Ca2+-triggered peptide secretion in single cells imaged with green fluorescent protein and evanescent-wave microscopy

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**Abstract**

Green fluorescent protein fused to human chromogranin B or neuropeptide Y was expressed in PC12 cells and caused bright, punctate fluorescence. The fluorescent points colocalized with the endogenous secretory granule marker dopamine beta-hydroxylase. Stimulation of live PC12 cells with elevated [K+] or of permeabilized PC12 cells with Ca2+, led to Ca2+-dependent loss of fluorescence from neurites. Ca2+ stimulated secretion of both fusion proteins equally well. In living cells, single fluorescent granules were imaged by evanescent-wave fluorescence microscopy. Granules were seen to migrate; to stop, as if trapped by plasmalemmal docking sites; and then to disappear abruptly, as if through exocytosis. Evidently, GFP fused to secreted peptides is a fluorescent marker for dense-core secretory granules and may be used for time-resolved microscopy of single granules.

**Reference**


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**Ca^{2+}-Triggered Peptide Secretion Neurotechnique in Single Cells Imaged with Green Fluorescent Protein and Evanescent-Wave Microscopy**

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**Summary**
Green fluorescent protein fused to human chromogranin B or neuropeptide Y was expressed in PC12 cells and caused bright, punctate fluorescence. The fluorescent points colocalized with the endogenous secretory granule marker dopamine β-hydroxylase. Stimulation of live PC12 cells with elevated [K+]o, or of permeabilized PC12 cells with Ca^{2+}, led to Ca^{2+}-dependent loss of fluorescence from neurites. Ca^{2+} stimulated secretion of both fusion proteins equally well. In living cells, single fluorescent granules were imaged by evanescent-wave fluorescence microscopy. Granules were seen to migrate; to stop, as if trapped by plasmalemmal docking sites; and then to disappear abruptly, as if through exocytosis. Evidently, GFP fused to secreted peptides is a fluorescent marker for dense-core secretory granules and may be used for time-resolved microscopy of single granules.

**Introduction**
Noninvasive optical studies of fluorescently stained, single live neurons have significantly advanced our understanding of how presynaptic terminals function (e.g., Betz and Bewick, 1992; Ryan et al., 1993; Wu and Betz, 1996). In these studies, synaptic vesicles are selectively stained with bath-applied, membrane-impermeant lipidic styryl dyes such as FM 1-43, while presynaptic terminals perform exo- and endocytosis. The method works because the membranes of synaptic vesicles, once in contact with the external fluid, are recycled in minutes to form new synaptic vesicles. A similar strategy has been used for selective immunostaining of synaptic vesicles (Krassowski et al., 1995). Besides synaptic vesicles, presynaptic terminals contain dense-core secretory granules for the secretion of hormones and peptide neurotransmitters, in neuroendocrine cells, such granules are the dominant secretory organelles. In vivo, dense-core granules cannot easily be stained with styryl dyes or antibodies, because their membranes and proteins are packaged together early in granule biogenesis; and it is not clear that, after exo- and endocytosis, their membrane components are ever specifically reunited with new peptides to form new secretory granules. Hence, staining by cycling, so successful with synaptic vesicles, has not worked with dense-core granules. This is unfortunate, because the relatively large size of dense-core granules promises that their exocytosis could be studied optically at the single-vesicle level, a goal that has been difficult to achieve with the much smaller synaptic vesicles.

To address this problem, we have turned to green fluorescent protein (GFP) (Chalfie et al., 1994; Cubitt et al., 1995). GFP has proved a useful tool for visualizing cellular events and cellular compartments in vivo (Kaether and Gerdes, 1995; Gerisch et al., 1995; Rizzuto et al., 1995; Cole et al., 1996; Wacker et al., 1997). It could be similarly useful for studying Ca^{2+}-triggered secretion or the sorting of proteins into the regulated secretory pathway. To this end, GFP must be expressed in fluorescent form in dense-core granules. Early mutants of GFP were not fluorescent in secretory granules, possibly because of the acidic environment and dense protein matrix (Gerdes and Kaether, 1996). Here we have used a GFP mutant with improved optical and protein folding characteristics (Cormack et al., 1996). We targeted it to dense-core secretory granules of PC12 cells by recombinant fusion to the neurotransmitter neuropeptide Y and to the dense-core granule matrix protein human chromogranin B. Expression of both fusion proteins caused bright fluorescence in dense-core granules that allowed their secretion to be imaged. Using evanescent-wave fluorescence microscopy, granules near the plasmalemma were readily observed for extended periods in living cells. The approach allows the study of transport, docking, and exocytosis at the level of single granules.

**Results**

**Expression of Two GFP Fusion Proteins in PC12 Cells**
We constructed mammalian expression plasmids coding for a mutant GFP (Cormack et al., 1996) fused to the C-terminal ends of either human chromogranin B (hCGB) or human pro-neuropeptide Y (hNPY). Within 48 hr after transfection with the plasmids, PC12 cells fluoresced brightly, even after fixation with parafomaldehyde. Figure 1 shows fluorescence micrographs of cells that were allowed to differentiate in the presence of nerve growth factor and extended neurites. Neurites showed the brightest GFP fluorescence (Figure 1A). They also contained the majority of the dense-core granules, as was confirmed in electron micrographs (not shown) and in fluorescence micrographs immunostained with antibodies against the membrane-associated dense-core granule protein dopamine-β-hydroxylase (DβH; Figure 1B). At the level of an entire cell, the distribution of GFP fluorescence is consistent with hCGB–GFP being targeted to dense-core granules. GFP fluorescence is also readily seen in live PC12 cells transfected with hCGB–GFP (Figure 1C) or with hNPY–GFP (Figure 1D).

**Localization of GFP**
We tested whether hCGB–GFP fluorescence also colocalizes with dense-core granule markers at the level of

Figure 1. Fluorescence from hCGβ-GFP and hpNPY-GFP is concentrated in neurites of NGF-differentiated PC12 cells. GFP fluorescence (A) and immunofluorescence (B) with anti-DβH antibody. Aldehyde-fixed cells (see Experimental Procedures). GFP fluorescence (C and D) in two live cells. Transfection with hCGβ-GFP (A-C) and hpNPY-GFP (D).

single organelles. Figure 2 presents high magnification confocal scans through single neurites showing hCGβ-GFP fluorescence (Figure 2A) and fluorescence of an antibody against DβH (Figure 2B). Both Figures show punctate fluorescence suggestive of single granules, with GFP and DβH apparently colocalizing even at the level of single spots in this section. Colocalization was not perfect. In other cells (e.g., Figures 3A and 3B), there were clear examples (arrows) where DβH-positive structures showed no GFP label, and other examples where GFP-labeled structures failed to react with anti-DβH. To quantitate the degree of colocalization, we drew 500 nm diameter circles around spots of GFP fluorescence (Figure 3C), duplicated the circles into the DβH image at identical pixel locations (Figure 3D), and then determined whether the new circle contained a fluorescent point concentric to within 150 nm. Circles were scored as positive if they contained a fluorescent spot.

Figure 2. Colocalization of GFP and a Dense-Core Granule Marker
Localization of hCGβ-GFP (A) and DβH (B) in an aldehyde-fixed neurite of a hCGβ-GFP-transfected cell. Confocal high magnification scan; some areas of extensive colocalization are outlined. In (B) (lower left), note fluorescence from a neighboring and apparently untransfected cell.
in the DβH image (numbered 1, 5, 6, 8, 9), negative if they did not (2, 3, 10), and neutral if the image did not allow a determination (4, 7, 11). In eight cells, GFP fluorescent spots were examined in both cell bodies and neurites; 65% ± 6% of the GFP spots were positive, 22% ± 5% were negative, and 13% ± 2% were neutral, suggesting that 65% to 78% of all GFP resided in dense core granules.

DβH fluorescence without GFP fluorescence could result if cells contained "old" GFP-free granules, synthesized before transfection. Furthermore, GFP requires posttranslational processing and folding, which may not proceed to completion in all granules. Conversely, GFP fluorescence in structures without DβH label could result either because the antibody did not have access to all granules or because hCgB-GFP also reaches organelles that are negative for DβH. This was tested by comparing, as in Figure 3, hCgB-GFP fluorescence with labeling by a polyclonal antibody against GFP. Colocalization was complete (99 ± 0.5%, 7 cells; not shown). Thus, immunochemistry suggests that GFP occasionally reaches organelles other than dense-core secretory granules.

**Ca2+-Stimulated GFP Secretion**

If hCgB-GFP fluoresces in dense-core granules of living cells, its fluorescence should diminish when an increase in cytosolic [Ca2+] causes cells to secrete. Figure 4 shows digitized images of a single neurite of a differentiated cell, in brightfield (upper) and in epifluorescence (lower). The neurite is shown both before (Figures 4A and 4C) and after 3 min in a buffer whose elevated [K+] is expected to depolarize the plasma membrane, open voltage-gated Ca2+ channels, and raise cytosolic [Ca2+] (Figures 4B and 4D). Depolarization led to a loss of hCgB-GFP fluorescence (Figures 4C and 4D), with little or no change in morphology (Figures 4A and 4B).

To quantitate the effect, the neurite in Figure 4C was outlined, and the outline was digitally transferred to the same pixel locations in Figure 4D. Light within the outlines of both images was measured and compared. The results are summarized in Figure 4E. About 30% of the fluorescence was lost when cells were exposed to the stimulation buffer, and none was lost when Mg2+ replaced Ca2+. Evidently the loss of fluorescence requires Ca2+ influx.

Depolarization-triggered secretion requires the opening of voltage-gated Ca2+ channels, which are not always strongly expressed in the PC12 cells used here (Sankaranaranajah, personal communication). Thus, elevated external [K+] may not raise cytosolic [Ca2+] strongly enough in all PC12 cells. To see whether higher cytosolic [Ca2+] caused more vigorous secretion of GFP, we applied Ca2+-directly to permeabilized PC12 cells. Intact cells were first imaged in a buffer of low [Ca2+] and then permeabilized for 2 min in a Ca2+-containing buffer, kept there for a further 3 min, and returned to a low [Ca2+] buffer. They were then imaged a second time. Images were analyzed as in Figure 4. In both hCgB-GFP and hNPY-GFP transfected cells, Ca2+ caused at least half of the GFP fluorescence to be lost. When Ca2+ was not elevated after permeabilization, loss of GFP was minimal (Table 1). Taken together, the results indicate that approximately half of the GFP fluorescence is lost in a Ca2+-dependent manner.

**Table 1. Loss of GFP Fluorescence in Permeabilized Cells**

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<thead>
<tr>
<th>Construct</th>
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<tr>
<td>Fluorescence Lost in 5 min (%)</td>
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<td></td>
<td>[Ca2+] = 17 μM</td>
<td>[Ca2+] = 0.037 μM</td>
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<tr>
<td>hpNPY-GFP</td>
<td>74.5 ± 4.5 (n = 10)</td>
<td>17.8 ± 4.3 (n = 12)</td>
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<tr>
<td>hCgB-GFP</td>
<td>53.3 ± 4.8 (n = 8)</td>
<td>5.5 ± 3.9 (n = 10)</td>
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**Tracking Dense-Core Granules in Live Cells**

We used video microscopy to test whether GFP allows the observation of single fluorescent granules in living cells. Because granules in neurites are often too crowded to be singly resolved by light microscopy, we examined cells that were grown in the absence of NGF and had no neurites. Cells adhering tightly to a glass coverslip were chosen. To selectively illuminate a thin layer of cytosol immediately adjacent to the coverslip, we used the evanescent wave formed by a laser beam experiencing total reflection at the glass–cytosol interface (Stout and Axelrod, 1989; Figure 5). Compared with confocal microscopy, this method allows for imaging a thinner layer and, more importantly, avoids the extensive loss of fluorescent light at the confocal pinhole. Figure 6A shows a region where a live, round, hCgB-GFP-transfected cell adhered to a coverslip. Fluorescence is punctate, with some spots fluorescing brightly and
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Figure 5. Fluorescence Excitation with an Evanescent Wave
The method takes advantage of the refractive index, n, being lower in the cell than in the glass substrate to which it adheres tightly. A laser beam enters the coverslip through the microscope objective at an angle causing it to be totally reflected at the glass-buffer interface, or at the interface between the coverslip and the cell. Although dense-core granules in a cell are uniformly labeled, only those within the evanescent wave fluoresce and are detected by the microscope.

others dimly. Two hundred exposures could be taken without significant photobleaching or signs of photodamage, allowing time-resolved studies at the level of single granules. Because the intensity of the evanescent wave declines with distance from the glass substrate, structures closest to the adhering plasmalemma are expected to be the brightest, while those more than 300 nm away (Steyer, personal communication) are too dim and out-of-focus to be resolved. Indeed, some spots slowly appeared and faded from view while they moved into and out of the evanescent wave; other spots were stationary. Some spots migrated parallel to the plasma membrane over micron distances. Figures 6B–6F are examples from a permeabilized cell containing buffer with sufficient $[\text{Ca}^{2+}]$ to support exocytosis. One granule was tracked in 32 successive frames; four of them are shown in Figures 6C–6F (arrows). Figure 6B tracks the path of the granule at 2 s intervals. For the first 42 s, the granule traveled extensively, covering a distance of over 2 mm. Then it stopped and disappeared abruptly, 20 s later. It is tempting to suggest that the granule stopped because it was captured by a plasmalemmal docking site, and that it disappeared when it performed exocytosis and the GFP, thus released, diffused away.

Discussion

We have fused GFP to two proteins that are secreted in the regulated pathway. PC12 cells expressing the fusion proteins fluoresced and concentrated the fluorescence in neurites as they differentiated. They lost GFP fluorescence under conditions that raise cytosolic $[\text{Ca}^{2+}]$ and stimulate secretion. Since dense-core secretory granules are the only organelles in PC12 cells known to secrete proteins in a Ca$^{2+}$-dependent fashion, we assume that all GFP, thus released, comes from dense-core granules.

solution where all Ca$^{2+}$ was replaced with Mg$^{2+}$ and no Ca$^{2+}$ influx could take place. Fluorescence was lost in the Ca$^{2+}$-containing but not the Mg$^{2+}$-containing solution. The difference is significant at $p < 0.07$ (one-tailed t test).
How efficiently are our fusion proteins targeted to secretory granules? In immunofluorescence, hCgB-GFP and dense-core granule markers colocalized only imperfectly. Though difficult to quantitate, our immunofluorescence results suggest that 65%–78% of the hCgB-GFP is targeted to DβH-containing organelles. Ca\(^{2+}\)-stimulated secretion of GFP is a more quantifiable measure of targeting. Intact differentiated cells lost 30% of their GFP in 3 min when they were stimulated with elevated [K\(^+\)]. This is no less than the amount of secretogranin released by the same PC12 clone under conditions that were similar, except that the cells were not induced to differentiate by NGF (20% in 15 min; Bauerfeind et al., 1995). More vigorous GFP secretion was seen when Ca\(^{2+}\) was supplied directly to permeabilized cells (~50% in 5 min; Table 1). Since only dense-core granules are expected to release GFP in response to Ca\(^{2+}\), this organelle must contain at least half of the fluorescent GFP. The actual fraction is higher, since norepinephrine, though selectively accumulated in dense-core granules, is never released completely even under maximal stimulation. Norepinephrine release in permeabilized PC12 cells was ~45% in 5 min in undifferentiated cells (Hay and Martin, 1992) and 40% in 10 min in differentiated cells (Banerjee et al., 1993). NGF-treatment did not increase norepinephrine secretion by permeabilized PC12 cells (Banerjee et al., 1993). Apparently, Ca\(^{2+}\) stimulated the release of hNPY-GFP and hCgB-GFP from neurites as efficiently as it stimulated the release of more conventional markers of secretion from whole cells. The comparison is consistent with most or all of the fluorescent GFP being contained in dense-core granules.

GFP will be generally useful for labeling secreted proteins or dense-core secretory granules. First, an endocrine cell targets to dense-core granules just about any protein secreted through the regulated pathway, even when the protein is not normally expressed in that cell. For example, ACTH-secreting AT20 cells target the foreign pro-insulin to secretory granules as effectively as ACTH (Moore et al., 1983; for other examples, see Halban and Irminger, 1994). Second, to the targeting mechanism, even a small peptide is dominant over a medium-sized attached protein such as GFP, as shown by our results on hNPY-GFP. We have not tested what fraction of the pro-NPY-GFP was processed to NPY-GFP in our experiments, but even pro-NPY is only 69 amino acids long, compared with the 238 added amino acids of its fusion partner, GFP. Evidently the addition of GFP does not easily confuse the targeting mechanism.

By GFP labeling, we viewed single organelles in live cells. This generally requires imaging a thin layer of cytosol and collecting photons at high density from the small region occupied by a granule. Confocal microscopy accomplished both tasks (Figures 2 and 3) by exciting strong fluorescence and then rejecting all but the in-focus fluorescence with a confocal pinhole. The intense illumination required for our samples bleached most GFP in <10 scans, and likely would have caused unacceptable photodamage to a living cell. Bleaching and photodamage may be reduced by keeping the illumination weak, by restricting it to the focal plane, and by increasing the efficiency of light collection. To this end, we used evanescent-wave illumination to excite fluorescence in a thin layer of cytosol. Like two-photon microscopy, this method excites little or no out-of-focus fluorescence; no confocal pinhole was required, and we could collect all fluorescence reaching our objective. Up to 200 exposures were readily captured without obvious bleaching or photodamage. Combined with the efficient light collection possible with evanescent-wave excitation, the GFP tag provides enough fluorescence for studying the transport, docking, and exocytosis of single granules in living or permeabilized cells.

**Experimental Procedures**

**Cell Culture and Transfection**

PC12 cells (clone 251; Heumann et al., 1983) were maintained and propagated as described (Toozé and Huttner, 1990) in 75 cm\(^2\) uncoated flasks containing growth medium (DMEM with 4.5 g/l glucose, 10% fetal calf serum [FCS], 5% horse serum, 100 U/ml penicillin, and 0.1 mg/ml streptomycin) at 37° C in 10% CO\(_2\). For electroporation, cells were detached from their substrate with trypsin and EDTA, triturated, pelleted, and resuspended in cytomix (van der Hoff et al., 1992; 120 mM KCl, 10 mM KH\(_2\)PO\(_4\), 0.15 mM CaCl\(_2\), 2 mM EGTA, 5 mM MgCl\(_2\), 2 mM ATP, 5 mM glutathione, and 25 mM K-HEPES [pH 7.6]). The ATP and glutathione were added immediately before the experiment. Cytomix (350 µl) containing 2 × 10\(^5\) cells was placed into an electroporation cuvette, then 50 µg plasmid in 50 µl of 0.1 Tris-EDTA was added. A single 1150 V shock was applied through 50 µF and 24 Ω. The cells were allowed to recover for 15 min at room temperature and then were diluted into 2.7 ml growth medium. The aliquots (0.5 ml) were placed onto five 20 mm glass coverslips, and the cells were allowed to settle...
for 5 min. An additional 1.7 ml growth medium was added, and the cells were placed in the incubator. Within 24 hr, the medium was changed once more. The cells were used 48 hr after transfection. Unless otherwise indicated, the cells were allowed to differentiate and grow neurites by including neurite growth factor (NGF, 100 ng/ml; human recombinant NGF-β chain, Sigma) in the growth medium continuously after electroperoration.

**Plasmids**

The cDNAs encoding chimeras of hCgB and hNPY with GFP (GFPmut2, Cormack et al., 1996) were cloned in mammalian expression vectors under the control of the cytomegalovirus promoter. For hCgB–GFP, a construct cloned in the pCM8 vector (Invitrogen) was already available with a GFP bearing a S65T mutation (Kaether and Gerdes, 1995; Wacker et al., 1997), and we simply replaced that GFP with the newer GFPmut2. We modified the GFPmut2 cDNA (originally received from Dr. B. Cormack in an e. coli expression vector, pKEN) by PCR to introduce a Kpn I site in-frame with the GFPmut2 ORF; this site allowed subsequent cloning in-frame with the C-terminal end of the CgB. The cDNA coding for hNPY was changed once more. The cells were used 48 hr after transfection. morph (Universal Imaging Co).

KH2PO4, 4.2 mM NaHCO3, 5.6 mM glucose, 0.8 mM MgCl2, and 10 mM PIPES (pH 7.0). The calculated concentrations were [Ca2+]i – 0.033 mM and [MgATP] – 0.06 mM. After the first set of images were taken, the buffer was replaced with buffer B (63 mM K glutamate, 50 mM K-EGTA, 5 mM MgCl2, 3.5 mM MgCl2, 1 mM K-ATP, 5 mM glucose, and 20 mM PIPES [pH 7.0]). Concentrations were [Ca2+]i – 17 µM (measured with a Ca2+-selective electrode) and [MgATP] – 0.86 mM (calculated). Digitonin was removed after 2 min, and the cells remained for an additional 3 min in buffer B. Then, buffer A was applied again, and the cell was imaged a second time. Controls were carried out identically, except that buffer A replaced buffer B during and after the permeabilization step.

**Video Microscopy**

After transfection, the cells were grown on glass coverslips without NGF. A Zeiss Axiosvert microscope was modified for evanescent-wave excitation as described (Stout and Axelrod, 1989). Fluorescence was excited with a 488 nm argon laser, which was directed in epifluorescence mode through the objective, such that it suffered total reflection at the interface between the coverslip and cytosol or bathing medium (Figure 5). Measurements with 280 nm diameter fluorescent beads showed that the microscope collected fluorescent light from a 300 nm thin aqueous layer adjacent to the coverslip carrying the cells (Steyer, personal communication). The images were captured as above and analyzed with Metamorph (Universal Imaging Co). To track the position of organelles, we high pass filtered the images at a spatial frequency of 1 µm and identified the organelles by a thresholding algorithm (Metamorph). We considered an organelle's image as a cloud whose thickness represented the local fluorescence intensity. The cloud’s center of mass was determined by an algorithm in Metamorph and localized the organelle to within ~50 nm.

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**References**


Exocytosis Imaged with Green Fluorescent Protein


Note Added in Proof

In a further paper, we will show that PC-12 cells do not cleave hCgB-GFP and, on stimulation, release the protein intact into the medium (Kaether, C., Salm, T., Giombik, M., Almers, W., and Gerdes, H.-H., Eur. J. Cell Biol., in press).