Nerve Growth Factor-Induced Differentiation Changes the Cellular Organization of Regulated Peptide Release by PC12 Cells

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PC12 cells, like endocrine chromaffin cells, undergo neuronal-like differentiation in response to nerve growth factor (NGF). Here we report that this phenotype conversion produces major changes in release of a green fluorescent protein-tagged neuropeptide–hormone. First, the spatial distribution of the releasable pool is altered; peptide release from untreated cells is supported predominantly by membrane-proximal vesicles, whereas a diffuse pool at the ends of processes is used by NGF-treated cells. Second, the time course of release evoked by photolysis of caged Ca^{2+} is faster after differentiation. High-resolution measurements suggest that a slow step before

membrane fusion dominates the kinetics of release in untreated cells. Finally, the effect of actin microfilament depolymerization on total release is altered by NGF treatment. This implies that the mechanism that limits the size of the releasable pool is altered by phenotype conversion. Therefore, the cellular organization of peptide release is plastic and changes in response to NGF. This flexibility may be used to generate cell-specific release properties.

Key words: neuropeptide release; hormone release; GFP; caged calcium; actin; secretory vesicle; neuronal differentiation; releasable pool

Traditionally, neuropeptide secretion mechanisms have been deduced indirectly from biochemical or electrophysiological release measurements. However, recently it has become possible to use green fluorescent protein (GFP)-based imaging approaches to follow the trajectories of secretory granules in live PC12 cell growth cones (Burke et al., 1997; Abney et al., 1999; Han et al., 1999b). This has facilitated the study of neuropeptide release by directly imaging intracellular and, for the first time, intravesicular (Han et al., 1999a) events. For example, studies with GFP-tagged peptides have demonstrated that neuropeptide release is dominated by recruitment of a diffuse pool of slowly moving secretory vesicles (Burke et al., 1997; Han et al., 1999b). This explains how neuropeptide release can proceed with few initially docked secretory vesicles (Leenders et al., 1999; Karhunen et al., 2001). However, a large pool of membrane-proximal vesicles supports the first minutes of stimulated exocytosis by chromaffin cells (Steyer et al., 1997; Oheim et al., 1998). This difference may underlie variations found among neurons (Whim and Lloyd, 1994; Ohnuma et al., 2001) or could reflect phenotype-dependent cellular organization of peptide release.

Interestingly, the phenotype of PC12 cells, like adrenal chromaffin cells, can be converted by nerve growth factor (NGF) from an endocrine-like state to a sympathetic neuron-like state (Tis-

chler and Greene, 1980; Doupe et al., 1985). Ultrastructure revealed that NGF-induced PC12 processes contain dense core granules (Greene and Tischler, 1976). However, electron microscopy studies could not follow depletion of secretory granules in real time. Therefore, we used fluorescence microscopy to test whether differentiation with NGF changes release from a recently developed PC12 cell clone (here called ANF-GFP cells) that constitutively expresses GFP-tagged proANF (atrial natriuretic factor) (Han et al., 1999b). This construct is particularly appealing because ANF (also known as atrial natriuretic peptide, ANP) is secreted by neurons and endocrine cells (Gutkowska et al., 1997). Therefore, its targeting to peptidergic secretory granules should not be affected by differentiation. In addition to studying the distribution of the releasable pool, the effects of uncaging Ca^{2+} and depolymerizing filamentous actin (F-actin) were measured. These studies reveal major changes in peptide release with NGF treatment. Thus, live cell imaging establishes that the cellular organization of release is plastic.

MATERIALS AND METHODS

Cells and constructs. The ANF-GFP PC12 clone that constitutively expresses Emerald GFP-tagged proANF was described recently (Han et al., 1999b). Cell culture was performed in the presence or absence of NGF on poly-lysine-coated coverslips for 2 or 3 d in accordance with previous reports (Burke et al., 1997; Han et al., 1999a,b). For imaging experiments, the extracellular medium contained (in mM): 140 NaCl, 5.4 KCl, 5 CaCl_2 , 0.8 MgCl_2 , 10 glucose, and 10 NaHEPES, pH 7.5. Release was evoked by addition of 10 μM Br-A23187, replacement of 100 mM Na^+ with K^+ , replacement of 100 mM Na^+ with K^+ and Ca^{2+} with Ba^{2+} , or by photolysis of caged Ca^{2+} (see below).

Epifluorescence and confocal microscopy. Conventional epifluorescence experiments were performed on a Nikon (Tokyo, Japan) Diaphot inverted microscope equipped with a 75 W xenon lamp and a shuttered filter wheel. A 60 $\times$ 1.4 numerical aperture (NA) Olympus Optical (Tokyo, Japan) oil immersion objective was used for measuring release evoked by depolarization or the ionophore. Standard fluorescein optics were used when only GFP was imaged. For experiments with Ca^{2+}

Received Dec. 19, 2001; revised Feb. 21, 2002; accepted March 1, 2002.

This research was supported by National Institutes of Health Grants R01 GM53395 (G.C.R.E.-D.) and R01 NS32385 and an Established Investigator Award from the American Heart Association (E.S.L.). We thank Dr. G. Romero for his comments.

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measurements, a 40× UV/340 1.3 NA Olympus oil immersion objective was used along with a fura/fluo dichroic mirror (Chroma Technology, Brattleboro, VT), and excitation was varied between 480, 340, and 380 nm. Photolysis was accomplished with a 350 ± 30 nm excitation filter from Omega Optical (Brattleboro, VT). Epifluorescence data were collected with a cooled CCD camera (Photometrics, Tucson, AZ) and analyzed with Ratiotool and Isee software (Inovision, Raleigh, NC). Single wavelength confocal experiments were performed on a Molecular Dynamics (Sunnyvale, CA) 2001 scanning laser confocal microscope. Confocal experiments with GFP and FM4-64 used fluorescein and rhodamine optics, respectively, of a Leica (Nussloch, Germany) NT TCS scanning laser confocal microscope. A $100\times$ 1.4 NA objective and a $0.05 \mu\text{m}$ pixel size were used for these experiments. All imaging experiments were performed at room temperature.

Ratiometric Ca^{2+} measurements and photolysis of dimethoxynitrophenyl-EGTA-4. Initially, the Ca^{2+} indicator fura-2 FF was used. However, this indicator responds to both Ca^{2+} and Mg^{2+} . The sensitivity to Mg^{2+} is not problematic for $[\text{Ca}^{2+}]$ measurements when the concentration of Mg^{2+} is fixed and known. However, fura-2 measurements in the presence of Ca^{2+} chelators showed that the concentration of Mg^{2+} was different in growth cones than in the dialyzing pipette solution. Therefore, fura-2 was only used for qualitative assessments of $[\text{Ca}^{2+}]$.

Quantitative Ca^{2+} measurements were performed with the Mg^{2+} -insensitive Ca^{2+} indicator fura-2 FF. Calibration of the indicator was based on the equation from Grynkiewicz et al. (1985), with a K_d of $10 \mu\text{M}$: $[\text{Ca}^{2+}] = (K_d)(F_{\text{max}}/380/F_{\text{min}}/380)(R - R_{\text{min}})/(R_{\text{max}} - R)$, where R is the ratio of the fluorescence signals obtained at 340 and 380 nm (F_{340}/F_{380}), max refers to data obtained from a solution containing $10 \text{ mM } \text{Ca}^{2+}$, and min refers to *in vivo* data obtained with a solution with $<5 \text{ nM}$ free Ca^{2+} . R_{min} did not change with photolysis of dimethoxynitrophenyl-EGTA-4 (DMNPE-4) and only differed by 5% when measured in dialyzed cells or in a droplet on a coverslip. The latter method was used to determine max values.

Photolysis illumination was provided by the epifluorescence light source. Dialysis with a patch pipette solution (in mM: 90 K-aspartate, 10 KCl, 3 MgATP, 0.3 GTP, 0.5 MgCl_2 , 5 glutathione, 10 $\text{K}_2\text{DMNPE-4}$, 5 CaCl_2 , 1 $\text{K}_4\text{fura-2 FF}$, and 42 K-HEPES, pH 7.2) was used for intracellular delivery of the Ca^{2+} indicator and caged Ca^{2+} . The cell was voltage clamped to -70 mV in the whole-cell configuration for at least 5 min before photolysis. Illumination times were typically 400 msec for uncaging (350 nm) and 50 msec for GFP (480 nm) and fura-2 FF (340 and 380 nm). One complete cycle of GFP and Ca^{2+} measurements was completed every 2 sec.

Some experiments used a flash lamp light source (Rapp Optoelektronik, Hamburg, Germany) for uncaging within 1 msec. Output of the lamp was collected with a quartz fiber optic whose output was focused on to the cells with a beam probe (Oriol, Madison, WI). To ensure robust increases in $[\text{Ca}^{2+}]$, the pipette solution contained $8 \text{ mM } \text{CaCl}_2$. A Hamamatsu (Bridgewater, NJ) cooled CCD camera collected data at 16 Hz with continuous GFP illumination. Then a set of fura-2 FF ratios was measured ~ 14 sec after discharging the flash lamp. To take into account photobleaching, a GFP response to a flash was recorded before patch clamping. This response was then subtracted from subsequent data obtained after whole-cell patch clamping.

Texas Red-X phalloidin labeling of F-actin. Mycalolide B was dissolved in DMSO to yield a 2 mM stock solution. Cells were treated with vehicle or $2 \mu\text{M}$ mycalolide B for 30 min at room temperature. They were then fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, incubated with 33 nM Texas Red-X phalloidin (Molecular Probes, Eugene, OR), washed twice with PBS, and viewed with standard rhodamine optics by epifluorescence microscopy.

For all experiments described above, statistical significance was measured with the *t* test. Error bars show SEM.

RESULTS

Distribution of releasable secretory granules

Confocal microscopy was used to examine the distribution of GFP-tagged ANF in control untreated and NGF-differentiated clonal ANF-GFP cells. Horizontal and vertical scans reveal that untreated cells are round and display an abundance of fluorescence near the cell surface ($n = 5$) (Fig. 1A). Besides this characteristic ring of fluorescence, punctate fluorescence is evident throughout the cytoplasm that likely is produced by secre-

Figure 1. NGF-induced differentiation of ANF-GFP PC12 cells alters the intracellular distribution of a secreted neuropeptide-hormone. Horizontal (left) and vertical (right) confocal sections are shown from an untreated (A) and an NGF-treated (B) ANF-GFP cell. Scale bars, $2 \mu\text{m}$.

tory vesicles. In transiently transfected cells, large concentrations of fluorescence are found in a region near the nucleus that likely reflects peptide that has not yet been packaged into secretory vesicles (e.g., peptide that is in the Golgi apparatus). However, such fluorescence localization was not evident in stable ANF-GFP cells indicating that very little nonvesicular fluorescent peptide is present at steady state. This is expected because secretory vesicles have a very long half-life and, thus, dominate steady-state expression.

Horizontal and vertical confocal scans also show that peptide distribution is not affected by a 1 hr treatment with NGF, which is too short to induce phenotype conversion. Furthermore, ANF-GFP cells treated with NGF for 2 or 3 d still display punctate fluorescence in the cell body cytoplasm (Fig. 1B). However, the characteristic ring of fluorescence is absent. Instead, fluorescence is marked at the ends of processes ($n = 5$). Thus, neuronal-like differentiation causes a redistribution of secretory vesicles away from the cell surface of the cell body into the ends of processes.

This redistribution led us to examine the spatial organization of the releasable peptide pool with respect to the plasma membrane in untreated and NGF-treated cells. Time lapse confocal microscopy was used to measure depletion of peptidergic secretory granules in live ANF-GFP cells. Figure 2A shows that membrane-proximal granules in untreated cells are markedly depleted during stimulation of release by a Ca^{2+} ionophore ($n = 7$). Similar results are found with depolarization with Ba^{2+} ($n = 5$), indicating that the releasable pool is spatially delimited regardless of the stimulus. Indeed, measurements from regions of interest show that depletion is far less efficient and more delayed from the bulk cytoplasm (Fig. 2B). To determine the position of releasable secretory vesicles relative to the cell surface, the outer leaflet of the plasma membrane of untreated cells was labeled with the red fluorescent dye FM4-64. Although it is not possible to image the actual thickness of the plasma membrane because of diffraction, this limit does not apply to localizing the center of the subresolution membrane. These measurements show that peak GFP fluorescence is $59 \pm 24 \text{ nm}$ from the membrane, indicating

Figure 2. Depletion of peptidergic secretory granules in untreated ANF-GFP cells. *A*, Horizontal confocal images showing peptide depletion evoked by the Ca^{2+} ionophore Br-A23187. Note the decrease in the characteristic ring of fluorescence with little change in the cytoplasm. Scale bar, 2 μm . *B*, Time course of depletion from regions of interest in confocal images (see *inset*) near the plasma membrane (*Region 1*, open circles) and from the cytoplasmic region of untreated cells (*Region 2*, filled circles) after K^+ depolarization with Ba^{2+} (indicated with bar) ($n = 5$). *C*, Quantification of peptide fluorescence relative to the plasma membrane in nine regions of interest from five cells. Note that depletion after 2 min of stimulation (filled circles) is most marked near the plasma membrane when compared with controls (open circles). In accordance with the results in *B*, some depletion is seen farther away from the cell surface after 10 min ($\times$ symbols).

that secretory vesicles are very close to the cell surface in live, untreated cells. Furthermore, GFP-tagged ANF loss using the plasma membrane label as a landmark revealed that depletion is initially most robust within 0.5 μm of the cell surface (Fig. 2*C*). Similar results were obtained after a 1 hr exposure to NGF, indicating that the distribution of the releasable pool is not

acutely regulated by NGF. Thus, peptide release from undifferentiated ANF-GFP cells is supported initially and predominantly by vesicles that are very close to the plasma membrane.

This spatially delimited depletion was then compared with the release from the same clonal cells after differentiation. We reasoned that, if vesicles within a half micrometer of the membrane in growth cones dominate release (i.e., as is found in untreated cells), depletion of this ring of fluorescence would be detectable because the axial (i.e., z -axis) resolution of our confocal instrument is much less than the thickness of the growth cones studied here. However, both horizontal xy (Fig. 3*A,B*) and vertical xz (Fig. 3*C,D*) confocal scans showed that depletion of peptide from ANF-GFP cell growth cones is not limited to membrane-proximal regions. Specifically, in contrast to untreated cells, depletion 1–2 μm from the nearest membrane (i.e., in the center of growth cones) is robust ($n = 3$). Thus, the role of cytoplasmic vesicles initially located far from the plasma membrane is much greater with NGF-induced growth cones than with untreated ANF-GFP cells.

Responses to photolysis of caged Ca^{2+}

Peptide release by untreated ANF-GFP cells or growth cones is slow in response to depolarization or Ca^{2+} ionophore (data not shown). Because space-averaged free $[\text{Ca}^{2+}]$ within PC12 growth cones typically reaches only submicromolar levels with depolarization (Reber and Reuter, 1991) (X. Lu and E. S. Levitan, unpublished observations), this might be a consequence of intracellular $[\text{Ca}^{2+}]$ not being high enough to evoke rapid exocytosis. Photolysis of caged Ca^{2+} compounds (i.e., photolabile Ca^{2+} chelators) produces large global increases in $[\text{Ca}^{2+}]$ without the spatial heterogeneities associated with channels. Indeed, release of catecholamines from untreated PC12 cells has been evoked after uncaging of Ca^{2+} in the absence of intracellular Mg^{2+} and nucleotides (Ninomiya et al., 1997). Therefore, we set out to compare the secretory responses of untreated and NGF-treated cells to large steps in $[\text{Ca}^{2+}]$. However, to ensure that Mg -nucleotide-dependent processes were not inhibited, cells were dialyzed with a pipette solution containing a ratiometric low-affinity Ca^{2+} indicator, a photolabile Ca^{2+} chelator that has low-affinity for Mg^{2+} called DMNPE-4 (Ellis-Davies, 1998; Del-Principe et al., 1999), Mg^{2+} , GTP, and ATP. In the absence of intracellular Ca^{2+} , photolysis of DMNPE-4 by epi-illumination or by a flash lamp does not induce ANF-GFP release (Fig. 4*A,B*). Thus, uncaging illumination and the products formed by photolysis need not influence the fluorescence-based measurement of peptide release.

Initially, Photolysis of Ca^{2+} -loaded DMNPE-4 by epi-illumination was used to raise intracellular free $[\text{Ca}^{2+}]$. Figure 5*A* shows that increasing $[\text{Ca}^{2+}]$ to $\sim 2 \mu\text{M}$ induces slow release from growth cones. However, increases to $\geq 10 \mu\text{M}$ evoke responses that were nearly complete within 1 sec after completion of a 400 msec photolysis (i.e., the first time point after uncaging in these experiments) in 10 of 11 growth cones (Fig. 5*B*). Thus, the rate of release depends on Ca^{2+} and becomes too fast to resolve with this methodology. The relatively small size of these bursts likely reflects the limited number of membrane-proximal vesicles in growth cones and the minimal time for vesicle diffusion to the growth cone plasma membrane (Han et al., 1999b). Thus, uncaging of Ca^{2+} establishes that the readily releasable neuropeptide pool is small and, hence, supports the conclusion that the sustained release produced by Ca^{2+} influx must depend on

Figure 3. Depletion of peptide in neuronal growth cones. *A*, Horizontal confocal images of a growth cone 0, 2, and 10 min after K⁺ depolarization with Ba²⁺. The plane of focus was adjusted to be midway through a >2-μm-thick growth cone. Scale bar, 2 μm. *B*, Quantification from *A* showing the distribution of peptide in the boxed region in *A* 0 min (open circles), 2 min (filled circles), and 10 min (x symbols) after stimulation. *F* is presented in terms of arbitrary units of fluorescence intensity. The x-axis of the plot corresponds to the x-axis of the box. *L* and *R* indicate left and right. *C*, Top panel shows horizontal confocal image of a growth cone, with the position of vertical scans indicated by a line. Bottom panels show vertical confocal images acquired 0 and 10 min after K⁺ depolarization with Ba²⁺. Scale bars, 2 μm. *D*, Quantitation from the same experiment showing the distribution of peptide in the boxed region before (open circles) and 10 min after (x symbols) stimulation. The x-axis of the plot corresponds to the y-axis of the box. *T* and *B* indicate top and bottom.

recruitment of cytoplasmic secretory vesicles, as has been suggested previously (Burke et al., 1997; Han et al., 1999b).

Strikingly, although untreated cells have relatively more membrane-proximal vesicles, the extent of release in the first seconds after the uncaging of Ca²⁺ is not greater than from growth cones (Fig. 5C). In addition, release from untreated cells induced by uncaging Ca²⁺ occurs over a longer time course, with release still ongoing 20 sec after photolysis. Importantly, the dissimilarities in the time courses of release by growth cones and untreated cells cannot be attributed to differences in [Ca²⁺]_i (Fig. 5B,C, right panels). Thus, many of the abundant membrane-proximal secretory granules found in untreated ANF-GFP cells undergo exocytosis slowly, even in the presence of high [Ca²⁺]_i. In contrast, the overall time course of release of the few membrane-proximal granules in growth cones is relatively fast.

The fact that the time course of release by untreated cells with high Ca²⁺ is similar to release by growth cones with low Ca²⁺ led us to explore whether NGF changes the sensitivity of the Ca²⁺ sensor for exocytosis (i.e., differentiation speeds up the Ca²⁺-dependent rate-limiting step). The Ca²⁺ sensitivity hypothesis predicts that the initial rate of release by untreated cells should be limited by Ca²⁺. Thus, it should become faster in untreated cells when [Ca²⁺]_i is further elevated. However, we could not further raise [Ca²⁺]_i with more illumination. Therefore, time courses like those shown in Figure 5 could not be constructed with very high [Ca²⁺]_i (i.e., >100 μM). However, we could assess whether low Ca²⁺ sensitivity underlies the slow rate of release by untreated cells in Figure 5C by measuring the dependence of the initial rate of release on [Ca²⁺]_i in individual cells. Figure 6 shows that the rate of release from untreated cells in the first second after uncaging Ca²⁺ is apparently independent of [Ca²⁺]_i.

This is also true for later time points. Because release is evoked by Ca²⁺, this implies that there must be two kinetic steps involved in release, with the slowest step being independent of Ca²⁺ (i.e., it does not involve the Ca²⁺ sensor).

To explore the conclusion that two kinetic steps are involved, we made high-resolution measurements of peptide release evoked by flash photolysis of caged Ca²⁺. Figure 7A shows that a 1 msec flash photolysis is sufficient to evoke increases in [Ca²⁺]_i that are comparable with those obtained with epi-illumination. As can be seen in Figure 7B, rapid uncaging of Ca²⁺ evokes two kinetic phases of release in untreated cells. The presence of a large slow component is in accordance with previous experiments on untreated PC12 cells (Ninomiya et al., 1997). However, GFP-based measurements also reveal a very small and rapid burst of release for the first time. The rapid phase of release may have not been detected previously because of the absence of magnesium and nucleotides in past experiments. Also, past measurements of release from PC12 cells relied on local amperometry, a technique that may not be well suited for measuring fast release from a whole cell. Most importantly, the simplest conclusion from these experiments is that release from untreated cells is slow because it is dominated by a Ca²⁺-independent step that precedes function of the Ca²⁺ sensor and exocytosis.

This conclusion is further supported by high-resolution release measurements from cells after differentiation. An example from a growth cone shows again that two components are detectable. However, in this case, the fast phase is prominent (Fig. 7C). The Ca²⁺ sensitivity hypothesis predicts that, for a given [Ca²⁺]_i, the maximal rate of release should be faster after differentiation. However, exponential fitting of high-resolution data show that the time constant of the fast component of release is unaffected by

Figure 4. Photolysis of DMNPE-4 in the absence of Ca^{2+} does not evoke release. Responses to photolysis (indicated by arrow) by epi-illumination for 400 msec (*A*) or by a 1 msec flash (*B*) are shown. Data shown are from growth cones. Note that GFP fluorescence is not depleted.

differentiation (Fig. 7*D*). Thus, all predictions of the Ca^{2+} sensitivity hypothesis are not satisfied. Therefore, NGF treatment does not change release kinetics by altering the function of the Ca^{2+} sensor. Rather, differentiation changes the overall time course of release by affecting a Ca^{2+} -independent step preceding fusion (e.g., recruitment or priming of vesicles). This step is slow for the majority of abundant membrane-proximal vesicles found in untreated cells. In contrast, although membrane-proximal vesicles in growth cones are relatively small in number, most are prepared to undergo rapid exocytosis.

Actin microfilaments and peptide release

Preliminary experiments suggested that actin microfilaments influence release by growth cones (Ng et al., 2001). To test whether the role of actin microfilaments in regulated peptide release depends on phenotype, basal and NGF-treated ANF-GFP cells were incubated with the actin depolymerizing agent mycalolide B (Saito et al., 1994). Texas Red-X phalloidin labeling of microfilaments demonstrated that mycalolide B depolymerizes F-actin in untreated cells (Fig. 8*A*) and in NGF-differentiated cells (Fig. 8*B*). This depolymerization does not evoke peptide release and does not increase total stimulated release from untreated cells (Fig. 8*C*, *left*). In contrast, total release evoked by growth cones stimulated with a Ca^{2+} ionophore or by depolarization is increased by mycalolide B (Fig. 8*C*, *right*). An opposite effect is produced by jaspilakolide, which induces actin polymerization, (Fig. 8*C*, *right*), indicating that the effect of mycalolide B in

Figure 5. Peptide release evoked by photolysis of caged Ca^{2+} . *A*, Slow release (*left*) evoked by a moderate elevation $[\text{Ca}^{2+}]_i$ (*right*) in growth cones ($n = 5$). *B*, Faster release is evoked from growth cones by large $[\text{Ca}^{2+}]_i$ increases ($n = 8$). *C*, Release responses evoked by large $[\text{Ca}^{2+}]_i$ increases from untreated cells ($n = 7$). Note that, although $[\text{Ca}^{2+}]_i$ is comparable in *B* and *C*, the time courses of peptide release differ.

Figure 6. The initial rate of release induced by epi-illumination-induced Ca^{2+} uncaging is not Ca^{2+} dependent. Scatter plot showing the percentage of decrease in GFP fluorescence by the first time point 1 sec after photolysis from individual experiments with untreated cells.

NGF-treated cells involves F-actin. Therefore, despite their abundance at the periphery, actin microfilaments do not affect the total extent of peptide release from the large pool of membrane-proximal granules found in untreated cells. However, actin microfilaments limit the size of the distributed pool of releasable secretory granules after neuronal-like differentiation.

DISCUSSION

In this report, we set out to test whether peptide release changes with nerve growth factor-induced phenotype conversion. Our studies took advantage of two features of the ANF-GFP PC12 cell clone. First, the ability to image secretory vesicles in live cells

Figure 7. High time resolution measurements of release after flash photolysis of caged Ca^{2+} . *A*, Change in $[\text{Ca}^{2+}]_i$ in response to a single flash ($n = 5$ growth cones). *B*, Release data from an untreated cell. Line shows a double-exponential fit with the time constants indicated. $[\text{Ca}^{2+}]$ was $7 \mu\text{M}$ after acquisition of this release data. *C*, High time resolution measurement of release after flash photolysis of caged Ca^{2+} from a growth cone. Line shows a double-exponential fit with the time constants indicated. $[\text{Ca}^{2+}]$ was $5 \mu\text{M}$ after acquisition of this release data. *D*, Fast time constants for untreated cells and growth cones are similar. Ca^{2+} levels measured after acquisition of ~ 14 sec of release data were $7.8 \pm 1.8 \mu\text{M}$ for growth cones ($n = 3$) and $8.7 \pm 2.7 \mu\text{M}$ for untreated cells ($n = 3$). Thus, $[\text{Ca}^{2+}]_i$ was similar in both preparations.

with a GFP-based approach made it possible to assess the impact of NGF on the distribution of the releasable pool, the role of F-actin in determining the size of the releasable pool, and the rate of peptide release in response to photolysis of caged Ca^{2+} . Second, NGF alters the phenotype of these cells in a manner that is reminiscent of the NGF-induced transdifferentiation of young

Figure 8. Depolymerization of actin microfilaments and peptide release. Texas Red-X phalloidin labeling of untreated (*A*) and NGF-treated (*B*) ANF-GFP cells. *Top*, No mycalolide B treatment. *Bottom*, Treated with $2 \mu\text{M}$ mycalolide B for 30 min at room temperature. Scale bars, $2 \mu\text{m}$. Wide-field epifluorescence images are shown. *C*, Actin polymerization drugs mycalolide B (*M*) and $10 \mu\text{M}$ jasplakinolide (*J*) affect peptide release from growth cones evoked by the Ca^{2+} ionophore Br-A23187 (*I*) or high K^+ (*K*) ($*p < 0.05$). In contrast, no mycalolide B effect was seen with untreated cells. Release was measured after stimulation (18 min for *M* and 16 min for *J*) in the continued presence of the drugs. $n \geq 5$ for each case. *C*, Control. $*p < 0.05$; $***p < 0.001$.

chromaffin cells into sympathetic neurons (Doupe et al., 1985). Although untreated ANF-GFP cells express fewer cytoplasmic secretory granules than native chromaffin cells, they appeared to be endocrine-like because they use a membrane-proximal releasable pool like chromaffin cells (Steyer et al., 1997; Oheim et al., 1998) whose size is independent of F-actin, as is found with melanotrophs (Chowdhury et al., 1999). However, these properties, as well as the time course of release, change with prolonged NGF treatment. Thus, the cellular organization of peptide release is plastic and changes with phenotype conversion. This plasticity may reflect differences in the geometries and secretory requirements of endocrine and neuronal cells or, alternatively, may be used to generate variation in neuropeptide release among individual identified neurons (Whim and Lloyd, 1994).

We found that peptide release from untreated cells uses almost exclusively vesicles that are very close to the cell surface, whereas neurosecretion is supported by a more evenly distributed pool at the ends of processes. In the latter case, mobile vesicles must be

captured to support secretion (Burke et al., 1997; Han et al., 1999b). Given that most releasable vesicles are not freely mobile in untreated PC12 cells (our unpublished results) (Lang et al., 2000), it is evident that NGF changes the spatial distribution and dynamics of the releasable peptide pool.

The second effect of neuronal-like differentiation is to change the time course of release. Caged Ca^{2+} experiments show that the burst of release evoked by large increases in $[\text{Ca}^{2+}]$ by growth cones is essentially complete within 1 sec, whereas slow kinetics dominate release by untreated ANF-GFP cells. Because the latter result agrees with previous experiments obtained in the absence of Mg^{2+} , ATP, and GTP (Ninomiya et al., 1997), the slowest component of release in untreated cells must not involve nucleotide dependent proteins (e.g., rab3 and *N*-ethylmaleimide-sensitive factor). Our high-resolution measurements imply that the slowest component is produced by a Ca^{2+} -independent step preceding fast membrane fusion. Apparently, this step dominates the overall time course of peptide release from the abundant pool of membrane-proximal vesicles in untreated cells. Recent experiments on chromaffin cells have shown that secretory vesicle movement to the plasma membrane is restricted (Johns et al., 2001). Therefore, it is possible that hindered motion slows release of membrane-proximal vesicles in endocrine-like cells. Alternatively, a slow priming step may be required before most membrane-proximal vesicles can undergo exocytosis. Importantly, the slow step, rather than a difference in the Ca^{2+} dependence of the maximal rate of release, accounts for the striking finding that, although untreated cells have far more membrane-proximal vesicles, the amount of release seen in the first seconds after uncaging of Ca^{2+} is not greater or faster than from growth cones. In contrast, it appears that use of the few docked vesicles after neuronal-like differentiation is not limited by a slow pre-fusion priming or recruitment step. Hence, phenotype conversion does not dramatically affect the initial burst of release that may represent emptying of the readily releasable pool of docked vesicles. Instead, it appears that Ca^{2+} -independent refilling of the readily releasable pool that supports release seconds after uncaging Ca^{2+} is altered. This effect could be a consequence of the change in the spatial distribution of releasable vesicles described above.

Finally, we found that the effect of polymerized actin on the size of the releasable pool in ANF-GFP cells changes with NGF treatment. The lack of an effect in the undifferentiated state implies that actin microfilaments that are abundant near the cell surface do not interact avidly with the membrane-proximal secretory granules that support peptide secretion in untreated cells. This is consistent with the conclusion that actin microfilaments influence endocrine release by limiting the kinetics rather than the amount of exocytosis (Chowdhury et al., 1999) and the finding that F-actin depolymerization does not increase vesicle mobility in untreated PC12 cells and chromaffin cells (Lang et al., 2000; Oheim and Stuhmer, 2000). However, F-actin does affect release after phenotype conversion. Currently, the reason for this difference is not apparent. One potential explanation could be that vesicle size is larger after differentiation, but this has not been evident in our preliminary ultrastructure studies (our unpublished results). Alternatively, the structure of F-actin or the expression of tethers may change. Finally, this difference may be a consequence of an F-actin-independent barrier that changes with phenotype conversion. This barrier could be rate determining in untreated cells but could be insignificant at the ends of processes so that the role of F-actin becomes dominant. Given that neu-

ropeptide release is limited by the availability of mobile cytoplasmic vesicles (Burke et al., 1997; Han et al., 1999b), it will be of interest to test whether depolymerization of actin microfilaments liberates immobile neuropeptidergic vesicles after phenotype conversion with NGF.

The many changes in release with phenotype conversion reported here indicate that the release process is plastic. In addition to being relevant for understanding the cellular basis of peptide release, this result is of interest because PC12 cells are used avidly for studies of release. In many cases, altering the amount or function of proteins thought to be involved in exocytosis does not affect release by untreated PC12 cells (Sugita et al., 1999). This may reflect that such perturbations have not changed either the size of the releasable pool or the speed of the rate-limiting step for release evoked in those experiments. Our results show that the distribution of the releasable pool, the kinetic step that dominates release in the presence of high $[\text{Ca}^{2+}]$, and the role of the actin cytoskeleton change with neuronal-like differentiation. Thus, future biochemical and molecular studies of release by PC12 cells may decide to explore whether the role of a protein of interest in release changes with NGF treatment.

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