Journal of Cellular Biochemistry 97:1393–1406 (2006)

Light-Induced Exocytosis in Cell Development and Differentiation

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Abstract Calcium-dependent exocytosis of fluorescently labeled single secretory vesicles in PC12 cells and primary embryonic telencephalon cells can be triggered by illumination with visible light and imaged by TIRF or epifluorescence microscopy. Opsin 3 was identified by quantitative PCR expression analysis as the putative light receptor molecule for light-induced exocytosis. In primary chicken telencephalon cells, light-induced exocytosis is restricted to a specific period during embryonic development, and involves fusion of rather large vesicles. Strictly calcium-dependent exocytosis starts after a delay of a few seconds of illumination and lasts for up to 2 min. We analyzed the frequency, time course and spatial distribution of exocytotic events. Exocytosis in PC12 cells and telencephalon cells occurs at the periphery or the interface between dividing cells, and the duration of single secretion events varies considerably. Our observation strongly supports the idea that light induced exocytosis is most likely a mechanism for building plasma membrane during differentiation, development and proliferation rather than for calcium-dependent neurotransmitter release. J. Cell. Biochem. 97: 1393–1406, 2006.

Key words: PC12 cells; exocytosis; ryanodine; total internal reflection microscopy (TIRF); secretory granules; opsin 3

Light-sensing was recently observed in PC12 cells where growth cones followed the path of a moving laser beam [Ehrlicher et al., 2002]. The underlying mechanism is unclear but the existence of light receptor, which triggers plasma membrane dynamics appears to be very likely. Growth, neurite, and process formation in astrocytes, neurons, and neuroendocrine cells takes place by integration of vesicular membrane into the plasma membrane by exocytosis [Shapira et al., 2003]. The directional growth is determined by the relocation of specific fusion sites, characterized by accumulation of docking and fusion proteins [Lang et al., 2001]. It is still unknown, how the proteins are directed towards the correct target area, but there is evidence that the cytoskeleton rearrangement and calcium signaling is the basis of this process [Hsu et al., 2004]. Since alteration of neural connections constitutes the central mechanism of a developing brain and is required for maintenance of proper neuronal activity, dysfunctional plasticity causes psychiatric and neuronal diseases, such as depression. The positive effect of light in depression therapy has long been established. Recently several publications found strong evidence that the cause of the disease is a transient defect of brain

This article contains supplementary material, which may be viewed at the Journal of Cellular Biochemistry website at http://www.interscience.wiley.com/jpages/0730-2312/suppmat/index.html.

Grant sponsor: Max–Planck-Society; Grant sponsor: Department of Psychiatry and Psychotherapy, University of Erlangen-Nuremberg.

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Received 21 September 2005; Accepted 1 November 2005
DOI 10.1002/jcb.20751

plasticity [Duman et al., 2000; Husum et al., 2000; Vaidya et al., 2000]. Moreover, extraocular light perception and subsequent modulation of melatonin secretion was observed in blind patients [Czeisler et al., 1995].

It is well accepted that sensation of light via the rhodopsin (opsin 1)—transducin pathway in higher organisms is restricted to retinal cells, like rods and cones. However, light sensitive molecules such as pinopsin and melanopsin (opsin 2) are expressed in the pineal gland of birds and mammals, an organ preferentially involved in circadian rhythmus. Recently, encephalopsin/panopsin (opsin 3) was identified in specific regions of the human brain [Blackshaw and Snyder, 1999]. Unlike rhodopsin, which is only present in adult retinal cells, opsins 2 and 3 are expressed early in embryonic development [Tarttelin et al., 2003].

The molecular mechanism of retinal signal transduction has been analyzed intensively during the last 20 years. By now, almost all the major molecular steps are deciphered. The central pathway consists of a booster cascade that is mediated via transducin, a G-protein, and the activation of a cGMP-phosphodiesterase. This molecular system regulates the calcium concentration by acting on cGMP-dependent calcium channels in the plasma membrane and via IP_3 activated calcium channels in intracellular stores [Gotow and Nishi, 1991]. Currently it is still unclear, if extraocular light perception is based on the same signal transduction mechanism.

In this study, we describe the properties of light-induced exocytosis and present experimental evidence that its activation is mediated most likely by opsin 3 and intra- and extracellular calcium. The molecular mechanism appears to be similar to the retinal rod signal transduction pathway.

MATERIALS AND METHODS

PC12 Cell Culture and Staining

PC12 cells, clone 251 [Heumann et al., 1983] were maintained and propagated as described [Tooze and Huttner, 1990] in 75 cm^2 uncoated flasks containing growth medium (DMEM with 4.5 g/L glucose, 10% fetal calf serum, 5% horse serum, 100 U/ml penicillin, and 0.1 mg/ml streptomycin) at 37°C in 10% CO_2. The aliquots (0.5 ml) were placed onto 20 mm glass coverslips, and the cells were allowed to settle for 5 min. An additional 2.5 ml growth medium was added, and the cells were placed in the incubator. The cells were used within 24 h after plating and transferred into standard bath medium (in mM: 150 NaCl, 2 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 20 HEPES-NaOH, pH 7.2) for imaging.

Acidic compartments like dense core granules were stained for 5 min with 0.3 or 3 μM acridine orange (AO), (Molecular Probes, Eugene) at room temperature, because it was reported that AO concentrations of 10 μM or higher caused photolysis of vesicles [Brunk et al., 1997]. The cover-slips were washed three times for 5 min.

Changes in intracellular calcium concentrations were detected by calcium green-AM (Molecular Probes). Cells were incubated for 1 h at room temperature by 10 μM of the calcium sensor and washed three times for 10 min.

Culture of Chicken Telencephalic Cells

Fertilized White Leghorn chicken eggs were obtained from the institutional animal care facility, and all procedures using animals were approved by the Institutional Animal Care and Use Committee. Telencephalic cortices were dissected from 14-day-old chicken embryos and cultured [Pettmann et al., 1979]. In brief, after removing the meninges, telencephalic hemispheres were digested by incubation with trypsin/EDTA solution (0.05%/0.02%) for 15 min at 37°C and than dissociated by aspiration through a 20-gauge needle. The homogenate was centrifuged and resuspended into culture medium consisting of Dulbecco's Modification of Eagle's Medium supplemented with 5% fetal calf serum and 5% chicken serum. To remove undissociated tissue the suspension was passed through a sterile nylon sieve (80 μm) and cells were finally seeded at a density of 3.75×10^5 cells/cm^2 onto polylysine-coated 24-well culture plates. After 30 min, following cellular attachment, incubation medium was renewed. Cultures were grown at 37°C in a humidified atmosphere of 95% air and 5% CO_2, and experiments were performed between the second and fifth day in vitro.

Quantitative RNA Analysis

Total RNA was extracted from cultivated PC12 cells using a modified Qiagen-protocol: A phenol-extraction in Qiazol (Qiagen, Hilden, Germany) was followed by column-purification with "Rneasy Mini Kit" (Qiagen), including DNase digestion, and isolated RNA was

transcribed into cDNA. Reverse transcription was done by using poly-dT primers and "AMV Reverse Transcriptase" from the Roche cDNA Synthesis System (Roche, Basel, Switzerland). Quantitative PCR was performed by means of SYBR Green I Master Mix (Applied Biosystems, Darmstadt, Germany) on a real-time PCR-system "i-cycler" (BioRad, Munich, Germany), according to a three-step standard protocol at an annealing temperature of 66°C. Beta-actin was used as internal standard, and ΔCT values were calculated from differences between opsin 3 and beta-actin. We used the following primer pairs: beta-actin-F (CTG GAA CGG TGA AGG TGA CA), and beta-actin-R (AAG GGA CTT CCT GTA ACA ATG CA), rat opsin 3-F (TCT TCA TGA TCA GAA AGT TTC G), and rat opsin 3-R (CCT GTC CCC ATC TTT CTG TGA C).

Image Processing

Differential image stacks were generated by duplicating an image stack and removing the first frame from the copy. Then corresponding image planes of the copy were subtracted with "Metamorph" imaging software (Universal Imaging Co., Downingtown), creating a new stack that held only brightness changes. By this technique, exocytotic flashes become easily visible. The images were median-filtered; numbers and positions of exocytotic events were measured by our self-written software "Exo-Count." These coordinates were used to determine quantity of released AO and duration of individual exocytotic events. Duration of events were calculated from the original image stacks by measuring the time, until the brightness dropped below 10% of the maximal value. For calculation of AO cloud brightness, corresponding to total release of AO, the background-image right before the event was subtracted from the following sequence and the fluorescence around the cloud was integrated within this time interval.

TIRF and Epifluorescence Microscopy

A Zeiss "Axiovert 135" TV microscope (Zeiss, Jena, Germany) was modified for evanescent wave excitation and described elsewhere (Upmann, 2002). Fluorescence was excited with the 488 nm line of an argon laser, which was directed through the objective "Planchromat," 100×, 1.4 NA (Olympus, Hamburg, Germany), such that it suffered total reflection at the interface between the cover-slip and cytosol or bathing medium. Images were illuminated for 100 ms by a CCD-camera "SensiCam" (PCO, Kehlheim, Germany) with "TILLvisION" software (T.I.L.L. Photonics, Gräfelfing, Germany) at 10 Hz frequency after passing through an emission filter block "HQ 520-570" (AHF Analysentechnik AG, Tübingen, Germany). The laser power output was set to 200 mW and further reduced to 10% by a neutral density filter. Measurements with 100 nm and 4 μm diameter fluorescent beads showed that the microscope collected fluorescent light from a 100 nm thin aqueous layer adjacent to the cover-slip carrying the cells [Upmann, 2002].

Epifluorescence microscopy was performed on a different setup, consisting of a Zeiss "Axiovert 135" microscope, equipped with a "Fluar" objective, 100×, 1.3 NA, a FITC filter/dichroic mirror cube (Zeiss, Jena, Germany), a CCD camera "CoolSnap" (Photometrics, Tucson), and a polychromator (Visitron Systems, Puchheim, Germany) for single wavelength excitation. The images were captured as described above and processed with "Metamorph" imaging software (Universal Imaging Co.).

RESULTS

Membrane Fusion, Induced by Illumination of the Plasma Membrane

In order to monitor exocytosis by "total internal reflection microscopy" (TIRF), PC12 cells were stained with the fluorescent dye AO, a weak base that accumulates inside acidic compartments such as endosomes, vesicles and granules.

Figure 1A shows that loosely attached cells, characterized by their round shape, had no discrete individual granules and exhibited a rather unstructured appearance. All cells that were tightly attached to the cover-slip showed numerous bright spots (Fig. 1B) that corresponded to AO-stained vesicles and granules in TIRF microscopy mode as shown previously [Steyer et al., 1997].

Figure 1C illustrates that illumination of cells with blue (488 nm) light triggered many bright flashes after a short delay of between 15 and 30 s (see also *PC12exo01.mov* in the Online Supplemental Material section). Excitation at 514 nm revealed a very similar effect (data not shown). These flashes corresponded to exocytotic release of AO into the extracellular space, illuminated

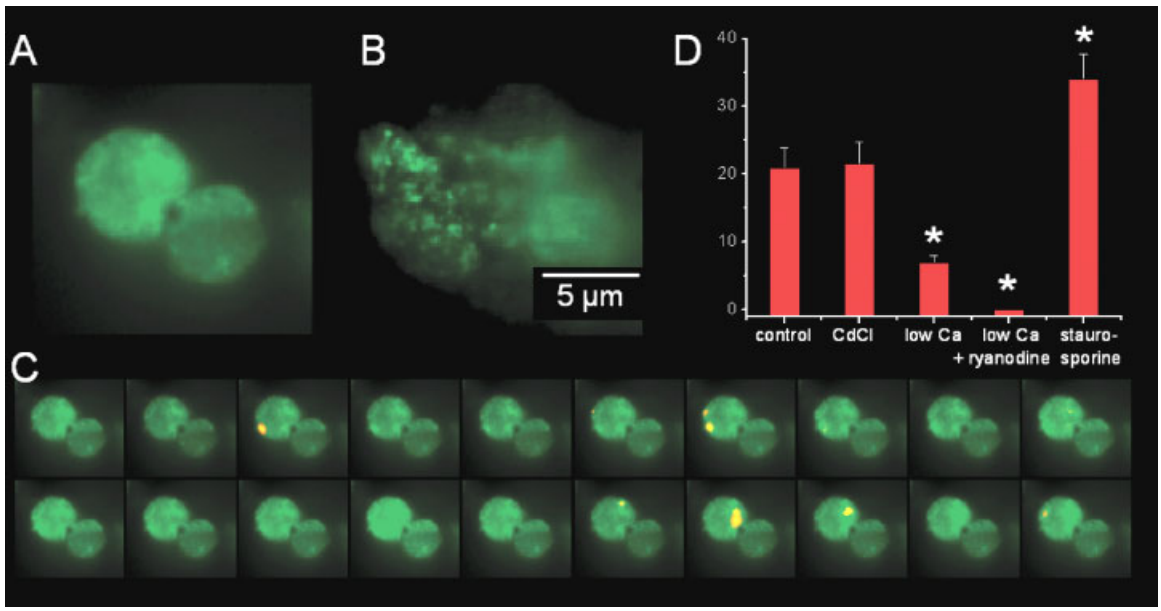

Fig. 1. Exocytosis in PC12 cells, imaged by TIRF microscopy. **A**: Two dividing, loosely attached PC12 cells show diffuse acridine orange staining. **B**: Individual vesicles are visible in one tight attached PC12 cell (left), while the right cell remains not in full contact with the coverslip. The magnification in panels A and B is identical. **C**: Time frame series, recorded within 20 s, from a movie of the cells in panel A. shows exocytotic events (yellow). The excitation wavelength in all experiments was 488 nm. **D**: The diagram shows the frequency of exocytotic events under the specified conditions ($CdCl_2 = 20$ µM, staurosporine = 1 µM, ryanodine = 10 µM, $Ca^{2+} = 156$ nM). Asterisks' indicate significant differences from control (Student's *t*-test, unpaired, $P < 0.01$). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

by the evanescent wave. This activity lasted for up to 2 min. Interestingly, also cells that were loosely attached to the cover-slip and whose granules were not directly exposed to the light exhibited exocytosis of AO.

In granules and vesicles, AO's green fluorescence at 530 nm is quenched by formation of dimers and multimers. It becomes much brighter by dequenching when released into the extracellular environment [Palmgren, 1991]. Exocytotic activity did strictly depend on exposure to light. AO-stained cells could be kept in the dark on the microscope stage for at least up to 45 min without any significant loss of fluorescence of either the background or the vesicular stain.

Only 20%–30% of the cells in the field of view showed light-induced exocytotic activity while the rest remained unaffected. Almost all inactive cells lay solitary, indicating no dividing activity, while cells with longer processes, with neighbors or actively dividing cells exhibited exocytosis after illumination very frequently.

Flash-like fluorescence signals were rarely seen when cells were stained with Lysotracker yellow-blue (Molecular Probes, Eugene), a lysosomotropic dye that specifically stains acidic lysosomes (data not shown).

Figure 1D summarizes the analysis of flash frequency under varied experimental conditions. First we looked if the activity depended on calcium, since calcium is necessary for many if not for all modes of exocytosis. In order to test if calcium enters the cell by light-induced depolarization of the plasma membrane, calcium currents through voltage-gated channels were blocked with cadmium, a rather unspecific blocker of calcium channels. Cadmium blocks all types of calcium channels non-specifically at concentrations above 500 µM but is also toxic for the cells. At a concentration of 20 µM (used in our experiments) cadmium blocks mainly voltage-gated calcium channels (IC 50 = 4 µM) and exhibits no significant cell toxicity [Hinkle et al., 1987]. Exocytotic activity was not affected by this treatment, arguing against the participation of voltage-sensitive calcium channels in this process. Addition of carbachol, a cholinergic activator of exocytosis in chromaffin cells and simultaneous elevation of calcium to 10 mM in the bath did not increase flash frequency significantly, an observation that argues against the participation of muscarinic acetylcholine receptors in exocytotic activity.

In calcium free bath the cells did not showed any flashes, but this environment caused most

cells to die within a few minutes, indicated by detachment from the cover-slip and morphological changes in cell shape. Reduction of calcium to 156 nM resulted in a profound decrease in number of flashes but maintained cell's normal morphology. Additional administration of ryanodine, a calcium channel inhibitor that blocks release of calcium from intracellular stores, inhibited the exocytotic activity entirely. Ryanodine and low extracellular calcium showed no cytotoxic effects within the timeframe of the experiments.

Spontaneous Calcium Flashes During Exposure to Light

Figure 2A shows cells on a TIRF microscope stage, stained with the membrane permeable calcium sensor Calcium Green AM. Some tightly attached cells showed flickering flashes that probably corresponded to single calcium channels after a short delay (15–30 s after start of illumination). Since Calcium Green AM fluorescence is excited with 488 nm light like AO, we could not image calcium and AO simultaneously. Depolarization of the cells by perfusion with 60 mM KCl caused large increase of calcium at these locations (see the movie $PC12Ca0_2$.mov in the supplemental material). No calcium flashes were observed in low calcium (156 nM) medium. However, some diffuse structures and calcium containing organelles appeared visible (data not shown). Three areas were analyzed for brightness fluctuation in Figure 2B. The traces indicate a random flickering that corresponds to calcium entry through single calcium channels or channel clusters into the cell.

Preferred Sites of Exocytosis

Thereafter we analyzed the spatial distribution of exocytotic events by overlay projection of all flashes onto a cell. Figure 3 gives two examples of cells. Flashes were not randomly distributed but appeared located at the periphery of the cell and at the interface between dividing cells.

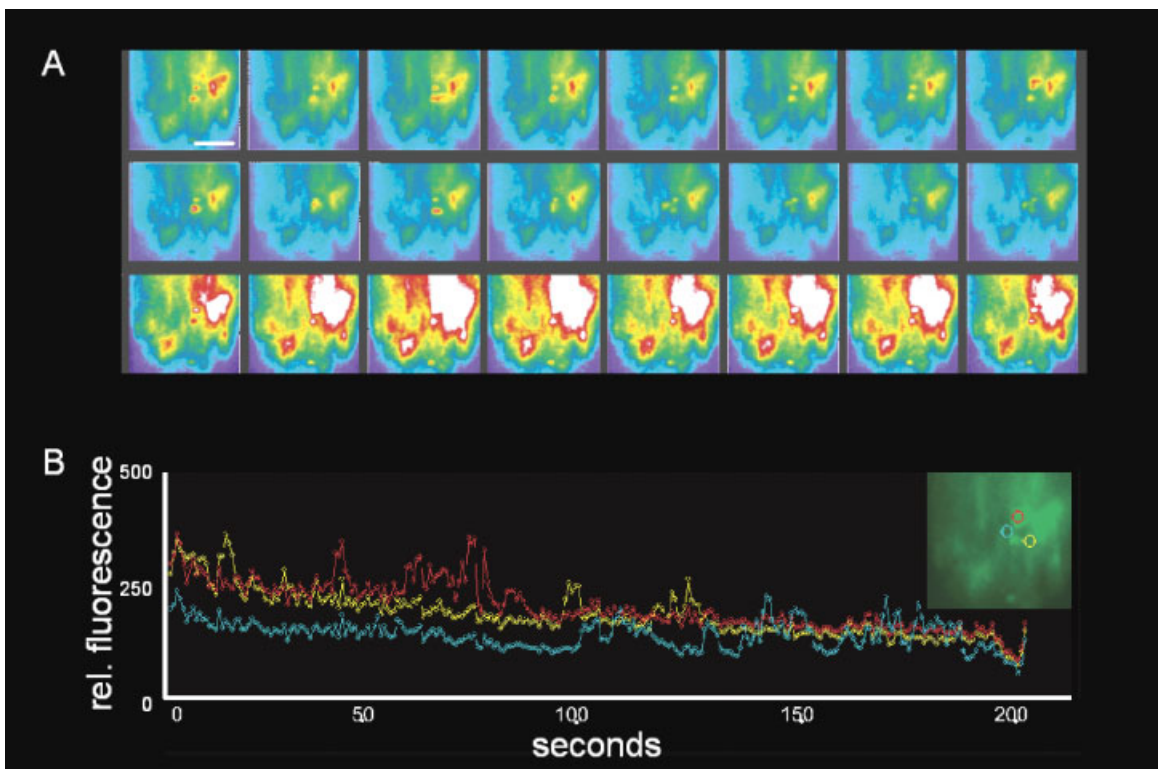

Fig. 2. Calcium imaging reveals single channel activity by TIRF microscopy. **A**: Pseudo color time frame series of a tightly attached PC12 cell stained with the calcium sensor Ca green-AM. The sequence was recorded at a frequency of 10 Hz. Individual fluctuating flashes represent localized calcium entries (two upper rows). Perfusion with 60 mM KCl triggered massive calcium influx into the cell (lower row). Scale bar = 2 μm. **B**: Ca green-AM brightness measurements of a PC12 cell from 3 small regions marked in the inset picture, show transient fluorescence calcium fluctuations within 20 s. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

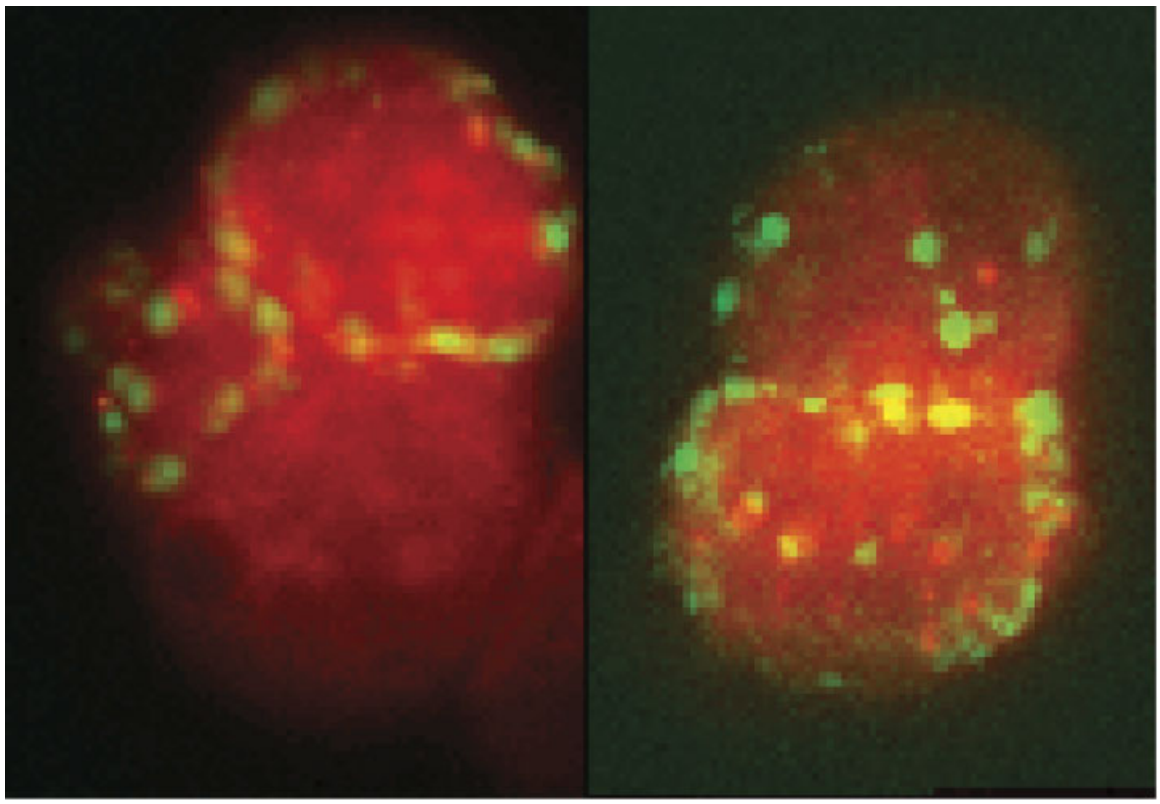

Fig. 3. Distribution of exocytotic events. All individual exocytotic events (green) are overlaid onto PC12 cells. Most events occur at the periphery or at the border between dividing cells. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

In addition, there are small specific sites were multiple flashes occurred in rapid succession so called "hot spots." In contrast to genuine "kiss-and-run" events, where one vesicle connects repetitively with the plasma membrane, a hot spot corresponds to a site were many individual vesicles undergo fusion. No genuine kiss-and-run events were observed in PC12 cells, but some cells had restricted areas, where many exocytotic events occurred within 1 min. Hot spots frequency varied considerably from cell to cell, but these events were never observed when the external calcium concentration was low.

Expression of Opsin 3 in PC12 Cells

mRNA of PC12 cells was collected after 1 day, 1 week, and 1 month after replating and quantitative expression analysis of opsin 3 was performed by real time PCR. Light-induced exocytosis was highest during the first 3 days and declined after this period until no exocytosis could be triggered after 1 week in culture. This time course corresponded very well with the expression of opsin 3. Table I shows the results of quantitative real time PCR. One-day-old cells expressed 2.33-fold more opsin 3 than 7-day-old cells while mRNA was not detectable in cells that were kept in culture for 1 month.

Embryonic Chicken Telencephalon Cells Exhibit Light-Induced Exocytosis During Development

Besides PC12 cells, several other primary cells and cell lines were tested for light response too. Table II shows that besides PC12 cells only embryonic chicken telencephalon cells (CTCs) exhibited light-induced exocytosis. The

TABLE I. Expression of Opsin 3 in PC12 Cells

Days after replating	ΔCT (opsin 3-β-actin)	Normalized opsin 3 RNA (%)	Experiments (n)
1	7.4 ± 0.46	100	3
7	8.62 ± 0.59	42.9	16
30	Not detectable	0	4

TABLE II. Light-Induced Exocytosis is Cell Type Specific

Cell type	Exocytotic activity
Astrocytes (rat)	–
Cerebellar Körner cells (rat)	–
Cortex cells (rat)	–
Hippocampus cells (rat)	–
PC12 cells (rat)	++
Telencephalon cells (chicken)	+++
H4 cells (human neuroblastoma)	–

phenomenon was development-dependent and disappeared after 4–5 days in culture. Figure 4A shows four single exocytosis events in a CTC that had very similar kinetic characteristics like in PC12 cells. Figure 4B shows a kymograph display of this cell. The green streaks represent single vesicles while the purple clouds indicate exocytotic events. The disappearance of the streaks after exocytosis indicates complete fusion of the corresponding vesicle. This kind of presentation offers an opportunity to see the temporal sequence and intensity in a picture, similar to a movie. Time course and magnitude are quantified in Figure 4C. In three exocytotic events the brightness dropped below the initial level after dissipation of the AO-cloud.

Kiss-and-Run Exocytosis in CTCs

Transient exocytosis "kiss-and-run" has been reported by several groups in neurons and endocrine cells [Duclos et al., 2000; Henkel et al., 2000; Stevens and Williams, 2000]. We analyzed individual exocytotic events in order to determine, if light induced exocytosis preferentially utilizes complete or "kiss and run" exocytosis. "Kiss-and-run" exocytosis from a single

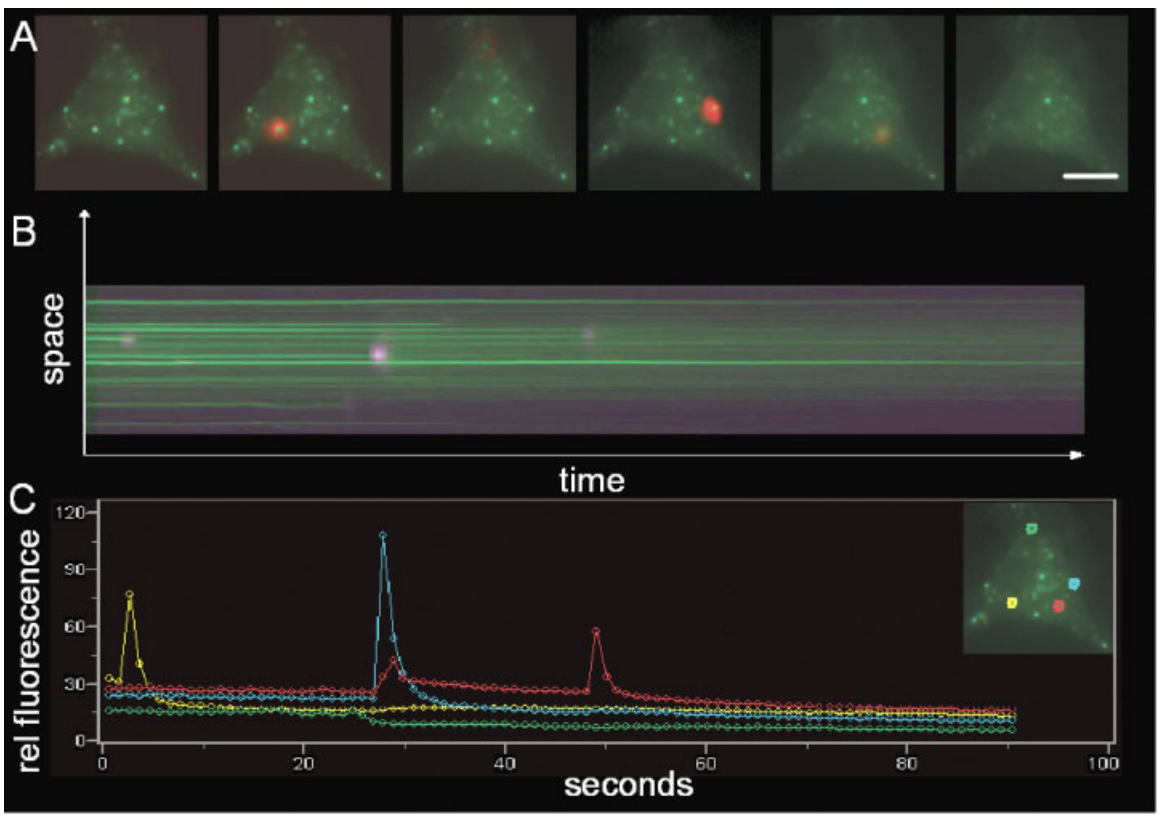

Fig. 4. Exocytotic events in chicken telencephalon cells (CTCs), imaged by epifluorescence microscopy. **A**: Single images of a CTC with a astrocyte-like morphology from a movie, show four exocytotic events (red colored) within 30 s. The original images were recorded at a frequency of 3 Hz and the focus plane was adjusted onto the lower plasma membrane (scale bar = 5 μm). **B**: Kymograph (space-time projection) of the movie. The green streaks represent individual vesicles. Fading of streaks represents photo-bleaching, while purple clouds at the end of some streaks indicate exocytosis of the corresponding vesicle. **C**: Fluorescence quantification of four individual vesicles shows that three vesicles (yellow, blue, and red traces) undergo complete exocytosis and release a cloud of AO while one vesicle (green trace) disappears with a very faint AO cloud (see third image in panel A). The peak in the red trace at 29 s corresponds to a cloud that was released from a nearby fusion (blue trace). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

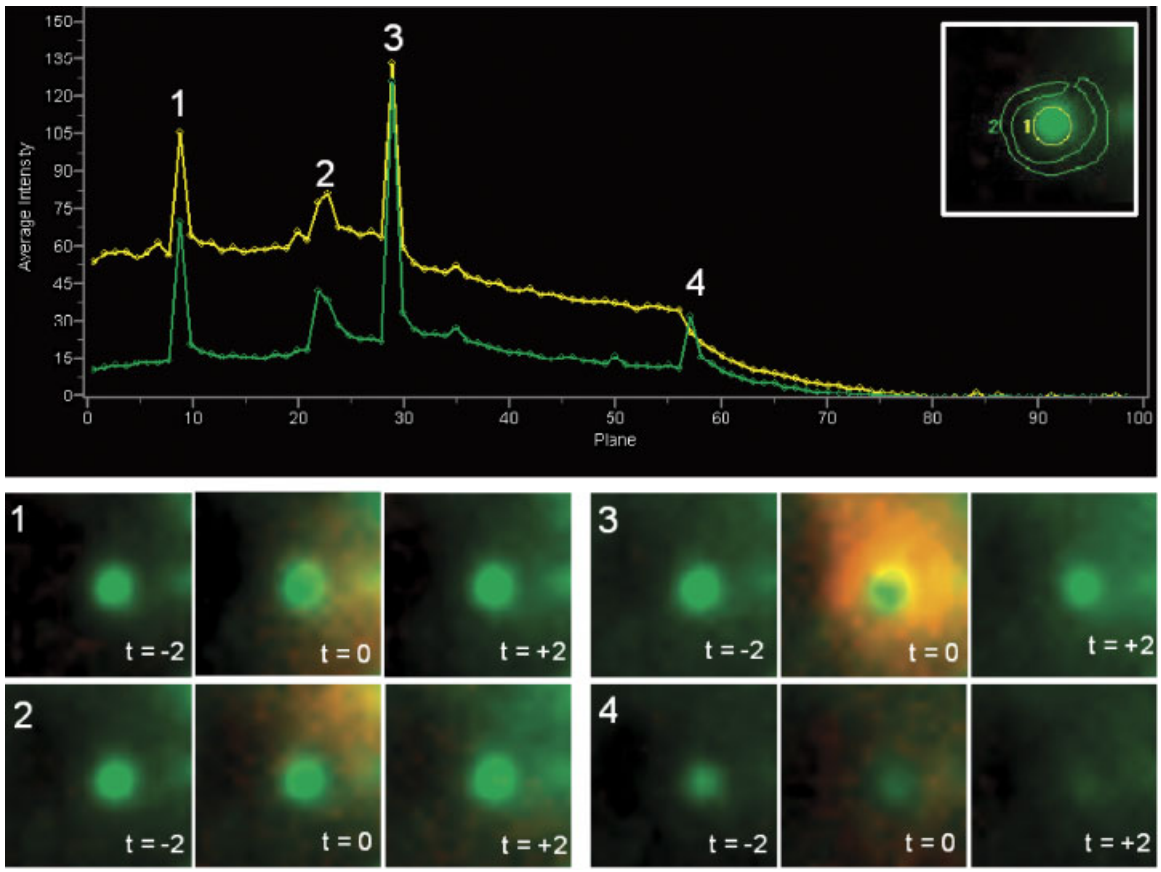

Fig. 5. 'Kiss and run' exocytosis by an individual vesicle. The yellow trace (one plane = 0.33 s) in the fluorescence intensity plots (**upper panel**) shows the average brightness of the vesicle in region 1 (inset) and the green trace an annular region 2 around it. The lower four **panels** (**1**–**4**) correspond to the fluorescence intensity peaks in the upper panel: AO clouds in events 1 and 2 originate from exocytosis of nearby vesicles. Event 3 represents a partial exocytosis that coincide with a drop in remaining vesicle brightness. Event 4 releases AO more slowly, indicated by a less intensive cloud that decayed within 2.66 s. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

granule occurred in only 26 of 543 (4.8%), of all observed events in CTCs. Figure 5 demonstrates that genuine "kiss-and-run" events may be obscured by exocytotic release from nearby vesicles. However, Figure 5 also shows that repetitive release from an individual vesicle can be separated from complete fusion by a drop of fluorescence below the baseline (events 3 and 4).

Differentiation Between Exocytosis and Photolysis of AO-Loaded Vesicles by Epifluorescence Microscopy

PC12 cells or CTCs were loaded with 10, 3, or 0.3 μM of AO in order to test, if illumination causes rupture of vesicles rather than fusion with the plasma membrane. When cells were loaded with 3 μM AO, two opposite phenomena were observed. First, one group, corresponding to 31.5% of all light responsive cells, went gradually dimmer and many flashes occurred when they were exposed to light. This is expected when AO is released from the cell and when the remaining dye is photo-bleached by exposure to light.

Figure 6A,B shows an experiment, where two CTCs and two extracellular areas were analyzed for fluorescence change during exocytotic activity by epifluorescence microscopy. The upper cell in Figure 6A did not respond to light while the lower cell exhibited a furious exocytotic activity. The results are presented in Figure 6B. The active cell shows many flashes and an overall increase in brightness (yellow trace). The single flashes caused a stepwise increase of brightness during the first part of the experiment. The dye becomes trapped under the cell and its diffusion is retarded. When the flash

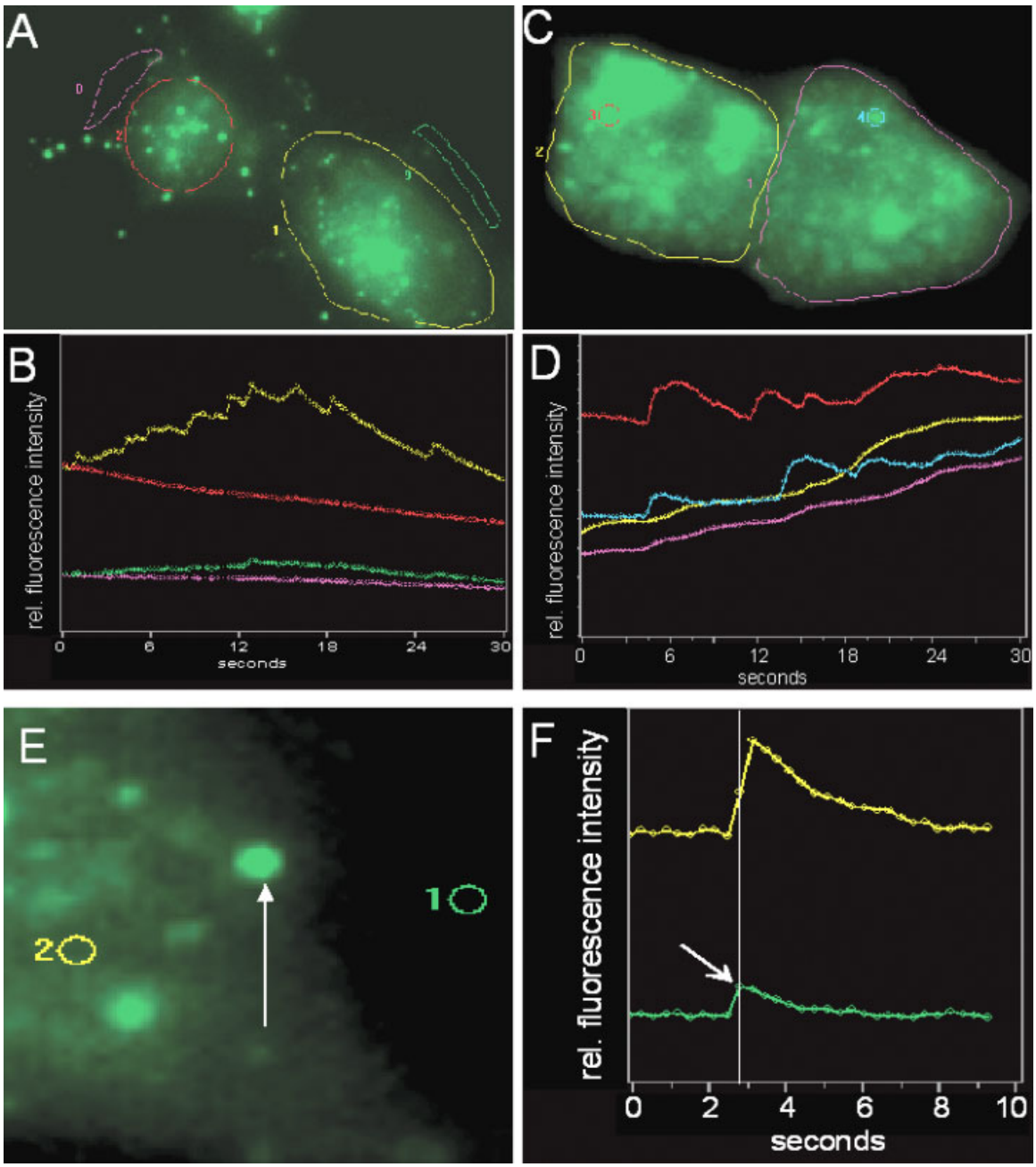

Fig. 6. Extra- and intracellular release of AO. Epifluorescence images of CTCs, loaded with 3 μM AO. The focus plane is adjusted to the coverslip-plasma membrane region. **A**: Release of AO into the extracellular space. The red encircled upper left cell is inactive, while the yellow encircled cell exhibits genuine exocytosis. The green and purple regions are located outside the cells. **B**: Fluorescence intensity plots of four regions in panel A. The brightness within the purple and red regions declined steadily due to photo-bleaching, while the brightness within the yellow region increases initially, due to release of AO from fusing vesicles. Brightness decreases fast by lateral diffusion of AO after exocytotic activity has ended. The green region outside the cell shows a transient increase when released AO diffuses away from the cell. **C**: Intracellular photolysis of AO-loaded vesicles, imaged in two PC12 cells (encircled in purple and yellow). Two red and the blue encircled regions enclose vesicles. **D**: Fluorescence intensity plots corresponding to panel C show that both cells exhibit dye leakage and become brighter, while the vesicles release the dye slowly into the cytoplasm. **E**: Extracellular release of AO from a CTC, imaged during exocytosis of a vesicle (arrow) that is located at the very periphery of the cell. **F**: Fluorescence intensity plots, obtained from the regions in panel E, show a exocytotic event and the diffusion of an AO cloud into the extracellular space (green trace) and the area between plasma membrane and cover slip (yellow trace). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

frequency and thus the AO supply went down during the second half of the experiment, the fluorescence declined sharply, much faster than expected for photo bleaching. For comparison, the rate of photo bleaching of the inactive cell (red trace) was much less steep. The extracellular area (encircled by the green line) that was located closer to the active cell shows also in transient increase of brightness, indicating AO concentration increase outside the cell.

Intracellular photolysis of AO-stained vesicles was observed in PC12 cells in epi-fluorescence mode; two examples are presented in Figure 6C,D. This second group, which corresponded to 68.5% of all light responsive cells, showed also many flashes, but the kinetic of these single events was much slower and took longer, compared to genuine exocytotic events. Additionally, the cells became much brighter during the course of the experiment. Interestingly, the cells stayed bright after the last flash and went finally dimmer due to slow photo bleaching. This phenomenon was only observed, when cells were imaged at the epifluorescence microscope and were never seen in TIRF microscopy. It can only be explained by rupture or blasting of vesicles within the cell and release of dye into the cytoplasm. The dequenching of AO after disruption of vesicles caused an increase of brightness that overcompensates photo bleaching. All cells ($n = 15$), loaded with 10 μM AO showed this brightening, an indication for phototoxicity and photolysis caused by AO at high concentrations.

Figure 6E shows an example for release of AO from a vesicle into the extracellular space. The vesicle is located at the cell periphery and secretes into the interspace between cover-slip and plasma membrane. As expected, the AO cloud outside the cell, dissipated much faster as it escaped out of the focal plane, compared to the dye trapped under the cell. When the extracellular fluorescence in Figure 6F was measured at increased distances from the origin, the maximal brightness was reached only 0.3 s after the fusion event. It stayed at a plateau during the time course of secretion activity.

When PC12 cells or CTCs were loaded with 0.3 μM AO, all cells showed only flashes and simultaneous dimming during exposure to light. At this low concentration, AO containing vesicles kept their structural integrity and the dye was exclusively released into the extracellular space.

DISCUSSION

An Extraocular Light Sensing Mechanism for Cell Development

The observation that a light sensing mechanisms exists in cells which are located within the body or even the brain, appears somewhat surprising at the first sight. However, there are photosensitive molecules present in the brains of many vertebrates, reaching back to lamprey eel [Yokoyama and Zhang, 1997]. Visible and infrared light (480–900 nm) can penetrate living tissue and even bones [Grinvald et al., 1986; Frostig et al., 1990]. Especially light of more than 700 nm is capable to penetrate even the scull of human babies completely, thou near-infrared light is clinically used for cerebral oxygenation measurements in newborns [Wyatt et al., 1986]. The light-sensitive molecule pinopsin in avian pineal gland is important for regulation of the circadian rhythm [Okano et al., 1994]. Extraocular light perception has also been suggested as the primary regulator of the circadian clock in humans [Czeisler et al., 1995; Cambell and Murphy, 1998]. Since these mechanisms are important for neuronal plasticity, they support a role of light for cell development and growth cone activity.

Light-Induced Exocytosis Occurs at the Cell's Periphery

In our experiments, we found that exocytotic events occurred preferentially at the periphery of the cells and at the border between dividing cells. This is in contrast to observations in PtK2 cells, which showed spontaneous fusions predominantly in the center of the cells [Toomre et al., 2000]. These cells posses a very different morphology and are much larger, up to 100 μm in diameter, than PC12 cells or CTCs. Interestingly, there are no vesicles located in the periphery of these cells at all. This might point to another mechanism for lateral growth or represent constituent exocytosis. Several other groups, working on TIRF setups, reported fusions occurring randomly all over the cells adhesive region under conditions of chemical or electrical stimulation [Steyer and Almers, 2001; Toomre and Manstein, 2001; Zenisek et al., 2003]. By contrast, light induced exocytosis was not seen in chromaffin cells which is not really surprising, since these cells do not divide anymore [Steyer et al., 1997; Oheim et al., 1998].

In PC12 cells, however, directional growth cone movement can be guided by long wavelength light from a laser [Ehrlicher et al., 2002]. Since growth cone growth requires highly elevated exocytotic activity at the position of extension, it appears likely that a photon-sensing mechanism is present in these cells. A Japanese group showed recently that NGF-dependent differentiation of PC12 cells into a neuron-like morphology is modified by exposure to light [Higuchi A et al., 2003]. Consequently, PC12 cells appear to have a light receptor that is involved in cell development. We found evidence that the light receptor in PC12 cells may correspond to opsin 3, a rat homolog to human encephalopsin [Blackshaw and Snyder, 1999]. The expression of opsin 3 in PC12 cells is highest during the first week after sub-culturing when the cells reside in the exponential "log" growth phase [Freshney, 2000]. We found that PC12 cells exhibited light-induced exocytosis only during the first few days after sub-culturing and CTCs only up to 4 days after isolation. When PC12 cells enter the "plateau" growth phase or embryonic CTCs stop dividing, light-induced vesicle fusions are not observed anymore. The close correlation of this effect with opsin 3 expression, measured in PC12 cells, strongly supports the idea that light may regulate cell proliferation.

Calcium is Required as Trigger and Regulator of Light-Induced Exocytosis

A further argument for a physiological role is based on the calcium dependence of light-induced exocytosis. Since all exocytotic events occurring in PC12 cells and CTCs depended on calcium and were restricted to a defined time window during CTC development, it appears unlikely that these signals are caused by simple photolysis of the vesicles [Brunk et al., 1997]. There is no evidence for calcium regulated or calcium dependent photolysis so far.

We were also able to show by direct calcium imaging that single calcium sparks occurred during illumination. These transient flashes were visualized by TIRF microscopy, which indicated a close localization to the plasma membrane. It is not clear, if the sparks originated from single calcium channels or from intracellular stores. Similar observations were interpreted as calcium microdomains [Becherer et al., 2003]. Exocytosis was completely blocked by additional inhibition of ryanodine-receptor mediated calcium release from internal stores. But which mechanism could be the basis for of the observed effects? In salamander rod outer segments, calcium concentration can also be increased by light if extracellular calcium and sodium are removed [Matthews and Fain, 2001]. It was shown earlier that the outer segment of rods and cones contains a calcium store within membrane disks, similar to smooth endoplasmic reticulum [Fain and Schroder, 1985; Schnetkamp and Bownds, 1987]. The mechanism of calcium release into the cytoplasm operates via phospholipase C, which generates inositol 1,4,5-triphosphate (IP3) activating an IP3-receptor or a ryanodine receptor located on these stores [Berridge, 1993; Mikoshiba et al., 1994]. It is conceivable that a similar mechanism operates in PC12 cells and CTCs during an initial phase of development.

Exocytosis Versus Photolysis

The major objection against light induced exocytosis is that it is not a biological but rather a physical or chemical phenomenon. We tested for several artifacts that could be responsible for the observed effects. The most important is light-induced photolysis of vesicles as reported earlier [Brunk et al., 1997]. In our TIRF experiments, some PC12 cells, which were only loosely attached to the cover-slip, exhibited turbulent exocytosis too. In these cases where no individual granules were seen, the evanescent wave transferred only a small fraction of energy, if any, to the granules. Therefore, the granules were never subjected to potentially photolysis that could have caused disruption of the vesicular membrane. Consequently, the light-sensing mechanism must have been located at the plasma membrane and the signal had to be transduced to the cell's internal calcium stores by a second messenger mechanism. The delayed onset (15–30 s) of exocytosis after light exposure strongly supports this conclusion. In the rod cone and in the pineal gland, light triggers vesicle fusion via a second messenger pathway that also involves the release of calcium from internal stores in order to stimulates synaptic neurotransmitter release [Brockerhoff et al., 2003]. Staurosporine, a rather unspecific kinase inhibitor increased the frequency of exocytotic events. This is most likely due to an increased frequency of "kiss-and-run" events, which have been observed before [Henkel et al., 2001].

We were able to demonstrate that AO is secreted from vesicles into the extracellular space. This has been shown by measuring a faster diffusion of an AO cloud in areas, located outside of secreting cells, compared to regions within cells or within the interface between plasma membrane and the cover-slip. An example is presented in Figure 6.

We are aware that the light intensities in our experiments were much higher than PC12 cells and CTCs would have been exposed in in-vivo situations. Therefore the magnitude and frequency of exocytosis is not considered physiological. However, we intended to determine the experimental parameters with regard to controls, in order to emphasize differences. Low light would have been much less efficient to trigger exocytotic events and this would have prevented us to quantify exocytotic events in a reasonable time frame.

Physiological Relevance of Light-Induced Exocytosis

In this study, we have shown that CTCs and PC12 cells possess a light-sensing mechanism that triggers exocytosis of large individual vesicles. The nature of the mechanism is not completely clear yet, however, possible candidates for light sensing could be members of the opsin family. Pinopsin from the bird pineal gland, for example, is most sensitive for blue light of 470 nm [Okano et al., 1994], about the same wavelength we used to trigger exocytosis. We have identified opsin 3 as a potential molecular candidate for light-sensing in PC12 cells by quantitative PCR. It was expressed preferentially during the proliferation phase and decreased when the cells became confluent. At the same time the cells lost the ability to respond to light-induced exocytosis.

The physiological meaning of light-induced exocytosis is most likely relevant during embryonic development. The molecular machinery for light sensing shows similarities to the signal transduction in retinal rod cells. The major difference in PC12 cells and CTCs is that light-induced exocytosis is the basis of plasma membrane surface increase, rather than for liberation of neurotransmitters. Recently an exocytotic vesicle type, the "enlargeosom," was identified in PC12 cells that has been suggested to be involved in calcium-dependent enlargement of the plasma membrane surface [Cocucci et al., 2004]. It is conceivable that this new cellular compartment corresponds to vesicles, undergoing light-dependent exocytosis. It is further conceivable that the mechanism is involved in the regulation of neural plasticity, since some psychiatric diseases like depression show a strong response to light therapy even in blind patients [Rosenthal et al., 1989]. Intrinsic emission of "biophotons" that originate from metabolic processes could also provide a light source for regulation of cell development [Niggli, 1992]. Consequently, high metabolic activity could promote cell growth and proliferation [Niggli et al., 2001].

In summary, our observations point to a novel mechanism for regulation of cellular development and/or differentiation by visible light.

ACKNOWLEDGMENTS

All TIRF experiments were conducted at the Max–Planck-Institute for Medical Research, Department of Biophysics, Heidelberg, Germany. We thank H.H. Gerdes for helpful discussions and for provision of PC12 cell cultures and D. Manstein for provision of the TIRF microscope setup.

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