Nnoc-DBHQ, a New Caged Molecule for Modulating Sarcoplasmic/Endoplasmic Reticulum Ca\(^{2+}\) ATPase Activity with Light Flashes*  

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We report the synthesis and characterization of \(O\)-[o-nitromandelloyxocarbonyl]-2,5-di(tert-butyl)hydroquinone (Nnoc-DBHQ), a new “caged” reagent for photorelease DBHQ, a membrane-permeant, reversible inhibitor of the sarcoplasmic/endoplasmic reticulum Ca\(^{2+}\) ATPase (SERCA). The Nnoc group is a new caging group developed for the current application. Photolysis of Nnoc-DBHQ proceeds with \(t_{1/2} = 126 \pm 2 \mu s\), and \(t_{1/2}\) for subsequent release of DBHQ is estimated to be \(-5\) ms. Nnoc-DBHQ thus allows rapid and reversible modulation of SERCA activity in living cells. Through its acetoxymethyl ester, Nnoc-DBHQ can be loaded into cells easily by incubation. We demonstrate the use of Nnoc-DBHQ for photomodulating SERCA activity in fibroblasts and vagal sensory neurons. We further demonstrate the utility of pulsed DBHQ photorelease for probing and manipulating dynamic phenomena such as [Ca\(^{2+}\)] oscillations in fibroblasts.

Intracellular concentration and distribution of the ubiquitous second messenger Ca\(^{2+}\) is tightly controlled by a number of pathways (1). The interaction of the pathways that mobilize and regulate free Ca\(^{2+}\) levels can result in highly complex and dynamic signaling patterns, such as Ca\(^{2+}\) oscillations and waves (1–5). Pulsed perturbation of the concentrations of various second messengers, achieved by flash photolysis of caged inositol-1,4,5-trisphosphate, diaicylglycerol, and Ca\(^{2+}\) (4–8), has yielded highly specific mechanistic information about these dynamic phenomena (5, 9). Although the role of second messengers themselves in dynamic signaling phenomena has been studied by photorelease techniques, the contribution of pathways that regulate second messenger levels remains unexplored.

The family of sarcoplasmic/endoplasmic reticulum Ca\(^{2+}\) ATPases (SERCA)\(^\dagger\) that sequester Ca\(^{2+}\) into the sarcoplasmic reticulum and endoplasmic reticulum are important regulators of cytosolic free Ca\(^{2+}\) levels (10). We reasoned that the effect of these pumps on Ca\(^{2+}\) oscillations and waves could be elucidated by the development of a method for the pulsed modulation of their activity. This could be accomplished by the preparation of a caged, reversible SERCA inhibitor. Of the known SERCA inhibitors (11), 2,5-di(tert-butyl)-1,4-hydroquinone (DBHQ, 1) (12, 13) is ideally suited for the development of a caged SERCA modulator because it is a structurally simple and reversible inhibitor that is commercially available in large quantities.

EXPERIMENTAL PROCEDURES

Synthesis—Reagents and solvents were ACS or high pressure liquid chromatography grade and were used as received from Aldrich or Fisher. Dimethylformamide and dichloromethane were stored over 3 Å molecular sieves. All oxygen- and water-sensitive reactions were performed under dry argon atmosphere. For water-sensitive reactions, glassware was dried at 130 °C for at least 3 h and cooled under a stream of argon or in a desiccator prior to use. Silica gel 60 (230–400 mesh, Merck) was used for column chromatography. Melting points were recorded on a Melt-temp II (Laboratory Devices) apparatus coupled to an Omega (Omega Engineering) HH23 digital thermometer and are uncorrected. The structures of all purified products were established by NMR spectral analysis. Spectra were recorded on a General Electric QE-300 (300 MHz) NMR spectrometer. Samples were dissolved in CDC\(_1\)\(_3\) (0.03% tetramethylsilane) unless otherwise stated and were referenced to tetramethylsilane. Samples in solvents other than CDC\(_1\)\(_3\) were referenced to the residual solvent peak. Resonances are reported in the following format: NMR (solvent): chemical shift in ppm downfield from tetramethylsilane, multiplicity (s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; b, broad), spin-spin coupling constant if appropriate, and integrated number of protons. High resolution mass spectrometric analysis was performed at the University of Maryland (College Park, MD) on a model VG70TE spectrometer (VG Analytical).

2-Hydroxy-2-[2-nitrophenyl]acetonitrile (4)—Sodium bisulfite (8.26 g, 79 mmol) was added to a suspension of 2-nitrobenzaldehyde (10 g, 66 mmol) in water (60 ml). After the reaction mixture had stirred for 10 min, it was cooled in ice water. A solution of potassium cyanide (5.16 g, 79 mmol; dissolved in 30 ml of water) was added dropwise. The mixture was stirred for 30 min, warmed to room temperature, and filtered. The solid was washed with water and air dried to give 10 g (85%) of 4.

3-Hydroxy-2-[2-nitrophenyl]acetic Acid (5)—Cyanohydrin 4 (9.5 g, 48 mmol) was refluxed in concentrated hydrochloric acid (100 ml) for 2.5 h. The solution was cooled to room temperature and extracted with ethyl acetate. The extract was dried (MgSO\(_4\)) and evaporated, leaving 12.98 g of crude acid, which had acetic acid as an impurity. This was taken on to the methyl ester without further purification.

To prepare pure 5, cyanohydrin 4 (4.00 g, 22.4 mmol) was refluxed in concentrated hydrochloric acid (40 ml) for 3 h. The solution was diluted with water (100 ml) and continuously extracted with ether overnight. Evaporation of solvent gave a brown solid, which was digested with isopropyl ether (10 ml), filtered, and washed with additional isopropyl ether to give 3.30 g (75%) of acid 5 as a tan, crystalline solid. mp. 137–139 °C. 1\(^H\)-NMR(acetone-\(d_2\)): 8.06 (d, \(J = 8.02\) Hz, 1H), 7.91 (d, \(J = 7.81\) Hz, 1H), 7.77 (t, \(J = 7.57\) Hz, 1H), 7.61 (t, \(J = 7.82\) Hz, 1H), 5.90

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\(\dagger\) The abbreviations used are: SERCA, sarcoplasmic/endoplasmic reticulum ATPase; AM, acetoxymethyl; DBHQ, 2,5-di(tert-butyl)hydroquinone; Nnoc, \(o\)-nitromandelloyxocarbonyl.
Methyl-2-hydroxy-2-[2-nitrophenyl]acetate (6)—Crude acid 5 (entire amount) was dissolved in methanol (100 ml). Sulfuric acid (5 drops) was added, and the mixture was refluxed for 2.5 h. The solvent was evaporated to dryness, and the residue was taken up in ethyl acetate (10 ml). The solution was acidified with 1N HCl (1 ml) and extracted with ethyl acetate (3 ml). The organic layer was dried (Na2SO4), filtered, and evaporated to give an oil. Crystallization from acetone/isopropanol (1:1) gave 9.13 g (90%) of crude ester, which was recrystallized from isopropyl ether to give 7.92 g (78%) of 6. mp 69–71 °C. 1H-NMR: 8.10 (d, J = 8.19 Hz, 1H), 7.70–7.63 (m, 3H), 7.55–7.49 (m, 1H), 5.81 (d, 1H), 4.82 (bs, 1H), 3.80 (s, 3H), 3.63 (d, 1H), 1.24 (s, 9H). HRMS: calculated for C11H17NO3 [M + H] + m/z = 212.0559, observed 212.0555.

Methyl-2-(2-nitrophenyl)acetate-2-oxycarbonylmidazolide (7)—Hydroxyester 6 (2.11 mg, 10 mmol) was dissolved in dichloromethane (30 ml). The reaction mixture was cooled to 0 °C, and carbonylimidazole (1.62 g, 10 mmol) was added. After 2 h, the reaction mixture was warmed to room temperature and extracted with water (5 × 30 ml). The organic layer was dried (MgSO4), filtered, and evaporated to give an oil. Crystallization from acetone/isopropanol (1:1) gave 1.86 g (61%) of 7. A second crop of crystals of equal purity weighed 0.68 g for an overall yield of 83%. mp 82–85 °C. 1H-NMR: 8.19 (s, 1H), 7.85 (d, J = 8.3 Hz, 1H), 7.79–7.63 (m, 3H), 7.54 (s, 1H), 7.00 (s, 1H), 3.83 (s, 3H), 3.80 (s, 3H). HRMS: calculated for C11H17NO3 [M + H] + m/z = 212.0559, observed 212.0555.

12-25 (D-tert-butyl)-4-hydroxyphenyl-2-(methyl-2-[2-nitrophenyl]acetate-2-y1) carbon (8) (Nmoc-DBHQ/Me)—2,5-Di-tert-butylhydroquinone (0.022 g, 1.0 mmol) and 4-dimethylaminopyridine (0.024 g, 0.2 mmol) were added to a solution of 7 (0.305 g, 1.0 mmol) in 2 ml of N,N-dimethylformamide. The reaction mixture was maintained at a temperature of 60 °C for 20 h, cooled, and taken up in ethyl acetate (25 ml). Extraction with water (3 × 25 ml) followed by drying (MgSO4) and evaporation of solvent gave an oil. Chromatography with hexane/ethyl acetate (3:1) followed by evaporation of solvent gave 0.165 g (36%) of 8 as a thick oil. 1H-NMR: 8.12 (d, J = 8.19 Hz, 1H), 7.78–7.56 (m, 3H), 6.95 (s, 1H) 6.92 (s, 1H), 6.64 (s, 1H), 4.79 (s, 1H), 3.79 (s, 3H), 1.37 (s, 9H), 1.23 (s, 9H). HRMS: calculated for C20H26NO3 [M + H] + m/z = 305.0668, observed 305.0648.

12-25 (D-tert-butyl)-4-hydroxyphenyl-2-(methyl-2-[2-nitrophenyl]acetate acid-2yl) carbon (9) (Nmoc-DBHQ/AM)—Sodium hydroxide (1N, 0.55 ml, 5.5 mmol) was added, and the mixture was stirred for 45 min. The solution was acidified with 1N HCl (1 ml) and extracted with ethyl acetate (3 × 3 ml). The organic layer was dried (Na2SO4), filtered, and evaporated to give crude 9 as an oil. This material was recrystallized from acetonitrile to give pure 9, mp 192–193 °C. 1H-NMR: 8.10 (d, J = 8.1 Hz, 1H), 7.78–7.57 (m, 3H), 6.93 (s, 1H), 6.63 (s, 1H), 5.30 (s, 1H), 1.35 (s, 9H), 1.29 (s, 9H). HRMS: calculated for C20H26NO3 [M + H] + m/z = 445.1764, observed 445.1768.

Results and Discussion

The three most commonly used SERCA inhibitors are thapsigargin (20, 21), cyclopiazonic acid (22), and DBHQ (12, 13). Because its inhibitory action is irreversible (23), thapsigargin is not a suitable target for a caged reagent to be used for reversible photomodulation of SERCA activity. Cyclopiazonic acid has a relatively complex molecular structure and, being a biosynthetic product of fungal origin, is available only in small quantities at high expense, which makes it an unattractive starting material for organic synthesis. In contrast, DBHQ is structurally simple, incorporating only one type of reactive functional group for caging purposes, and is commercially available in large quantities. These advantages, together with its reversibility, made DBHQ our preferred target for caging. The great majority of photoreleasable compounds have used caging groups structurally based on the 2-nitrobenzyl system.
(24). Although the simple parent 2-nitrobenzyl moiety is a common caging group, it was not appropriate for caging DBHQ; preliminary experiments indicated that UV irradiation of cells bathed in medium containing 2-nitrobenzyl alcohol resulted in irreversible inhibition of the SERCA pump. Because photolysis of any 2-nitrobenzyl-caged compound is expected to generate the same photochemical byproducts, we inferred that the byproducts of photolyzing a 2-nitrobenzyl-caged DBHQ would not be inert.

Hess and co-workers have shown that the α-carboxy-nitrobenzyl group is useful for caging neuroactive amino acids (25–29). Photodeprotection was shown to proceed rapidly and with high quantum yield (28). We recognized that the carboxylate on this caging group would reduce the reactivity of the photochemical byproduct. Furthermore, the presence of the carboxylate offers the added advantages of increasing the water solubility of the caged compound and allowing for the preparation of a caged AM ester, which could be passively loaded into cells.

Caging DBHQ directly with the α-carboxy-nitrobenzyl group requires the formation of a benzyl ether, which model studies indicated was problematic. For example, reaction of DBHQ with 2-nitrobenzyl chloride in the presence of K₂CO₃ yielded numerous compounds that were difficult to isolate and characterize. Reasoning that the difficulties encountered in benzyl ether formation were at least partially the result of the sterically congested environment surrounding the phenolic hydroxyl groups of DBHQ, we postulated that an efficacious caging reaction would need to proceed through a different mechanism. The Nmoc group was designed as a photocleavable caging group that would combine the desirable qualities of the α-carboxy-nitrobenzyl group with a caging reaction that proceeds via carbonyl substitution (30). Irradiation of Nmoc-DBHQ (2) with UV light would result in the formation of DBHQ-bicarbonate (3), which would rapidly decompose under physiological conditions to DBHQ (1) and carbon dioxide as shown in Scheme 1. The photochemical side product, 2-(2-nitrosophenyl)glyoxalate (3a) is the same as that generated by photolysis of α-carboxy-nitrobenzyl-caged molecules (25–29), for which no adverse biological effects have ever been reported. Carbon dioxide, liberated by decarboxylation of DBHQ-bicarbonate (3), is a normal product of metabolism and would thus also be innocuous.

Nmoc-DBHQ was prepared as outlined in Scheme 2. 2-Nitrobenzaldehyde was converted to the cyanohydrin (4), which was hydrolyzed to α-nitromandelic acid (5) by refluxing in concentrated hydrochloric acid. Methyl α-nitromandelate (6) was prepared by Fisher esterification of 5. Caging DBHQ necessitated the preparation of an activated α-nitromandelyl-oxy carbonyl group. The oxycarbonyl imidazole derivative (7) was considered superior to the chloroformate because of its stability and ease of preparation. Treatment of alcohol 6 with carbonyl diimidazole gave 7 in high yield as a stable crystalline solid. The desired carbonate (8) was formed when a solution of 7 and DBHQ in N,N-dimethylformamide was heated with a catalytic amount of 4-dimethylamino pyridine. Saponification of the methyl ester (8) followed by acidification gave Nmoc-DBHQ (2). To facilitate loading of the caged reagent into cells, the AM ester of Nmoc-DBHQ (9) was prepared. Neutralization of 2 with one equivalent of sodium hydroxide, followed by esterification with bromomethyl acetate in the presence of tetrabutylammonium iodide gave Nmoc-DBHQ/AM (9).

To demonstrate the photoreactivity of Nmoc-DBHQ, a series
of UV-visible absorption spectra were acquired from a solution of the sodium salt of Nmoc-DBHQ that was being photolyzed with 365 nm light (Fig. 1A). The spectroscopic changes resulting from photolysis are consistent with those expected from the classic 3-nitrobenzyl rearrangement. In particular, photolysis causes an increase in absorbance at longer wavelengths, a result of the long wavelength absorption by the highly conjugated byproduct (3a in Scheme 1). Because the spectra show good isosbestic points through the course of photolysis, we were able to determine the quantum efficiency of photolysis of Nmoc-DBHQ by analyzing the absorbance changes as a function of time (6, 14). Spectroscopic changes during photolysis followed an exponential time course, as shown in Fig. 1B. The quantum efficiency of photolysis, $Q$, was thus determined to be 0.10 (6, 14).

The kinetics for photolytic removal of the caging group were examined by monitoring the transient absorbance changes characteristic of the short-lived 3-nitro intermediate generated during photolysis of 3-nitrobenzyl moieties (30–33). The time course for the decay of the transient absorbance following laser pulse photolysis of Nmoc-DBHQ is shown in Fig. 2. The decay is dominated by an exponential component with a lifetime ($\tau$) of 182 ± 1 μs.2 These results imply that the photocleavage reaction is essentially complete in approximately 550 μs (τ in about three lifetimes).

Photocleavage of the nitromandelyl moiety leaves the carbonate monoester of DBHQ (3 in Scheme 1), which must lose CO$_2$ to liberate DBHQ. Although the rate of decarboxylation, $k_{\text{dec}}$, cannot be measured in the present system, it can be estimated from published studies of similar reactions. The decarboxylation rate ($k_{\text{dec}}$) of carbonate monoesters has been shown to follow the relationship: $\log(k_{\text{dec}}) = 15.1 - 1.16pK_a$, where $pK_a$ is the pK$_a$ of the hydroxyl group that is esterified to the carbonate (34, 35). Because the pK$_a$ of DBHQ is estimated to be 11.21 (36), the rate constant for decomposition of the carbonate monoester of DBHQ is expected to be $k_{\text{dec}} \approx 130$ s$^{-1}$.

There is a small contribution at short times from a fast minor component. The minor component accounts for less than 10% of the total decay amplitude and has $\tau = 29 \pm 2$ μs.

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**Fig. 2.** Transient absorbance changes following pulsed laser photolysis of Nmoc-DBHQ. $\Delta A_{290}$ of 860 μM Nmoc-DBHQ in 150 mM NaCl, 10 mM phosphate buffer, at pH 7.2, following photolysis by a 10-ns, 50-mJ, 308 nm pulse from a XeCl excimer laser. Trace shown is the sum of 45 individual pulse experiments. The decay comprises two exponential components. The faster component has a time constant of 29 ± 2 μs. The slower, dominant component, which accounts for >91% of the total amplitude, is characterized by a time constant of 182 ± 1 μs. Note that the trace does not return to the pre-photolysis baseline because the byproduct nitrosoketone absorbs more strongly than the starting material at wavelength longer than ~360 nm (compare pre- and post-photolysis spectra in Fig. 1A).

**Fig. 3.** Effect of DBHQ photorelease on [Ca$^{2+}$]. A, rat embryo fibroblast. B, ferret nodose neuron. Cells were loaded with Nmoc-DBHQ and fluo-3 indicator as described under “Experimental Procedures.” Fluorescence data are presented as the relative intensity $F(t)/F_0$, where $F(t)$ is the intensity at time $t$ during an experiment, and $F_0$ is the average intensity measured with the cell at rest (prior to photorelease). Arrowheads and associated numbers indicate, respectively, the times and durations of photolysis light flashes. Corresponding dips in the experimental traces are due to temporary interruption of fluorescence data acquisition by an electromechanical shutter to avoid exposure of the fluorescence photomultiplier tube to leakage from the high intensity photolytic flash. Each plus sign (+) on an experimental trace marks the first intensity datum collected after a photorelease episode. (t$_{1/2}$ ~ 5.3 ms) at 25°C. We demonstrate in subsequent biological experiments that DBHQ photorelease is sufficiently rapid for probing Ca$^{2+}$ signaling dynamics.

Biological efficacy of Nmoc-DBHQ was tested in living cells. Loading cells simultaneously with Nmoc-DBHQ and the fluorescent Ca$^{2+}$ indicator, fluo-3 allowed the effects of photorelease on intracellular Ca$^{2+}$ dynamics to be monitored through the Ca$^{2+}$-sensitive fluorescence of fluo-3. Results of DBHQ photorelease experiments, performed in rat embryo fibroblasts (REF52 cell line) as well as in acutely isolated nodose neurons of adult ferret, are shown in Fig. 3. As expected, the data in Fig. 3 show that photorelease of DBHQ within cells lead to rapid, dose-dependent transient increases in [Ca$^{2+}$]. The resting cytosolic [Ca$^{2+}$] is maintained through a dynamic balance of active pumping processes that remove Ca$^{2+}$ from the cytosol and passive leaks that introduce Ca$^{2+}$ into the cytosol. When photoreleased DBHQ disrupts the pump-leak balance by inhibiting the SERCA pumps, an increase in [Ca$^{2+}$], is observed. The [Ca$^{2+}$], rise is transient because SERCA inhibition by DBHQ is reversible and because free DBHQ is a small, uncharged, membrane-permeant molecule that is cleared from the cell by diffusion. Once DBHQ diffuses out of the cell and is
lytic light flashes. Cell loading with Nmoc-DBHQ and fluo-3 was as two cell types (rise times of 7.8 s). Onset of the response to DBHQ photorelease are similar for the pear more protracted in the nodose neuron. Whereas decay of the response to photorelease is of the order of 10–15 s in the fibroblast, in the nodose neuron the value increases to 40–50 s.

In summary, we have synthesized and characterized Nmoc-DBHQ, a caged inhibitor of the SERCA pump. Nmoc-DBHQ allows rapid and reversible modulation of the SERCA activity in living cells and should be useful for probing systems whose rapidly varying Ca\(^{2+}\) dynamics make study inaccessible through conventional techniques of reagent delivery.

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\(^3\) The perturbation effects are the result of photorelease and are not observed when light flashes are delivered in the absence of caged reagent, as has been demonstrated previously (5, 9, 37).
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