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Protein kinases and light: unlikely partners in a receptor localization puzzle

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Abstract

Brief flashes of light directed at neuronal cell bodies and proximal dendrites of neurons in culture can enhance whole-cell electrophysiological responses mediated by NMDA and $GABA_A$ receptors. In experiments aimed at identifying the molecular moieties responsible for mediating this phenomenon, we observed that broad-spectrum protein kinase inhibitors substantially amplified the actions of light. Kinase inhibitors, however, were surprisingly ineffective in altering light-induced potentiation of recombinant NMDA receptors expressed in Chinese hamster ovary (CHO) cells. Furthermore, receptors assembled from truncated NMDA receptor subunits, previously shown to be relatively insensitive to modulation via phosphorylation, remained light sensitive. Phosphatase inhibitors had no effects of light-induced NMDA receptor potentiation in neurons, and nucleated patches excised from neuronal somata behaved similarly to CHO cells. Taken together, these data suggests that the effects of kinase inhibitors were unrelated to the molecular mechanism of light-induced potentiation. We propose a model whereby kinase inhibition promotes an enrichment of NMDA receptors in the neuronal cell body vs. the distal dendrites. Under these conditions, NMDA receptor redistribution elicited by kinase inhibitors would increase the number of receptors exposed to light and, as a consequence, the whole cell response. These observations support a critical role for protein kinases in the rapid redistribution of neurotransmitter receptors, with profound physiological significance.

Keywords: NMDA; $GABA_A$; Receptors; Light; Protein kinase; Staurosporine; K252c; Cerebral cortex; Tissue culture; Chinese hamster ovary; Recombinant

1. Introduction

NMDA receptor activation has been intimately linked to critical physiological processes in the brain, including changes in synaptic plasticity that are thought to underlie learning and memory [1]. In addition, overactivation of NMDA receptors can lead to neuronal cell death, and this phenomenon is thought to be responsible for the neurodegenerative changes seen in a large number of neurological disorders, including stroke and epilepsy [2]. Given the multiple roles of the NMDA receptor, the activity of this ion channel is tightly regulated. A large number of intracellular and extracellular modulators of NMDA receptor function have been described, including protons, zinc, polyamines, pH, magnesium, redox agents, mechanical stretch and protein kinases [3,4]. We recently described light as a novel modulator of NMDA receptor activity. We observed that brief flashes of light could rapidly and dramatically enhance currents mediated by NMDA receptors, but not by other ionotropic glutamatergic receptors [5]. Although the physiological consequence of light regulation of these receptors is not known, we have hypothesized it to be important for retinal function. For one, light of wavelengths and intensities that normally reach the retina are effective in altering NMDA receptor properties [5] and, secondly, NMDA receptors in isolated retinal ganglion cells are sensitive to light [5].

In an effort to better understand light regulation of NMDA receptor function, we designed experiments aimed at identifying the light sensitive molecular moiety within the receptor or an associated structure. In the course of these studies, we observed that (i) currents mediated by $GABA_A$ receptors were also light sensitive and (ii) the effects of light on both NMDA and $GABA_A$ receptors were substantially amplified following inhibition of protein kinase activity in neurons. Although at first we thought this effect might help us resolve the molecular mechanism of light modulation, we discovered that it was a reflection of a putative change in the

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relative localization of receptors following kinase inhibition. Here, we describe the experimental results that led us to this conclusion.

2. Methods

2.1. Tissue culture

Cortical neurons were dissociated from E16 Sprague–Dawley rats as described previously [6]. Growth media contained 80% Dulbecco's modified eagle medium with 10% heat-inactivated, iron-supplemented bovine calf serum, 10% Ham's F-12 media, as well as 25 mM HEPES, 24 U/ml penicillin, 24 U/ml streptomycin and 2 mM L-glutamine. Two weeks after plating, nonneuronal cell growth was arrested with a 72-h treatment with 2 μM cytosine arabinoside, after which the growth media contained only 2% serum and no F-12. Cells were used for electrophysiology experiments in the fourth week after dissociation.

2.2. Heterologous expression system

Chinese hamster ovary (CHO) cells were grown in Ham's F-12 media containing 10% fetal bovine serum and 2 mM L-glutamine. The cDNAs for NMDA receptor subunits NR1 and NR2A had been previously ligated into mammalian expression vectors containing the cytomegalovirus promoter [7,8]. CHO cells were seeded at 2.8×10^5 cells/well into 6-well plates 24 h prior to transfection. Cells were transfected in serum-free medium with 6 μl Lipofectamine reagent (Gibco BRL) and a total of 1.4 μg of DNA/well. A ratio of 0.3:1:3 green fluorescent protein:NR1:NR2 subunit ratio was employed. Four hours after transfection, cells were refed with media containing 10% serum and, within 24 h of transfection, 300 μM ketamine was added to prevent the excitotoxic cell death that occurs following functional receptor expression [7]. Recordings were performed 2 days after transfection.

2.3. Electrophysiological recordings

Electrophysiological recordings were performed at room temperature using the whole-cell configuration of the patch-clamp technique. Cells were bathed in a solution containing (in mM): 150 NaCl, 1.0 $CaCl_2$, 2.8 KCl, 10 HEPES, 10 glycine, 0.25 tetrodotoxin and pH was adjusted to 7.2 with NaOH. Electrodes were pulled to a resistance of 1.5–3 MΩ when filled with internal solution containing (in mM): 140 CsF, 10 EGTA/CsOH, 1 $CaCl_2$ and 10 HEPES (pH adjusted to 7.2 with CsOH). Signals were amplified using an Axopatch 1D patch-clamp amplifier, filtered at 1 kHz and digitized at 2 kHz. The reference electrode was an Ag/AgCl wire bridged with 2 M KCl/1% agarose. NMDA was applied via a perfusion system with a stepper motor for fast solution changes.

2.4. Light stimulation

Light was directed onto cells as previously described [5]. Briefly, light from a 100 W mercury lamp was guided onto the preparation utilizing a 50-μm fused-silica fiber. A 280-nm long pass filter was placed in the path of the light beam to minimize UV damage to the cell. A computer-controlled shutter was utilized to determine the duration of the light stimulus. The fiber produced a $\sim$35-μm radius light spot covering the soma and proximal dendrites of a cultured cortical neuron.

3. Results and discussion

3.1. Effects of protein kinase inhibitors on light modulation of NMDA and $GABA_A$ receptors

A brief application of 10 μM NMDA-elicited nondesensitizing currents in voltage-clamped cortical neurons. These currents were enhanced by approximately 20% (1.2 ± 0.04-fold potentiation, mean ± S.D., $n = 9$) immediately following a 1-s flash of light directed towards the soma and proximal dendrites (Fig. 1A). This effect of light is essentially the same as was described in our earlier study [5]. Surprisingly, the effects of light were significantly enhanced in cells that had been previously exposed for 2 h to 40 μM K-252c, a dual inhibitor of protein kinases A and C (PKA and PKC). In K-252c-treated neurons, light more than doubled the NMDA-induced currents (Fig. 1A and B). Staurosporine (40 μM), a nonselective protein kinase inhibitor, had a similar effect to K-252c, albeit not as pronounced (Fig. 1A and B). Selective inhibitors of PKA (14–22 amide; 40 nM) and PKC (bisindolylmaleimide I; 20 μM) did not mimic the actions of either K252c or staurosporine, suggesting that inhibition of each of these enzymes by themselves was not sufficient to elicit the enhancement in light sensitivity.

We [9] and others [10] have previously reported that $GABA_A$ receptors are also light sensitive. We thus sought to determine if the effects of kinase inhibition would also be observed with this receptor. Indeed, light-induced potentiation of GABA (10 μM)-elicited responses was also substantially enhanced in K-252c-treated neurons (Fig. 1A,B, inset). Based on these observations, we concluded that the light-sensitive moieties in the NMDA and $GABA_A$ receptors behaved similarly, and that they may be structurally related or influenced by the state of phosphorylation of the receptor.

3.2. Light effects on recombinant NMDA receptors

With the previous results in mind, we decided to explore the actions of light on recombinant NMDA receptors. These experiments would allow us to employ molecular techniques to dissect the mechanisms of light modification of

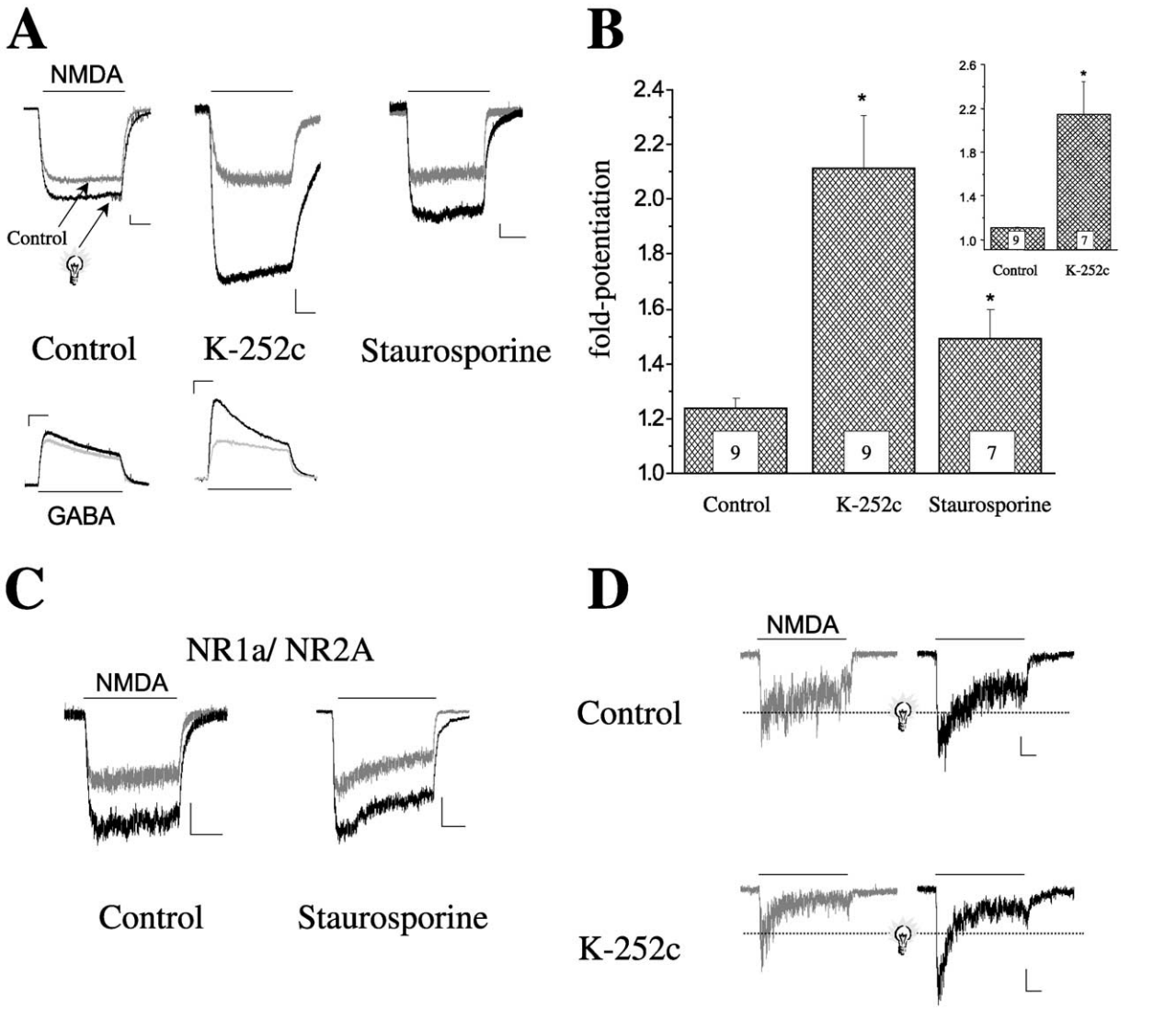

Fig. 1. (A) Light modulation of NMDA and $GABA_A$ receptors is influenced by kinase inhibitors. Whole cell responses elicited by a brief application of either 10 μM NMDA (top traces) or 10 μM GABA. Cells were voltage clamped at either − 60 mV (NMDA) or − 40 mV (GABA). Pre-flash responses are in light gray, while darker traces are those obtained immediately following a 1-s flash of light (light bulb symbol). Note that in cells that had been previously treated with either K-252c (40 μM) or staurosporine (40 μM) for 2 h prior to recordings, the effects of light were dramatically enhanced. (B) Summary of observation for experiments similar to those shown in (A) for NMDA and GABA (inset) responses. Values represent the mean ± S.D. for the number of cells indicated in each bar. * $P < .05$; ANOVA/Tukey (NMDA) or unpaired *t*-test (GABA). (C) Light effects on recombinant NR1a/NR2A NMDA receptors expressed in CHO cells remain unaltered following kinase inhibition. Whole cell currents mediated by recombinant NMDA receptors are nearly doubled in amplitude following a light flash. Note that staurosporine-treated CHO cell shows a similar response to light when compared to control (− 60 mV). (D) Effects of light in excised neuronal nucleated patches. Whole-cell responses from nucleated patches in response to 30 μM NMDA before and after a light flash (− 60 mV). Note that light induces nearly a doubling of the current amplitude and that kinase inhibition does not enhance this effect further. Scale bars: 250 pA and 1 s (A, C); 25 pA and 0.5 s (D).

NMDA receptor function. Whole-cell currents recorded from CHO cells transiently expressing NMDA receptors assembled by the NR1a/NR2A subunit configuration were light sensitive (Fig. 1C). We observed, however, that responses obtained under these conditions were enhanced by light nearly 100% (2.1 ± 0.8-fold potentiation, $n = 9$). The magnitude of this effect was similar to the actions of light on K-252c-treated neurons. We hypothesized that perhaps the NMDA receptors expressed in CHO cells were overall less phosphorylated, and hence naturally more sensitive to light. Indeed, NMDA-induced responses obtained from staurosporine-treated CHO cells were no more sensitive to light than in control CHO cells (1.7 ± 0.8-fold potentiation, $n = 4$). We then evaluated whether recombinant NMDA receptors assembled by an NR2A subunit truncated at its c-terminus, previously shown to be insensitive to phosphorylation [11], would be less sensitive to light. Unexpectedly, receptors assembled by NR1a/NR2AΔ_{880} were equally sensitive to light modulation, when compared to wild-type receptors (1.7 ± 0.2-fold potentiation, $n = 5$).

3.3. Nucleated neuronal patches: resolution of the paradox

The experiments described above did not support a role for the phosphorylation site(s) on the NMDA receptor as the light-sensitive moiety. However, they did not rule out the possibility that a receptor in the dephosphorylated state is most sensitive to light, as it is possible that truncation of NR2A is akin to a dephosphorylated state, at least with regards to light modulation. However, this scenario proved unlikely, as cells treated with 1 mM orthovanadate, a phosphatase inhibitor, are no more sensitive to light than untreated controls (1.2 ± 0.4-fold potentiation, $n = 12$). We therefore hypothesized that kinase inhibitors might be amplifying the actions of light not by modifying the light-sensitive site per se, but by changing the relative distribution of NMDA receptors in neurons. Under control conditions, the light flash illuminates only a fraction of the total NMDA receptor population. We have previously shown that most of the receptors in the cell are located in the distal dendrites [12], and the flash does not directly illuminate this population of receptors. Following kinase inhibition, a redistribution or enrichment of NMDA receptors in the soma vs. the distal dendrites would result in light reaching a relatively larger population of receptors and apparently amplifying the actions of light. In fact, protein kinases have been implicated in the translocation and redistribution of NMDA receptors in cultured hippocampal neurons [13]. In order to test our proposed model, we performed an additional set of studies on excised nucleated neuronal patches obtained from neurons. This macropatch configuration [14] essentially separates the cell body from the dendrites and allows recording from somatic receptors [12]. We found that NMDA-evoked responses from these nucleated patches were nearly as sensitive to light as what was observed in our CHO cell experiments (1.6 ± 0.2-fold potentiation, $n = 4$; Fig. 1D). In addition, nucleated patches excised from K252c-treated cells were equally sensitive to light as controls (1.6 ± 0.2-fold potentiation, $n = 5$). This result strongly argues in favor of our model.

4. Conclusions

In support of previous observations [13], the results presented in this study suggest that NMDA receptors can undergo mobilization and translocation following changes in their phosphorylation status. A recent study suggests that these receptors can be re-arranged laterally at the cell surface within synaptic and extrasynaptic regions [15]. At this point, we cannot determine whether new receptors have been inserted in the soma following kinase inhibition, or whether the phenomena described here can be attributed to such lateral mobility. Nonetheless, our studies further demonstrate that neurotransmitter receptors in neurons represent a dynamic entity that can rapidly influence synaptic activity following modulation by kinase activity. The regulation of this mobility is likely to play an important role in the complex integration of neuronal signals.

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