POSSIBLE INVOLVEMENT OF NITRIC OXIDE IN THE MODULATION OF PHOTOLYTIC FLASH-INDUCED INTERCELLULAR CALCIUM WAVES IN CULTURED ASTROCYTES

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SUMMARY

Waves of elevated intracellular free calcium that propagate between neighboring astrocytes are important for the intercellular communication between astrocytes as well as between neurons and astrocytes. In this study, intercellular calcium waves were evoked by focal photolysis of a caged calcium ionophore in cultured astrocytes. The focal photolysis of the caged compound resulted in an increase of intracellular calcium in the flashed cells, and this increase then propagated to neighboring astrocytes. The evoked calcium increase was inhibited by incubating cells with an inhibitor of nitric oxide synthase as well as with a scavenger of nitric oxide（NO）. In addition, treatment of cultures with an NO donor resulted in the marked enhancement of the photolytic flash-induced calcium rise in astrocytes. This enhancement was reversed by treatment with inhibitors of soluble guanylyl cyclase as well as of protein kinase G.

KEY WORDS: calcium waves; caged ionophore; photolysis; astrocyte; nitric oxide

INTRODUCTION

Waves of elevated intracellular free calcium that propagate between neighboring astrocytes constitute a newly discovered form of nonsynaptic long-range signaling in the brain (1). Although the function of such waves is not yet fully understood, glial calcium waves possibly provide an information-processing system operating in parallel with neuronal circuits within the nervous system (2). It is now becoming apparent that intercellular calcium waves might be the result of a combined contribution of intracellular and extracellular calcium signaling pathways in glial cells (3). Recently, we have demonstrated that propagating calcium waves can be evoked by focal photolysis of a caged calcium ionophore in cultured astrocytes from newborn

rats (4). Both the conduction distance and conduction velocity seem to be markedly different from those in the previously described calcium waves evoked by mechanical or electrical stimulation. However, the mechanisms involved in the initiation and propagation of the photolytic flash-induced calcium waves in cultured astrocytes remain largely unknown.

Nitric oxide (NO) is generated in various mammalian tissues, and acts as an intercellular messenger associated with various physiological and pathological events (5). Recently, we have demonstrated that the activity of nitric oxide synthase (NOS) varies associated with the differentiation of a neuronal cell line (NG108-15 cells), suggesting that NO functions as an important signaling molecule in differentiated NG108-15 cells (6). Recent studies have suggested that NO is involved in the regulation of glial calcium waves (3,7). NO mobilized calcium from intracellular stores of astrocytes via a NO-cGMP dependent protein kinase (PKG) pathway, and NO is an effective mediator of intercellular calcium waves in dissociated glial cultures.

In this study, we evoked intercellular calcium waves by focal photolysis of a caged calcium ionophore in cultured astrocytes, and investigated whether NOS-NO signaling is involved in the modulation of the evoked waves.

MATERIALS and METHODS

Glial cells from postnatal day 1-3 rat pups were obtained using a modified version of a method reported previously (8). In brief, the cortical hemispheres were removed, cleaned, and dissociated using a 0.01 % papain (Boehringer Mannheim)-cysteine solution and mechanical trituration. Cells were placed on poly-L-lysine-coated glass coverslips at 5,000 cells/cm^2 and were maintained in 80 % Dulbecco's Modified Eagle's Medium (DMEM; Gibco BRL, Grand Island NY), 10 % Ham's F-12 Nutrient Mixture (F-12; Gibco BRL) and 10 % fetal bovine serum at 37℃ in a humidified atmosphere of 95 % air and 5 % CO_2. The medium was removed from coverslips and replaced with fresh culture medium 48 hr later and twice a week thereafter. The experiments described here were performed on astrocytes maintained for 2-3 weeks in culture.

The method of loading of astrocytes with calcium indicator and caged calcium ionophore was described previously in detail (4). In brief, the cultures were incubated at 37℃ for 60 min with the acetoxymethyl ester of fluo-3 (fluo-3-AM, 10 μM; Molecular probes, Eugene, OR) and 0.02 % Pluronic F-127 (Sigma). All experiments were performed in EBSS containing 1.5 mM Ca^{2+} and 1.5 mM Mg^{2+}, supplemented with HEPES (25 mM) and D-glucose (5.5 mM), pH 7.3. The loading of astrocytes with the caged calcium ionophore was done by incubating cells in EBSS containing 20 μM DMNPE-caged Br A23187 for 15 min at 37℃.

After the loading of astrocytes with fluo-3 AM, the cultures were rinsed two times in experimental solutions, and allowed to de-esterify for an additional 15 min at 37℃. Fluorescent images were acquired at 2 sec intervals with a cooled CCD camera (C4880-80; Hamamatsu Photonics, Hamamatsu). An analysis of the acquired images was made with an image processing and measuring system (AQUACOSMOS; Hamamatsu Photonics). The control image (F0) was that of the target astrocyte 5 sec before the focal photolytic flash. Changes in the calcium concentration within the astrocytes were monitored by examining

changes in the fluorescence intensity (F) of each cell relative to the control image. The relative fluorescence intensity (F/F_0) was used for evaluating the astrocytic response. The propagation of calcium waves was analyzed using a series of images taken at 2 sec intervals. Distances were measured from the center of the flashed circular area (diameter, 50 μm) for uncaging DMNPE-caged Br A23187. The arrival of the calcium wave front was defined as a rise of relative fluorescent intensity above 1.20.

To investigate whether NOS-NO signaling is involved in the modulation of photolytic flash-induced calcium waves, astrocytic cultures were incubated with 1 mM N^G-monomethyl-L-arginine (L-NMMA, Sigma), a competitive inhibitor of NOS, for 30 min or 10 μM 2-(4-carboxyphenyl)-4,4,5,5-tetramethyl-imidazoline-1 oxyl 3-oxide (carboxy-PTIO, Dojindo Chem., Kumamoto, Japan), a scavenger of NO for 20 min prior to the photolytic flash, and after the flash. In some experiments, the cultures were exposed to 100 μM S-nitroso-N-acetyl-DL-penicillamine (SNAP, Dojindo Chem.) for 20 min prior to the photolytic flash, and after the flash. In addition, to investigate the possible involvement of NO-cGMP-PKG signaling in the regulation of the waves, astrocytic cultures were treated with either 10 μM 1*H*-[1,2,4-]Oxadiazole[4,3-a]quinoxalin-1-one (ODQ, Dojindo Chem.), an inhibitor of sGC, or 10 μM KT-5823 (Calbiochem, San Diego, CA), an inhibitor of PKG for 20 min prior to the photolytic flash, and after the flash.

Data were statistically analyzed using Student's *t*-test as indicated in the text. P values < 0.05 were considered statistically significant differences.

RESULTS

We first investigated whether the increase in the production of NO affected the initiation and/or propagation of the photolytic flash-induced intercellular calcium waves. Propagating calcium waves were induced by the photolysis of DMNPE-caged Br A23187 (caged calcium ionophore) in our astrocytic cultures (4). The duration of the photolytic flash for uncaging the caged-ionophore was set at 1 sec. The photolytic flash resulted in an immediate increase in the concentration of intracellular calcium in the flashed astrocyte (Fig. 1A1, A4). The flashed area is indicated by a pink circle (diameter; 50 μm) in Figure 1A1. The intracellular calcium increase then propagated to neighboring astrocytes (Fig. 1A2, A3). Treatment of cultures with 100 μM carboxy-PTIO, a scavenger of NO, resulted in a marked reduction in the increase in intracellular calcium in cultured astrocytes (Fig. 1B1-B4). Figures C, D, and E summarize the effects of carboxy-PTIO and L-NMMA, a competitive inhibitor of NOS, on the photolytic flash-induced intercellular calcium waves. In the presence of 10 μM carboxy-PTIO or 1 mM L-NMMA, the percent increase of intracellular calcium in neighboring astrocytes within a radius of 100 μm from the center of the flashed area significantly decreased compared with that of control (Fig. 1C). The conduction velocity of the waves calculated from the distance between the center of the flashed area and the time of the calcium response in other astrocytes was also reduced significantly by the treatment with 10 μM carboxy-PTIO or with 1 mM L-NMMA compared with that of the control (Fig. 1E). In contrast, the treatments did not result in a significant change in the conduction distance of the photolytic flash-induced calcium

waves

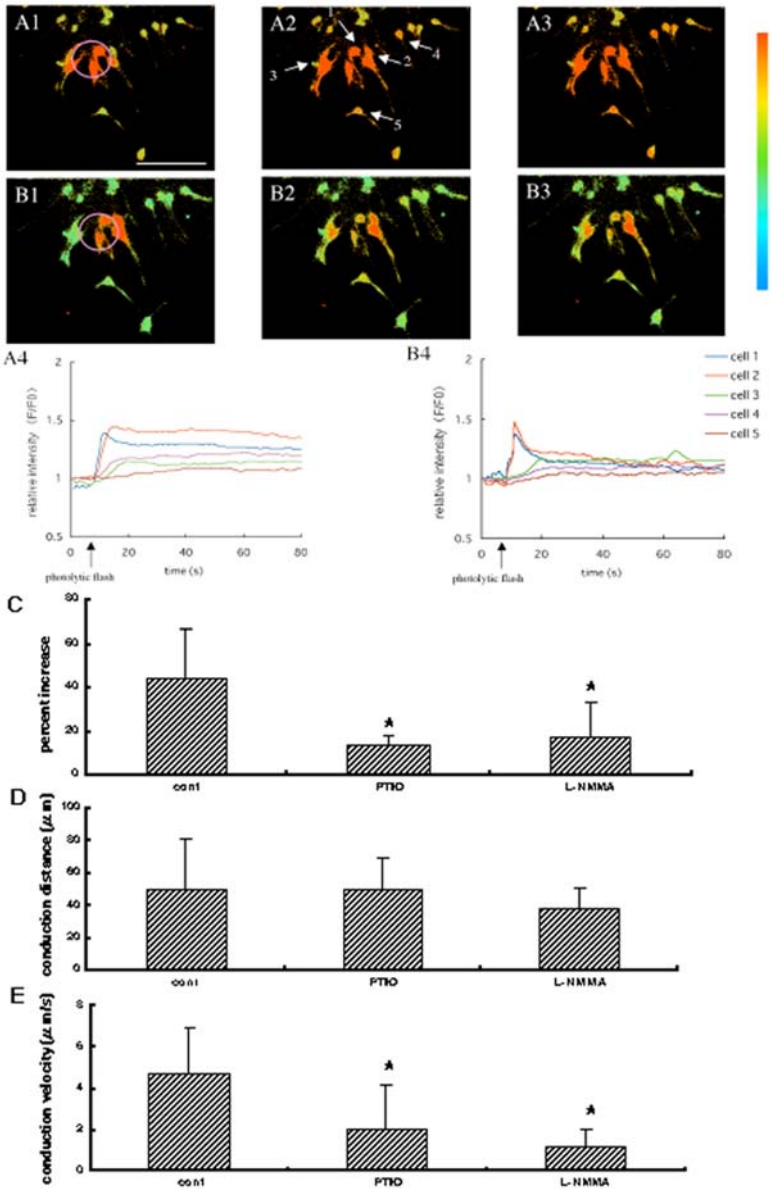

Fig. 1. Nitric oxide (NO)-induced modulation of astrocytic calcium waves triggered by focal photolysis of the caged calcium ionophore. Propagating intercellular calcium waves induced by photolysis of the caged calcium ionophore at three different time points; 6 sec (A1, B1), 30 sec (A2, B2), and 60 sec (A3, B3) after the photolytic flash. Treatment of cultures with carboxy-PTIO markedly reduced the photolytic flash-induced increase in the magnitude of fluo-3 fluorescent intensity (B1-B4) compared with the intensity without the treatment (control) (A1-A4). Relative fluorescence intensity (F/F0) increases from dark blue to red through yellow. The flashed area (diameter, 50 μm) is indicated by a pink circle in A1 and B1. Scale bar 100 μm. Figures C, D, and E show the changes in the calcium response (Δ%), conduction distance and velocity of photolytic flash-induced calcium waves on treatment with carboxy-PTIO and L-NMMA, respectively. Error bars indicate SD (*$p < 0.05$ vs control; n=4 ~12). Abbreviations: cont, control (without drug treatment); PTIO, treatment with 10 μM

carboxy-PTIO; L-NMMA, treatment with 1 mM L-NMMA.

(Fig. 1D). These results suggested that the increased production of NO induced by the photolysis of the caged calcium ionophore modulated the calcium response to the photolytic flash, although the conduction distance remained unchanged by the production of NO. If so, then treatment of the cultures with an NO donor such as SNAP is expected to increase the intracellular calcium response to the photolytic flash.

We then investigated whether treatment with an NO donor SNAP affected the photolytic flash-induced intercellular calcium waves in cultured astrocytes (Fig. 2). Pre-treatment of cultures with 100 μM SNAP for 20 min resulted in a marked enhancement of the photolytic flash-induced rise in the fluo-3 fluorescence intensity (Fig. 2B1-B4), reflecting the rise in intracellular calcium, as compared with the control (Fig. 2A1-A4).

Figure 2C summarizes the effects of SNAP treatment on the astrocytic calcium waves evoked by photolysis of the caged calcium ionophore. Treatment with 100 μM SNAP from 20 min before the application of the photolytic flash resulted in a significant enhancement of the percent increase of intracellular calcium in neighboring astrocytes within a radius of 100 μm from the center of the flashed area compared with that of control, and the enhancement was completely reversed by co-treatment with SNAP and 10 μM carboxy-PTIO (data not shown).

Previous studies (3,7) have suggested that NO-G-kinase signaling is coupled to calcium mobilization in glial cells and that this pathway plays a fundamental role in the initiation and propagation of intercellular calcium waves induced by the mechanical stimulation of astrocytes. Thus, we then investigated whether NO-G-kinase signaling was also responsible for the SNAP-induced modulation of photolytic flash-induced calcium waves observed in this study.

Treatment of cultures with 10 μM ODQ, an inhibitor of soluble guanylyl cyclase (sGC) (9,10), or with 10 μM KT5823, a selective inhibitor of cGMP-dependent protein kinase (PKG) (11), resulted in a marked suppression of the SNAP-induced enhancement of the calcium response in astrocytes in the flashed area and in neighboring cells (Fig. 2C). These results suggested that NO-G-kinase signaling was crucially involved in the SNAP-induced enhancement of the calcium response to the photolytic flash.

DISCUSSION

This study demonstrated the possible involvement of NO in the modulation of intercellular calcium waves evoked by the photolysis of a caged calcium ionophore. Treatment of cultures with L-NMMA or carboxy-PTIO significantly decreased the photolytic flash-induced rise in intracellular calcium in cultured astrocytes (Fig. 1), suggesting that endogenously produced NO

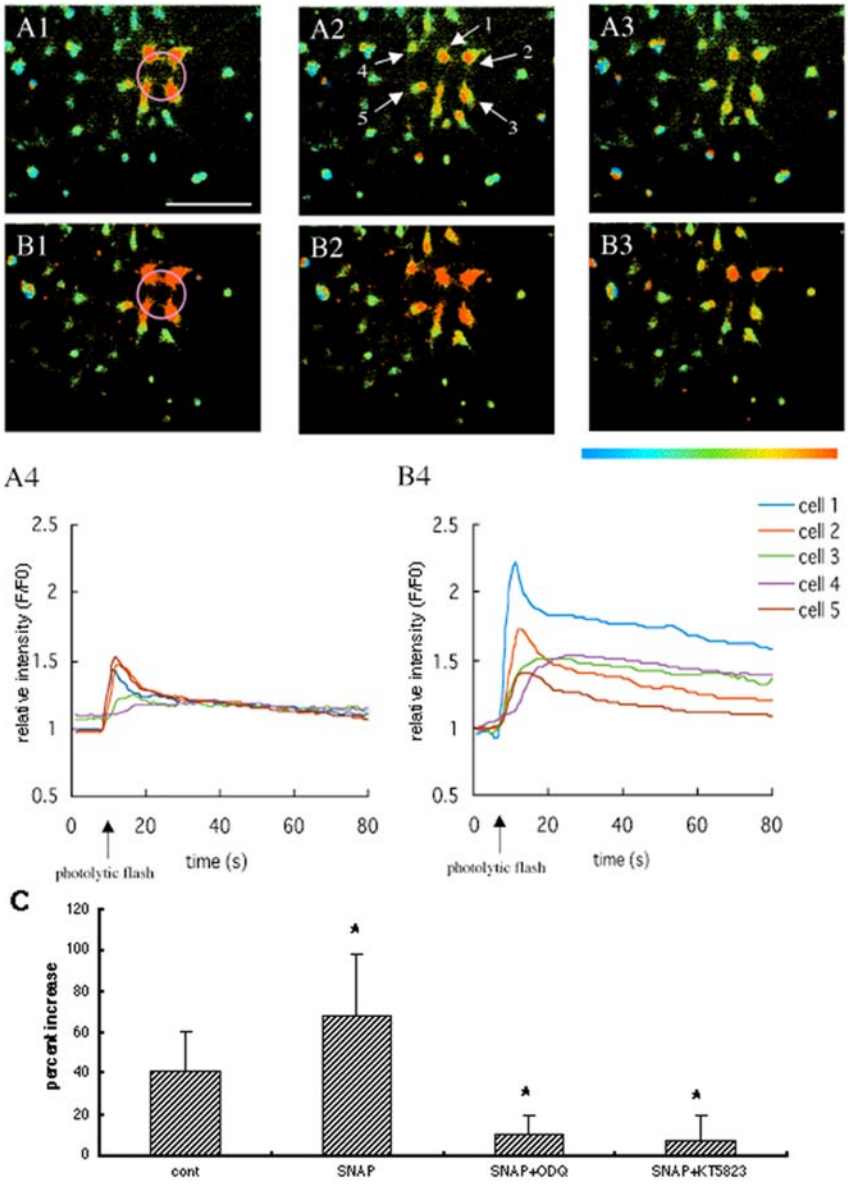

Fig. 2. SNAP-induced enhancement of the calcium response to the photolysis of the caged calcium ionophore in cultured astrocytes. Figures A and B show the calcium response of the same culture to the photolytic flash in the absence (control) and presence of SNAP, respectively. The fluo-3 fluorescence images are shown at three different time points; 2 sec (A1, B1), 30 sec (A2, B2), and 120 sec (A3, B3) after the flash. Relative fluorescence intensity reflecting intracellular calcium in A and B increases from dark blue to red through yellow. The flashed area is indicated by a pink circle in A1 and B1 (diameter, 50 μm). Scale bar 100 μm. Figure C shows the changes in the calcium response of photolytic flash-induced calcium waves on treatment with SNAP. The percent increase (Δ%) became significantly greater than that in control after treatment with 100 μM SNAP. The SNAP-induced enhancement of calcium response was completely reversed by co-treatment with 100 μM SNAP and 10 μM ODQ or 10 μM KT5823. Error bars indicate SD (*$p < 0.05$ vs control; n=4~6).

was responsible for the response. Previous studies have demonstrated that NO enhances calcium release from ryanodine-sensitive calcium stores; that is, a calcium-induced calcium release (CICR) via a NO-cGMP-G-kinase cascade (12,13) or by direct nitrosylation of the regulatory thiols of ryanodine receptors (14). Therefore, there is a possibility that the reduced cytosolic NO caused by the treatment with carboxy-PTIO or L-NMMA attenuates the calcium release from ryanodine-sensitive calcium stores.

In this study, SNAP treatment markedly increased the magnitude of the increase in intracellular calcium (Fig.2), and the SNAP-induced enhancement of the calcium response was almost completely reversed by co-treatment with SNAP and ODQ, an inhibitor of sGC, or KT5823, an inhibitor of PKG (Fig. 2C). SNAP treatment, however, did not significantly increase the conduction distance of the photolytic flash-induced intercellular calcium waves (data not shown). These results suggested that exposure of cultured astrocytes to an NO donor SNAP did not affect the propagation of calcium waves, but enhanced the calcium response to the photolitic flash via a NO-cGMP-G-kinase signaling cascade. The exact mechanisms downstream of this signaling cascade are currently unknown, but previous studies have suggested that NO enhances the synthesis of cyclic ADP ribose (cADPR) via the cGMP-G-kinase signaling cascade (13,15), and then increases calcium release from ryanodine-sensitive intracellular stores.

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