Communicative & Integrative Biology 5:3, 240–242; May/June 2012; © 2012 Landes Bioscience

Caged intracellular NMDA receptor blockers for the study of subcellular ion channel function

James E. Reeve,[1] Michael M. Kohl,[2] Antonio Rodríguez-Moreno,[2,3] Ole Paulsen[2,*] and Harry L. Anderson[1,*]

[1]Department of Chemistry; University of Oxford; Oxford, UK; [2]Department of Physiology, Development and Neuroscience; University of Cambridge; Cambridge, UK; [3]Department of Physiology, Anatomy and Cellular Biology; University Pablo de Olavide; Seville, Spain

Keywords: NMDA, MK801, ion channel, cage, photolysis

We have previously synthesized a caged form of the use-dependent N-methyl-D-aspartate (NMDA) receptor ion channel blocker MK801 and used intracellular photolysis of this compound to demonstrate the subcellular location of NMDA receptor ion channels involved in synaptic plasticity. Here, we discuss considerations regarding the choice of caging molecule, synthesis and the potential uses for caged ion channel blockers in neurophysiology.

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Introduction

Pharmacological interventions are a valuable tool for the dissection of biological functions. In the study of excitable cells, such as neurons, drugs that block ion channels are of particular use. In acute brain slices, drug effects are commonly restricted to three levels: (1), the whole tissue preparation through addition of the drug to the incubation medium; (2), a small volume of tissue through local pressure injection or microiontophoresis; (3), a single cell through addition of the drug to the whole-cell patch-clamp pipette. Through photolysis of a novel caged NMDA receptor antagonist, added via a patch pipette, we recently demonstrated control of ion channel activity at the subcellular level[1] (**Fig. 1**). This is of particular interest when studying the localization of ion channel function in highly compartmentalised cells such as neurons.

Cage Choice Considerations

A number of photolabile compounds have been used in the past to achieve improved spatial and temporal control of free calcium, neurotransmitters, intracellular signaling molecules and ion channel blockers (for review, see ref. 2). We based our design on the nitrophenyl-based photolabile protecting group (cage) nitroveratryloxycarbonyl (NVOC) rather than other commonly employed cages such as methoxy nitroindolinyl (MNI)[3] or nitrophenyl ethyl (NPE)[4] since it could be easily derivatised at its phenolic positions. Due to the relatively low uncaging efficiency of the NVOC group, it may be handled under ambient light conditions and excellent contrast can be achieved between subcellular compartments. NVOC cages are neuropharmocologically compatible; we showed that the cage itself does not cause any channel blocking, unlike MNI which interacts with $GABA_A$ receptors.[5] We synthesized both NVOC-protected MK801 (cMK801, **Fig. 2A**) and a novel triethylene glycol-substituted nitrophenyl cage-protected MK801 (TcMK801, **Fig. 2B**). Formulation of cMK801 with Polysorbate 20 afforded sufficient water-solubility, though was detrimental to quick patch-clamp seal formation. Water solubility conferred instead by the triethylene glycol chains of TcMK801 permitted a formulation-free solution of caged MK801, which permits seal formation, as with standard aqueous intracellular solutions.

Compound Synthesis and Improvement of Water-Solubility

4,5-Bis{2-[2-(2-methoxyethoxy)ethoxy]ethoxy}-2-nitrobenzaldehyde. This compound was prepared according to an adapted literature procedure.[6] A solution of 4,5-dihydroxy-2-nitrobenzaldehyde (60 mg, 327 μmol), 2-[2-(2-methoxyethoxy)ethoxy]ethyl tosylate (209 mg, 655 μmol) and K_2CO_3 (90 mg, 655 μmol) in anhydrous acetonitrile (5 mL) was refluxed for 16 h under N_2. The mixture was evaporated to dryness and purified by flash chromatography (ethyl acetate). The combined fractions were evaporated to dryness to yield **1** as a yellow oil (92 mg, 59%). ^1H NMR (400 MHz, $CDCl_3$) δ_H 10.43 (s, 1H), 7.71 (s, 1H), 7.45 (s, 1H), 4.32 (m, 4H), 3.94 (m, 4H), 3.75 (s, 4H), 3.66 (m, 8H), 3.55 (m, 4H), 3.38 (s, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ_C 188.16, 153.39, 152.23, 144.19, 125.99, 111.87, 109.61, 72.35, 71.43, 71.13, 71.03, 69.91, 69.71, 59.48. *m/z* (ESI+) 498.18 ($[M+Na]^+$ 100%, $C_{21}H_{33}ClNNaO_{11}^+$ requires 498.20).

|4,5-Bis{2-[2-(2-methoxyethoxy)ethoxy]ethoxy}-2-nitrophenyl|methanol. This compound was prepared according to an adapted literature procedure.[6] $NaBH_4$ (18.5 mg, 490 μmol) was added portionwise to a solution of 4,5-bis{2-[2-(2-methoxyethoxy)ethoxy]ethoxy}-2-nitrobenzaldehyde **1** (46.6 mg, 98 μmol) in

*Correspondence to: Ole Paulsen and Harry L. Anderson; Email: op210@cam.ac.uk and harry.anderson@chem.ox.ac.uk
Submitted: 11/17/11; Revised: 01/17/12; Accepted: 01/17/12
http://dx.doi.org/10.4161/cib.19400

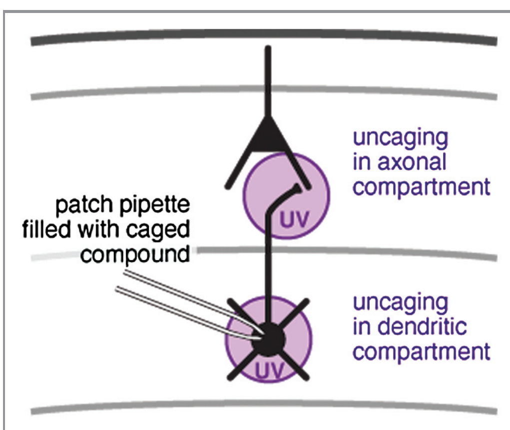

Figure 1. Spot photolysis of a caged ion-channel blocker with UV light allows for precise temporal and spatial control of the drug effect.

anhydrous methanol (1.5 mL) at 5°C. The reaction mixture was allowed to warm to room temperature and stirred for 3 h. The reaction was quenched with $HCl_{(aq)}$ (5 mL, 1.0 M) and extracted with chloroform (3 × 10 mL). The combined organic layers were washed with saturated NaCl solution (5 mL) and evaporated to dryness to yield **2** as a colorless oil (30 mg, 64%). ^{1}H NMR (400 MHz, $CDCl_3$) δ_H 7.76 (s, 1H), 7.35 (s, 1H), 4.95 (s, 2H), 4.33 (m, 2H), 4.23 (m, 2H), 3.90 (m, 4H), 3.74 (m, 4H), 3.66 (m, 8H), 3.55 (m, 4H), 3.37 (s, 3H), 3.36 (s, 3H), 1.25 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ_C 153.86, 147.25, 139.44, 133.19, 112.74, 110.56, 71.90, 71.87, 70.90, 70.79, 70.69, 70.65, 70.55, 70.43, 69.78, 69.51, 69.14, 68.89, 62.50, 59.01. *m/z* (ESI+) 500.16 [(M+Na)$^+$ 100%, $C_{21}H_{35}NNaO_{11}^+$ requires 500.21].

4,5-Bis{2-[2-(2-methoxyethoxy)ethoxy]ethoxy}-2-nitrobenzyl chloroformate. A mixture of |4,5-bis{2-[2-(2-methoxyethoxy) ethoxy]ethoxy}-2-nitrophenyl|methanol **2** (30 mg, 63 µmol) and triphosgene (28 mg, 94.5 µmol) in chloroform (5 mL) was stirred for 2 h. Further triphosgene (18 mg, 63 µmol) was added and the mixture stirred for 30 min. When the reaction was complete by thin layer chromatography (TLC), the mixture was evaporated to dryness and purified by column chromatography on SiO_2 (1% MeOH in ethyl acetate). Combined fractions were evaporated and dried *in vacuo* to yield the product **3** as a colorless oil (20 mg, 59%). ^{1}H NMR (400 MHz, $CDCl_3$) δ_H 7.83 (s, 1H), 7.06 (s, 1H), 5.70 (s, 2H), 4.26 (m, 4H), 3.93 (m, 4H), 3.73 (m, 4H), 3.67 (m, 8H), 3.57 (m, 4H), 3.38 (s, 6H). *m/z* (ESI+) 562.18 [(M+Na)$^+$ 100%, $C_{22}H_{34}ClNNaO_{12}^+$ requires 562.16]

TcMK801. This compound was prepared according to an adapted literature procedure.[7] A solution of 4,5-bis{2-[2-(2-methoxyethoxy)ethoxy]ethoxy}-2-nitrobenzyl chloroformate **3** (20 mg, 37 µmol) in tetrahydrofuran (THF, 0.5 mL) was added to a solution of (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d] cyclohepten-5,10-imine maleate (MK801, 12.4 mg, 37 µmol), Na_2CO_3 monohydrate (18.4 mg, 148 µmol) and dioxane (0.5 mL) in water (1.0 mL). The mixture was stirred for 48 h. The reaction mixture was poured onto saturated $Na_2CO_{3(aq)}$ (2 mL), extracted with chloroform (2 × 5 mL) and washed with water (2 × 5 mL). The organic layer was evaporated to dryness and purified by column chromatography on SiO_2 (1% MeOH in ethyl acetate). Combined fractions were evaporated and dried *in vacuo* to yield the product **4** as a colorless oil (8 mg, 29%). ^{1}H NMR (400 MHz, $CDCl_3$) δ_H 7.75 (m, 1H), 7.33 (m, 1H), 7.31 (m, 2H), 7.19 (m, 2H), 7.10 (m, 2H), 7.08 (m, 1H), 6.92 (m, 1H), 5.51 (s, 1H), 5.49 (s, 1H), 4.22 (m, 2H), 3.88 (m, 2H), 3.82 (m, 4H), 3.74 (m, 4H), 3.65 (m, 8H), 3.55 (m, 4H), 3.38 (s, 3H), 3.37 (s, 3H), 2.69 (d, *J* = 17.1 Hz, 1H), 2.26 (s, 3H), 1.26 (s, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ_C 207.00, 167.75,

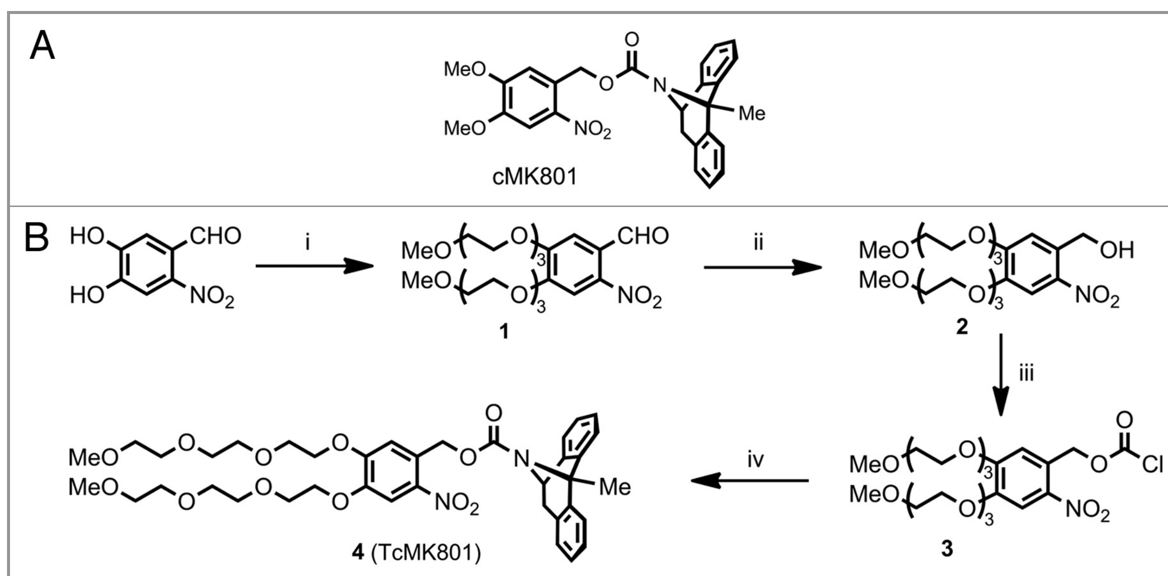

Figure 2. (A) Nitroveratryloxycarbonyl-protected MK801 (cMK801). (B) Synthesis of TEG-substituted caged MK801 (TcMK801) from 4,5-dihydroxy-2-nitrobenzaldehyde. (1) Triethylene glycol monomethyl monotosylate ether, K_2CO_3, MeCN, reflux, 16 h. (2) $NaBH_4$, MeOH, rt, 3 h. (3) $(Cl_3CO)_2CO$, Et_3N, $CHCl_3$, rt, 2.5 h. (4) Dizocilpine maleate (MK801), Na_2CO_3, dioxane, THF, water, rt, 48 h.

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153.33, 143.27, 139.16, 130.86, 130.44, 128.78, 127.64, 127.52, 127.41, 126.13, 121.28, 118.60, 110.79, 71.90, 70.91, 70.66, 70.54, 69.47, 69.21, 68.79, 67.95, 67.06, 59.86, 59.01, 38.70, 30.91, 30.33, 29.67, 28.89, 25.58, 23.72, 22.96, 22.67, 14.03, 10.94. *m/z* (ESI+) 747.3100 [$(M+Na)^+$ 100%, $C_{38}H_{48}N_2NaO_{12}^+$ requires 747.3099].

Potential Uses

A wide variety of caging groups exists, making it possible to tailor the photolysis of a caged compound to suit desired experimental conditions. Judicious choice of caging group allows specification of photolysis wavelength, temporal release behavior and light sensitivity. Photolysis can be achieved with millisecond precision, enabling the study of fast events like calcium[8,9] and neurotransmitter[10-12] signaling and slower events such as the expression of synaptic plasticity.[1]

The caged compounds cMK801 and TcMK0801 presented here are effective under one-photon activation. Greater spatial selectivity and depth penetration may be achieved by two-photon excitation due to the reduction in scattering and excitation out of the focal plane. Advances in two-photon photolysis[8] promise to improve spatial resolution and allow the precisely defined uncaging of compounds in volumes as small as 1 μm^3. A two-photon-optimized caged potassium channel blocker is commercially available[13] and we are directing efforts toward preparing two-photon-optimized variants of our caged NMDA receptor channel blocker. Using these compounds to selectively block neuronal conductances via two-photon photolysis will enable the dissection of neuronal signal integration in dendritic subcompartments. This approach can be extended to other pharmacologically active substances, allowing the study of cellular function and signaling at unprecedented levels of detail.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

This work was supported by the Biotechnology and Biological Sciences Research Council, UK and the Wellcome Trust OXION initiative. A.R.M. was supported by a short-term fellowship from the Alicia Koplowitz Foundation.

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