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**Possible involvement of ATP-purinoceptor signalling in the
intercellular synchronization of intracellular Ca^{2+} oscillation
in cultured cardiac myocytes**

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Summary

Isolated and cultured neonatal cardiac myocytes contract spontaneously and cyclically. The contraction rhythms of two isolated cardiac myocytes, each of which beats at different frequencies at first, become synchronized after the establishment of mutual contacts, suggesting that mutual entrainment occurs due to electrical and/or mechanical interactions between two myocytes. The intracellular concentration of free Ca^{2+} also changes rhythmically in association with the rhythmic contraction of myocytes (Ca^{2+} oscillation), and such a Ca^{2+} oscillation was also synchronized among cultured cardiac myocytes. In this study, we investigated whether intercellular communication other than via gap junctions was involved in the intercellular synchronization of intracellular Ca^{2+} oscillation in spontaneously beating cultured cardiac myocytes. Treatment with either blockers of gap junction channels or an un-coupler of E-C coupling did not affect the intercellular synchronization of Ca^{2+} oscillation. In contrast, treatment with a blocker of P2 purinoceptors resulted in the asynchronization of Ca^{2+} oscillatory rhythms among cardiac myocytes. The present study suggested that the extracellular ATP-purinoceptor system was responsible for the intercellular synchronization of Ca^{2+}

oscillation among cardiac myocytes.

Keywords:

cultured cardiac myocytes; calcium oscillation; intercellular synchronization; gap

junctions; purinoceptors

Introduction

Isolated and cultured neonatal cardiac myocytes contract spontaneously and cyclically (Harary and Farley, 1963). The contraction rhythm in the myocytes has the properties of non-linear oscillation, because the rhythm is entrained to the externally applied rhythmic electrical stimulation (Glass et al., 1983). In addition, the contraction rhythms of two isolated cardiac myocytes, each of which beats at different frequencies at first, become synchronized after the establishment of mutual contacts (Jongsma et al., 1987), suggesting that mutual entrainment occurs due to electrical and/or mechanical interactions between two myocytes. The intracellular concentration of free Ca^{2+} also changes rhythmically in association with the rhythmic contraction of myocytes (Ca^{2+} oscillation). Such a Ca^{2+} oscillation was also synchronized among cultured cardiac myocytes (Nakayama et al., 2005b). It is generally believed that gap junctional intercellular communication plays an important role in the intercellular synchronization of intracellular Ca^{2+} oscillation (Kimura et al., 1995). However, our recent preliminary study has revealed that the Ca^{2+} oscillation was synchronized not only among myocytes in an aggregate, but also among cells without apparent physical contact with each other,

suggesting that intercellular communication other than via gap junctions was involved in the synchronization.

Extracellular ATP acts as a potent agonist on a variety of different cell types, including cardiomyocytes (Kunapuli and Daniel, 1998), inducing a broad range of physiological responses. The cellular effects mediated by ATP are determined by the subtypes of P2 purinergic receptors expressed in the particular cell type. In cardiomyocytes, the expression of ionotropic P2X1–P2X7 receptors and metabotropic P2Y1, P2Y2, P2Y4, P2Y6, and P2Y11 receptors have been described (Vassort, 2001). The sensitivity of these receptors toward extracellular ATP is characterized by their EC₅₀-ATP values, which are reported to be in the low micromolar range (Vassort, 2001). The diversity of ATP receptors expressed in cardiomyocytes reflects the variety of ATP effects described for single cells as well as for the whole organ. In the single cardiomyocyte, micromolar levels of extracellular ATP increase plasma membrane permeabilities for cations (Puceat et al., 1991), intracellular calcium transients (Vassort, 2001; Zhang et al., 1996), and the contraction amplitude (Mei and Liang, 2001; Podrasky et al., 1997). In addition, ATP can stimulate phospholipase C (PLC)

(Podrasky et al., 1997). On the organ level, ATP acts as a positive inotropic agent

(Mei and Liang, 2001) and can induce various forms of arrhythmia (Vassort, 2001).

In this study, we investigated whether intercellular communication other than via gap junctions was involved in the intercellular synchronization of intracellular Ca^{2+} oscillation in spontaneously beating cultured cardiac myocytes. Here we suggested that the extracellular ATP-purinoceptor system was responsible for the intercellular synchronization of Ca^{2+} oscillation among cardiac myocytes.

Materials and Methods

Culture of cardiac myocytes

The method of culture was described elsewhere in detail (Kawahara et al., 2002; 2006; Kohashi et al., 2003; Yamauchi et al., 2002). In short, cardiac myocytes were prepared from ventricles of 1 to 3-day old neonatal Wistar rats removed after decapitation. The ventricles were rinsed in a 25 mM HEPES-buffered minimum salt solution (MSS) to remove contaminating blood cell components and then minced with scissors into fragments to be digested with 0.1% collagenase (Wako Chemical, Tokyo, Japan) in MSS at 37 °C for 10 min. The digested fragments were centrifuged at 1000 rpm for 2 min (LC-100, TOMY, Japan) and precipitated cell components were washed twice with MSS to terminate the effects of collagenase. The cell components were suspended in MCDB 107 (Research Institute for Functional Peptides, Yamagata, Japan) containing 5% FCS (MBL, Nagoya, Japan), and then passed through a wire mesh screen (90 µm porosity) to remove large aggregates of cells; the filtered suspension contains cardiac myocytes and fibroblasts. To separate cardiac myocytes from fibroblasts based on the selective adhesion technique, the cell suspension was poured into petri dishes (φ

60 mm, Falcon), and incubated for 60 min at 37 °C, in 5% CO₂ and 95% air. By virtue of the procedure, most of the fibroblasts adhere to the dish. After the incubation, the suspension, mostly containing cardiac myocytes, was collected. The suspension was centrifuged at 700 rpm for 5 min to separate the remaining blood cell components in the supernatant. The precipitated cells were resuspended in MCDB 107 containing 5% FCS, transferrin (10 µg/ml, Sigma, St. Louis, MO), and insulin (10 µg/ml, Yamanouchi, Tokyo, Japan). The cell suspension was passed through a fine wire mesh screen (25 µm porosity) to remove remaining small aggregates of myocytes, and finally the isolated myocytes remaining were cultured at a density of about 3.0×10⁵ cells/ml in a petri dish (ϕ30 mm, Falcon) coated beforehand with fibronectin (10 µg/ml, Sigma).

Usually, cultured cardiac myocytes begin to contract spontaneously and rhythmically at around 2 DIV (2 days in vitro). At that time, the cells are not fully matured and the Ca²⁺ oscillatory rhythm of the cells was markedly irregular and not perfectly synchronized with each other. In the present study, Ca²⁺ oscillations were recorded in cells cultured for 4 days (4 DIV), because the oscillatory rhythm became relatively stable at that time. The Ca²⁺ oscillation in almost all the cultured cells at 4

DIV was synchronized with each other.

Image analysis

In this study, the rhythm of spontaneous contraction of cultured myocytes was evaluated using a video image recording method. This procedure is described elsewhere in detail (Kawahara et al., 2002; Yoneyama and Kawahara, 2004). In short, images of beating myocytes were recorded with a CCD camera (WV-BD400, Panasonic, Japan) through a phase-contrast microscope (IX70, OLYMPUS, Japan). A spontaneously beating myocyte was arbitrarily selected from myocytes in the video image. A small area (a square of about 20 pixels) of the myocyte where brightness changed considerably with contraction was selected, and the video signals were digitized to an 8-bit number every video frame (30 frames/s) by a video capture board in a personal computer (Power Macintosh 7500/100, Apple). A reference frame was arbitrarily selected and cross-correlograms were calculated between pixels of the reference frame and those of other frames, to represent the temporal variation of brightness in the selected area corresponding to the contraction rhythm of the cardiac myocyte.

Cellular Ca²⁺ measurements

Changes in the cytosolic concentration of free Ca²⁺ were measured using fluo 4. Cardiac myocytes in culture were loaded with the fluorescent calcium indicator during a 30 min incubation with acetoxymethyl ester of fluo 4 (fluo 4/AM, 5 μM; Molecular probes, Eugene, OR) in MCDB medium at room temperature. Fluo 4 was excited at 490 nm, and emission intensity was measured at 525 nm. Fluorescent images were acquired at about 200 msec intervals with a cooled CCD camera (C4880-80; Hamamatsu Photonics, Hamamatsu, Japan). An analysis of the acquired images was done with an image processing and measuring system (AQUACOSMOS; Hamamatsu Photonics). Fluorescent intensity (F) was normalized with the initial value (F₀), and the changes in the relative fluorescent intensity (F/F₀ - 1) were used to assess those in cellular free Ca²⁺.

Immunocytochemistry

The cardiac myocytes were fixed with 4% paraformaldehyde at room temperature. The cells were then incubated with a primary anti-Cx 43 antibody (Sigma) overnight at 4 °C using a dilution of 1:6000. After being washed with phosphate-buffered saline

(PBS), the cells were incubated with a secondary anti-rabbit IgG antibody (Vector Laboratories, Burlingame, CA). Bound antibodies were detected by the avidin-biotin-peroxidase complex (ABC) method using a commercial ABC kit (Vector Laboratories). Observation of the peroxidase activity was made possible by incubation with 0.1% 3,3'-diaminobenzidine tetrahydrochloride (DAB) in a 50 mM Tris-HCl buffer (pH 7.4) supplemented with 0.02% H₂O₂.

Statistics

The data are expressed as the mean±S.D. Comparisons were performed using the one-way analysis of variance (ANOVA) followed by a paired t-Test. A *P* value of less than either 0.01 or 0.05 was considered statistically significant.

Results

Isolated and cultured neonatal cardiac myocytes started to contract spontaneously and cyclically usually after 2 to 4 days *in vitro* (2-4 DIV). The intracellular concentration of free Ca^{2+} also changed cyclically in association with the spontaneous rhythmic contraction of cardiac myocytes (Nakayama et al., 2005b). We first investigated whether the spontaneous cyclic changes in the concentration of free Ca^{2+} (Ca^{2+} oscillation) in cultured cardiac myocytes were synchronized among myocytes using a fluorescent Ca^{2+} indicator, fluo 4/AM (Fig. 1). The relative intensity of fluo 4 fluorescence in three cells (Fig. 1A1 and 1A2) fluctuated cyclically and the Ca^{2+} oscillation was synchronized among myocytes (Fig. 1B). In almost all of the cultures tested, the Ca^{2+} oscillation in cardiac myocytes was synchronized among cultured cells at 4 DIV. To further confirm the existence of intercellular synchronization among cardiac myocytes, a cross-correlation analysis was conducted (Fig. 1C). The correlation analysis also revealed that cyclic changes in the intracellular concentration of Ca^{2+} in cardiac myocytes were almost synchronized with each other. It should be noted that the intercellular synchronization of Ca^{2+} oscillation was observed not only

among myocytes within an aggregate (cell 2 and cell 3), but also between cells without apparent physical contact with each other (cell 1 and cell 2 or cell 3). This finding seems very interesting, since it has been generally believed that the intercellular communication among cardiac myocytes is mainly attained via gap junctional intercellular communication. That the cultured cardiac myocytes without apparent physical contact with each other showed synchronized intracellular Ca^{2+} oscillation suggested the existence of additional mechanisms other than gap junctional intercellular communication. In fact, an immunocytochemical analysis using an anti-Cx 43 antibody suggested that the gap junctional communication among cells was not fully developed in cultured cardiac myocytes at least at 4 DIV (Fig. 1D). A dye transfer analysis by microinjecting the lucifer yellow dye into a single myocyte also supported our inference that gap junction channels were not fully developed in cultures at 4 DIV (Nakayama et al., 2005a). Thus, we next investigated whether treatment of cultured cardiac myocytes at 4 DIV with blockers of gap junction channels resulted in the asynchronization of the intracellular Ca^{2+} oscillation among myocytes.

Cultured cardiac myocytes were treated with 12-doxyl stearic acid (DSA, 200 μM), a reversible blocker of gap junction channels (Burt, 1989), for 20 min (Fig. 2). A higher concentration of DSA, more than 200 μM , occasionally terminated the contractile activity of the myocytes (data not shown). Treatment at 4 DIV with DSA did not affect the intercellular synchronization of intracellular Ca^{2+} oscillation among cardiac myocytes (Fig. 2B). To confirm this, a cross-correlation analysis was conducted. The analysis also demonstrated that the intercellular synchronization of intracellular Ca^{2+} oscillation among cardiac myocytes persisted even after the gap junctional communication was blocked with DSA (Fig. 2C and D). Treatment of cultured cardiac myocytes with this concentration of DSA results in asynchronization of beating rhythms among cardiac myocytes when gap junction channels are functional (Kawahara et al., 2002). These results suggested that gap junction channels in cultured cardiac myocytes at 4 DIV were not fully functional; that is, the channels did not primarily contribute to the intercellular communication among cardiac myocytes. In addition, treatment with heptanol (7 mM), a blocker of gap junction channels, for 5 min

did not significantly affect the intercellular synchronization of intracellular Ca^{2+} oscillation among cardiac myocytes either (Fig. 2D).

Previous studies have demonstrated that cardiac myocytes have mechano-sensitive ion channels such as stretch-activated channels (Kawakubo et al., 1999; Zeng et al., 2000), indicating the possibility that cyclic mechanical movements associated with the rhythmic contraction of myocytes would affect the intercellular synchronization of the intracellular concentration of Ca^{2+} . Therefore, we then investigated whether the intercellular synchronization of Ca^{2+} oscillation in cardiac myocytes changed when the contractile activity was suppressed by treatment with 2,3-butanedione monoxime (BDM), a reversible blocker of cardiac contraction (Cheng et al., 1997; Gwathmey et al., 1991; Kurihara et al., 1990). Loading of cultured cardiac myocytes with 7.5 mM BDM for 10-20 min resulted in the termination of spontaneous rhythmic contraction (Fig. 3B2), but Ca^{2+} oscillation was still observed and the treatment did not affect the intercellular synchronization of Ca^{2+} oscillation at all (Fig. 3C2). The cross-correlation analysis also demonstrated that the intercellular synchronization of intracellular Ca^{2+} oscillation among cardiac myocytes persisted even

after the termination of contractile activity with BDM (Fig. 3D and E), suggesting that mechanical factors associated with rhythmic contraction did not affect the intercellular synchronization of intracellular Ca^{2+} oscillation. Treatment with this concentration of BDM terminated the rhythmic contraction of almost all the myocytes observed.

All the above results have demonstrated the possibility that some form of extracellular signaling other than gap junctional communication was involved in the intercellular synchronization of intracellular Ca^{2+} oscillation. Although intercellular gap junction channels generally provide the main pathway for the intercellular communication among cardiac myocytes, a paracrine route, mediated by the diffusion of an extracellular messenger such as ATP, might also operate in parallel with the direct cytosol-to-cytosol coupling via gap junction channels (Suadicani et al., 2000).

Therefore, we then investigated whether and how the extracellular ATP-purinoceptor system was involved in the intercellular synchronization of intracellular Ca^{2+} oscillation among cultured cardiac myocytes.

Cultured cardiac myocytes were treated with suramin, a blocker of P2 purinoceptors (Scemes et al., 2000), for 20 min. The concentration of suramin was adjusted to 100 μM . This concentration was high enough to block P2 purinoceptors, leading to a marked reduction in the cell's responses to ATP (Scemes et al., 2000). A higher concentration of suramin, more than 100 μM , occasionally terminated the contractile activity of cardiac myocytes (data not shown). Exposure of cultured cardiac myocytes at 4 DIV to suramin (100 μM) resulted in the asynchronization of Ca^{2+} oscillation between myocytes without physical contact with each other (Fig. 4B2). The cross-correlation analysis between cells also demonstrated that suramin treatment significantly decreased the cross-correlation coefficient between Ca^{2+} oscillations in cardiac myocytes (Fig. 4C2 and D), suggesting that the extracellular ATP-purinoceptor system was involved in the intercellular synchronization of intracellular Ca^{2+} oscillation in cultured cardiac myocytes.

Discussion

The present study suggested that the ATP-purinoreceptor signaling pathway was involved in the intercellular synchronization of intracellular Ca^{2+} oscillation in cultured cardiac myocytes.

This study demonstrated that cyclic changes in the concentration of intracellular Ca^{2+} (Ca^{2+} oscillation) were almost synchronized with each other among cultured cardiac myocytes (Fig. 1). It should be noted that the intercellular synchronization of intracellular Ca^{2+} oscillation was observed not only among myocytes within an aggregate, but also between cells without physical contact with each other. This finding seems very interesting, since it has been generally believed that the intercellular communication among cardiac myocytes is mainly attained via gap junctional intercellular communication. The fact that the mutually-separated cultured cardiac myocytes showed synchronized intracellular Ca^{2+} oscillation indicated a possible existence of additional mechanisms other than gap junctional intercellular

communication responsible for the synchronization of Ca^{2+} oscillation among cardiac myocytes.

Each gap junction channel is formed through the docking of two hemichannels (connexons) located in the membranes of two contacting myocytes (van Veen et al., 2001). Each connexon is formed by six transmembrane monomeric proteins known as connexins (Cx) (Garcia-Dorado and Ruiz-Meana, 2000). The expressions of four different Cx (Cx 43, Cx 40, Cx 45, and Cx 37) have been identified in the adult mammalian myocardium (Garcia-Dorado and Ruiz-Meana, 2000). In ventricular myocytes, gap junction channels are mainly formed by Cx43 (Dhein et al., 2000). Previous immunohistochemical studies have revealed that cultured rat neonatal cardiac myocytes retain typical features of the native rat ventricular myocardium (Darrow et al., 1995; Kwak et al., 1999). The present immunocytochemical analysis using an anti-Cx 43 antibody has revealed a possibility that gap junction channels were not fully developed in cultured cardiac myocytes at 4 DIV (Fig. 1D1). Taken altogether, the experimental findings that the intercellular synchronization of Ca^{2+} oscillation among cardiac myocytes persisted even after blocking gap junction channels (Fig. 2) have

raised a possibility that the intercellular communication via gap junction channels did not primarily contribute to the intercellular synchronization of Ca^{2+} oscillation at least in cardiac myocytes at 4 DIV.

Exogenously applied ATP evoked an increase in the concentration of intracellular Ca^{2+} in astrocytes (Bruner and Murphy, 1993) and in cardiac myocytes (Vassort, 2001; Zhang et al., 1996). Studies on the potency of ATP analogs have suggested that the increase in the concentration of intracellular Ca^{2+} in astrocytes is due to activation of the P2Y subtype (Kastritsis et al., 1992; Salter and Hicks, 1994). ATP-evoked Ca^{2+} responses could play important roles in physiological or pathological processes within the central nervous system. Increases in the concentration of intracellular Ca^{2+} produced by ATP have been correlated with increases in inositol phospholipids turnover and with inositol 1,4,5 trisphosphate (IP3) itself (Kastritsis et al., 1992; Pearse et al., 1989). This correlation has led to a speculation that IP3 may be a mediator of Ca^{2+} release from the intracellular Ca^{2+} store (sarcoplasmic reticulum, SR) produced by ATP, although a causal relationship has not been established. Evidence indicates that P2Y receptors may be coupled to additional signal transduction pathways that might affect

intracellular Ca^{2+} secondarily (Bruner and Murphy, 1993). In addition, a previous study has revealed that activation of P2 purinergic receptors raises the concentration of intracellular Ca^{2+} via the phospholipase C /IP3 pathway (Podrasky et al., 1997), and this pathway possibly contributes to the intercellular synchronization of Ca^{2+} oscillation in cultured cardiac myocytes.

In conclusion, the present study demonstrated a possibility that the ATP-purinoceptor signaling pathway was involved in the intercellular synchronization of intracellular Ca^{2+} oscillation. The involvement of this extracellular signaling system in the intercellular synchronization of Ca^{2+} oscillation in myocytes seems dependent on the period of the culture. Treatment of cardiac myocytes cultured for more than 5 days (>5 DIV) with suramin did not produce an asynchronization of Ca^{2+} oscillation (data not shown), probably because of the full development of intercellular communication via gap junction channels. However, the extracellular ATP-purinoceptor system, which once disappears as myocytes mature, would become functional again and play an important role in the coordination of oscillatory rhythm among cardiac myocytes when the intercellular communication via gap junction channels ceases under conditions such

as cardiac ischemia.

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Figure captions

Fig. 1

Intercellular synchronization of intracellular Ca^{2+} oscillation in cardiac myocytes in culture. The Ca^{2+} oscillation in cultured neonatal cardiac myocytes at 4 days *in vitro* (4 DIV) indicated by arrows in the phase-contrast image of the culture (A1) and in the fluorescence image (A2) was synchronized among myocytes (B). Cyclic changes in the intracellular concentration of free Ca^{2+} were measured by loading myocytes with the fluorescent Ca^{2+} probe fluo 4/AM (5 μM). The scale bar indicates 100 μm , and applies to both A1 and A2. Cross-correlation analysis of intracellular Ca^{2+} oscillation between cell 1 and cell 2 (C1), cell 2 and cell 3 (C2), and cell 1 and cell 3 (C3) also revealed the intercellular synchronization of intracellular Ca^{2+} oscillation among cardiac myocytes. An immunocytochemical analysis on 4 and 7 DIV cultures using an anti-Cx43 antibody to identify the expression and distribution of gap junction proteins in cultured cardiac myocytes revealed that Cx 43 proteins were distributed in clusters in cardiac myocytes at 4 DIV (D1), but myocytes at 7 DIV had a dot-like Cx43 distribution linking adjacent cells (D2), suggesting that gap junctional coupling among

myocytes was not fully developed in cardiac myocytes at 4 DIV. The scale bar indicates 50 μm , and applies to both D1 and D2.

Fig. 2

Changes in the intercellular synchronization of Ca^{2+} oscillation on the blocking of gap junction channels. Treatment of cardiac myocytes at 4 DIV with DSA (200 μM) for 20 min did not affect the intercellular synchronization of Ca^{2+} oscillation among cultured cardiac myocytes at 4DIV. Cross-correlograms between intracellular Ca^{2+} oscillations among cardiac myocytes are shown in C1-C3. Treatment of cultured cardiac myocytes with DSA (200 μM) for 20 min did not produce marked changes in the amplitude of the cross-correlograms, suggesting that the intercellular communication among cells via gap junction channels did not significantly contribute to the intercellular synchronization of Ca^{2+} oscillation. Treatment with heptanol (7 mM) for 5 min did not affect the intercellular synchronization either (D). Figure D shows a statistical comparison of the mean of the peak-to-peak amplitude of the

cross-correlation coefficient when the cultures were treated with heptanol. The amplitude is normalized with that of the control. Data are expressed as the mean + SD (n=4 different cultures). The scale bar indicates 100 μm , and applies to both A1 and A2.

Fig. 3

Persistence of intercellular synchronization of intracellular Ca^{2+} oscillation in quiescent cultured cardiac myocytes treated with BDM. The spontaneous rhythmic contraction of a cultured cardiac myocyte (cell 2 in A1) was almost completely terminated by the loading of cells with 7.5 mM BDM for 20 min (B2). After the washout of BDM, the rhythmic contraction gradually recovered (B3). The cyclic changes in the concentration of intracellular Ca^{2+} in two cardiac myocytes indicated by arrows in the phase-contrast image (A1) and in the fluo 4-fluorescent image (A2) were synchronized before the onset of treatment with BDM (C1), during the treatment (C2), and 20 min after the washing out (C3). Cross-correlograms between intracellular Ca^{2+}

oscillations among cardiac myocytes are shown in D1-D3. Treatment of cultured cardiac myocytes with BDM did not produce marked changes in the amplitude of the cross-correlograms, suggesting that cyclic mechanical movements did not significantly contribute to the intercellular synchronization of Ca^{2+} oscillation. Figure E shows a statistical comparison of the mean of the peak-to-peak amplitude of the cross-correlation coefficient. The amplitude is normalized with that of the control. Data are expressed as the mean + SD (n=4 different cultures). The scale bar indicates 100 μm , and applies to both A1 and A2.

Fig. 4

ATP-purinoreceptor system involved in the intercellular synchronization of intracellular Ca^{2+} oscillation in cultured cardiac myocytes. Treatment of cultured cardiac myocytes with suramin (100 μM), a blocker of P2 purinoreceptors, for 20 min resulted in the asynchronization of Ca^{2+} oscillation among cardiac myocytes (B2). Changes in the cross-correlograms between intracellular Ca^{2+} oscillations in cardiac

myocytes by treatment with suramin are shown in C1-C3. Treatment with suramin resulted in a decrease in the peak-to-peak amplitude of the cross-correlograms (C2), suggesting that the ATP-purinoceptor signaling system was responsible for the intercellular synchronization of Ca^{2+} oscillation. Figure D shows a statistical comparison of the mean of the peak-to-peak amplitude of the cross-correlation coefficient. The amplitude is normalized with that of the control. Data are expressed as the mean + SD (n=4 different cultures). * $p < 0.05$. The scale bar indicates 100 μm , and applies to both A1 and A2.







