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Optical Nanosensors for Chemical Analysis inside Single Living Cells. 2. Sensors for pH and Calcium and the Intracellular Application of PEBBLE Sensors

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Optical nanosensors, or PEBBLEs (probes encapsulated by biologically localized embedding), have been produced for intracellular measurements of pH and calcium. Five varieties of pH-sensitive sensors and three different calcium-selective sensors are presented and discussed. Each sensor combines an ion-selective fluorescent indicator and an ion-insensitive internal standard entrapped within an acrylamide polymeric matrix. Calibrations and linear ranges are presented for each sensor. The photobleaching of dyes incorporated into PEBBLEs is comparable to that of the respective free dye that is incorporated within the matrix. These PEBBLE sensors are fully reversible over many measurements. The leaching of fluorescent indicator from the polymer is less than 50% over a 48-h period (note that a typical application time is only a few hours). The PEBBLE sensors have also been applied to intracellular analysis of the calcium flux in the cytoplasm of neural cells during the mitochondrial permeability transition. Specifically, a distinct difference is noted between cells of different types (astrocyte vs neuron-derived cells) with respect to their response to the toxicant *m*-dinitrobenzene (DNB). Use of PEBBLE sensors permits the quantitative discrimination of subtle differences between the ability of human SY5Y neuroblastoma and C6 glioma to respond to challenge with DNB. Specifically, measurement of intracellular calcium, the precursor to cell death, has been achieved.

Changes in intracellular ion concentrations accompany many processes in living cells, including transport, signaling, and enzyme function.[1−5] Two of the most widely studied ions, calcium and hydrogen, have been found to be ubiquitous in these and other cellular processes. Several methods have been developed for studying intracellular calcium and pH, one of the most common being fluorescence spectroscopy. Addition of exogenous fluorescent indicators to the intracellular environment has proven to be useful for qualitative monitoring of ion fluxes, as the emission intensity of the indicator changes with intracellular ion concentration, leading to greater insight into cellular processes. Although there are many fluorescent indicators for both calcium and pH, and much effort has been invested developing methods for their accurate quantitative use, there still exist problems that prevent the dyes from being ideal for such intracellular measurements.

Fluorescent indicators for pH have been used longer and more widely for intracellular measurements than any other fluorescent indicators.[5] Fluorescein has been used for many years and has been modified and derivatized to make it more useful in cellular applications. Other families of pH indicators have also been developed, and most of these pH probes are commercially available.[6] Thus, pH indicators are probably the most advanced in their ability to accurately report intracellular levels of hydrogen ions. However, there are still many questions about the validity of some of the measurements made with these indicators. Even a ratiometric dye can provide erroneous readings due to interactions between the cell and the dye. It has been shown previously[7,8] that even low levels of proteins can bind dye molecules and lead to large errors in measurements. To circumvent this problem, methods have been developed to calibrate the dyes inside the cellular environment, but these calibrations can be laborious and time-consuming, frequently incurring experimental error. Most importantly, many pH indicators rapidly leak out from cells, thereby significantly affecting the measurements and severely limiting the amount of time in which the cells can be analyzed.[9]

Use of calcium indicators for intracellular measurements can also be problematic.[3,4] Fura-2 is the most commonly used calcium indicator,[9] due to its ratiometric properties and commercial availability. Fura-2, like almost any cytoplasmic indicator, distributes throughout the entire cell. The calcium concentration

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that is reported is thus a distribution of signals from all areas of the cell, including organelles and membranes. Efforts to minimize errors due to intracellular distribution, such as cold incubations and mathematical deconvolution,[10] may help, but the contribution from sequestration of the indicator dye into organelles is often ignored. Other calcium ionophores have been developed, such as Calcium Green and its family of indicators.[6,11] The principal reason for using these dyes instead of Fura-2 is that their longer excitation wavelength allows the use of lasers as the excitation light source. Other advantages of using longer wavelengths are both reduced autofluorescence and minimized photodamage to the cells. Unfortunately, the Calcium Green family of indicators displays only a single emission peak (not ratiometric), which makes it difficult to differentiate between high concentrations of calcium in a cell and high levels of dye in the cell.

The probes encapsulated by biologically localized embedding (PEBBLE) sensor was devised in order to provide a fluorescence-based method for intracellular analysis that circumvents many of the above-mentioned problems. The PEBBLE is a submicrometer, spherical polymer that contains one or more fluorescent dyes that are trapped within the pores of the matrix. The polymer provides a protective coating for the dyes, so that they are not affected by the cell, thereby minimizing error from protein binding and membrane/organelle sequestration. Thus, PEBBLEs can be calibrated in vitro and used in vivo, with the response of the sensors remaining consistent regardless of environment. Multiple dyes can be combined in a single PEBBLE sensor, so that a reference standard can be used to minimize errors from uneven dye distributions throughout the cell. Another advantage of PEBBLEs is that they are small enough to be biocompatible, yet large enough to be retained in the cytoplasm of the cell with no leakage of the sensor out of the cell, even after several days. Furthermore, many toxic effects of the dye are eliminated.

Each sensor discussed in this paper contains a combination of dyes, one selective for the ion of interest and one used as a reference. The dual dye combination provides the ability to compensate for areas of high dye concentration in an intracellular environment, so that they can be distinguished from areas of high ion concentration. Calibration also becomes easier than that of a single-intensity dye or sensor. Many dye calibrations used to date rely on the ability to pipet equal amounts of dye into buffers of known ion concentration, imparting a significant error upon the calibration. The dual intensity PEBBLE sensors are easy to fabricate and calibrate, as it does not matter if there are different amounts of sensors in each buffer solution or if a single stock solution is diluted in order to change the ion concentration or pH.

The sensors developed in this paper are selective for pH and calcium, due to the intracellular importance of each. The PEBBLE properties that are specific to the dye incorporated within the matrix are discussed herein. These properties include calibration, leaching of the dye from the matrix, photobleaching of the sensors, reversibility of the measurements, and interference from other ions. Properties that are not specific to the dye entrapped in the sensor, i.e., properties that are true for any PEBBLE sensor, such as fabrication, size, shape, macromolecule interference, and response time as well as methods for inserting PEBBLEs into living cells, have been discussed in the preceding paper.[8] A biological application of the calcium-selective PEBBLE sensors is also presented here. In this application, the mitochondrial permeability transition (MPT) is stimulated in neural cells and astrocytes using the neurotoxicant, *m*-dinitrobenzene (*m*-DNB). The *m*-DNB induces mitochondrial depolarization and subsequent release of calcium reserves from these organelles into the cytoplasm of the cell. Calcium-selective PEBBLE sensors were used to monitor mitochondrial calcium release, using both spectroscopy and confocal microscopy to characterize this intracellular phenomenon leading to cell death.

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EXPERIMENTAL METHODS

Instrumentation. All measurements, except for confocal imaging, were taken on a FluoroMax-2 spectrofluorometer (ISA Jobin Yvon-Spex, Edison, NJ), slits set to 5 nm for both the emission and excitation.

Photobleaching. Measurements were performed on a solution of PEBBLEs or dye of approximately equal concentrations. The solution was pipetted into a glass capillary and mounted in the fluorometer at a 30° angle to the excitation source. The intensity of the fluorescence signal was monitored vs time.

Leaching. A solution of PEBBLEs was injected into a 10 kDa dialysis cartridge (Pierce, Rockford, IL) and immersed in aqueous buffer, pH 7.0, for 48 h. Aliquots of the buffer surrounding the dialysis cartridge were monitored in a fluorometer over the 48-h period and replaced after each measurement. The PEBBLE solution from inside the dialysis cartridge was analyzed before and after the experiment to find the total dye loss, to correct for differences in concentration and quantum efficiency between the PEBBLE matrix and the surrounding buffer solution. After a week's time, it was determined that all the dye had leached from the PEBBLEs, and this dye was compared to known concentrations of dye to determine the total amount of dye contained within the PEBBLE sensors.

Calibrations. PEBBLEs were calibrated in a standard spectrophotometric cuvette, both by placing PEBBLEs in buffers of varying ion concentrations and by varying the ion concentration in a single sample of PEBBLEs. Both methods yield similar results. Free dyes were calibrated similarly, with a reference dye added to each sample to make calibrations more accurate and adjust for dilutions.

Cell Culture. Human C6 glioma and SY5Y neuroblastoma cells were cultured in Dulbecco's modified Eagle medium (DMEM) containing 4500 mg/L D-glucose, 2 mM L-glutamine, and 20% (C6 glioma) or 10% (SY5Y neuroblastoma) fetal bovine serum. Cells were released from culture dishes by trypsin treatment 1 day prior to experiments and plated on uncoated 22-mm glass cover slips in 35-mm culture dishes.

Laser Scanning Confocal Microscopy. Fluorescence of the PEBBLE sensors was monitored using a Noran laser scanning confocal microscope equipped with an argon−krypton laser. Green fluorescence of Calcium Green and red fluorescence of sulforhodamine 101 were excited simultaneously by reflecting the 488- and 568-nm lines of the argon−krypton laser onto the specimen using a double dichroic mirror. Emitted fluorescence was divided by a second dichroic reflector into light with wavelengths above

Table 1. Results of Calibrations of Five pH-Sensitive Dyes and the Corresponding PEBBLE Sensors[a]

pH indcator[b]	linear range (pH units)	slope[c] ± SD	intercept	r^2	n
CNF (dye)	7.0−7.7	7.8 ± 0.4	−53	0.98	6
CDMF + SR (dye)	6.2−7.4	3.3 ± 0.5	−19	0.96	12
BCPCF + SR (dye)	6.5−7.6	1.3 ± 0.07	−7.3	0.99	6
FSA + SR (dye)	5.8−7.2	3.1 ± 0.3	−17	0.99	7
SNAFL (dye)	7.2−7.7	−0.75 ± 0.01	6.8	0.99	4
CNF (PEBBLEs)	7.0−7.7	0.24 ± 0.01	−0.66	0.96	5
CDMF + SR (PEBBLEs)	6.2−7.4	0.67 ± 0.05	−3.0	0.99	6
BCPCF + SR (PEBBLEs)	6.2−7.2	0.43 ± 0.07	−1.6	0.90	6
FSA + SR (PEBBLEs)	5.8−7.0	3.4 ± 0.4	−17	0.99	5
SNAFL (PEBBLEs)	7.2−8.0	−0.49 ± 0.02	4.6	0.99	7

[a] An internal standard, sulforhodamine 101 was added to each dye solution and was contained within the polymeric matrix of each PEBBLE sensor. Measurements were made at the optimum emission wavelength for each individual indicator and dye. [b] CNF, 5-(and 6-)carboxynaphthofluorescein; CDMF, 5-(and -6-)carboxy-4′,5′-dimethylfluorescein; BCPCF, 2′,7′-bis-(2-carboxypropyl)-5-(and 6)carboxyfluorescein; FSA, fluorescein-5-(and 6)sulfonic acid; SNAFL, 5-(and-6)-carboxy SNAFL-1; SR, sulforhodamine 101. [c] Ratio of normalized fluorescence intensity vs pH.

and below 560 nm. Calcium Green fluorescence was detected by passage through a 500-nm (25-nm band-pass) barrier filter and red fluorescence of sulforhodamine 101 through a 590-nm long-pass filter. Laser dwell time was 100 ns. A 60×, 1.4 NA oil immersion objective was used to image Calcium Green and sulforhodamine 101 fluorescence. Data were acquired with a Silicon Graphics Indy workstation (SGI, Mountainview, CA) and analyzed using Photoshop Adobe Systems, (Mountainview, CA) software packages.

Measuring Calcium Release in Cell Culture. Cells were grown on 22-mm cover slips and suspended at a 30° angle in a standard cuvette. This angle was used in order to minimize the amount of scatter from the fluorometer excitation source into the PMT. The fluorescence intensity was monitored for 10 min after an aliquot of *m*-DNB was added through an addition port on the fluorometer. A new coverslip of cells was used for each dose of *m*-DNB. PEBBLEs were introduced by lipofection.[8]

RESULTS AND DISCUSSION

Table 1 summarizes the linear ranges, slopes, intercepts, and linear fits for five different pH dyes and their corresponding PEBBLE sensors. Each PEBBLE contained an inert internal standard, sulforhodamine 101, and this dye was also added to each dye solution as an internal standard, to aid in calibration and to be a consistent comparison to the PEBBLE sensors. The slopes and intercepts correspond to the normalized fluorescence intensity, which is the fluorescence intensity of the pH indicator divided by the fluorescence intensity of the internal standard. The ratio of intensities is then normalized as R/R_0 (R is the ratio of the fluorescence intensities, and R_0 is the ratio at the lowest pH in the linear range of the pH calibration) for consistency in comparing one pH indicator to another. The fluorescence intensity ratio was normalized to compensate for variations in the amount of internal standard used from one type of pH sensor to another, so that the slopes could be compared directly. It is evident that the slope of the pH indicator changes significantly when it is immobilized in the PEBBLE sensor. The slope of the calibration becomes much less steep, reducing the sensitivity of the measurement. This slope change is apparent in each of the pH sensors, except for the FSA PEBBLE, which retains a slope comparable to the free dye. The linear ranges do not change significantly when the dyes are incorporated in the PEBBLE, even when the slope changes. In the case of CNF and CDMF, the range stays the same, and in the case of the rest of the dyes, the range is only reduced by about 0.2 pH unit.

The comparison of several pH dyes and their corresponding PEBBLE sensors was made so that an optimal PEBBLE sensor could be chosen for intracellular use. The qualities that were deemed most important for an intracellular sensor were quantum efficiency and resistance to photobleaching. First, the quantum efficiency of the dye must be high, both so that the spectral interference of autofluorescence is minimized and so that fewer PEBBLEs can be used in the cell, to minimize cellular perturbations. For these reasons, CNF was rejected since its quantum efficiency is orders of magnitude lower than that of any of the other dyes. Finally, of the remaining dyes, the photobleaching of CDMF was the least severe over a few minutes of exposure and most closely matched the photobleaching properties of the internal standard, sulforhodamine 101 (SR). This close match in photobleaching properties between the pH dye and the internal standard is important in order to be able to obtain an accurate pH measurement in the cell even after photobleaching has taken place. For these reasons, the PEBBLEs containing CDMF and SR were chosen for further characterization.

Table 2 summarizes the linear ranges, slopes, intercepts, and linear fit for three different calcium-selective dyes and their corresponding PEBBLE sensors. The slopes were calculated as above for the pH sensors. The slopes and linear ranges are again affected when the dye is immobilized in the polymer matrix. It should be noted that while the linear ranges are not severely affected when the dye is immobilized in the polymer matrix, the linear ranges of free dye are not as broad as reported in the literature [5,6,12] due to spectral overlap with the internal standard (SR). Thus, since Calcium Orange has a reduced linear range, and the Calcium Green 5N has a very flat slope, meaning a high limit of detection, neither of these PEBBLEs was used for further study. For the calcium dyes, which were all similar in quantum efficiency and photobleaching properties, the Calcium Green PEBBLE was chosen for further characterization due to its linear range and slope.

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Table 2. Results of Calibrations of Three Calcium Selective Dyes and the Corresponding PEBBLE Sensors[a]

calcium indicator	linear range (μM calcium)	slope ± SD	intercept	r^2	n
Calcium Green + SR (dye)	0−0.15	30 ± 0.7	1.7	0.94	4
Calcium Orange + SR (dye)	0−0.15	1.5 ± 0.03	1.0	0.95	6
Calcium Green 5N + SR (dye)	3−30	0.010 ± 0.05	0.99	0.95	7
Calcium Green + SR (PEBBLEs)	0−0.15	7.3 ± 0.05	0.97	0.99	6
Calcium Orange + SR (PEBBLEs)	0−0.1	1.3 ± 0.05	1.0	0.79	5
Calcium Green 5N + SR (PEBBLEs)	0−5	0.022 ± 0.007	1.0	0.99	4

[a] An internal standard, sulforhodamine 101 was added to each dye solution and was contained within the polymeric matrix of each PEBBLE sensor. Measurements were made at the optimum emission wavelength for each individual indicator and dye.

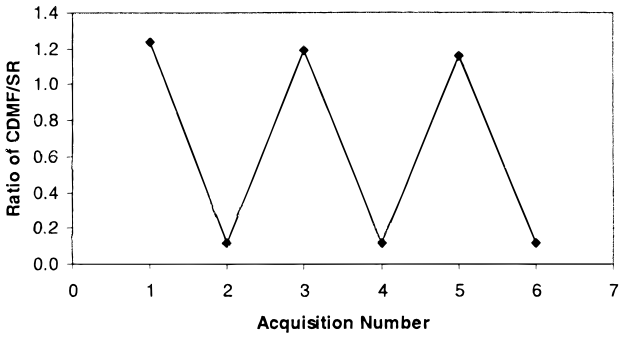

Figure 1. Reversibility of PEBBLE sensors. The pH of a solution of CDMF/SR PEBBLEs was changed from pH 6.4 to pH 7.0, and back and forth for several measurements. The ratio of the two peaks in the spectrum attained from each of the measurements was then plotted, illustrating that the PEBBLE sensors are reversible.

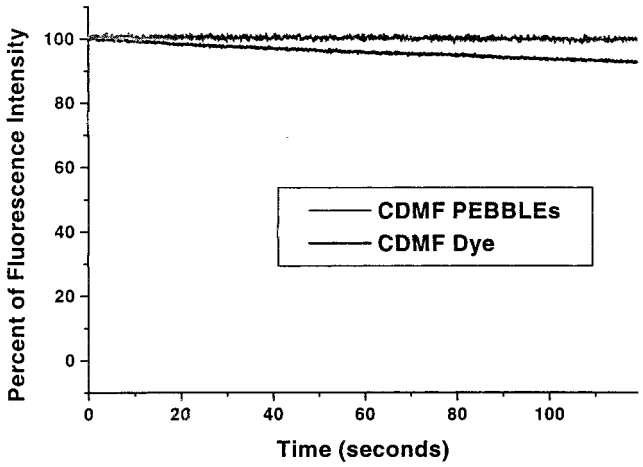

Figure 2. Comparison of photobleaching. Photobleaching of CDMF/SR PEBBLE sensors (top) vs the free dye in solution was studied by monitoring the emission of the solution under constant illumination for 2 min. The photobleaching of the PEBBLEs (1%) is 8 times less than that of the corresponding free dye in solution (8%).

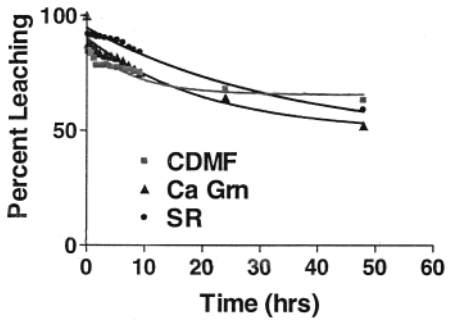

Figure 3. Leaching of dye out of PEBBLE sensors. PEBBLEs were assessed for leaching by monitoring the amount of dye that escaped from the polymer matrix over a 48-h period. Less than 50% of the dye leached from these 20- and 200-nm sensors over 2 days.

Both the calcium-selective PEBBLE (Calcium Green) and the pH-sensitive PEBBLE (CDMF) sensors are completely reversible. Figure 1 shows the reversibility of the pH PEBBLEs containing CDMF and SR. In this experiment, the pH was changed from pH 6.4 to pH 7.0, and vice versa, for several measurements. The ratio of the two peaks in the spectrum, attained from each of the measurements, was then plotted. Even though the PEBBLE solution is being diluted with each pH change (with the addition of HCl or NaOH), the measurements are still accurate due to the internal standard, which compensates for any dilution of the solution. The excellent reversibility indicates that the polymer is not significantly restricting the binding of the indicator to the ion and the indicators are free to bind and are not physically restricted within the pores of the matrix.

The photobleaching properties of the dyes entrapped within the polymer matrix are at least as good as those of the dye not in the matrix. The PEBBLE sensors show photobleaching properties similar or better than those of the free dye. In some cases (Figure 2), the PEBBLE sensor shows significantly less photobleaching than the dye alone. This may be, in part, due to the reduced quantum efficiency of the dye once it is in the matrix, which lends itself to less rapid damage upon excitation, or due to stabilization by the matrix (e.g., partial shielding from oxygen). Photobleaching of the PEBBLE sensors is an aspect that cannot be prevented; it is determined by the dye the PEBBLE contains. Depending on the experiment, an apparently inferior PEBBLE, such as the CNF PEBBLE, could be utilized, because even though the quantum yield is significantly lower than for the other dyes on the list, the photobleaching is significantly less. Thus, when PEBBLEs are used for measuring intracellular ion concentrations, a sensor can be evaluated on its physical properties, such as photobleaching, without any concern regarding biocompatibility, while the latter is usually the deciding factor for the selection of free dyes.

Leaching of dye from the polymer is very dependent on the dye. Factors such as dye molecule size (small dyes can more readily diffuse through the pores of the matrix) and dye hydrophilicity (a hydrophobic dye may stay on the surface of the sensor rather than reside deep in the matrix) play a significant role. These factors must be taken into consideration during the polymerization of the sensors, since the pore size can be tightened by varying the type of cross-linker, or the pH of the polymerization solution can be changed to ensure that a charged molecule stays deeper in the core of the polymer. The CDMF and Calcium Green (both with SR) sensors show rapid leaching during the first minutes in

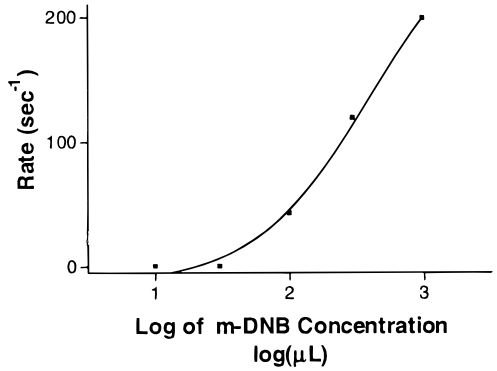

Figure 4. Rate of calcium release from the mitochondria of C6 Glioma cells plotted vs the dose of *m*-DNB used to stimulate the mitochondrial permeability transition (fluorometric measurement).

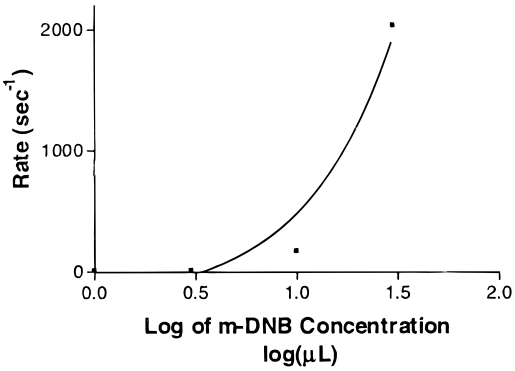

Figure 5. Rate of calcium release from the mitochondria of SY5Y neuroblastoma cells plotted vs the dose of *m*-DNB used to stimulate the mitochondrial permeability transition (fluorometric measurement).

aqueous solution (Figure 3), but then the leaching slows. One possibility is that the dye in the outer region of the polymer is lost and the dye in the core is retained. Another explanation is that the larger (200-nm) PEBBLEs are leaching at a slower rate than the 20-nm PEBBLEs. The indicators CDMF and Calcium Green show less than 50% leaching of the dye from the matrix over a 48-h period. Through leaching experiments, it was found that there are approximately 3000 indicator molecules and 4500 reference dye molecules in a 200-nm PEBBLE, and about 3 indicator dye molecules and 5 reference molecules in the 20-nm PEBBLEs. It should be noted that in the cell lines that are used in our experiments, after a time period of 24 h, the biggest influence on the signal of the sensors in the cell is not due to leaching but rather the natural division of the cells, dividing the cellular contents (including the sensors) in half with each division. Leaking of the PEBBLE sensors out of the cells has not been seen, unlike for the cytosolic dyes, where there is a very limited time for measurements to be completed (in our experience little more than a couple of hours), due to leaking of the free dyes out of the cell.

Ion interference is also a dye-dependent phenomenon, and in contrast to protein, which is size excluded, any small ion or small molecule is free to diffuse through the matrix and potentially interfere with the sensor. While, allegedly, CDMF is not affected by ion interferences,[7] it has been shown previously[12] that sensors containing Calcium Green suffer from the same effects that interfere with the free dye, and this behavior has been observed in our experiments also. For instance, known interferences of Calcium Green-1, such as high levels of magnesium, high ionic strength, and pH below 6.8,[6,13] can cause errors in calcium measurements when PEBBLE sensors that contain Calcium Green are used. However, it should be noted that, in the cytoplasm, the pH does not fall below 6.8[5] and magnesium does not fluctuate much,[14] and thus interference from these ions should be minimal. Development of dyes that are more selective for calcium or usage of a different polymer matrix (such as a lipophilic polymer matrix[15−18]) could probably solve these problems, but for now these potential interferences should be considered when intracellular measurements are made.

Measurement of the Rate of Calcium Release during the Mitochondrial Permeability Transition. Numerous disease states of the central nervous system, including cancer,[19] ischemia−reperfusion injury,[20] and neurodegenerative disorders,[21] involve specific subpopulations of cells. In particular, susceptibility to injury following oxidative insults is widely varied throughout the brain. For example, damage resulting from reperfusion injury after global forebrain ischemia is associated primarily with destruction of neurons in the hippocampal CA1 and neocortical layers 3, 5, and 6.[22] Exposure to *m*-DNB, a compound used widely as an industrial intermediate in the manufacture of dyes, plastics, and explosives, induces brain stem gliovascular lesions in nuclei with high-energy requirements, particularly those in the auditory pathway.[23] Neurotoxicity associated with *m*-DNB exposure is thought to be mediated by loss of ATP production secondary to disruption of cellular redox potential.[24] Single-electron reduction of *m*-DNB by the flavoprotein nitroreductase yields the nitroxyl anion radical, which can reduce molecular oxygen to superoxide anion radical, leading to further production of reactive oxygen species and alteration of cellular redox potential through depletion of reduced pyridine nucleotides and glutathione.[25] Thus, differences in vulnerability of central nervous system (CNS) cells to oxidative injury may be explained, in part, by the capability of a particular cell to withstand alterations in redox potential and energy metabolism that result from increases in reactive oxygen species and the ensuing drop in molecular oxygen and reducing equivalents.

Considering the potential of *m*-DNB to undergo redox cycling in the reducing environment of the mitochondrion, we investigated whether disruption of mitochondrial function after *m*-DNB expo-

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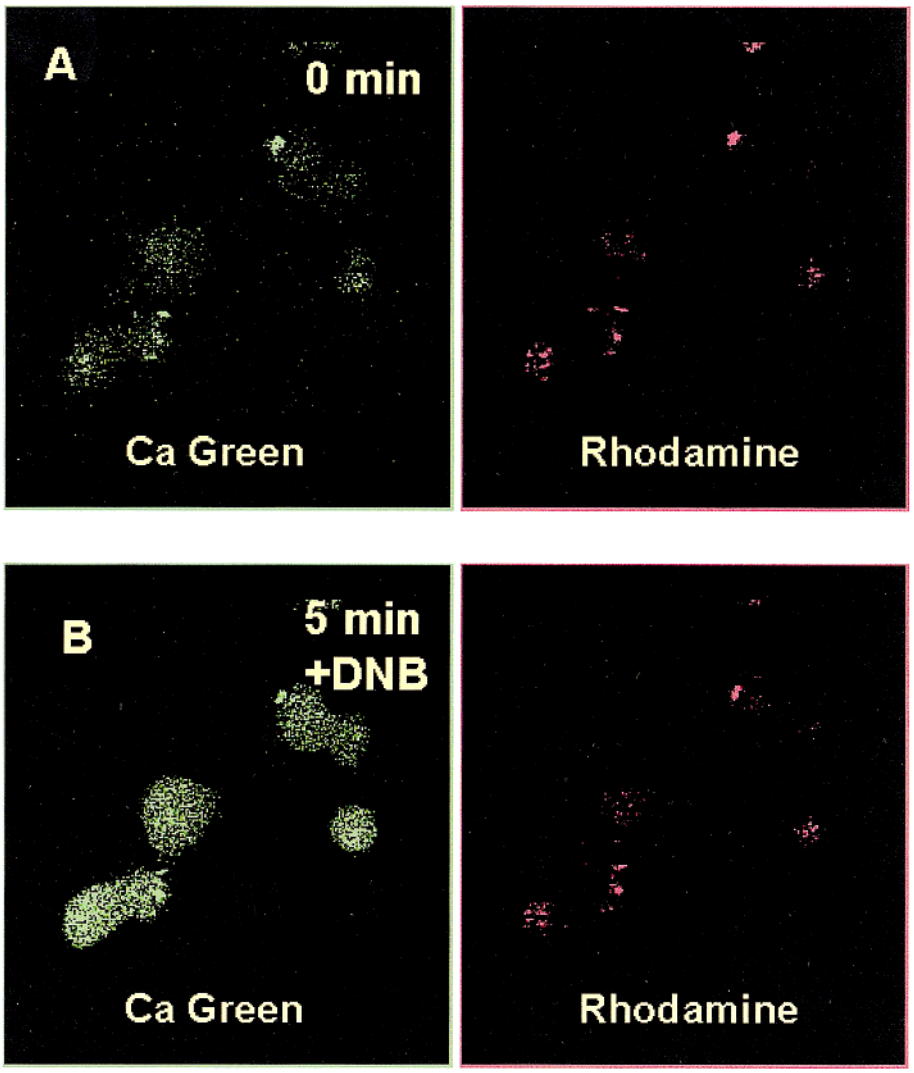

Figure 6. Laser scanning confocal microscopy images of *m*-DNB-stimulated calcium release in SY5Y neuroblastoma cells lipofected with Calcium Green/sulforhodamine 101 PEBBLEs. Neuroblastoma cells were incubated in Dulbecco's phosphate buffered saline supplemented with glucose (0.5 g/L) at 37 °C and treated with 10 μM *m*-DNB. Calcium Green and rhodamine fluorescence were monitored at time zero, prior to addition of *m*-DNB (A) and 5 min following addition (B). The calcium level increased, as can be seen in the increased fluorescence intensity of the Calcium Green. The internal standard, as expected, remained constant.

sure resulted from uncontrolled release of calcium associated with onset of the mitochondrial permeability transition (MPT). The MPT is characterized by opening of a high conductance pore across the inner mitochondrial membrane following matrix calcium accumulation, resulting in dissipation of mitochondrial membrane potential ($\Delta\iota_m$), high-amplitude swelling, and demise of the mitochondrion.[26,27] Onset of MPT also results in release of potent apoptogenic factors upon disruption of the outer mitochondrial membrane, including cytochrome *c*, APAF-1, and calcium.[28] Though the role of mitochondrial injury in necrotic and apoptotic cell death is now extensively documented, differential regulation of the MPT in varying tissue and cell types is not well understood. We postulated therefore that resistance or sensitivity to *m*-DNB toxicity in C6 glioma (glial) and SY5Y neuroblastoma (neuronal) cells is mediated by the cell-specific propensity of mitochondria to undergo a permeability transition associated with increases in intracellular calcium.

We have established that *m*-DNB differentially induces the MPT in C6 glioma and SY5Y neuroblastoma cells by evaluating mitochondrial depolarization and cell viability using both a fluorometer and a laser scanning confocal microscopy method.[29] Since it is known that calcium stores are released from the mitochondria during permeability transition,[30] PEBBLE sensors selective for calcium were used to measure the rate of calcium release in the presence of increasing concentrations of *m*-DNB. A dose-dependent increase in cytosolic calcium was observed upon treatment with *m*-DNB in both C6 and SY5Y cells at concentrations comparable to those which induced MPT in each cell line, suggesting that mitochondria are a likely source for the observed

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calcium increases through onset of MPT. This is illustrated in Figures 4 and 5, which depict the rate of cytosolic calcium increase in C6 and SY5Y cells as a function of *m*-DNB concentration. Utilizing calcium-selective PEBBLE sensors, it was discovered that the half-maximal rate of calcium release (EC_{50}) occurred at a 10-fold lower concentration of *m*-DNB in SY5Y cells than in C6 cells. This finding supports our hypothesis that sensitivity to *m*-DNB-induced calcium release is associated with differential regulation of the MPT in the cell lines evaluated.

To further illustrate that the observed increases in cytosolic calcium resulted from release from intracellular stores rather than disruption of the plasma membrane by *m*-DNB, cells containing calcium-selective PEBBLE sensors were imaged using laser scanning confocal microscopy (Figure 6). As in the fluorometry studies, increased cytosolic calcium was observed upon *m*-DNB exposure, as indicated by an increase in fluorescence intensity of Calcium Green-containing PEBBLE sensors. The fluorescence intensity of the internal standard dye, sulforhodamine 101, did not change following treatment with *m*-DNB, since it is inert with respect to ion concentration, demonstrating the utility of ratiometric PEBBLE sensors in measuring intracellular calcium fluxes. The use of confocal microscopy illustrates that the PEBBLEs can be used to monitor microdomains within a single cell, in addition to monitoring a large number of cells as with the fluorimeter. Furthermore, the PEBBLEs cross neither external nor internal cell membranes.

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