Biochemical and Biophysical Research Communications 397 (2010) 564–568

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Gap junction-mediated spontaneous Ca^{2+} waves in differentiated cholinergic SN56 cells

Nishani T. Hettiarachchi [a], Mark L. Dallas [a], Hugh A. Pearson [b], Gareth Bruce [b], Susan Deuchars [b], John P. Boyle [a], Chris Peers [a,*]

[a] Faculty of Medicine and Health, University of Leeds, Leeds LS2 9JT, UK
[b] Biological Sciences, University of Leeds, Leeds LS2 9JT, UK

ARTICLE INFO

Article history:
Received 25 May 2010
Available online 4 June 2010

Keywords:
Calcium oscillation
Gap junction
Cholinergic
Patch clamp

ABSTRACT

Neuronal gap junctions are receiving increasing attention as a physiological means of intercellular communication, yet our understanding of them is poorly developed when compared to synaptic communication. Using microfluorimetry, we demonstrate that differentiation of SN56 cells (hybridoma cells derived from murine septal neurones) leads to the spontaneous generation of Ca^{2+} waves. These waves were unaffected by tetrodotoxin (1 μM), but blocked by removal of extracellular Ca^{2+}, or addition of non-specific Ca^{2+} channel inhibitors (Cd^{2+} (0.1 mM) or Ni^{2+} (1 mM)). Combined application of antagonists of NMDA receptors (AP5; 100 μM), AMPA/kainate receptors (NBQX; 20 μM), nicotinic AChR receptors (hexamethonium; 100 μM) or inotropic purinoceptors (brilliant blue; 100 nM) was also without effect. However, Ca^{2+} waves were fully prevented by carbenoxolone (200 μM), halothane (3 mM) or niflumic acid (100 μM), three structurally diverse inhibitors of gap junctions, and mRNA for connexin 36 was detected by PCR. Whole-cell patch-clamp recordings revealed spontaneous inward currents in voltage-clamped cells which we inhibited by Cd^{2+}, Ni^{2+} or niflumic acid. Our data suggest that differentiated SN56 cells generated spontaneous Ca^{2+} waves which are propagated by intercellular gap junctions. We propose that this system can be exploited conveniently for the development of neuronal gap junction modulators.

1. Introduction

Whilst it is long established that synaptic activity governs communication between central neurones, evidence continues to accumulate indicating that gap junctions are an important additional form of interneuronal communication, accounting for direct electrical signalling [1–3]. Such coupling permits neuronal activity to be synchronised over substantial networks, and may represent an important target for future therapeutic intervention, since neuronal gap junctions are implicated in, for example, spreading cortical depression which may underlie development of migraine [4,5]. Exploration of their significance to co-ordinated neuronal network activity has been delayed by the lack of tools available for their study. Thus, although the characterisation of the connexins and related proteins underlying gap junctions are being elucidated [6], the lack of specific pharmacological modulators available to probe the role of gap junctions without additional cellular actions has hindered progress. Here, we describe a preparation of murine septal neurone-derived (SN56) cells which, upon differentiation, generate spontaneous Ca^{2+} waves which are mediated by gap junction formation between cells.

2. Materials and methods

The SN56 hybridoma cell line (kindly provided by A. Szutowicz, Medical University of Gdańsk, Poland) is derived from murine septal neurones fused with murine neuroblastoma (N18TG2) cells, and retains cholinergic properties of septal neurones [7,8]. Indeed, they have been proposed previously to represent an amenable model for studying aspects of central cholinergic neurones, and therefore of potential use in the study of neurodegenerative disorders such as Alzheimer's disease [9]. These cells were cultured and maintained in 75-cm^2 flasks using DMEM, 10% (v/v) heat inactivated foetal calf serum (inactivated by heating for 1 h at 50 °C) and 1% (v/v) penicillin–streptomycin. To differentiate the SN56 cells, adenosine 3′,5′-cyclic monophosphate (cAMP, 1 mM, Sigma–Aldrich, Poole, UK) and retinoic acid (1 μM, Sigma) was added to the cells. This media was replaced every 2 days. After 2 days in the differentiating media the cells start developing numerous processes and the experiments were carried out after this time period.

* Corresponding author. Address: Faculty of Medicine and Health, University of Leeds, Worsley Building, Leeds LS2 9JT, UK. Fax: +44 113 343 4803.
E-mail address: c.s.peers@leeds.ac.uk (C. Peers).

0006-291X/$ - see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2010.05.159

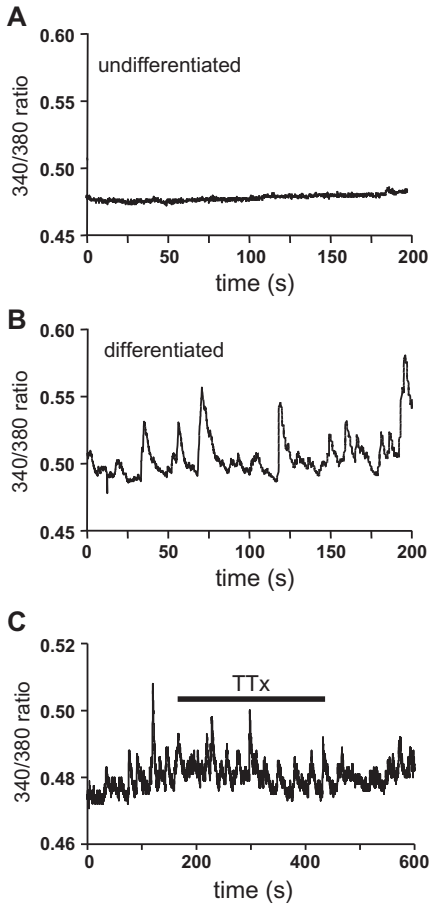

Fig. 1. Differentiated SN56 cells display spontaneous Ca^{2+} *waves.* Representative example recordings of $[Ca^{2+}]_i$ obtained from Fura-2 loaded SN56 cells which were either undifferentiated (A) or chemically differentiated for at least 2 days (B). In (C) and all subsequent figures, data presented are from differentiated cells. (C) Example recording of $[Ca^{2+}]_i$ shown on a slower time base than shown in (A) or (B), illustrating the lack of effect of bath application of 1 μM tetrodotoxin (TTx; applied for the period indicated by the horizontal bar).

$[Ca^{2+}]_i$ was monitored fluorimetrically following incubation of cells with HEPES buffered perfusate containing 4 μM Fura-2-AM at 20 °C for 40 min. Fragments of coverslip with attached cells were transferred to a perfusion chamber and perfused with a solution of (in mM): NaCl, 135; KCl, 5; $MgSO_4$, 1.2; $CaCl_2$, 2.5; HEPES, 5; D-glucose, 10 (pH 7.4, adjusted with NaOH; 21–24 °C). $[Ca^{2+}]_i$ was measured using a Cairn Research ME-SE Photometry system (Cairn Research, Cambridge) as the ratio of fluorescence emitted at 510 nm due to alternating excitation at 340 nm and 380 nm using a monochromator. Ca^{2+}-free perfusate contained 1 mM EGTA and no added $CaCl_2$. Drugs employed to probe Ca^{2+} signalling were added to the perfusate as indicated below.

In order to investigate expression of connexins in SN56 cells, RNA was extracted from differentiated and undifferentiated cells using an Aurum Total RNA Mini Kit (Bio-Rad Laboratories) and 2 μl of this RNA converted to cDNA using an iScript cDNA Synthesis Kit (Bio-Rad Laboratories). Both kits were used as per manufacturer's instructions. Amplification and detection of the Cx36 gene was performed in a 25 μl reaction mixture containing 12.5 μl HotStarTaq Master Mix (Qiagen), 1.25 μl DMSO, 4.75 μl H_2O, 5 μl cDNA template and 0.5 μl each of the primers Cx36USP-1 (5′-TAA GTG CAA TAA AGG GGG AGG GCC TCG-3′), Cx36DSP-1 (5′-GAG ACA GGA GAA GGT ATT CCC AAG GGC-3′) and DSP-CFP (5′-AAG AAG TCG TGC TGC TTC ATG TGG-3′), to produce a 311 bp product for the Cx36 allele [10].

For electrophysiological recordings fragments of coverslip with attached cells were similarly transferred to a continuously perfused recording chamber and whole-cell patch-clamp recording were then obtained in voltage clamp mode with either differentiated or undifferentiated SN56 cells clamped at −70 mV. The standard perfusate (pH 7.4) was composed of (in mM): NaCl, 150; KCl, 5; $MgCl_2$, 2; HEPES, 10; $CaCl_2$, 2; D-glucose, 10. Patch pipettes had resistances 5–7 MΩ when filled with the following solution (in mM): KSCN, 140; EGTA, 5; $MgCl_2$, 1; $CaCl_2$, 0.5; HEPES, 10; MgATP, 3; NaGTP, 0.3 (pH 7.25). Series resistance was monitored after breaking into the whole cell configuration throughout the duration of experiments. If a significant increase occurred (>20%), the experiment was terminated. Signals were acquired using a Axopatch 200B amplifier (Axon Instruments Inc., Foster City, CA) controlled

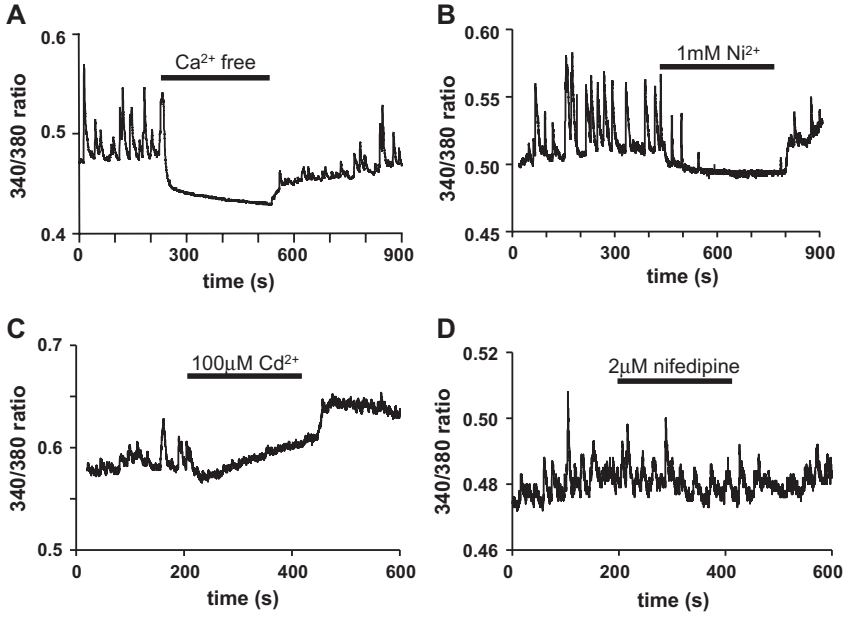

Fig. 2. Inhibition of Ca^{2+} influx prevents spontaneous Ca^{2+} *waves.* Representative example recordings of $[Ca^{2+}]_i$ obtained from Fura-2 loaded differentiated SN56 cells. For the periods indicated by the horizontal bars in each case, perfusate was exchanged for one which contained either (A) no added Ca^{2+} (replaced with 1 mM EGTA), (B) 1 mM Ni^{2+}, (C) 100 μM Cd^{2+} or (D) 2 μM nifedipine, as indicated. Note the abolition of spontaneous Ca^{2+} waves in (A–C) but no such effect in (D).