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Functional link between tyrosine phosphorylation and human serotonin transporter gene expression

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Abstract

Treatment of the JAR human placental choriocarcinoma cells with herbimycin A, an inhibitor of tyrosine kinases, led to an increase in the activity of the serotonin transporter. This effect was accompanied by an increase in the serotonin transporter density and in the steady-state levels of the serotonin transporter mRNA. A treatment time of >4 h was necessary for herbimycin A to elicit its effect. Actinomycin D and cycloheximide blocked the effect. There was no increase in the steady-state levels of the serotonin transporter mRNA when cells were treated with herbimycin A in the presence of actinomycin D. The herbimycin A-induced increase in the transporter activity was abolished by genistein, another inhibitor of tyrosine kinases. But the increase in the transporter mRNA levels caused by herbimycin A was not affected by genistein. Treatment of cells with herbimycin A resulted in an increase in the tyrosine phosphorylation of specific cellular proteins, suggesting that herbimycin A directly or indirectly activates specific tyrosine kinases. It is concluded that tyrosine phosphorylation is an essential component in the signaling pathways participating in the regulation of the human serotonin transporter gene expression. © 1997 Elsevier Science B.V.

Keywords: 5-HT (5-hydroxytryptamine, serotonin) transporter; Tyrosine kinase inhibitor; Tyrosine phosphorylation; Transcription; Choriocarcinoma cell, human

1. Introduction

The Na^+- and Cl^--coupled serotonin transporter is primarily expressed in serotonergic neurons, platelets and placenta (Rudnick and Clark, 1993; Ganapathy and Leibach, 1995). Molecular cloning studies have established that the transporter expressed in these tissues is identical, encoded by the same gene (Ramamoorthy et al., 1993a; Lesch et al., 1993a,b). The serotonin transporter is the target for antidepressant drugs such as fluoxetine (Prozac) and also for abusable drugs such as cocaine and amphetamines (Rudnick and Clark, 1993). Because of its clinical and pharmacological relevance, regulation of the serotonin transporter function is currently an area of intense research. For these regulatory studies, the JAR human placental choriocarcinoma cells which constitutively express the serotonin transporter (Cool et al., 1991) have proved to be an extremely useful model system. Two other human placental choriocarcinoma cells, BeWo and JEG, also express the transporter, but at a much lower level (Jayanthi et al., 1994; unpublished results). To our knowledge, the choriocarcinoma cells are the only cell lines of human origin which natively express the serotonin transporter. Human platelets can be employed to investigate the regulation of the serotonin transporter, but there are limitations with this system. Platelets are nonnucleated cells and therefore it is not feasible to study the transporter regulation at the gene level. The choriocarcinoma cells are suitable for these studies. With the JAR choriocarcinoma cells, we have demonstrated that the expression of the serotonin transporter gene is up-regulated by cAMP (Cool et al., 1991; Ramamoorthy et al., 1993b), staurosporine (Ramamoorthy et al., 1995a) and interleukin-1β (Ramamoorthy et al., 1995b).

cAMP-induced serotonin transporter gene expression is blocked by protein kinase A inhibitors (Cool et al., 1991), indicating participation of the enzyme in the regulatory process. Staurosporine induces the transporter gene expression via a cAMP-independent mechanism, but the effects

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of cAMP and staurosporine are not additive, suggesting convergence of the two pathways to a common point (Ramamoorthy et al., 1995a). In the case of interleukin-1β, there is no evidence for involvement of cAMP, cGMP, nitric oxide or ceramide (Ramamoorthy et al., 1995b). Moreover, the effects of cAMP and interleukin-1β are additive. Similarly, the effects of staurosporine and interleukin-1β are also additive. Thus, the interleukin-1β pathway appears to be distinct from that of cAMP or of staurosporine.

The current study was undertaken to investigate the role of tyrosine phosphorylation in the serotonin transporter expression. The results of the study show that tyrosine kinases are intimately involved in the regulation of the expression of the human serotonin transporter.

2. Materials and methods

2.1. Materials

5-[1,2-^{3}H]Hydroxytryptamine (serotonin) binoxalate (specific radioactivity 26.3 Ci/mmol) and [^{125}I]RTI-55 ([^{125}I]2β-carbomethoxy-3β-(4-iodophenyl)tropane) (specific radioactivity 2200 Ci/mmol) were purchased from DuPont-NEN (Boston, MA, USA). [α-^{32}P]dCTP (specific radioactivity 3000 Ci/mmol) was purchased from Amersham (Arlington Heights, IL, USA).

The JAR human placental choriocarcinoma cells were originally obtained from the American Type Culture Collection (Rockville, MD, USA). RPMI 1640 culture medium, penicillin, streptomycin and trypsin were from Life Technologies (Gaithersburg, MD, USA). Fetal bovine serum, imipramine, iproniazid, serotonin, prostaglandin E_1, apotransferrin, thyroxine, cycloheximide and actinomycin D were obtained from Sigma (St. Louis, MO, USA). Human recombinant insulin was from Novo Nordisk Pharmaceuticals (Princeton, NJ, USA). Herbimycin A, genistein, erbstatin and tyrphostin were obtained from Research Biochemicals International (Natick, MA, USA). The anti-phosphotyrosine antibody PY20 was purchased from Transduction Laboratories (Lexington, KY, USA). All other chemicals were of analytical grade.

2.2. Cell culture

JAR cells were cultured as described previously (Ramamoorthy et al., 1995a) in a hormonally defined medium which consisted of RPMI 1640, supplemented with insulin (5 μg/ml), apotransferrin (5 μg/ml), prostaglandin E_1 (2.5×10^{-5} mg/ml) and thyroxine (5×10^{-12} M). Hydrocortisone was not included in the culture medium in the current study because of the recent evidence for modulation of biological effects of cAMP and interleukin-1β by glucocorticoids (Kunz et al., 1994). Treatment with different reagents was carried out for indicated time periods in the defined medium prior to transport measurements.

2.3. Transport measurements in cells

The dishes containing monolayer cultures of the cells were taken out of the incubator and let stand at room temperature for 2 h. This 2 h incubation at room temperature was necessary because without this incubation the serotonin transport activity was found to increase significantly (approx. 40%) without any treatment over a period of 1 h following the removal of the cells from the incubator. The reasons for this increase are not known. The culture medium was then aspirated, and the cells were washed once with the transport buffer. Transport buffer (1 ml) containing radiolabeled serotonin was added to the cells and incubated for 3 min. Transport was terminated by aspirating the buffer and subsequently washing the cells three times with fresh transport buffer. The cells were lysed with 1 ml of 0.2 M NaOH/1% SDS, and the lysate was transferred to scintillation vials for quantitation of radioactivity. The composition of the transport buffer was 25 mM HEPES-Tris, pH 7.5, 140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5 mM glucose and 0.1 mM iproniazid, an inhibitor of monoamine oxidases. Serotonin transport that occurred independent of the serotonin transporter (i.e., diffusion) was determined by measuring the transport in the presence of imipramine (0.1 mM), and this component was always $< 10\%$ of total transport measured in the absence of imipramine.

2.4. RTI-55 binding to JAR cell plasma membranes

JAR cells were cultured and treated with or without herbimycin A (1 μg/ml) as described for transport measurements except that 150-cm^2 flasks were used instead of 35-mm dishes. Plasma membranes were prepared as described previously (Ramamoorthy et al., 1995a). Equilibrium binding of [^{125}I]RTI-55 to the plasma membranes was assayed by incubating the membranes (40 μg of membrane protein) with varying concentrations of the ligand (0.1–1 nM) at room temperature for 1 h at pH 9.5 as previously detailed (Ramamoorthy et al., 1995a). Non-specific binding was determined in the presence of 5 μM paroxetine.

2.5. Immunoblot analysis of phosphotyrosine-containing proteins

JAR cells were cultured and treated with herbimycin A (1 μg/ml) for varying time periods (0–6 h). Following the treatment, the medium was removed and the cells were lysed with 400 μl of SDS-PAGE sample buffer (62.5 mM Tris-HCl, pH 6.8, 50 mM dithiothreitol, 2.5% SDS, 10% glycerol) containing 1 mM orthovanadate. The samples were boiled for 5 min at 100°C and used for sodium

dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The size-fractionated proteins were transferred onto a nitrocellulose membrane (Bio-Rad). The blots were blocked with 3% fetal bovine serum, 1% nonfat dry milk and 0.5% Tween-20. The blots were probed with anti-phosphotyrosine antibody (1 μg/ml) and the immunoreactive proteins were detected using the Enhanced ChemiLuminescence Western blotting detection kit (Amersham).

2.6. Isolation of poly(A)$^+$ RNA and Northern blot analysis

Poly(A)$^+$ RNA was isolated from JAR cells treated under different conditions. The FastTrack mRNA isolation kit (Invitrogen, San Diego, CA, USA) was used for this purpose. Northern blot hybridization was carried out as described earlier (Jayanthi et al., 1994; Ramamoorthy et al., 1993b, 1995a) under high stringency conditions using ^{32}P-labeled human serotonin transporter (SERT) cDNA as the probe. The same membrane blot was used for probing with ^{32}P-labeled human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA as the probe by sequential hybridization. This was done as an internal control for RNA loading and transfer efficiency. Quantification of transcript signals in autoradiograms was done with a Hewlett-Packard densitometer (Scan Jet II CX).

The serotonin transporter cDNA probe was a 2.2 kb fragment obtained by digestion with *Eco*RI from the human SERT clone (Ramamoorthy et al., 1993a). The GAPDH cDNA probe was a 0.78 kb fragment obtained from a human GAPDH clone (American Type Culture Collection) by digestion with *Pst*I and *Xba*I. The cDNA probes were radiolabeled with [α-^{32}P]dCTP by random priming.

2.7. Data analysis

Experiments were routinely carried out in duplicate or triplicate and each experiment was repeated two or three times. The results are expressed as means ± S.E. The kinetic constants were calculated by nonlinear regression methods and confirmed by linear regression (Eadie-Hofstee and Scatchard transformations) using a commercially available computer program (Biosoft, Cambridge, UK).

3. Results and discussion

3.1. Differential influence of tyrosine kinase inhibitors on the expression of the serotonin transporter in JAR cells

There are several structurally dissimilar compounds which are currently used as potent inhibitors of different classes of tyrosine kinases (Levitzki and Gazit, 1995). Previous studies from our laboratory have shown that treatment of JAR cells with the tyrosine kinase inhibitors genistein and tyrphostin for 16 h has no effect on the expression of the serotonin transporter (Ramamoorthy et al., 1995a). Recently, our attention was drawn to a report by Doherty et al. (1994) which demonstrated opposing biological effects of some tyrosine kinase inhibitors. These investigators have shown that neurite outgrowth from cerebellar granule cells is stimulated by the tyrosine kinase inhibitor herbimycin A and that this effect is blocked by other tyrosine kinase inhibitors such as erbstatin. Interestingly, these other tyrosine kinase inhibitors (e.g., genistein, tyrphostins, erbstatin and lavendustin A) by themselves have no effect on neurite outgrowth. Therefore, we investigated the influence of herbimycin A on the expression of the serotonin transporter in JAR cells and compared the herbimycin A effect with that of three other tyrosine kinase inhibitors (Table 1). Treatment of JAR cells with herbimycin A for 16 h led to marked stimulation of the serotonin transporter activity. This stimulatory effect was however not observed with genistein, erbstatin and tyrphostin. These results demonstrate the differential influence of various tyrosine kinase inhibitors on the serotonin transporter activity in JAR cells and indicate participation of tyrosine phosphorylation in the regulation of the transporter.

Table 1
Differential influence of tyrosine kinase inhibitors on serotonin transporter expression

Tyrosine kinase inhibitor	Serotonin transport	
	pmol/mg of protein per 3 min	%
None	0.96 ± 0.07	100
Genistein (75 μM)	0.77 ± 0.07	80
Erbstatin (2 μg/ml)	0.84 ± 0.08	88
Tyrphostin (100 μM)	0.79 ± 0.06	82
Herbimycin A (1 μg/ml)	1.94 ± 0.04	202

Confluent monolayer cultures of JAR cells were treated for 16 h at 37°C with or without tyrosine kinase inhibitors. Following the treatment, the medium was removed and the cells were washed with the transport buffer. Imipramine-sensitive transport of serotonin (50 nM) was then measured in these cells with a 3 min incubation. Concentration of each compound used here is within the range reported in the literature for these compounds to inhibit tyrosine kinases in several cell types.

3.2. Characterization of the herbimycin A-induced activation of the serotonin transporter

Fig. 1A describes the influence of treatment time on the ability of herbimycin A to enhance serotonin transport activity in JAR cells. Exposure of the cells to herbimycin A up to 4 h did not change the transport activity. However, when the treatment time was increased to 16 h, the stimulatory effect of herbimycin A became evident. Furthermore, the herbimycin A-induced increase in the serotonin transporter activity was found to be sensitive to inhibitors of protein and RNA synthesis (cycloheximide and actinomycin D respectively) (Fig. 1B). These results suggest that

increased de novo synthesis of the serotonin transporter might underlie the increase in the serotonin transport activity induced by herbimycin A.

Even though the herbimycin A-induced increase in the transporter activity was observed only with the exposure of the cells to herbimycin A for 16 h because of the involvement of de novo synthesis of the transporter protein, the initial effect of herbimycin A on the signaling events which trigger the de novo synthesis may actually occur at a much earlier time period. To investigate this possibility, JAR cells were exposed to herbimycin A for shorter time periods after which the cell cultures were washed to remove herbimycin A and then treated with herbimycin A-free medium for the remainder of the total treatment time of 16 h. At the end of this time period, the activity of the serotonin transporter was measured (Fig. 2). A significant stimulatory effect was observed even when the cells were exposed to herbimycin A only for 2 h and the effect was maximal within an exposure time of 6 h.

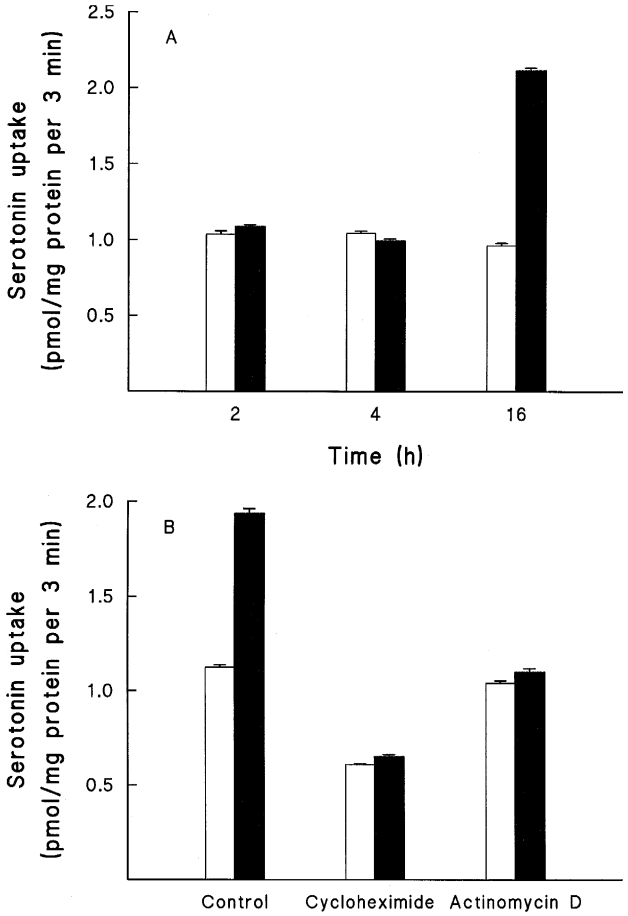

Fig. 1. (A) Time dependence of herbimycin A-induced expression of the serotonin transporter and (B) effect of actinomycin D and cycloheximide on herbimycin A-induced expression of the serotonin transporter. (A) Confluent monolayer cultures of JAR cells were treated with (closed bars) or without (open bars) herbimycin A (2 μg/ml) for 2, 4 or 16 h at 37°C. Following the treatment, the medium was aspirated and the cells were washed with the transport buffer. Imipramine-sensitive transport of serotonin (50 nM) was then measured in these cells with a 3 min incubation. (B) Confluent monolayer cultures of JAR cells were treated with (closed bars) or without (open bars) herbimycin A (1 μg/ml) in the absence or presence of cycloheximide (40 μM) or actinomycin D (0.03 μg/ml). Treatment was for 16 h at 37°C. Following the treatment, the medium was aspirated and the cells were washed with the transport buffer. Imipramine-sensitive transport of serotonin (50 nM) was then measured in these cells with a 3 min incubation.

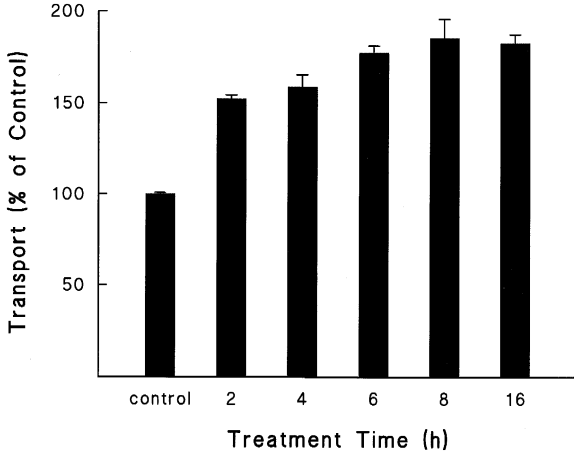

Fig. 2. Time dependence of the initiation of herbimycin A effect. Confluent monolayer cultures of JAR cells were treated with herbimycin A (1 μg/ml) for 0, 2, 4, 6, 8 or 16 h at 37°C. Following the treatment, the medium was aspirated and the cells were washed with herbimycin A-free culture medium and incubated in the same medium to complete a total incubation period of 16 h. Imipramine-sensitive transport of serotonin (50 nM) was then measured in these cells with a 3 min incubation. Results are given as percent of transport in control cells (no treatment with herbimycin A). Value for control transport was 0.66 ± 0.01 pmol/mg of protein per 3 min.

The influence of herbimycin A on the serotonin transporter activity was dose-dependent, with the maximal effect occurring at a concentration of 1–2 μg/ml (Fig. 3A). Fig. 3B describes the influence of herbimycin A on the kinetic parameters of the serotonin transporter. The increase in transport activity induced by herbimycin A was found to be primarily due to an increase in the maximal velocity of the transport system. In control cells, the values for the kinetic parameters K_t and V_{max} were 0.34 ± 0.02 μM and 9.9 ± 0.2 pmol/mg of protein per 3 min. The corresponding values in herbimycin A-treated cells were 0.28 ± 0.02 μM and 17.5 ± 0.5 pmol/mg of protein per 3 min. Thus, herbimycin A increased the maximal velocity but had little effect on the affinity of the transporter for serotonin. To see whether the observed increase in the maximal velocity of the transport process was associated with a parallel increase in the density of the serotonin transporter protein in the plasma membrane of the cells, the binding of [^{125}]RTI-55 to the plasma membranes prepared from control and herbimycin A-treated cells was analyzed. RTI-55 is a cocaine analog which is a high affinity ligand to the serotonin transporter as well as to the dopamine and norepinephrine transporters (Boja et al., 1992; Wall et al., 1993; Jayanthi et al., 1993; Prasad et al., 1994). However, since the JAR cells do not express the

dopamine and norepinephrine transporters, RTI-55 is regarded as a selective ligand for the serotonin transporter in these cells. The equilibrium binding of RTI-55 to JAR cell plasma membranes was measured over a concentration range of 0.1–1 nM. The results given in Fig. 4 as Scatchard plots revealed that the apparent dissociation constant (K_d) was 0.20 ± 0.03 nM and the maximal binding capacity (B_{max}) was 38.8 ± 2.2 fmol/mg of protein in control membranes. The corresponding values for the binding in membranes from herbimycin A-treated cells were 0.22 ± 0.02 nM and 91.2 ± 4.0 fmol/mg of protein. These data show that herbimycin A treatment caused a 2.4-fold increase in the serotonin transporter density in the JAR cell plasma membrane without altering the K_d. These results together with the sensitivity of the herbimycin A effect to cycloheximide and actinomycin D (Fig. 1B) strongly suggest that herbimycin A might activate the expression of the serotonin transporter gene in JAR cells.

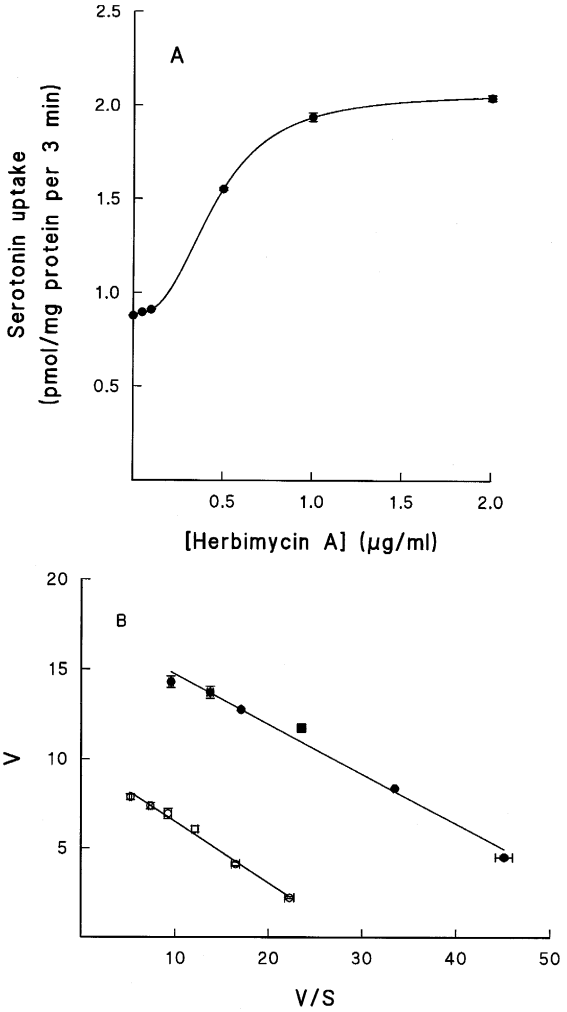

Fig. 3. (A) Dose-response relationship for the influence of herbimycin A on the expression of the serotonin transporter and (B) influence of herbimycin A treatment on the saturation kinetics of the serotonin transporter. (A) Cells were treated with increasing concentrations of herbimycin A (0–2 μg/ml) for 16 h a 37°C, following which the medium was removed, the cells were washed and imipramine-sensitive serotonin (50 nM) transport was measured (3 min incubation). (B) Cells which were treated with (●) or without (○) herbimycin A (1 μg/ml) for 16 h at 37°C were used for measurement of imipramine-sensitive serotonin transport. Concentration range for serotonin was 0.1–1.5 μM and incubation time was 3 min. Results are given as Eadie-Hofstee plots (V/S versus V). V, serotonin transport in pmol/mg of protein per 3 min; S, serotonin concentration in μM.

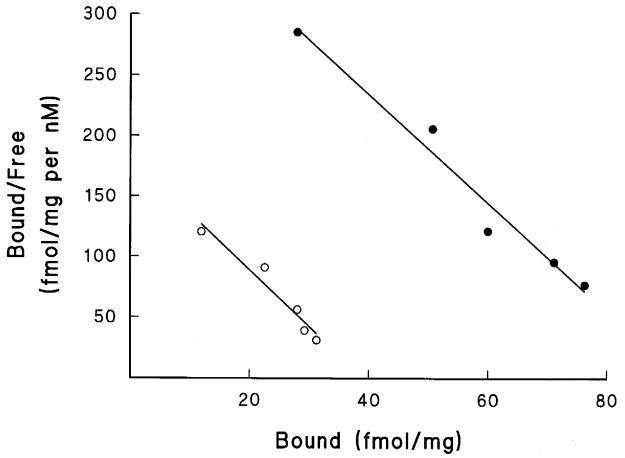

Fig. 4. Influence of herbimycin A (1 μg/ml, 16 h treatment) on the kinetics of [^{125}I]RTI-55 binding. Plasma membranes were prepared from control and herbimycin A-treated cells and [^{125}I]RTI-55 binding to the membranes was measured over an RTI-55 concentration range of 0.1–1 nM. Results given as Scatchard plots (○, control; ●, herbimycin A) represent only the paroxetine-sensitive binding.

Recently, Helmeste and Tang (1995) reported that serotonin uptake in human platelets is inhibited by tyrosine kinase inhibitors. This effect is however distinct from the effect observed in JAR cells. In the case of platelets, herbimycin A as well as other tyrosine kinase inhibitors reduced serotonin uptake. Thus, there is no difference between herbimycin A and other tyrosine kinase inhibitors in their effect on the platelet serotonin transporter in contrast to what was observed in our present study with the JAR cell serotonin transporter. In addition, the inhibition of the platelet transporter by herbimycin A and other inhibitors is observed within 5 min treatment time and there is no change in the serotonin transporter density as a result of treatment. Since platelets are nonnucleated cells, the inhibitory effects of herbimycin A and other tyrosine kinase inhibitors on serotonin uptake apparently involve mechanisms other than changes in gene expression.

3.3. Blockade of the herbimycin A effect on the serotonin transporter by genistein

Prompted by the findings that the influence of herbimycin A on neurite outgrowth in cerebellar granule cells is abolished by other tyrosine kinase inhibitors (Doherty et al., 1994), we investigated the influence of genistein on herbimycin A-induced stimulation of the serotonin transporter activity in JAR cells. The results of the experiments

Table 2
Blockade of herbimycin A-induced serotonin transporter expression by genistein

Treatment	Serotonin transport	
	pmol/mg of protein per 3 min	%
None	1.12 ± 0.01	100
Herbimycin A	1.94 ± 0.03	173
Genistein	0.88 ± 0.01	79
Herbimycin A plus genistein	0.77 ± 0.01	69

Confluent monolayer cultures of JAR cells were treated for 16 h at 37°C as follows: (a) no addition, (b) 1 μg/ml herbimycin A, (c) 75 μM genistein and (d) 75 μM genistein plus 1 μg/ml herbimycin A. Following the treatment, the medium was removed and the cells were washed with the transport buffer. Imipramine-sensitive transport of serotonin (50 nM) was then measured in these cells with a 3 min incubation.

showed that while treatment of the cells with herbimycin A led to a marked stimulation of serotonin transport activity, cotreatment with genistein abolished the stimulation completely (Table 2).

3.4. Influence of herbimycin A on steady-state levels of the serotonin transporter mRNA

The JAR cells were treated under four different conditions (control, genistein, herbimycin A and herbimycin A plus genistein) and steady-state levels of the serotonin transporter mRNAs were analyzed by Northern blot hybridization (Fig. 5). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA levels served as internal control

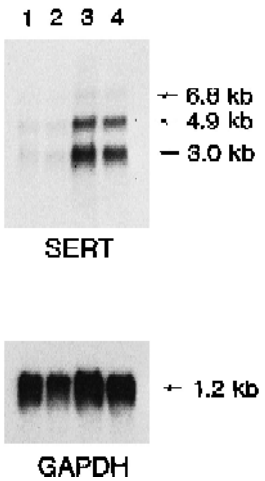

Fig. 5. Influence of herbimycin A and genistein on steady-state levels of serotonin transporter mRNAs. Poly(A)$^+$ RNA was prepared from control cells (lane 1) and from cells treated for 16 h at 37°C with genistein (lane 2), herbimycin A (lane 3) and herbimycin A plus genistein (lane 4). Concentration of genistein and herbimycin A was 75 μM and 1 μg/ml respectively. mRNA was size-fractionated by denaturing formaldehyde-agarose gel electrophoresis, transferred onto a nylon membrane and probed with the serotonin transporter (SERT) cDNA and with the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA by sequential hybridization.

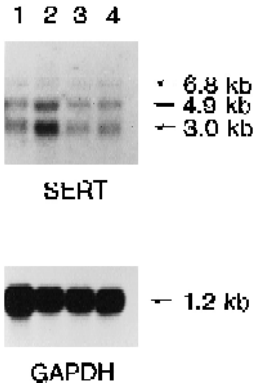

Fig. 6. Influence of actinomycin D on herbimycin A-induced increase in serotonin transporter mRNAs. Poly(A)$^+$ RNA was prepared from control cells (lane 1) and from cells treated for 16 h at 37°C with herbimycin A (lane 2), actinomycin D (lane 3) and herbimycin A plus actinomycin D (lane 4). Concentration of actinomycin D and herbimycin A was 0.03 μg/ml and 1 μg/ml respectively. mRNA was size-fractionated on denaturing formaldehyde-agarose gel electrophoresis, transferred onto a nylon membrane and probed with the serotonin transporter (SERT) cDNA and with the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA by sequential hybridization.

for RNA loading and transfer efficiency in these experiments. Treatment of the cells with herbimycin A resulted in a 4- to 6-fold increase in the levels of all three transcripts (6.9 kb, 4.8 kb and 3.0 kb) of the serotonin transporter mRNA. Genistein by itself had no significant effect on the transcript levels. Interestingly, even though genistein completely blocked the herbimycin A-induced increase in the serotonin transporter activity (Table 2), the increase in mRNA levels induced by herbimycin A was not affected by genistein. In contrast, the effect of herbimycin A on the mRNA levels was completely blocked in the presence of actinomycin D, a transcription inhibitor (Fig. 6). These results show that the herbimycin A-mediated increase in serotonin transporter activity is accompanied by an increase in the steady-state levels of the serotonin transporter mRNA, contributing to an increase in the serotonin transporter density and that the observed increase in the mRNA levels is due to transcriptional activation. However, the increase in the mRNA levels is much greater than the increase in the transporter activity and the transporter density (4- to 6-fold versus 2- to 2.5-fold). It is also apparent from these results that the site of action of genistein in this pathway lies somewhere beyond the mRNA stage in the de novo synthesis of the transporter protein.

3.5. Influence of herbimycin A on protein tyrosine phosphorylation

The ability of genistein to block the effect of herbimycin A on the serotonin transporter activity suggests that herbimycin A might mediate its effect by enhancing protein tyrosine phosphorylation in JAR cells. To test this

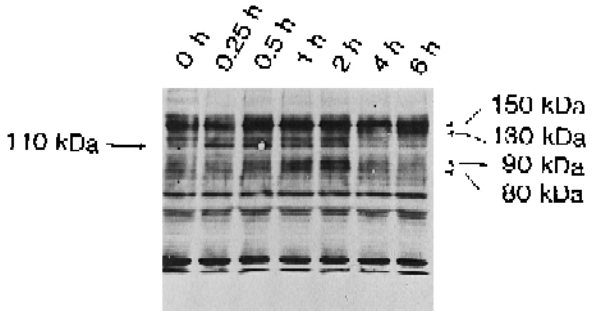

Fig. 7. Influence of herbimycin A on the phosphotyrosine content of cellular proteins. Confluent cultures of JAR cells were treated with herbimycin A (1 μg/ml) for 0–6 h following which phosphotyrosine-containing proteins in the cell extract were detected by immunoblotting using the PY20 anti-phosphotyrosine antibody. The immunoreactive proteins were detected using the Enhanced ChemiLuminescence method.

possibility, JAR cells were treated with herbimycin A for varying time periods (0–6 h), following which phosphotyrosine-containing proteins in the cell extract were analyzed by immunoblot using anti-phosphotyrosine antibody (Fig. 7). Several proteins were found to have increased phosphotyrosine content following treatment of the cells with herbimycin A and the temporal patterns varied significantly among these proteins. The earliest increase in phosphotyrosine content was noted in a 110 kDa protein which occurred within 15 min of herbimycin A treatment. The phosphotyrosine content in three other proteins (150, 90 and 80 kDa) also increased as a result of herbimycin A treatment, but at a much later time (0.5–2 h). The phosphotyrosine content of a 130 kDa protein was found to increase only after 6 h treatment. Even though the identity of these phosphotyrosine-containing proteins is not known, the results show that herbimycin A enhances tyrosine phosphorylation of specific proteins in these cells. It is likely that this effect is functionally linked to the observed increase in the serotonin transporter expression following treatment of the cells with herbimycin A.

The findings of the present study can be summarized as follows: (a) treatment of the JAR placental choriocarcinoma cells with herbimycin A, an inhibitor of tyrosine kinases, activates the expression of the serotonin transporter gene and (b) the inductive effect of herbimycin A is blocked by genistein, another inhibitor of tyrosine kinases. These findings unequivocally establish an essential role for tyrosine phosphorylation in the expression of the serotonin transporter gene. The opposing effects of herbimycin A and genistein on the serotonin transporter expression are of interest because both these agents are inhibitors of tyrosine kinases. Genistein is a competitive inhibitor of ATP in the tyrosine kinase reaction. Since the ATP binding site is conserved among various tyrosine kinases, genistein is capable of inhibiting a broad spectrum of tyrosine kinases. In contrast, herbimycin A irreversibly blocks several src-family members of tyrosine kinases (Uehara et al., 1989; June et al., 1990; Fukazawa et al., 1991) and receptor tyrosine kinases (Sepp-Lorenzino et al., 1995). There is evidence that herbimycin A irreversibly interacts with these tyrosine kinase proteins and targets them for proteasome- and ubiquitin-dependent degradation (Uehara et al., 1989; Sepp-Lorenzino et al., 1995). The results of the present study clearly show that tyrosine phosphorylation of specific proteins in JAR cells is increased by treatment of the cells with herbimycin A. Increased phosphotyrosine content of specific proteins upon treatment with herbimycin A has been demonstrated in other cell types (Doherty et al., 1994). However, there is no evidence for direct activation of any tyrosine kinase by herbimycin A. The findings that herbimycin A can increase the phosphotyrosine content of some proteins and that this effect can be blocked by genistein strongly suggest that herbimycin A may indirectly lead to activation of a specific tyrosine kinase by inhibiting other tyrosine kinases which negatively regulate the specific tyrosine kinase. One example of this kind of regulation is the inhibition of the $pp60^{c\text{-}src}$ tyrosine kinase by a recently cloned tyrosine kinase (Nada et al., 1991). The opposing effect of genistein would then indicate differential sensitivity of the involved tyrosine kinases to different tyrosine kinase inhibitors. The identity of the tyrosine kinase that is activated by herbimycin A and participates in the regulation of the serotonin transporter expression remains to be determined.

Acknowledgements

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