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Photochemically Knocking Out Glutamate Receptors in Vivo

James J. Chambers, Hiroaki Gouda, David M. Young, Irwin D. Kuntz, and Pamela M. England*

Departments of Pharmaceutical Chemistry and Cellular and Molecular Pharmacology, University of California, San Francisco, California 94143-2280

Received March 23, 2004; E-mail: england@picasso.ucsf.edu

Photochemical manipulation of small molecules provides a means of probing biological systems with unparalleled spatiotemporal resolution. Photoreactive protecting groups have been used to control the release of neurotransmitters and second messengers on the millisecond time scale, and photoreactive ligands have been used to identify receptors and define binding site residues.[1] Here we describe the design of a photoreactive AMPA (α-amino-3-hydroxy-5-methyl-4-isooxazole) receptor antagonist that provides a means of photochemically "knocking out" AMPA receptors in living cells.

AMPA receptors (AMPARs), a major subtype of ionotropic glutamate receptors (iGluRs), mediate the majority of the fast excitatory communication at synapses in the brain and have been clearly implicated in mammalian learning and memory.[2] In particular, memory, a form of synaptic plasticity, is thought to involve an activity-dependent increase in the number of AMPARs at synapses, thus strengthening the communication between connected neurons.[3]

Much effort has been directed toward developing a detailed understanding of the activity-dependent trafficking of AMPARs into synapses. Ehlers demonstrated that the fast endocytic trafficking of AMPARs takes place with a half-time of 10−15 min in cultured neurons.[4] This study involved biotinylating surface-exposed receptors and biochemically quantifying the number of AMPARs on the surface of cells relative to intracellular stores in response to pharmacological manipulation. While this approach offers sufficient temporal resolution to study AMPAR receptor trafficking, it does not provide a means of selectively monitoring the trafficking of AMPARs in individual neurons, a desirable feature given the intrinsic heterogeneity of neuronal preparations. Malenka, Tsien, and co-workers used tetracysteine-tagged GluR subunits in combination with biarsenical dyes, which become fluorescent upon binding the cysteine motif, to monitor the activity-dependent synthesis and localization of new AMPARs in individual neurons using fluorescence imaging.[5] While this approach offers sufficient spatial resolution, it does not provide the requisite temporal resolution to monitor the kinetics of receptor trafficking, as ∼1 h is required to label the receptors with the dyes. Other methods for monitoring AMPAR trafficking have also been described and typically involve introducing recombinant proteins into neurons (e.g., enhanced green fluorescent protein-tagged iGluRs providing an optical signal, mutant iGluRs providing a unique electrophysiological signal).[6] These approaches, while informative, perturb the normal complement of iGluRs in the cell and/or do not provide the combined level of temporal and spatial resolution required to precisely characterize the trafficking kinetics of native AMPARs.

To provide a means of monitoring the trafficking of native AMPARs with the requisite level of both temporal and spatial resolution, we have developed a membrane-impermeable, photoreactive AMPAR antagonist. Specifically, with this antagonist, ultraviolet light can be used to photoinactivate surface-exposed wild-type AMPARs on the minute time scale in individual cells (through focused illumination) while ionic current recordings from the same cell are simultaneously used to functionally monitor the rate of insertion and the level to which AMPARs are inserted into the synaptic membrane from intracellular stores (Figure 1). This approach can be used to address several key questions. For example, what are the trafficking kinetics of native AMPARs, and are the kinetics influenced by increased synaptic activity? Further, are memories encoded by the number of functional AMPARs located in the postsynaptic membrane?

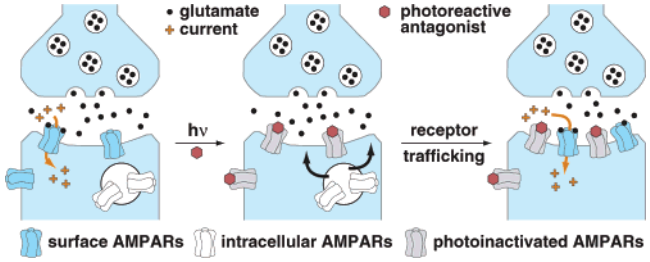
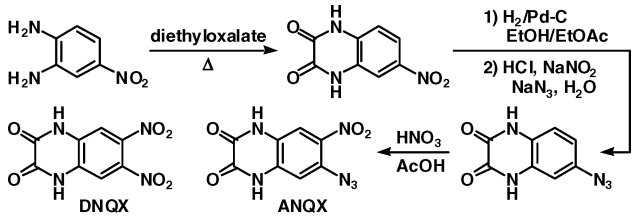


Figure 1. Using photoinactivation of surface-exposed AMPARs to study receptor trafficking from intracellular stores into synapses.

Scheme 1



We envisioned designing such a photoreactive AMPA receptor antagonist by replacing the C-6 nitro group on the established AMPAR-specific antagonist 6,7-dinitroquinoxaline-2,3-dione (DNQX) with a photoreactive azido ($-N_3$) group to form 6-azido-7-nitro-1,4-dihydroquinoxaline-2,3-dione (ANQX) (Scheme 1). Aryl azides are inert until photoactivated by ultraviolet (UV) light, whereupon the azido group loses dinitrogen ($-N_2$) to become a highly reactive nitrene.[7] The nitrene can insert into either the peptide backbone or the amino acid side chains of the protein to which it is bound, forming a covalent linkage and rendering the receptor permanently inhibited.

The crystal structure of an AMPAR ligand-binding core complexed with DNQX served as a template to initially evaluate ANQX binding in silico.[8,9] Computational docking of ANQX to the AMPAR ligand-binding core indicated a binding orientation virtually indistinguishable from that of DNQX, with the ANQX C-6 azido group buried deep within a receptor cleft.

Once it was established that ANQX would theoretically bind to AMPARs, the antagonist was synthesized in four steps (Scheme 1). The quinoxalinedione heterocycle was prepared by condensing 4-nitro-phenylenediamine with diethyloxalate. Catalytic reduction

10.1021/ja048331p CCC: $27.50

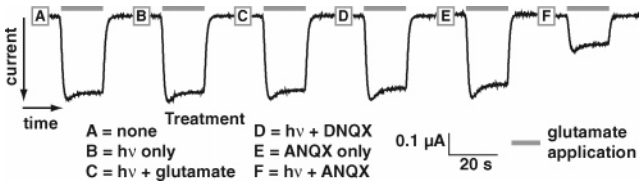


Figure 2. Glutamate-evoked current traces from a single oocyte *following* the indicated (A−F) treatments. Currents are irreversibly knocked down following irradiation with UV light (100 W, 30 s) in the presence of ANQX (F). Control experiments (B−E) show that peak currents are unaffected following treatment with UV light alone, UV light in the presence of glutamate or DNQX, or ANQX in the absence of UV light (E).

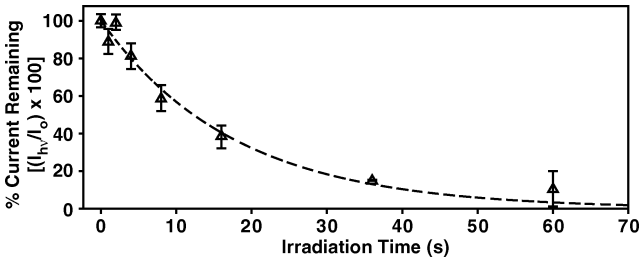


Figure 3. Time course for photochemically knocking out AMPAR currents with ANQX. *I* represents the peak glutamate-evoked currents recorded from untreated oocytes (I_o) and oocytes irradiated with UV light (1000 W) in the presence of ANQX ($I_{h\nu}$).

of the nitro group to the amine, followed by diazotization and displacement of the arenediazonium ion with azide, afforded 6-azido-1,4-dihydroquinoxaline-2,3-dione. Finally, nitration with fuming nitric acid provided ANQX in 44% overall yield.

The activity of ANQX was experimentally evaluated using two-electrode voltage clamp recordings from *Xenopus* oocytes heterologously expressing AMPARs (GluR1 homomers). The measured IC_{50} value of ANQX (1.0 μM) was comparable to the IC_{50} of DNQX (0.5 μM), demonstrating that ANQX is a potent AMPAR antagonist. As shown in Figure 2, glutamate-evoked currents were *irreversibly* inhibited upon photolysis in the presence of ANQX. In control experiments, glutamate-evoked currents were unaffected by exposure to UV light alone or in the presence of either glutamate or the non-photoreactive antagonist DNQX (Figure 2). Glutamate-evoked currents from oocytes expressing the other iGluR subtypes, kainate and *N*-methyl-D-aspartate receptors, were unaffected by photolysis in the presence of ANQX (Supporting Information).

Finally, irradiation of batches of oocytes in the presence of ANQX demonstrated that the glutamate-evoked currents decayed at a single exponential rate with nearly complete inhibition observed after ~60 s of irradiation (Figure 3).[10]

In summary, we have designed, synthesized, and evaluated a photoreactive AMPAR antagonist that provides a means of directly characterizing the role of AMPAR trafficking in synaptic plasticity. The membrane impermeability of ANQX will allow for exceptional spatial resolution, targeting only surface-exposed receptors, and the photoreactivity of the azido group will allow for unparalleled temporal resolution, knocking out receptors on the minute time scale.[11]

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Supporting Information Available: Molecular docking details, GluR1 DNA and mRNA preparation protocols, electrophysiological recording and photolysis conditions, bar graph showing the mean currents (±SEM) from seven oocytes treated as described in Figure 2, and bar graph showing mean currents (±SEM) from oocytes expressing kainate and *N*-methyl-D-aspartate before and after treatment with ANQX + UV light. This material is available free of charge via the Internet at http://pubs.acs.org.

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(10) The apparent cross-linking efficiency of ANQX in these experiments was likely limited by the size (~1 mm diameter), shape (spherical), and opacity of the oocytes. In preliminary experiments on cultured neurons expressing endogenous AMPARs, irradiation in the presence of ANQX completely inactivated AMPAR currents after only 2 s of photolysis and with significantly less UV light (data not shown).

(11) The measured octanol/buffer (pH 7.5) partition coefficient (−0.72) suggests that ANQX is a fairly hydrophilic compound. In addition, the calculated polar surface area (171.33 Å^2) for ANQX predicts that ANQX is membrane impermeable. Finally, the photochemical knock-out experiments require only brief (<1 min) exposure to ANQX—a period too short to permit penetration of this compound through the plasma membrane.

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