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Use of calcium imaging for analysis of neuronal gap junction coupling

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Abstract

We recently used western blots for connexin 36 and neuronal dye coupling with neurobiotin to measure developmental decrease in neuronal gap junction coupling in cell cultures. To ask whether Ca^{2+} imaging also can be used to measure changes in the amount of neuronal gap junction coupling, we defined a Ca^{2+} coupling coefficient as the percentage of neurons with bicuculline-induced increases in intracellular Ca^{2+} that are suppressed by blocking gap junctions. We demonstrate in rat and mouse hypothalamic neuronal cultures that the Ca^{2+} coupling coefficient decreases during culture development, this decrease is prevented by manipulations that also prevent developmental decrease in neuronal gap junction coupling, and the coefficient is low in cultures lacking connexin 36. The results indicate that Ca^{2+} imaging is a useful tool to quantify the amount of neuronal gap junction coupling in cultures.

Keywords

Connexin; gap junctions; calcium imaging; NMDA receptors; CREB; cultures

Introduction

Coupling of neurons by gap junctions (electrical synapses) transiently increases in the mammalian central nervous system (CNS) during embryonic and/or early postnatal development and plays a role in a number of developmental events [7]. The coupling subsequently decreases marking the transition of the CNS from immature structure with a primitive form of synchronized network-driven spontaneous activity (which includes a cooperation of immature chemical synapses and gap junctions) to the mature structure where chemical synaptic transmission in neuronal circuits predominates [6,13,18,22].

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We recently demonstrated [1] that developmental uncoupling of neuronal gap junctions is regulated by *N*-methyl-D-aspartate (NMDA) receptors via Ca^{2+} /cAMP response element binding protein (CREB)-dependent down-regulation of connexin 36 (Cx36), that is a neuron-specific gap junction protein [2,19,21]. For that study, we analyzed neuronal gap junction coupling in primary rat and mouse hypothalamic neuronal cultures using two traditional and reliable methods, dye coupling with a gap junction-permeable dye (neurobiotin), and western blots for Cx36. However, we began to develop a new approach for measuring neuronal gap junction coupling in cultures, a fura-2 Ca^{2+} imaging method that defines and measures a Ca^{2+} coupling coefficient. The results reported here demonstrate that the fura-2 Ca^{2+} coupling method reliably replicates results obtained by the more traditional techniques. We have thus established an accurate screening method for analysis of the amount of neuronal gap junction coupling in cultures.

Materials and methods

Animals

The experiments were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. The formal approval of the described experiments has been obtained from the animal review board of Tulane University (New Orleans, LA). All efforts were made to minimize the number of animals used and their pain or discomfort. Sprague-Dawley rats, wild-type mice, NMDA Receptor Subunit 1 (NMDAR1) knockout mice, and Cx36 knockout mice were used in these experiments. The Cx36 knockout was originally created by Dr. David Paul (Harvard Medical School) [12] and was donated to us by Dr. Marla Feller (University of California, San Diego). NMDAR1 knockout animals were generated using an established NMDAR1 knockout line (Dr. Susumu Tonegawa, Massachusetts Institute of Technology) [16]. The animals were raised and genotyped by PCR as described [1,11].

Culture preparation and treatments

Neuronal cultures were prepared as described [5] from embryonic day 18–19 (E18–19) medial hypothalamus. Pregnant animals were anesthetized with nembutal (70 mg/kg), the embryos were removed, the hypothalamus was dissected and the tissue was treated with papain to obtain a single cell suspension. Cells were plated on glass coverslips and raised in glutamate- and glutamine-free minimal essential medium (Invitrogen, Carlsbad, CA, USA) with supplements [5] and cytosine β -D-arabinofuranoside (1 μM). In rat cultures, the material obtained from several embryos was combined for plating; in mouse cultures, each plating (coverslip) contained the hypothalamic material obtained from a single embryo. All coverslips were randomly labeled, tested blindly, and then matched with the genotyping results using a database [1]. The culture medium was changed twice a week. The treatments were performed using sister cultures. Cell survival was determined using a toxicity assay (Live/Dead Kit; Invitrogen) as described previously [5], and none of the chronic treatments reduced neuronal survival. Only CREB viral vectors induced neurodegeneration in cultures after 7–10 days; therefore, cells were exposed to viral vectors for no more than 5 days. CREB antisense oligodeoxynucleotide (CREB ODN) and CREB viral vectors were used as described [1]. CREB ODN (obtained from Oligo's Etc. Inc., Wilsonville, OR, USA) was administered to the cultures daily at the concentration of 2 nmols per treatment. A herpes simplex virus CREB vector (HSV-CREB) and a dominant-negative mutant form of CREB (HSV-mCREB; that contains a single point mutation Ala for Ser at residue 133) were prepared as described [8]. The estimated titer of recombinant viral stocks was 1×10^8 infectious units/ml. HSV-CREB and HSV-mCREB were added to cultures once (0.25 μl stock/ml) on, respectively day *in vitro* 16 (DIV16) and DIV24. The used CREB constructs effectively change CREB expression in hypothalamic neuronal cultures [1]. The drug concentrations (including the concentration of carbenoxolone) were chosen based on data from the literature and our previous studies [1,3] and, as a rule, the low

range of previously reported agent concentrations were used in this study. All drugs were obtained from Sigma-RBI unless otherwise specified.

Fura-2 Ca^{2+} digital imaging

Fura-2 Ca^{2+} imaging was performed in cultures as described [5]. Perfusion solution contained: 137 mM NaCl, 25 mM glucose, 10 mM HEPES, 5 mM KCl, 1 mM MgCl_2 , 3 mM CaCl_2 and 1 μM glycine (pH 7.4, 22°C). Cells were incubated with fura-2 AM (5 μM , Invitrogen) for 30 min and examined using a Nikon TE2000s inverted microscope. Conventional dual wavelength ratios (at 340 and 380 nm excitation) were obtained using a Sutter DG-4 filter changer and Axon Workbench software (Axon Instruments, Inc., Foster City, CA, USA). Ca^{2+} standards from Invitrogen were used to calibrate the imaging system. Calibrated Ca^{2+} data were analyzed with Igor Pro (WaveMetrics, Lake Oswego, OR, USA) and InStat (GraphPad Software, San Diego, CA, USA) software. The used cultures contained both neurons and glial cells. Neurons had the characteristic rounded cell body, small size (~20 μm), and multiple tiny processes and were easily distinguished from astrocytes which did not have a clear-cut cell body and processes, were spread, and had a bigger size (40–100 μm). Recordings were made from neuronal cell bodies. Further, for each experimental group, recordings were made from one microscope field per coverslip (from 30–45 neurons per field) in at least 3 coverslips (each from an independent culture preparation). Drugs were applied to neurons using a flow pipe perfusion system [5]. A γ -aminobutyric acid A (GABA_A) receptor antagonist, bicuculline (50 μM), was applied for 3–4 min to induce intracellular Ca^{2+} increases and the increases were detected in 92–98% of neurons in all different groups of cell cultures. A neuron was considered as responding to bicuculline if, during bicuculline application, Ca^{2+} increased by >10 nM from the initial background level or intracellular Ca^{2+} oscillations appeared (also >10 nM in amplitude) and the Ca^{2+} activity returned to the background level after bicuculline wash-out. In cultures that were chronically (DIV4–32) treated with an NMDA receptor antagonist, D,L-2-amino-5-phosphonovalerate (AP5; 100 μM), or were subjected to ODN CREB treatments, the bicuculline-induced Ca^{2+} increases were induced 30–40 min after wash out of the treating agents. In all culture groups in different neurons, the amplitude of bicuculline-induced Ca^{2+} increases was in the range of 85–350 nM.

Statistical analysis

Data were analyzed using InStat software and ANOVA with post hoc Tukey test or the unpaired two-tail Student's *t*-test. All data are reported as mean $\pm$ s.e.m. for the number of samples indicated.

Results

We previously demonstrated [4,5] that neuronal disinhibition with bicuculline, a GABA_A receptor antagonist, induces cytoplasmic Ca^{2+} increases in neurons in primary hypothalamic cultures. In control (non-treated) cultures, these increases are glutamate-dependent as are completely suppressed by ionotropic glutamate receptor antagonists, AP5 plus 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (100/10 μM). In contrast, in cultures that are chronically (2–3 weeks) treated with AP5 (100 μM) or AP5+CNQX (100/10 μM), neurons display the bicuculline-induced Ca^{2+} increases despite the continued presence of glutamate receptor antagonists. These glutamate-independent Ca^{2+} increases are suppressed by acetylcholine receptor antagonists and represent up-regulation of cholinergic functions in neurons by chronic NMDA receptor blockade [4,5]. Moreover, in AP5+CNQX-chronically treated cultures, gap junctions contribute to the generation of bicuculline-induced Ca^{2+} increases as a significant amount of these Ca^{2+} transients also is suppressed by carbenoxolone, a general gap junction blocker [3]. Here, we utilize Ca^{2+} imaging and the analysis of Ca^{2+} coupling coefficient, i.e., the percentage of bicuculline-responsive neurons in which the bicuculline-induced Ca^{2+}

activity is suppressed by carbenoxolone, to determine whether this approach can be used to characterize the amount of neuronal gap junction coupling in different cell culture conditions and at different developmental periods.

The present evaluation of the Ca^{2+} coupling coefficient approach is based on the previous observation that neuronal gap junction coupling, measured using traditional methods, initially increases in developing rat and mouse hypothalamic neuronal cultures between DIV1-16 and then decreases between DIV16-32 [1]. The initial increase is not affected by chronic blockade of NMDA receptors, but the subsequent developmental decrease is prevented by chronic (DIV4-DIV32) inactivation of NMDA receptors. These published results were based on analyses of neuronal dye coupling (with neurobiotin) and Cx36 protein expression [1].

In the present study, we first determined whether the Ca^{2+} coupling coefficient detects and quantitatively corroborates the developmental decreases in dye coupling and Cx36 expression that occur in hypothalamic cell cultures, and, if it does, whether the decrease in the Ca^{2+} coupling coefficient can be prevented by chronic inactivation of NMDA receptors. The tests were conducted in DIV16 and DIV32 control (non-treated) rat hypothalamic neuronal cultures and cultures that were chronically treated (starting DIV4) with an NMDA receptor antagonist, AP5 (100 μM). In these tests, bicuculline (50 μM) was applied to neurons to induce intracellular Ca^{2+} increases (Fig.1; in AP5 chronically-treated cultures, bicuculline was applied in the absence of AP5, i.e., 30–40 min after AP5 was out). In both control and AP5-treated culture groups, the bicuculline-induced Ca^{2+} increases were detected in 92–98% of the neurons at both DIV16 and DIV32. During the same experiment, carbenoxolone (25 μM) was administered to these cultures to determine the percentage of bicuculline-responsive neurons in which Ca^{2+} activity is suppressed by blocking gap junction coupling, i.e., to determine Ca^{2+} coupling coefficient. For purposes of quantifying via the Ca^{2+} coupling coefficient, only neurons with complete suppression of the bicuculline-induced Ca^{2+} activity by carbenoxolone were counted as having gap junction-dependent Ca^{2+} responses (e.g. as illustrated in Fig.1b). The Ca^{2+} coupling coefficient decreased between DIV16–32 in control cultures, and this decrease was reduced by the chronic administration of AP5 (Fig.2a). These results agree qualitatively and quantitatively with previously established changes in dye coupling and Cx36 expression in rat hypothalamic neuronal cultures in this paradigm [1].

We performed experiments in mouse hypothalamic cultures prepared from wild-type, NMDAR1 knockout, and Cx36 knockout animals to test further our hypothesis that the Ca^{2+} coupling coefficient can reproduce results obtained by traditional methods for measuring gap junctions. As in a previous study [1], which demonstrated the developmental decreases in dye coupling and Cx36 expression in wild-type mouse cultures and the preclusion of these decreases by pharmacological (with AP5) or genetic (NMDAR1 knockout) inactivation of NMDA receptors, in the present study, the Ca^{2+} coupling coefficient also decreased in wild-type cultures between DIV16–32 (Fig.2b). As expected, this decrease was prevented by AP5, and did not occur in NMDAR1-deficient cultures (Fig.2b). Moreover, at both the time points studied (i.e., DIV16 and DIV32) the Ca^{2+} coupling coefficient was low in hypothalamic cultures prepared from Cx36 knockout mice (Fig.2b), which is consistent with low or absent neuronal gap junction coupling in the hypothalamus of Cx36-deficient animals [17].

We also demonstrated previously [1] that developmental uncoupling of neuronal gap junctions is mediated by NMDA receptors via the activation of CREB-dependent signaling pathways that down-regulate Cx36 expression. Specifically, we reported that in rat hypothalamic neuronal cultures administration of CREB ODN (on DIV16–32; that blocks CREB translation [15]) or overexpression of HSV-mCREB (on DIV24-28; that prevents CREB phosphorylation/transactivation [8]), both prevented developmental decreases in dye coupling and Cx36 expression. In the meantime, the treatment of cultures with HSV-CREB (on DIV16–19; that

causes CREB overexpression [8]) accelerated developmental decreases in these two parameters. Here we used these established CREB manipulations at the same developmental periods in rat hypothalamic cultures to test the validity of the Ca^{2+} coupling coefficient as an assay for the amount of gap junction coupling. The developmental decrease in Ca^{2+} coupling coefficient was prevented by CREB inactivation (Fig.3a,b) and was accelerated by increase in CREB activity (Fig.3c), further validating this new imaging-based assay.

Finally we analyzed the Ca^{2+} coupling coefficient in DIV32 control and AP5 chronically-treated rat hypothalamic cultures using two additional gap junction blockers, 18 α -glycyrrhetinic acid and halothane. Analyses yielded the results similar to those obtained using carbenoxolone, i.e., the higher levels of Ca^{2+} coupling coefficient in AP5-treated than in control cultures (Fig.4).

Discussion

Taken together, the data indicate strong agreement among results obtained for changes in gap junction coupling measured either by the Ca^{2+} coupling coefficient that we introduce here, or by traditional methods, neuronal dye coupling and Cx36 western blots [1]. For example, in developing rat hypothalamic cultures, the incidence of dye coupling, i.e. the percentage of primary-labeled neurons coupled to one or more secondary-labeled neurons, decreases from 28.6 on DIV16 to 2.5 on DIV32 [1], and the Ca^{2+} coupling coefficient decreases during the same time periods from 30.1 ± 1.4 to 8.8 ± 1.2 (present study). Further, in wild-type mouse hypothalamic cultures during the same time periods, these parameters decrease from 25.0 to 0 and from 22.5 ± 1.5 to 5.9 ± 0.7 , respectively. Similar parallels exist between dye coupling and Ca^{2+} imaging data for different treatments (e.g. NMDA receptor and CREB manipulations) and between values for the Ca^{2+} coupling coefficient (present study) and Cx36 expression [1].

The Ca^{2+} coupling coefficient compares levels of gap junction coupling between neurons at different stages of development or under different experimental conditions in cell cultures by measuring bicuculline-induced intracellular Ca^{2+} activity in the presence and absence of the established gap junction blocker carbenoxolone [9,10]. However, although carbenoxolone is used widely as a blocker of gap junction coupling, it is also known to have other effects on neurons, including inactivation of voltage-gated Ca^{2+} channels and inhibition of chemical synaptic transmission [20,23]. Therefore, concerns for non-specific effects of this agent exist, and results must be controlled and interpreted carefully when carbenoxolone is used. In the present study the specificity of carbenoxolone in terms of acting as a gap junction blocker is indicated by several results and controls. First, we demonstrate that carbenoxolone suppresses bicuculline-induced Ca^{2+} activity in a manner very similar to that of two additional gap junction blockers, 18 α -glycyrrhetinic acid and halothane (Fig.4). Second, the Ca^{2+} coupling coefficient measured using carbenoxolone decreases during neuronal development *in vitro*, and this developmental decrease is prevented or accelerated by the same pharmacological and genetic manipulations that prevent or accelerate developmental decreases in neuronal dye coupling and Cx36 expression [1]. Third, the Ca^{2+} coupling coefficient is very low in cultures lacking neuronal Cx36, as would be predicted by genetic deletion studies indicating that Cx36 is required for most gap junction coupling among neurons [17]. In addition, agreement of the data presented here with dye coupling data obtained using an intracellular injection of the gap junction-permeable tracer neurobiotin [1] also suggests that the Ca^{2+} coupling coefficient reflects changes in neuronal gap junctions and not in gap junction hemichannels, which have also been reported to contribute to the spread of Ca^{2+} waves under certain conditions [14].

Strictly speaking, the Ca^{2+} coupling coefficient quantifies gap junctions in neurons with Ca^{2+} transients that are mediated exclusively, or at least predominantly, by gap junctions. This

is because neurons with Ca^{2+} transients that are mediated by both gap junctions and chemical synapses are likely to continue to score positive for Ca^{2+} transients even after gap junctions are blocked. Such neurons would not contribute to any change in the coefficient. However, validity of the Ca^{2+} coefficient approach for assaying gap junctions is supported by the strong agreement between changes measured using the Ca^{2+} coupling coefficient (Fig.2 and Fig.3) and changes measured using the established dye coupling and Cx36 expression assays [1]. The proximity of the values obtained using all these different techniques suggests that the Ca^{2+} coupling coefficient approach is sufficiently accurate and sensitive to determine the level of neuronal gap junction coupling. However, the advantage of using Ca^{2+} imaging, relative to dye coupling and Cx36 western blotting, is its higher throughput potential: reliable measurements can be obtained for an experimental group or condition during a short period of time (as little as 1 day). This is in comparison to 2–3 weeks for dye coupling tests and to 2–3 days for western blots.

In conclusion, we propose that the Ca^{2+} imaging approach is reliable for analysis of the level of neuronal gap junction coupling in cultures. We also suggest that this approach has high throughput potential for gap junction-related drug screening tests.

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Fig.1.

Ca^{2+} imaging with fura-2 AM in rat hypothalamic neuronal cultures. (**a**, **b**) This figure demonstrates examples of measurements from single neurons in which the bicuculline-induced intracellular Ca^{2+} increases are not suppressed (**a**) and are suppressed (**b**) by carbenoxolone. Applications of bicuculline (Bic, 50 μM) and carbenoxolone (Cbx, 25 μM) are indicated by bars above the recordings. The recordings are made from neurons in DIV16 control (**a**) and AP5-treated (**b**) cultures. Neuron in **b** was tested in the absence of AP5 (i.e., 35 min after AP5 wash out).

Fig.2.

Changes in Ca²⁺ coupling coefficient in hypothalamic neuronal cultures during development and under different experimental conditions. (**a**, **b**) Results represent the data from rat (**a**) and mouse (**b**) cultures. In both rat and wild-type (WT) mouse cultures (**a**, **b**), Ca²⁺ coupling coefficient decreases between DIV16 and DIV32 and the decrease is reduced by chronic inactivation of NMDA receptors with AP5 (100 μ M). The decrease in the Ca²⁺ coupling coefficient does not occur in NMDAR1-deficient cultures (**b**) and the coefficient is low on both DIV16 and DIV32 in Cx36-deficient cultures (**b**). In both graphs, statistical significance was analyzed using ANOVA with post hoc Tukey test; n=3 independent cultures (>100 neurons

per group); data are reported as mean $\pm$ s.e.m. In all groups, 92–98% of the neurons were bicuculline-responsive.

Fig.3.

Changes in Ca^{2+} coupling coefficient in rat hypothalamic cultures during changes in CREB activity. Treatments with CREB ODN (a), HSV-mCREB (b) and HSV-CREB (c) were conducted on the indicated days. Statistical significance was analyzed relative to the corresponding controls using the unpaired two-tail Student's *t*-test; $n=3$ independent cultures (>100 neurons per group; 92–98% were bicuculline-responsive); data are reported as mean $\pm$ s.e.m.

Fig.4.

Ca²⁺ coupling coefficients in rat hypothalamic cultures at DIV32 as determined by two alternate gap junction blockers, 18 α -glycyrrhetic acid (18-GA, 20 μ M) and halothane (5 M). Bicuculline was used to induce Ca²⁺ increases in control and AP5-treated cultures. Statistical significance was analyzed relative to the corresponding controls using ANOVA with post hoc Tukey test; n=3 independent cultures (92–128 neurons per group; >92% were bicuculline-responsive); data are reported as mean $\pm$ s.e.m.