Tyrosine Phosphorylation Regulates Rapid Endocytosis in Adrenal Chromaffin Cells

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Secretion of neurotransmitter at the synapse and in secretory cells depends on the availability of vesicles for exocytosis. Rapid endocytosis is responsible for initiating local vesicle recycling and is essential during sustained neurotransmission. Although exocytosis is triggered by Ca$^{2+}$ influx and modulated by serine/threonine kinases, relatively little is known about the regulation of rapid endocytosis. Our data suggest that rapid endocytosis is controlled by tyrosine phosphorylation. Treatment of bovine adrenal chromaffin cells with tyrphostin 23, a protein tyrosine kinase inhibitor, dramatically slowed the time course of rapid endocytosis. In contrast, there was no effect on either the amount or rate of exocytosis. Application of orthovanadate, Zn$^{2+}$, or poly(Glu, Tyr) (4:1), each of which is a tyrosine phosphatase inhibitor, reversed the effect of tyrphostin 23 on rapid endocytosis. Thus rapid endocytosis, like exocytosis, is subject to regulation by intracellular signaling pathways.

Key words: rapid endocytosis; tyrosine phosphorylation; adrenal chromaffin cells; exocytosis; tyrphostin 23; capacitance measurements

The plasma membrane of neurosecretory cells is in a state of dynamic equilibrium in which exocytosis is balanced by membrane reuptake via endocytosis (Betz et al., 1992; Ryan and Smith, 1995; Stevens and Tsujimoto, 1995; Betz and Angleon, 1998). Disruption of endocytosis is lethal in the Drosophila mutant shibire as a result of a defect in dynamin, a GTPase involved in the final steps of endocytosis (Chen et al., 1991). Recent work has suggested that the activity of proteins involved in rapid endocytosis, as in exocytosis, is determined by their phosphorylation state (Neher and Zucker, 1993; Robinson et al., 1994; Henkel and Betz, 1995; Vitale et al., 1995; Smith et al., 1998). Dynamin, for example, is phosphorylated by protein kinase C and may undergo stimulus-dependent dephosphorylation by calcineurin (Liu et al., 1994, 1996). The GTPase activity of dynamin, crucial for exocytosis, is inhibited by dephosphorylation [Robinson et al. (1993); but see Sever et al. (1999) for an alternate point of view]. The dephosphorylation of dynamin, synaptojanin, and amphiphysin enhances their formation into complexes (Bauerfeind et al., 1997; Slepnev et al., 1998). Calcineurin, a Ca$^{2+}$- and/or calmodulin-dependent phosphatase, promotes rapid endocytosis in some studies but acts as an inhibitor in others (Artalejo et al., 1996; Kuromi et al., 1997; Engisch and Nowycky, 1998; Marks and McMahon, 1998). Thus, protein kinase activity may represent a means by which endocytosis is coupled to excitation.

Ligand-dependent endocytosis, which is not coupled to secretion, is known to be regulated via tyrosine phosphorylation. Autophosphorylation of receptor tyrosine kinases initiates a cascade that leads to clathrin-mediated membrane internalization (Yarden and Schlessinger, 1987; Chang et al., 1993). Recently it was shown that dynamin, required for both ligand-dependent endocytosis and rapid endocytosis, undergoes tyrosine phosphorylation after activation of the insulin receptor (Baron et al., 1998). Likewise, eps15, which may also be involved in endocytosis, is tyrosine phosphorylated by the epidermal growth factor (EGF) receptor kinase (Benmerah et al., 1995).

To determine whether rapid endocytosis is regulated in a manner similar to ligand-dependent endocytosis, we treated adrenal chromaffin cells with inhibitors of tyrosine kinases and phosphatases and observed the effects on rapid endocytosis. The tyrosine kinase inhibitor tyrphostin 23 (Yaish et al., 1988) caused a dramatic slowing in the kinetics of rapid endocytosis. The slowing could be reversed via the addition of orthovanadate, Zn$^{2+}$, or poly(Glu, Tyr) (4:1), which are tyrosine phosphatase inhibitors (Swarup et al., 1982; Tonks et al., 1988; Walton and Dixon, 1993). The effect of tyrosine phosphorylation was specific to rapid endocytosis, because exocytosis was unaffected. In addition, tyrosine phosphorylation altered the kinetics but not the amount of rapid endocytosis. Our data suggest that rapid endocytosis is the specific target of one or more tyrosine kinase regulatory pathways.

MATERIALS AND METHODS

Cell culture. Bovine adrenal chromaffin cells were prepared from 18-week-old animals as described previously (Artalejo et al., 1992). Adrenal glands were obtained from a local abattoir, digested with collagenase, and purified by density gradient centrifugation. Cells were plated with 10 μM arabinoside at a density of 0.15 × 10⁶ cells cm⁻² on collagen-coated glass coverslips and maintained for up to 96 hr in an incubator at 37°C in an atmosphere of 92.5% air and 7.5% CO₂ with a relative humidity of 90%. One-half of the incubation medium was changed every day. Experiments were performed 24–96 hr after preparing the cells. Cultures included both adrenaline- and noradrenaline-containing cells, although adrenaline constituted 65% of the total catecholamine content.

Electrophysiology. Stimulation consisted of a train of 10 depolarizations to either +10 or +20 mV (as noted) from a holding potential of −90 mV. Depolarizations lasted 160 msec and were separated by an interpulse interval of 380 msec. Capacitance was measured using the phase-tracking technique (Joshi and Fernandez, 1988; Fidler and Fernandez, 1989), in which a 60 mV peak-to-peak sine wave applied at 1.4 kHz was...
Significance tests were calculated using Student’s value. If this value was negative, the plateau time was defined as 0 sec. The exponential decay fitting function reached the same capacitance difference between the time of peak capacitance and the time at which isolated backward in time until its capacitance was equal to the peak capacitance value during exocytosis. The amount of membrane retrieval approximately matched the total exocytosis. The time course of rapid endocytosis was fit with either one or two exponentials during the falling phase was fit as a double exponential with time constants of 5.8 sec and 59 sec. To measure the plateau phase, we estimated total exocytosis and retrieval because of overlap between exocytosis and rapid endocytosis. In this experiment, the amount of membrane retrieval approximately matched the amount of exocytosis. The time course of rapid endocytosis during the falling phase was fit as a double exponential with time constants of 5.8 sec and 59 sec. To measure the plateau phase, we calculated the difference between the time at which the capaci- tance trace peaked and the time at which the double exponential used to fit rapid endocytosis reached the same capacitance value. By the use of this procedure, the calculated “plateau time” in Figure 1A was 4 sec.

Rapid endocytosis was strikingly different in a cell stimulated after 6 min of treatment with the tyrosine kinase inhibitor tyrphostin 23 (100 μM), as shown in Figure 1B. In this case the time course was fit with a single time constant of 26 sec. The plateau time was 41 sec. The longer plateau time is likely to indicate

\[ \text{Plateau} = 41 \text{ s} \]

\[ \tau_1 = 5.8 \text{ s} \]

\[ \tau_2 = 59 \text{ s} \]

\[ 100 \text{ fF} \]

\[ 10 \text{ s} \]

**Figure 1.** Tyrophostin 23 slowed the kinetics of rapid endocytosis. Exocytosis and rapid endocytosis were monitored using the capacitance technique on whole-cell patch-clamped bovine adrenal chromaffin cells. A, Capacitance recording of a control cell that underwent exocytosis during stimulation. Rapid endocytosis began after a short plateau phase. B, Capacitance recording of a cell treated with 100 μM tyrphostin 23, a protein tyrosine kinase inhibitor. Exocytosis was similar to that in the control cell, but the plateau time was longer, and the fast time constant of rapid endocytosis was slower. Exponential fits to the data are indicated by the solid lines.
either a delay in the triggering of rapid endocytosis or a slowing of its initial components. The amount of endocytosis was approximately equal to that of exocytosis, indicating that tyrphostin 23 primarily affected the kinetics of rapid endocytosis. The maximum rate of exocytosis in this cell was 560 fF/sec, similar to the value observed in Figure 1A. These results suggest that some component of rapid endocytosis is subject to continuous tyrosine phosphorylation, even under basal conditions.

To ensure that the response to tyrphostin 23 observed in Figure 1B was specifically caused by an inhibition of tyrosine kinase activity, we attempted to reverse the response by inhibiting tyrosine phosphatases. Because our results suggest that ongoing tyrosine phosphorylation is necessary for rapid endocytosis, we expected tyrosine phosphatase inhibitors to counteract the effects of tyrosine kinase inhibitors. Figure 2 shows that the changes caused by tyrphostin 23 could be reversed by protein tyrosine phosphatase inhibition. The cell in Figure 2A was pretreated for 25 min with 1 mM orthovanadate, a protein tyrosine phosphatase inhibitor, after which 100 μM tyrphostin 23 was applied for 6 min in the continued presence of orthovanadate. After stimulation, both the time course of rapid endocytosis (time constants of 4.9 and 27 sec) and the plateau time (0 sec) were comparable with those in Figure 1A. A different protein tyrosine phosphatase inhibitor, 250 μM Zn²⁺, was dialyzed into the cell via the intracellular patch pipette (Fig. 2B) while 100 μM tyrphostin 23 was simultaneously applied to the bath. Rapid endocytosis was similar to that in Figure 1A, with time constants of 4.9 and 120 sec. In this experiment the plateau time was 3 sec. In addition, 10 μM poly(Glu, Tyr) (4:1), a peptide that inhibits tyrosine phosphatases, was dialyzed into the cell shown in Figure 2C before application of tyrphostin 23. Rapid endocytosis had time constants of 2.9 and 54 sec. The plateau time was 2 sec. Thus, when protein tyrosine phosphatases were inhibited, tyrphostin 23 no longer altered either the kinetics of rapid endocytosis or the plateau phase.

The findings related to tyrosine phosphorylation are summarized in Figure 3. Rapid endocytosis was fit with either a single or double exponential; the data describing the fastest time constant are plotted in Figure 3A. The mean fast time constant of rapid endocytosis in control cells was 12 ± 3 sec (n = 17). In the presence of tyrphostin 23, the fast time constant was 36 ± 5 sec (n = 22), a value that is significantly longer than the control value (p < 0.002). Tyrosine phosphatase inhibitors reversed this increase. Rapid endocytosis had an average fast time constant of 13 ± 3 sec when tyrphostin 23 was applied with orthovanadate (n = 20), 9 ± 4 sec when tyrphostin 23 was applied in the presence of Zn²⁺ (n = 6), and 16 ± 5 sec when tyrphostin 23 was applied with poly(Glu, Tyr) (4:1) (n = 6). (Only 71% of control cells required two time constants to fit endocytosis successfully, whereas the rest were well fit with a single time constant. In the cells that required a second slow time constant the average slow time constant was 105 ± 22 sec).

The mean plateau time in control cells was 0.8 ± 0.3 sec, as shown in Figure 3B (n = 17). In the presence of tyrphostin 23 the mean plateau time was 23 ± 5 sec (n = 22), a value significantly longer than that in control cells (p < 0.0006). In contrast, cells cotreated with tyrphostin 23 and orthovanadate had a mean plateau time of 0.7 ± 0.3 sec (n = 20). In cells cotreated with tyrphostin 23 and Zn²⁺ the mean plateau time was 2.2 ± 0.6 sec (n = 6), whereas cells cotreated with tyrphostin 23 and poly(Glu, Tyr) (4:1) had a mean plateau time of 8.7 ± 8.1 sec (n = 6). The ability of tyrosine phosphatase inhibitors to reverse the effects of tyrphostin 23 suggests that its actions were mediated by tyrosine phosphorylation.

By themselves the tyrosine phosphatase inhibitors did not affect rapid endocytosis. Figure 4A shows that cells treated with poly(Glu, Tyr) (4:1) had average fast time constants for rapid endocytosis that were not different from that of control cells. Figure 4B shows that the amount of endocytosis in poly(Glu, Tyr) (4:1)-treated cells was not different from that of control cells. Our data suggest that the effect of tyrosine phosphatase inhibitors was dependent on treating cells with tyrphostin 23 and reinforce the hypothesis that the actions were mediated by tyrosine phosphorylation.
Tyrosine kinase inhibition does not affect the amount of membrane retrieval or exocytosis

Although tyrphostin 23 had striking effects on the kinetics of rapid endocytosis, there was little effect on membrane retrieval. As shown in Figure 5A, the average amount of membrane retrieval during the falling phase of the capacitance trace was 480 ± 60 fF (n = 17) in control cells. In the presence of tyrphostin 23, the amount of rapid endocytosis was not significantly different (500 ± 80 fF; n = 24). Tyrphostin 23 also had no effect on either the amount or kinetics of exocytosis, as shown in Figure 5, B and C. Exocytosis in control cells caused a mean capacitance change of 470 ± 80 fF (n = 17; Fig. 5B), corresponding to the fusion of ~360 vesicles assuming that the average capacitance of an individual vesicle is 1.3 fF (Moser and Neher, 1997). The maximum rate of exocytosis was 720 ± 110 fF/sec (~550 vesicles/sec; Fig. 5C). In the presence of tyrphostin 23, exocytosis caused a mean capacitance change of 570 fF (~440 vesicles; n = 24), and its maximum rate was 850 ± 260 fF/sec (~650 vesicles/sec), values not significantly different from control values. Finally, the relationship between the total exocytosis and the amount of membrane retrieval was not affected by tyrphostin 23, as shown in Figure 5D. In both treated and untreated cells, the amount of membrane added to the cell via exocytosis was approximately equal to the amount of membrane retrieved.

The effect of tyrosine phosphorylation on rapid endocytosis is not Ca\(^{2+}\) dependent

The Ca\(^{2+}\) dependence of rapid endocytosis is still unclear (von Gersdorff and Matthews, 1994; Reuter and Porzig, 1995; Artalejo et al., 1996), although two recent reports suggest Ca\(^{2+}\) influx may trigger several components of rapid endocytosis (Smith and Neher, 1997; Engisch and Nowycky, 1998). We found no significant difference in Ca\(^{2+}\) influx between cells treated with tyrphostin 23 and control cells, suggesting that the changes in rapid endocytosis induced by tyrphostin 23 were not mediated by alterations in Ca\(^{2+}\) influx. Figure 6 shows representative Ca\(^{2+}\) currents in a single cell before and after treatment with 100 μM tyrphostin 23; treatment with tyrphostin 23 did not affect the Ca\(^{2+}\) current. Note that the early spike of inward current was carried by Na\(^+\). Although Ca\(^{2+}\) influx in cells treated with tyrphostin 23 and either orthovanadate or Zn\(^{2+}\) was a little larger than that in cells treated with tyrphostin 23 alone, similar to that in smooth muscle cells (Wijetunge et al., 1998), this did not account for the tyrosine phosphatase inhibitor effects. We tested the effect of slightly...
elevated Ca$^{2+}$ influx by using a modified stimulation protocol that maximized Ca$^{2+}$ currents. The test potential during the train of depolarizations was changed to +20 mV, the peak of the $I-V$ curve for Ca channels (the test potential in all the figures shown in this manuscript is +10 mV). The depolarization length, interpulse intervals, and external [Ca$^{2+}$] were otherwise unchanged. This small increase in Ca$^{2+}$ influx did not alter rapid endocytosis in tyrphostin 23–treated cells (data not shown).

In addition to being potent broad spectrum tyrosine kinase inhibitors, tyrphostins are effective inhibitors of certain GTPases (Wolbring et al., 1994). Although the tyrphostin 23–induced slowing of endocytosis is consistent with the inhibition of tyrosine kinases, we wanted to rule out possible effects on GTPases; experiments were performed using GTP-$\gamma$S to inhibit GTPases. Figure 7 shows a capacitance trace obtained from a cell dialyzed with 350 $\mu$M GTP-$\gamma$S.most cells treated in this way showed little or no endocytosis, consistent with a study by Artalejo et al. (1995), which showed that GTP-$\gamma$S inhibited endocytosis. Thus, GTP-$\gamma$S does not mimic tyrphostin 23, which slows endocytosis.
DISCUSSION

Since the discovery that nerve growth factor actions by stimulating the tyrosine kinase activity of a surface membrane receptor (Kaplan et al., 1991; Klein et al., 1991), interest has surged in the role of tyrosine phosphorylation in the nervous system. Receptor tyrosine kinases are now known to have a variety of roles in neuronal growth and development. For example, Eph-related receptor tyrosine kinases play a prominent part in establishing the topography of retinotectal projections (Drescher, 1997). There is also an increasing recognition of the regulatory actions of tyrosine phosphatases, such as DPTP69D. When mutated in Drosophila, this tyrosine phosphatase causes abnormal motor neuron development (Desai et al., 1996; Krueger et al., 1996). Our data indicate another important physiological role for tyrosine phosphorylation. In adrenal chromaffin cells tyrosine phosphorylation helps to regulate rapid endocytosis. Furthermore, this new regulatory function specifically affects the kinetics of rapid endocytosis, while leaving exocytosis unaffected.

Receptor tyrosine kinases have a well-established role in the initiation of ligand-dependent endocytosis. Mutations that reduce the tyrosine kinase activity of the EGF receptor may also inhibit ligand-dependent endocytosis (Lamaze and Schmid, 1995). After undergoing autophosphorylation, the EGF receptor has an increased affinity for the coated pit adapter protein in complex AP2 (Nesterov et al., 1995). The EGF receptor also phosphorylates eps15, an AP2-binding protein that promotes clathrin assembly (Benmerah et al., 1995, 1998). The GTPase dynamin, a key component of endocytosis, undergoes tyrosine phosphorylation after activation of the insulin receptor, although the functional consequences of phosphorylation are unknown (Chen et al., 1991; Baron et al., 1998).

Rapid endocytosis does not require binding of an extracellular ligand, because our fast perfusion system ensured that all potential ligands were rapidly washed away. Nevertheless, rapid endocytosis shares many similarities with ligand-dependent endocytosis. Dynamin, for example, is necessary in both types of endocytosis (Koenig and Ikeda, 1989; Morris and Schmid, 1995). Inhibition of dynamin with GTP-γ-S, which blocked endocytosis in chromaffin cells (Artalejo et al., 1995), an observation consistent with the data in this manuscript. Our work shows that rapid endocytosis, like ligand-dependent endocytosis, is regulated by tyrosine phosphorylation. Treatment of adrenal chromaffin cells with the tyrosine kinase inhibitor tyrphostin 23 slowed the time course of rapid endocytosis. This effect was reversed by the addition of three different tyrosine phosphatase inhibitors, indicating that the slowing of rapid endocytosis was mediated by changes in tyrosine phosphorylation rather than by tyrphostin-mediated changes in GTP metabolism (Wolbring et al., 1994). In contrast to GTP-γ-S, which blocked endocytosis in most cells, tyrphostin 23 slowed rapid endocytosis with no effect on the total amount of membrane retrieval. Slowing of rapid endocytosis was observed soon after application of tyrphostin 23, but there was no effect on the total amount of membrane retrieval. This indicates that tyrosine kinases specifically govern the kinetics, not the amount, of membrane retrieval. The kinetic effects of tyrosine kinases may be crucial under conditions of strong stimulation because rapid endocytosis may be rate-limiting in such circumstances (Klingauf et al., 1998). In contrast, on longer time scales rapid endocytosis may act to maintain surface area homeostasis (Ceccarelli et al., 1972; Heuser and Reese, 1973); our results suggest that tyrosine kinases are less likely to be directly involved in the regulation of this process.

Dynamic tyrosine phosphorylation plays an important part in modulating synaptic efficiency. The inhibition of N-type Ca2+ channels by GABA<sub>B</sub> receptor activation can be partially blocked by treatment with tyrosine kinase inhibitors (Diverse-Pierluissi et al., 1997). Tyrosine kinases also regulate voltage-dependent facilitation of T-type Ca<sup>2+</sup> channels (Arnoult et al., 1997) and Ca<sup>2+</sup> currents in smooth muscle cells (Wijetunge et al., 1998). Although there is evidence of a role for Ca<sup>2+</sup> in the control of rapid endocytosis (Neher and Zucker, 1993; Smith and Neher, 1997; Engisch and Nowacky, 1998), we did not observe significant changes in Ca<sup>2+</sup> influx in chromaffin cells after treatment with tyrphostin 23. Consequently, the slowing of rapid endocytosis by tyrosine kinase inhibition was probably mediated by a novel synaptic regulatory mechanism. This conclusion is supported by the finding that rapid endocytosis was slowed even in experiments specifically designed to increase Ca<sup>2+</sup> influx (data not shown).

Neither the rate nor the amount of exocytosis was significantly affected by tyrphostin 23. Because exocytosis is known to be a Ca<sup>2+</sup>-dependent process, this is consistent with our observations that Ca<sup>2+</sup> influx was not affected by tyrosine kinase inhibition. These findings are especially intriguing because a study by Cox et al. (1996) showed that tyrphostin 23 and other tyrosine kinase inhibitors significantly reduced catecholamine secretion by adrenal chromaffin cells. It is important to note that the stimulations in their work were approximately two orders of magnitude longer than those described in this paper. Long-term failure of catecholamine secretion after treatment with tyrphostin 23, which quickly interferes with rapid endocytosis, suggests that competent endocytosis may be necessary for cells to maintain exocytosis for extended periods, and interference with endocytosis may impair the mobilization of filled granules.

At least two kinetic parameters were altered by tyrphostin 23: the time constant of rapid endocytosis and the plateau time. The plateau phase most likely reflects a slowing or delay in the onset of rapid endocytosis. Alternately, the longer plateau time might reflect an upregulation of exocytosis that was precisely balanced by rapid endocytosis. The latter hypothesis seems unlikely because longer plateau times were associated with slower time constants of rapid endocytosis, and there was no evidence of a change in exocytosis during the rising phase of the capacitance trace. Furthermore, a recent study has shown that neurotransmitter release ends <1 sec after stimulation (Albillos et al., 1997). These findings argue that the primary effect of tyrphostin 23 was on the kinetics of rapid endocytosis, although the possibility remains that multiple regulatory components of rapid endocytosis are tyrosine phosphorylated.

What is the target of tyrosine phosphorylation in rapid endocytosis? It is tempting to speculate that dynamin is tyrosine phosphorylated in rapid endocytosis as well as in ligand-dependent endocytosis. Alternately, our observations may reflect a requirement for the phosphorylation of other proteins, such as eps15. Finally, although the kinase(s) required for rapid endocytosis has not yet been identified, many proteins involved in rapid endocytosis interact with src, including dynamin, synapsin Ia, and synapsin Ib (Onofri et al., 1997; Foster-Barber and Bishop, 1998). Src is thought to promote ligand-dependent endocytosis (Ware et al., 1997), and the src family kinase fyn is activated during...
stimulation in adrenal chromaffin cells (Allen et al., 1996). Thus, activation of src family kinases might represent a common pathway for ligand-dependent endocytosis and rapid endocytosis.

REFERENCES


