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Original article

Measurement of intracellular Ca^{2+} in cultured rat embryonic hippocampal neurons using a fluorescence microplate reader: potential application to biomolecular screening

Kamondanai Hemstapat, Maree T. Smith, Gregory R. Monteith*

School of Pharmacy, University of Queensland, St. Lucia Campus, Brisbane, Queensland, 4072, Australia

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Abstract

Introduction: Fluorescence microplate readers for the measurement of cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) are used as a drug screening tool, particularly for immortal cell lines. However, wider application of this methodological approach to more differentiated cells such as neurons would also be useful for the screening of compounds that modulate synaptic transmission. Such an approach has the potential to identify lead compounds for the development of novel drugs for the treatment of epilepsy, pathological pain states, Parkinson's disease, or other neurological disorders. **Methods:** In this paper, we describe the development of a microplate reader assay for the assessment of $[Ca^{2+}]_i$ in a primary culture of rat hippocampal neurons maintained in Neurobasal medium using the fluorescent calcium indicator, fluo-3. **Results:** The assay was appropriate for the screening of glutamate receptor agonists and antagonists. Furthermore, lowering the extracellular Mg^{2+} concentration ($[Mg^{2+}]_O$) produced consistent oscillations in neuronal $[Ca^{2+}]_i$ detected using the fluorescence microplate reader. These oscillations were inhibited by the $GABA_B$ agonist, baclofen, and the NMDA receptor antagonist, LY274614. **Discussion:** Our results indicate that assessment of the inhibitory effects of agents on spontaneous $[Ca^{2+}]_i$ oscillations in neurons may be useful for the identification of agents that act on targets for which specific screening methods are not currently available, or those which act via a previously unknown pathway to inhibit synaptic transmission. This technique also has the potential to increase the productivity of experiments designed to characterize changes in $[Ca^{2+}]_i$ (including calcium oscillations) in cultured neurons.

Keywords: Calcium; Calcium oscillations; Neurons

1. Introduction

Fluorescence microplate readers, particularly those designed for moderate to high throughput screening such as the Fluorescence Imaging Microplate Reader (FLIPR, Molecular Devices, Sunnyvale, CA, USA) and the NOVOstar (BMG LabTechnologies, Offenburg, Germany), are increasingly used in a range of biomolecular screening assays (Kassack et al., 2002; Rogers, Fong, Redburn, & Griffiths, 2002). Fluorescence microplate readers can measure a variety of cellular signals and intracellular ions including Na^+, H^+, and Ca^{2+}. Ca^{2+} is a particularly useful ion for biomolecular screening assays, because of its important role in processes as diverse as muscle contraction, synaptic transmission, hormone release, and gene transcription (Luebke, Dunlap, & Turner, 1993; Monteith & Roufogalis, 1995).

One of the most common uses of microplate readers, which involves the assessment of cytosolic free Ca^{2+} ($[Ca^{2+}]_i$), is the screening of agents for the modulation of G-protein-coupled receptors (GPCRs) (Kassack et al., 2002). Often, a particular GPCR is overexpressed in a convenient cell line that already has the necessary functional downstream signaling cascades (e.g., IP_3-dependent Ca^{2+} store release) (Patel et al., 2001). Agents can then be screened for their ability to activate the GPCR of interest. Such studies are easily adapted for the screening of potential therapeutic antagonists, whereby agents are tested for their ability to block Ca^{2+} transients initiated by a ligand acting at the overexpressed GPCR. These techniques are extremely powerful when the target is known and can be overexpressed.

Studies whereby Ca^{2+} responses are assessed in cells that more closely mimic those found in vivo or in disease

* Corresponding author. Tel.: +61-7-336-57442; fax: +61-7-336-51688.
E-mail address: G.Monteith@pharmacy.uq.edu.au (G.R. Monteith).

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states would also be useful. Indeed, a lung carcinoma cell line (NCI-H146) has been reported to be similar to neurons in that these cells express N- and P-type Ca^{2+} channels. Indeed, this cell line has been used to screen the effects of Australian plant extracts on K^+-depolarization-induced Ca^{2+} influx, to identify novel lead compounds for the treatment of neurological disorders (Rogers et al., 2002). However, such cell lines still fail to mimic the complexity of neuronal networks in vivo. Primary cultured neurons, however, retain many features similar to those found in vivo, such as morphology, development of synapses, together with spontaneous neurotransmitter release and $[Ca^{2+}]_i$ oscillations (Bacci, Verderio, Pravettoni, & Matteoli, 1999; Shen, Piser, Seybold, & Thayer, 1996). Inhibition of spontaneous Ca^{2+} oscillations in primary cultured neurons has been used to characterize the effects of a wide variety of agents including cannabinoids (Shen et al., 1996; Shen & Thayer, 1998) and these oscillations are inhibited by L-type, P/Q-type, and N-type Ca^{2+} channel blockers as well as TTX (Hemstapat, Monteith, Smith, & Smith, 2003; Tanaka, Saito, & Matsuki, 1996). Such studies have used fluorescence microscopy to assess $[Ca^{2+}]_i$ in individual or very small groups of neurons, a technique unsuited to screening.

In the studies described herein, we sought to determine whether $[Ca^{2+}]_i$ could be assessed in neurons cultured in media which promotes high purity (>95%) neurons (Neurobasal medium), using a microplate reader suitable for moderate throughput screening (NOVOstar). We also wanted to determine whether neuronal $[Ca^{2+}]_i$ oscillations could be assessed in a fluorescence microplate format, as a tool for the screening of novel inhibitors of synaptic transmission. Our technique, described here in detail, has the potential to be a useful initial screening method to determine if a compound is likely to modulate synaptic transmission. Such an evaluation could be done before a wide array of tests on specific targets is performed. Furthermore, this protocol should identify compounds which modulate synaptic transmission through a mechanism for which screening assays are not yet available, or which act on a target that has not been previously identified.

2. Methods

2.1. Materials

N-methyl-D-aspartate (NMDA), poly-D-lysine (PDL), papain, bovine serum albumin (BSA), dimethyl sulfoxide (DMSO), and HEPES Hank's Salt Solution (HHSS) components were obtained from Sigma (St. Louis, MO). Tissue culture medium was purchased from Invitrogen (Melbourne, Australia). LY274614 (±-decahydro-6-(phosphonomethyl)-3-isoquino-linecarboxylic acid) was a generous gift from Eli Lilly (Sydney, Australia). Baclofen (Lioresal intrathecal) vials (2 mg/ml), a product for intrathecal use in humans containing no preservatives (excipients: sodium chloride; water for injection) were purchased from the Royal Brisbane Hospital Pharmacy (Brisbane, Australia). Fluo-3/AM (Fluo-3) was from Molecular Probes (Eugene, OR). All test compounds were prepared in either HHSS containing an extracellular Mg^{2+} ($[Mg^{2+}]_O$) concentration of 0.1, 0.9, or 3 mM, depending upon the protocol.

2.2. Cell culture

Hippocampi were dissected and prepared from Sprague–Dawley rats at 18–19 days of gestation. Rat hippocampal neurons were cultured as described by Brewer, Torricelli, Evege, and Price (1993) with minor modifications. Hippocampi were dissociated using papain in Hibernate-E (2 mg/ml) at 30 °C for 30 min. Dissociated cells were plated in Neurobasal medium containing glutamate (2.5 μM), L-glutamine (0.5 mM) and 2% B-27. Cell suspension (100 μl) was plated at a density of $3–4\times10^5$ cells/ml onto 96-well plates (TTP 96) coated with PDL (0.1 mg/ml). Cell density of a typical neuronal culture at the time of experimentation in 96-well plates was approximately 500 cells/mm^2. For imaging experiments, hippocampal neurons were plated onto 25-mm round glass coverslips coated with PDL at a density of $6–7\times10^5$ cells/ml. Neurons were grown in a humidified atmosphere of 5% CO_2 at 37 °C, and cultures were fed on Day 3 by exchanging half of the medium with fresh Neurobasal medium without glutamate. Culture media were then exchanged for fresh media every 2 days for neurons cultured on microplates and weekly for cultures grown on coverslips. Neuronal cultures maintained in Neurobasal medium may be particularly useful for microplate studies, because they yield cultures of high neuronal purity (>95%) (Brewer et al., 1993). The neuronal content and glial contamination were examined using fluorescence immunocytochemistry as described previously by our laboratory, and glial contamination was less than 5% (Hemstapat et al., 2003).

2.3. $[Ca^{2+}]_i$ measurement

Increases in $[Ca^{2+}]_i$ were determined using the NOVOstar fluorescence microplate reader (BMG LabTechnologies). Cultures grown in 96-well plates aged 11–12 days in vitro, were loaded with 4 μM fluo-3 which was dissolved in DMSO (1:1000) for 45 min at 37 °C in HHSS containing BSA (3 mg/ml), and were subsequently washed twice with fresh HHSS containing BSA followed by a further incubation for 15 min. Each well was then washed twice with either HHSS containing 0.1 mM $[Mg^{2+}]_O$ or 3 mM $[Mg^{2+}]_O$, depending upon the protocol. All experiments were performed at room temperature. Data points were collected at 0.7-s intervals, unless otherwise indicated. For NOVOstar experiments, the volume in each well before adding test solution was 90 μl, and 10 μl of a solution of the appropriate

test compound was applied to each well using either the reagent pipettor or the reagent pump injector. Control experiments, where HHSS with no drug or NMDA was added, showed that cells did not respond to the addition of HHSS. Moreover, other controls included cells not loaded with fluo-3; these wells showed significantly less fluorescence and did not respond to the addition of any test compounds. The area of reading for NOVOstar experiments is approximately 30 mm^2 (James Balmer, BMG LabTechnologies, personal communication). Values for *n* refer to the number of wells examined, and traces shown are representative of results obtained for each well.

In separate experiments, fluorescence digital imaging was used to assess increases in $[Ca^{2+}]_i$ in the somatic region of neurons. Neurons aged 14–18 days in vitro were loaded with 2 μM fluo-3 and were superfused with HHSS containing 0.9 mM $[Mg^{2+}]_O$ at a flow rate of 3 ml/min.

Fluo-3 loaded cells were excited at 480 nm using a Sutter DG-4 wavelength selector (Sutter Instrument, Novato, CA). Emission was observed at 550 nm (band pass filter, Omega Optical, Brattleboro, VT). Cells were observed using a ×40 oil immersion objective. Fluorescence images were acquired using a MicroMAX 5-MHz cooled CCD Camera (Princeton Instruments, Trenton, NJ). Images were collected every 0.6 s or every 10–30 s during incubation or washout periods. This was done to reduce photobleaching. The camera exposure time was 150 ms, the image resolution was 12 bits and binning was set to 3. Images were analyzed using Metafluor software (West Chester, PA).

3. Results and discussion

Fig. 1 shows data obtained from three individual neurons using fluorescence digital imaging. $[Ca^{2+}]_i$ oscillations in HHSS (containing 0.9 mM Mg^{2+}) occurred spontaneously in these cultured rat hippocampal neurons and neurons in the field were synchronized. Oscillations were inhibited by the addition of baclofen (10 μM) (a $GABA_B$ agonist) and were dependent on extracellular Ca^{2+} (Hemstapat et al., 2003). However, high-resolution fluorescence microscopy is unsuited for screening of $[Ca^{2+}]_i$ and so we assessed the suitability of a fluorescence microplate reader to measure $[Ca^{2+}]_i$ in primary cultured neurons. A higher throughput assay using neurons may allow the identification of novel compounds useful for the treatment of diseases of the central nervous system such as epilepsy, pathological pain states, and Parkinson's disease.

Initially, we wanted to identify agents that could be effectively screened using primary cultured neurons *not* undergoing spontaneous oscillations in $[Ca^{2+}]_i$ in a microplate format. To achieve this, we used the NOVOstar to examine the effects of agents on both basal and NMDA-mediated increases in $[Ca^{2+}]_i$ in cultured neurons exposed to 3 mM Mg^{2+}. As expected, 3 mM Mg^{2+} prevented neurons from undergoing consistent rapid oscillations in $[Ca^{2+}]_i$, as this concentration of Mg^{2+} is well-documented to inhibit glutamate-mediated synaptic transmission (Kudo & Ogura, 1986). Addition of the NMDA receptor agonist, NMDA (100 μM), to cultured neurons grown in microplate format, produced sustained increases in $[Ca^{2+}]_i$ (n=3, Fig. 2A) that were completely blocked by pretreatment of neurons with the selective NMDA receptor antagonist, LY274614 (50 μM) (n=4, Fig. 2B). Importantly, preincubation of neurons with 10 μM baclofen (n=7, Fig. 2C) did not prevent NMDA-evoked increases in $[Ca^{2+}]_i$, confirming the fidelity of this methodological approach with respect to receptor specificity of the agents tested. Further reinforcing these observations, neither LY274614 nor baclofen significantly altered basal $[Ca^{2+}]_i$, confirming that prior exposure of the cultured neurons to an appropriate agonist was a prerequisite to observe the effects of LY274614, and that many drugs, acting via a non-NMDA mechanism (such as the $GABA_B$ agonist, baclofen) to inhibit $[Ca^{2+}]_i$ increases or synaptic responses, would be effectively excluded by using this screening technique to identify novel NMDA antagonist compounds.

Thus, in neuronal cultures *not* undergoing spontaneous synaptic transmission, the measurement of $[Ca^{2+}]_i$ in a microplate environment can be used to screen compounds with the ability to increase neuronal $[Ca^{2+}]_i$. Characterization of the specific mechanism (e.g., NMDA activation) by which $[Ca^{2+}]_i$ is increased could be determined using appropriate antagonists. However, such studies could also be performed in cell lines overexpressing a receptor target (e.g., NMDA receptors). Studies in non-oscillating neurons can also be used to assess neurotransmitter antagonist activity (e.g., LY274614) when used with the appropriate agonist (in this case NMDA). However, these protocols are ineffective in assessing the effects of baclofen and would be inappropriate for many other agents which act via inhibition of neurotransmitter release or are not associated with direct antagonism of neurotransmitter responses, in particular, agents which act via a presynaptic mechanism. Thus, we next sought to design a protocol which could screen for agents that had a modulatory effect on spontaneous neuronal $[Ca^{2+}]_i$, in a moderate throughput microplate reader.

Since spontaneous $[Ca^{2+}]_i$ oscillations arise from spontaneous synaptic neurotransmitter release, we developed an assay whereby spontaneous oscillations in $[Ca^{2+}]_i$ could be consistently observed. The assessment of spontaneous $[Ca^{2+}]_i$ in neuronal cultures grown in a microplate format would allow the higher throughput screening of a wider array of compounds capable of inhibiting synaptic transmission (e.g., baclofen and cannabinoids; Shen et al., 1996). Such an assay could also be used to identify agents that inhibit synaptic transmission via a novel, hitherto unidentified mechanism. It should be noted that the measurement of $[Ca^{2+}]_i$ in individual neurons is not possible with the NOVOstar; this makes the assessment of oscillations more difficult. Therefore, to monitor synaptic transmission in a microplate format, we induced consistent simultaneous

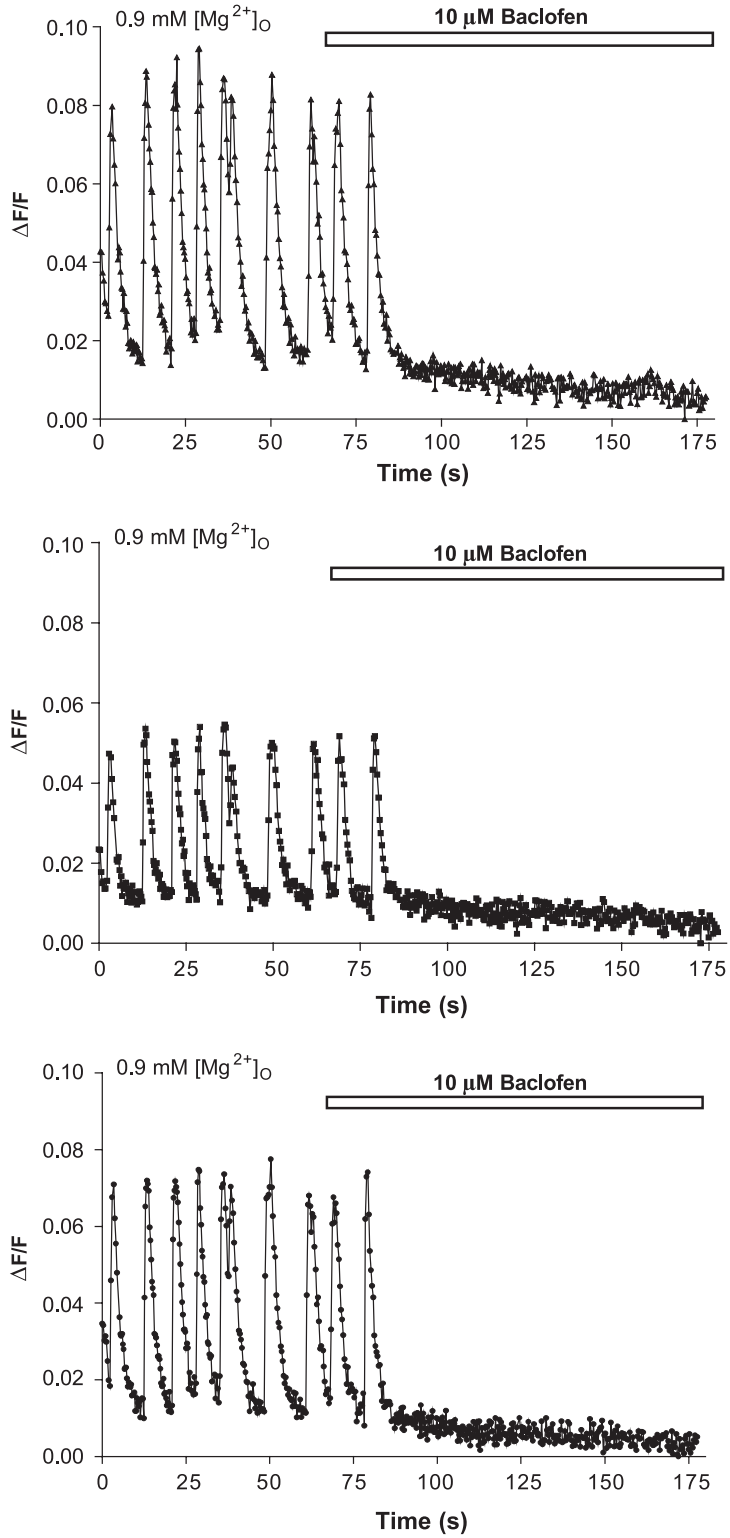

Fig. 1. Assessment of the effect of the $GABA_B$ agonist, baclofen, on three individual cultured neurons in which spontaneous synchronous $[Ca^{2+}]_i$ oscillations were observed, using fluo-3 fluorescence digital imaging. Baclofen (10 μM) inhibited spontaneous oscillations in $[Ca^{2+}]_i$ in these neurons.

oscillations in $[Ca^{2+}]_i$ in a large number of neurons by lowering the extracellular Mg^{2+} concentration to 0.1 mM (Shen et al., 1996). In HHSS containing 0.1 mM $[Mg^{2+}]_O$, spontaneous $[Ca^{2+}]_i$ oscillations were observed and these oscillations were markedly attenuated by 50 μM LY274614 (n=3, Fig. 3A), consistent with their well-documented NMDA-receptor dependence (Abele, Scholz, Scholz, & Miller, 1990; Kudo & Ogura, 1986). However, other gluta-

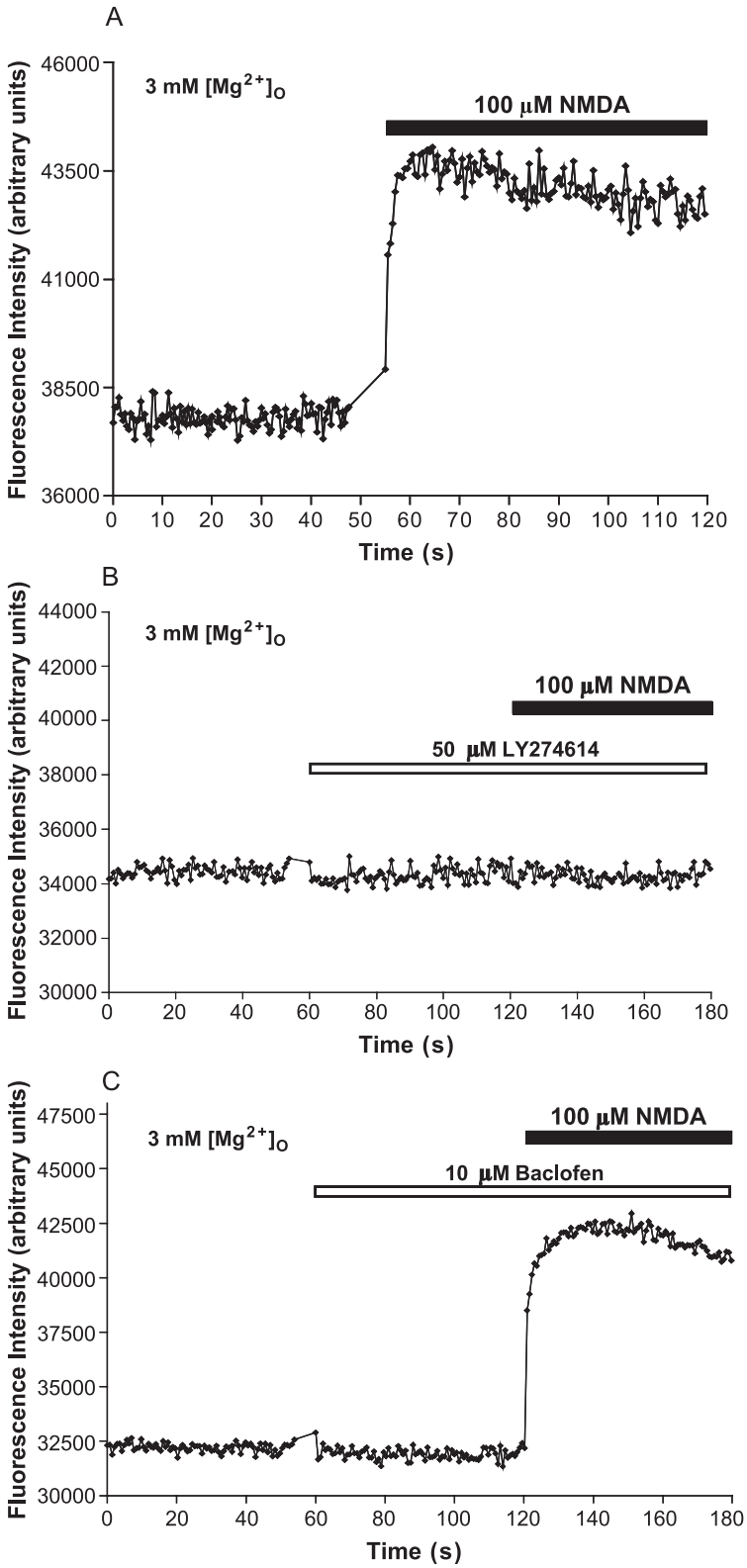

Fig. 2. Assessment of $[Ca^{2+}]_i$ in non-oscillating neuronal cultures using a microplate reader. (A) Addition of NMDA (100 μM) to the neuronal culture produced a rapid and sustained increase in $[Ca^{2+}]_i$, as measured by fluo-3 fluorescence. (B) The competitive NMDA antagonist, LY274614 (50 μM), completely inhibited NMDA-induced increases in $[Ca^{2+}]_i$ in non-oscillating neurons. (C) As expected, the $GABA_B$ agonist, baclofen (10 μM), did not block NMDA-mediated increases in neuronal $[Ca^{2+}]_i$.

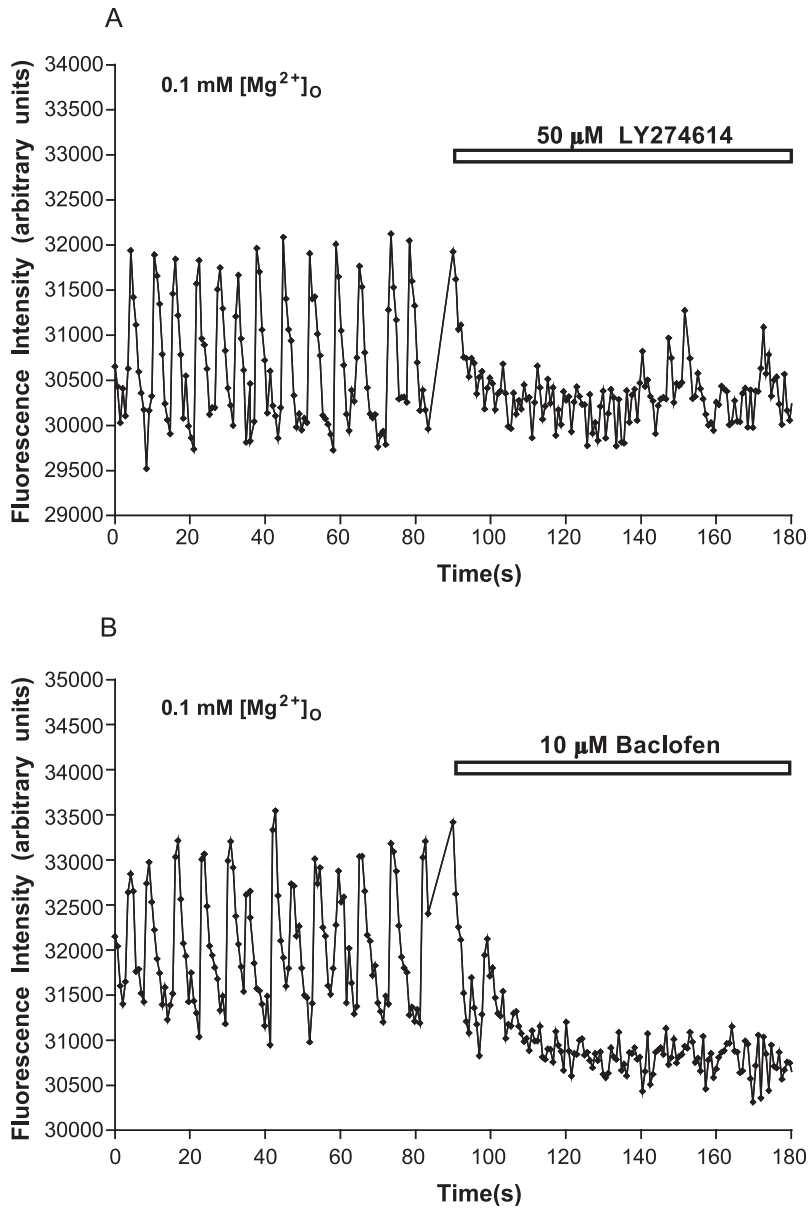

Fig. 3. Assessment of spontaneous oscillations in $[Ca^{2+}]_i$ in neuronal cultures, using a microplate reader. (A) The competitive NMDA antagonist, LY274614 (50 μM), inhibited spontaneous oscillations in neuronal $[Ca^{2+}]_i$ induced by 0.1 mM $[Mg^{2+}]_O$. (B) The $GABA_B$ agonist, baclofen (10 μM), abolished spontaneous oscillations in neuronal $[Ca^{2+}]_i$ induced by 0.1 mM $[Mg^{2+}]_O$.

mate receptors such as AMPA and metabotropic receptors may contribute to spontaneous $[Ca^{2+}]_i$ oscillations in these cultures; indeed, our previous experiments using fluorescence digital imaging have shown that these oscillations are also sensitive to AMPA receptor antagonists (Hemstapat et al., 2003).

In marked contrast to the inability of the protocol using 3 mM Mg^{2+} to detect exposure of cultured neurons to baclofen (10 μM), this latter protocol using 0.1 mM Mg^{2+} showed that baclofen (10 μM, n=4, Fig. 3B) effectively abolished spontaneous neuronal $[Ca^{2+}]_i$ oscillations. Hence, spontaneous oscillations in neuronal $[Ca^{2+}]_i$ have the potential to be used as a screening tool for compounds inhibiting synaptic transmission. Although the oscillations observed in 0.1 mM Mg^{2+} have a higher noise level than those measured using fluorescence digital imaging (Fig. 1), the lower Mg^{2+} concentration and the high purity of the neuronal culture clearly show that neuronal Ca^{2+} oscillations can be successfully assessed using the NOVOstar. One of the key differences in the data from imaging and microplate experiments presented herein is the use of perfusion in the imaging experiments, whereas the microplate experiments were undertaken using 'stop-flow' conditions. Importantly, our recent imaging study showed that perfusion is not a prerequisite for observing spontaneous Ca^{2+} oscillations using fluorescence digital imaging, since we observed that P/Q- and L-type Ca^{2+} channel blockers blocked

spontaneous Ca^{2+} oscillations under nonperfusion conditions (Hemstapat et al., 2003).

The assessment of spontaneous $[Ca^{2+}]_i$ oscillations in low Mg^{2+} HHSS has the potential to be more powerful in detecting agents which attenuate synaptic transmission, than studies which assess inhibition of responses to specific neurotransmitters.

In conclusion, oscillations in neuronal $[Ca^{2+}]_i$ can be characterized in Xenon lamp-based fluorescence microplate readers suitable for moderate throughput screening. However, the limitation of this approach compared with imaging experiments is that agents cannot be removed, limiting the ability to assess reversibility of effects. The assessment of spontaneous oscillations in neuronal $[Ca^{2+}]_i$ could potentially identify agents which act on a target for which specific screening methods are not widely available, or those which act via a previously unknown pathway to inhibit synaptic transmission.

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