^{2+} currents, by genistein and another tyrosine kinase inhibitor, tyrphostin B42 (and not by two of genistein inactive analogues), in hippocampal slices (Potier and Rovira, 1999). Since these results were obtained using whole cell patch clamp and applying the inhibitors to the whole slice, they cannot be easily compared with the results we obtained in our study.

Although our results do not support a meaningful constitutive regulation of the channels involved in neurotransmitter release by tyrosine phosphorylation, tyrosine kinase activation (or tyrosine phosphatase inhibition) following depolarization may lead to further tyrosine phosphorylation of the channels. This could enhance Ca^{2+} influx during high frequency pre-synaptic stimulation, resulting in a positive feedback mechanism. In fact, tyrosine kinase activity is required for the induction of LTP in the hippocampus (O'Dell et al., 1991; Huang and Hsu, 1999).

The synaptic vesicle proteins synaptophysin and synaptogyrin are substrates of the tyrosine kinases c-src and c-fyn (Pang et al., 1988; Stenius et al., 1995; Janz et al., 1999). Studies with synaptophysin and synaptogyrin knockout mice suggested an essential but redundant role for these proteins in the modulation of short- and long-term synaptic plasticity, without being required for neurotransmitter release (Janz et al., 1999). Although the physiological relevance of tyrosine phosphorylation of synaptophysin and synaptogyrin is still unknown, this associates two of the few known phosphotyrosine synaptic vesicle proteins with LTP but not with neurotransmitter release.

In conclusion, we found that although there was an increase in tyrosine phosphorylation following K^+-depolarization of isolated nerve endings, tyrosine kinases and phosphatases do not seem to be involved in the modulation of Ca^{2+} influx and concomitant glutamate release evoked by the same stimulus. Unlike the other tyrosine kinase inhibitors, genistein inhibited Ca^{2+} influx and glutamate release, through mechanisms most likely independent of tyrosine kinase inhibition. This work, together with a vast list of previous reports, rules out genistein as a suitable tool to study the involvement of protein tyrosine kinase activity in cellular events.

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