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Slow intercellular Ca^{2+} signaling in wild-type and Cx43-null neonatal mouse cardiac myocytes

SYLVIA O. SUADICANI,[1,3] MONIQUE J. VINK,[1] AND DAVID C. SPRAY[1,2]
[1]*Department of Neuroscience,* [2]*Division of Cardiology, Department of Medicine, Albert Einstein College of Medicine, Bronx, New York 10461; and*
[3]*University São Judas Tadeu, São Paulo 03166, Brazil*

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Suadicani, Sylvia O., Monique J. Vink, and David C. Spray. Slow intercellular Ca^{2+} signaling in wild-type and Cx43-null neonatal mouse cardiac myocytes. *Am J Physiol Heart Circ Physiol* 279: H3076–H3088, 2000.—Focal mechanical stimulation of single neonatal mouse cardiac myocytes in culture induced intercellular Ca^{2+} waves that propagated with mean velocities of ~14 μm/s, reaching ~80% of the cells in the field. Deletion of connexin43 (Cx43), the main cardiac gap junction channel protein, did not prevent communication of mechanically induced Ca^{2+} waves, although the velocity and number of cells communicated by the Ca^{2+} signal were significantly reduced. Similar effects were observed in wild-type cardiac myocytes treated with heptanol, a gap junction channel blocker. Fewer cells were involved in intercellular Ca^{2+} signaling in both wild-type and Cx43-null cultures in the presence of suramin, a P_2-receptor blocker; blockage was more effective in Cx43-null than in wild-type cells. Thus gap junction channels provide the main pathway for communication of slow intercellular Ca^{2+} signals in wild-type neonatal mouse cardiac myocytes. Activation of P_2-receptors induced by ATP release contributes a secondary, extracellular pathway for transmission of Ca^{2+} signals. The importance of such ATP-mediated Ca^{2+} signaling would be expected to be enhanced under ischemic conditions, when release of ATP is increased and gap junction channels conductance is significantly reduced.

calcium waves; connexin; gap junctions; purinergic receptors; intercellular communication

A GREAT VARIETY OF CELL TYPES, including cardiac myocytes, can communicate between each other and even with other cell types by means of an increase in intracellular Ca^{2+} levels, which propagate from cell to cell as slow intercellular Ca^{2+} waves (44, 47). Low-frequency spontaneous intercellular Ca^{2+} waves, distinct from the electrically mediated and rapidly transmitted waves of contraction that generate cardiac output, have been observed propagating on the ventricular surface of the normal working or arrested myocardium (32), in the multicellular ventricular trabeculae preparation (30, 33–34), between pairs of isolated adult cardiac myocytes (31, 54), and between neonatal cardiac myocytes in culture (52).

Although little is known about the functional importance of slow intercellular Ca^{2+} signaling, it is currently believed that the information transmitted by this signal is essential to attain the coordination required by certain cooperative cellular activities (1, 44). In the case of cardiac tissue, however, the physiological relevance of this form of slow intercellular Ca^{2+} signaling would be more difficult to foresee in the normal working myocardium, because the signal would presumably be obscured by the fast waves associated with action potential propagation and excitation-contraction coupling, and the duration of sustained Ca^{2+} elevations would be limited by requisite Ca^{2+} buffering and removal. It has been demonstrated, however, that the frequency of these spontaneous waves can be increased by abnormal elevation of extracellular Ca^{2+}, cellular damage (30, 32), and rapid stretch/release of cardiac tissue (30), events that when combined can lead to damage-induced cardiac arrhythmias (reviewed in Ref. 57). Such triggered arrhythmias have been extensively studied in ventricular trabeculae (11–13, 36, 56). These studies have shown that stretch and release of damaged cardiac tissue during regular twitches induce aftercontractions, which arise in the damaged region and propagate to the neighboring healthy myocardium as slow waves of contraction accompanied by nearly synchronous delayed afterdepolarizations. Such depolarizations may reach threshold for generation of action potentials and, in doing so, may induce triggered twitches and arrhythmias. These aftercontractions seem to result from a chain reaction of Ca^{2+}-induced Ca^{2+} release initiated in the damaged regions by stretch/release-mediated dissociation of Ca^{2+} from the myofilaments followed by Ca^{2+} release from the overloaded sarcoplasmic reticulum (SR) (13, 56). It has been assumed that the Ca^{2+} transient generated by these events is transmitted to the adjacent cells by a combination of Ca^{2+} diffusion and Ca^{2+}-induced Ca^{2+} release, propagating into the healthy regions as intercellular Ca^{2+} waves (2, 13, 36, 56). The transmission of this intercellular Ca^{2+} signal does not require an intact sarcolemma, but it does depend on

Address for reprint requests and other correspondence: D. C. Spray, Albert Einstein College of Medicine, Kennedy Center, Rm. 712, 1410 Pelham Parkway S., Bronx, NY 10461 (E-mail: spray@aecom.yu.edu).

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the functional presence of gap junction channels (11, 33, 63).

Intercellular gap junction channels generally provide the main pathway for the intercellular communication of Ca^{2+} signals in the majority of cell types in which this phenomenon has been described (44, 47). A paracrine route, mediated by the diffusion of an extracellular messenger (such as ATP, other adenosine nucleotides, glutamate, and other neurotransmitters or hormones), can also operate in parallel with the direct cytosol-to-cytosol transmission mediated by gap junction channels. In some cell types or under certain conditions, this extracellular route may be the primary pathway involved in the transmission of the Ca^{2+} waves (47).

Cardiac myocytes provide a cell type in which gap junction channels would be expected to be the primary route for such intercellular Ca^{2+} signaling. Not only is junctional conductance very high in cardiac tissue (6, 51, 53), but the major gap junction protein in cardiac myocytes is connexin43 (Cx43), which forms channels that are permeable to intracellular second messengers such as Ca^{2+}, inositol (1,4,5)-trisphosphate, cAMP, ATP, ADP, and cGMP (4, 43). Nevertheless, the degree of participation of paracrine routes in the communication of slow intercellular Ca^{2+} signals between cardiac myocytes has not been evaluated previously.

In this study, we have investigated the phenomenon and pathways involved in the communication of intercellular Ca^{2+} signals in cultured neonatal cardiac myocytes using focal mechanical stimulation and real-time confocal microscopy to initiate and record the propagation of intercellular Ca^{2+} signals. Through comparisons of myocytes obtained from mice lacking Cx43 and by pharmacological manipulations, we show that the transmission of the slow Ca^{2+} signal between cardiac myocyte cultures is mainly provided by gap junction channels, although it is significantly modulated by ATP-mediated activation of P_2 receptors. The involvement of this paracrine pathway is expected to be enhanced and assumed to be of pathophysiological importance under abnormal conditions, such as those imposed by ischemia or cardiac damage, when the release of ATP is increased (17) and junctional conductance is diminished (14, 15, 29, 38, 60–62).

MATERIALS AND METHODS

Dissociation of Neonatal Hearts and Preparation of Primary Cultures of Cardiac Myocytes

Wild-type cultures. Neonatal mice (C57BL/6NCr1BR, Charles River Laboratories, Wilmington, MA) were killed by decapitation, and the hearts were isolated and placed in 60-mm plastic culture dishes containing sterile ice-cold Dulbecco's phosphate-buffered saline (PBS; GIBCO-BRL, Grand Island, NY). After rinsing with PBS to remove the blood, we throughly minced the hearts in the dissociation solution [containing 1.25% pancreatin (GIBCO-BRL) and 300 mg of bovine serum albumin (BSA; Sigma, St. Louis, MO) diluted in (in g/100 ml) 8.0 NaCl, 0.2 KCl, 0.05 Na_2HPO_4, 1.0 $NaHCO_3$, and 2.0 dextrose; pH 7.1–7.2]. The homogenate was then transferred to a 25-ml Ehrlenmeyer flask with 7 ml of the dissociation solution and placed in a water bath (37°C) for 10 min under continuous stirring. The supernatant was collected in a conical 15-ml tube and spun at 1,500 *g* for 4 min, and the pellet was ressuspended in 3 ml of Dulbecco's modified Eagle's medium (DMEM) [containing 10% fetal bovine serum (GIBCO-BRL) and 1% penicillin/streptomycin (GIBCO-BRL)]. The tube with the dissociated cells was then placed in the incubator (36–37°C, 5% CO_2). This procedure was repeated five to seven times or until the heart tissue was totally dissociated. The cells were pooled and preplated in 100-mm plastic culture dishes, to which fibroblasts adhered, for 1 h. The nonadhered cells were plated onto confocal imaging dishes (Glass Bottom Microwells Uncoated Dishes, MatTek), placed in the incubator, and allowed to settle for 24 h. After this period, we washed the dishes with DMEM to remove the nonadherent cells and fed the cells with 2 ml of DMEM supplemented with cytosine β-D-arabino-furanoside (12.2 mg/50 ml media; Sigma) to inhibit fibroblast growth.

Cx43-null cultures. In the case of Cx43-null mice, which die shortly after birth (42), the litters bred from Cx43 heterozygous mice (C57BL/6J-Gja1^{tm1Kdr}, Jackson Laboratories) were used immediately after birth with the same procedure used for the wild-type animals except that the cardiac myocyte cultures were prepared individually from each of the siblings, which were subsequently genotyped from tail DNA as described previously (16).

Calcium Imaging and Data Analysis

The experiments were performed within 3–5 days after plating the cells. The neonatal mouse heart cells plated on confocal imaging dishes were incubated for 45 min at 37°C with 10 μM of the ratiometric Ca^{2+} indicator indo 1-acetoxymethyl ester (indo 1-AM; Molecular Probes, Eugene, OR) and rinsed three times with DMEM, which was replaced by Tyrode solution [(in mM) 137.0 NaCl, 2.7 KCl, 0.5 $MgCl_2$, 1.8 $CaCl_2$, 12.0 $NaHCO_3$, 0.5 NaH_2PO_4, 5.5 glucose, and 5 HEPES; pH 7.1–7.2] shortly before the experiments. The experiments were conducted at room temperature (21°C) and, in some cases (as specified), also at 34°C. The ratio of indo 1-AM fluorescence intensity emitted at two wavelengths (390–440 nm and >440 nm) was imaged using ultraviolet laser excitation at 351 nm. Ratio images were continuously acquired at 1 Hz after background and shading correction using a Nikon real-time confocal microscope (RCM 8000) with a large ultraviolet pinhole and a Nikon ×40 water immersion objective (numerical aperture 1.15; working distance 0.2 mm). The intercellular Ca^{2+} waves were induced by focal mechanical stimulation of one myocyte with a glass pipette (outer diameter 1–2 μm). Ratiometric images were saved on the optical disk recorder (OMDR) as the average of 32 frames. The images were further analyzed for measurements of changes in calcium level during playback using Polygon-Star software (Nikon), which averages the gray levels (number of pixels/area) within the regions of interest (circular spots placed on each cell) as a function of elapsed time. The data generated by this software were plotted in graphs as the indo 1-AM fluorescence ratio values versus time (in s) using Microsoft ORIGIN software. The phenomenon of intercellular Ca^{2+} signaling was analyzed in terms of velocity, amplitude, and efficacy of the Ca^{2+} wave propagation. The velocities of Ca^{2+} wave propagation between cardiac myocytes were calculated as the distance (in μm) between the stimulated and all nonstimulated cells present in the confocal field divided by the time interval (in s) between the half-maximal calcium increases within the stimulated and responding cells. The distances between one stimulated

cell and another cell in the field were calculated from the micrographs of the fields (see Fig. 2) as the length of a straight line connecting the centers of the regions of interest. Half-maximal calcium increases were obtained from sigmoidal curves fitted to the ascending phases of the indo 1-AM fluorescence ratio increases using ORIGIN software (Fig. 1). Amplitudes of Ca^{2+} waves were considered to be the maximal increments in intracellular calcium observed in responding cells, calculated for each cell as the value of the indo 1-AM fluorescence ratio rise at the peak of the response divided by the basal fluorescence ratio value acquired before the induction of the calcium waves. The efficacy of the Ca^{2+} signal communication is reported here as the number of cells responding with a detectable increase in intracellular calcium [>10% over basal; calibration with Ca^{2+} standards (see Ref. 48) indicates that this corresponds to an approximate doubling of intracellular Ca^{2+} concentration] during the propagation of the wave in relation to the total number of cells within the field. To compare the combined contribution of these three parameters in the communication of the Ca^{2+} signal under different conditions, we used the EVA factor, defined as the product of the relative values (experimental/control) obtained for the efficacy, velocity, and amplitude of the calcium waves (see Ref. 48).

Other Chemicals

Heptanol, ATP sodium salt, suramin (sodium salt), and apyrase (from potato, E.C., 3.6.1.5-Grade III) were purchased from Sigma Chemical.

Statistical Analysis

All values are expressed as means ± SE. Jandel Scientific SigmaStat software was used for the statistic analysis. The data sets were compared using ANOVA. The differences between the groups were evaluated using either the Tukey test or Dunn's method and considered different when $P < 0.05$.

RESULTS

Characteristics of Mechanically Induced Intercellular Ca^{2+} Signaling Between Wild-Type Cardiac Myocytes

Focal mechanical stimulation of single quiescent or spontaneously beating cardiac myocytes in culture induced a steep and sustained rise in the intracellular Ca^{2+} level of the stimulated cell (Fig. 2A, *cell A*, 0 s, and Fig. 3*A*). The mechanically induced increase in intracellular Ca^{2+} corresponded to indo 1-AM fluorescence ratio values that were twice those measured at nonstimulated basal conditions (2.12 ± 0.08-fold, $N = 36$) and were significantly higher than the systolic-diastolic variations in fluorescence observed during regular beating (1.36 ± 0.03-fold, $N = 22$). A very fast increase in the Ca^{2+} level of the neighboring myocytes immediately followed this initial response, spreading to all myocytes in direct or indirect contact with the mechanically stimulated cell and traveling with velocities much higher than 10 mm/s, too fast to be precisely quantified under our experimental conditions. However, after a brief delay (2.7 ± 0.2 s, $N = 40$, 2-s and 3-s photos), a second much slower intercellular Ca^{2+} wave (velocity 14.29 ± 1.16 μm/s, $N = 103$; Figs. 2*A* and 3*A*) followed the faster one, with the Ca^{2+} signal ultimately

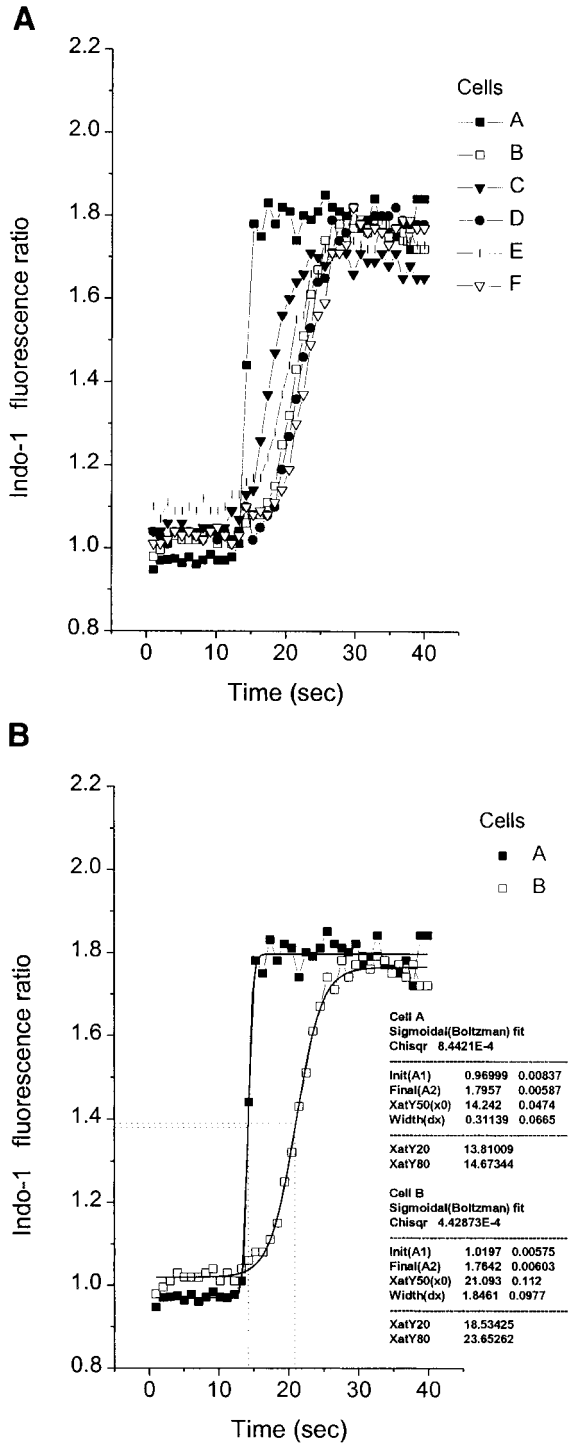

Fig. 1. Measurements of efficacy, velocity, and amplitude of slow Ca^{2+} wave spread. *A*: graphical representation of the changes in indo 1-acetoxymethyl ester (Indo 1-AM) fluorescence ratio as a function of time in 8 cardiac myocytes induced by focal mechanical stimulation of one of the cells (*cell A*). *B*: representative sigmoidal curve fittings for the stimulated cell (*cell A*) and for only one of the responding cells (*cell B*). The velocity of the intercellular Ca^{2+} signal propagation can be calculated from distance between the cells (in micrometers) divided by the time interval (in seconds) between the half-maximal increases in fluorescence (dashed lines, *XatY50*). For more detailed information, see text.

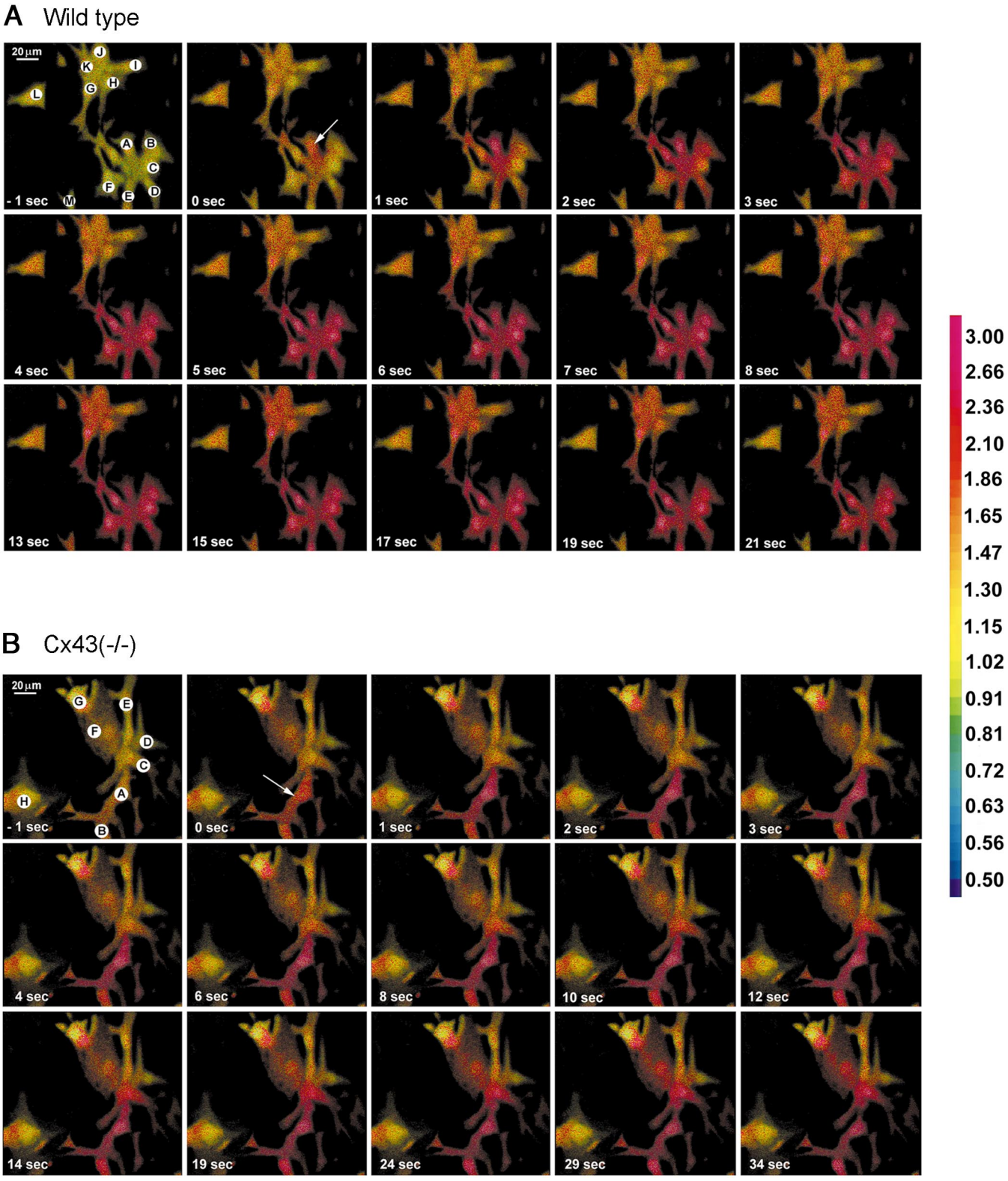

Fig. 2. Time-lapsed pseudocolored display of mechanically induced intercellular Ca^{2+} wave propagation between wild-type [connexin43 (Cx43) (+/+) mice; *A*] and Cx43-null [Cx43 (−/−) mice; *B*] neonatal mouse cardiac myocytes in culture. The cardiac myocytes were loaded with indo 1-AM and imaged with real-time confocal microscopy. Mechanical stimulation of a single myocyte (*cell A*, arrow) induces a steep increase in the intracellular Ca^{2+} level of the stimulated cell and triggers the propagation of the Ca^{2+} signal to the neighboring myocytes. The graphical representations of the phenomenon as a function of time are shown in Fig. 3.

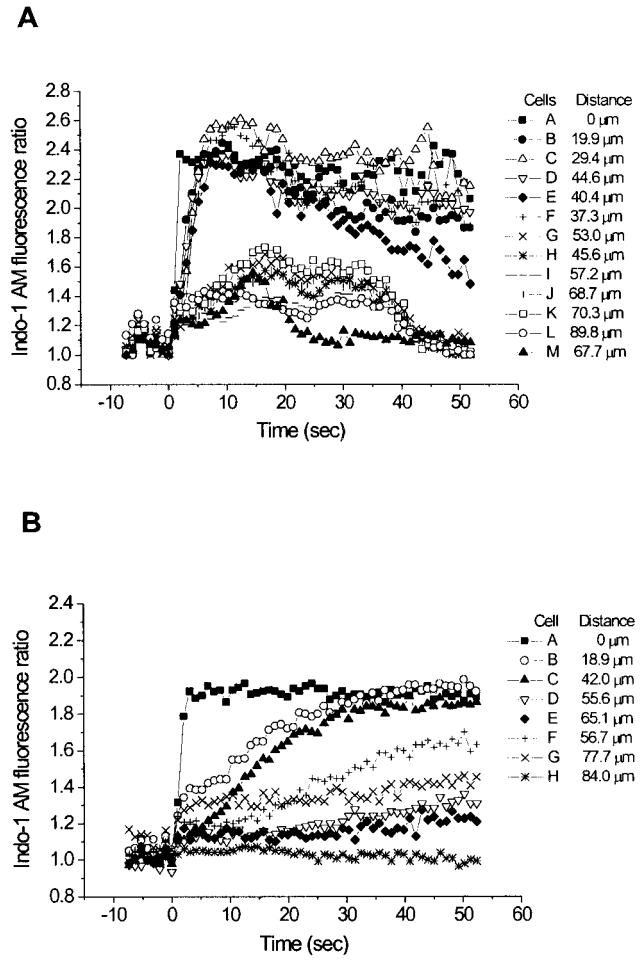

Fig. 3. Graphical representation of mechanically induced intercellular Ca^{2+} wave propagation between wild-type (*A*) and Cx43-null (*B*) neonatal mouse cardiac myocytes in culture. Time-lapsed pseudocolored display of the phenomenon is shown in Fig. 2.

spreading to ~80% of the cells in the confocal field, including those cells that were not in physical contact with the stimulated myocyte. There was no measurable delay in the transmission of the Ca^{2+} signal at the borders of the myocytes, and the conduction velocity as well as the amplitude (1.76 ± 0.12-fold basal levels, $N = 103$) of these slow intercellular waves were not attenuated as the Ca^{2+} signal traveled from myocyte to myocyte. The intracellular Ca^{2+} levels of the cells reached by the Ca^{2+} signal remained elevated for durations from <1 min to as long as 2 min, after which the cells resumed their rhythmic spontaneous activity (data not illustrated).

Pathways Involved in Communication of Slow Mechanically Induced Ca^{2+} Signaling Between Neonatal Cardiac Myocytes

Gap junction-mediated intercellular route. The participation of gap junction channels in the propagation of the slow intercellular Ca^{2+} waves between cardiac myocytes, providing a direct cytosol-to-cytosol route for the transmission of the Ca^{2+} signal, was investigated in cardiac myocytes obtained from transgenic mice with targeted disruption of the main cardiac gap junction protein, Cx43, and in wild-type cardiac myocytes exposed to heptanol, a gap junction channel blocker.

EFFECTS OF CX43 DELETION ON INTERCELLULAR CA^{2+} SIGNALING. Use of cultures from heterozygous mice [Cx43 (+/−)] indicated that the deletion of a single copy of the Cx43 gene did not measurably affect the transmission of the mechanically induced intercellular Ca^{2+} waves (Table 1). However, complete disruption of Cx43 expression [termed Cx43-null or Cx43 (−/−) mice] imposed a significant reduction in the ability of cardiac myocytes to communicate the Ca^{2+} signals (Table 1 and Figs. 2*B* and 3*B*). The increase in intracellular Ca^{2+} observed in the mechanically stimulated Cx43-null myocytes (Fig. 2B, *cell A*, 0-s photo) was not significantly different from that observed in the wild-type myocytes [Cx43 (+/+)] (2.12 ± 0.06-fold basal indo-1 fluorescence ratio, $N = 28$, and 1.97 ± 0.12-fold basal indo-1 fluorescence ration, $N = 15$, respectively). However, the intercellular transmission of the mechanically triggered Ca^{2+} signal was significantly attenuated in the Cx43-null cardiac myocyte cultures; the Ca^{2+} waves propagated between Cx43-null cells 3.7 times more slowly than in wild-type cell cultures, reaching only 40 versus 76% of the cells in the confocal field (Table 1 and Fig. 3*B*). Overall, the removal of Cx43-mediated intercellular communication imposed an overall 87% loss in the transmission of the Ca^{2+} signal between Cx43-null cardiac myocytes [Table 1, EVA factor Cx43 (−/−)/Cx43 (+/+)].

Table 1. *Effects of disruption of Cx43 gene expression on communication of slow mechanically induced intercellular Ca^{2+} signals between neonatal mouse cardiac myocytes*

Genotype	Efficacy, number responding cells/cells in field	Velocity, μm/s	Amplitude, fold basal	EVA Factor
Cx43 (+/+)	0.76 ± 0.06 (19 fields)	14.77 ± 1.64 (54)	1.69 ± 0.23 (54)	
Cx43 (+/−)	0.83 ± 0.05 (4 fields)	15.20 ± 3.28 (24)	1.49 ± 0.05 (24)	
Cx43 (+/−)/Cx43 (+/+)	1.09	1.03	0.88	0.99
Cx43 (−/−)	0.40 ± 0.06 (28 fields)*†	4.08 ± 0.62 (58)*†	1.42 ± 0.04 (58)*	
Cx43 (−/−)/Cx43 (+/+)	0.53	0.28	0.84	0.13

All values are expressed as means ± SE; numbers in parentheses represent the number of cells. Cx43 (+/+), Cx43 (+/−), and Cx43 (−/−) represent connexin43 (Cx43) wild-type, heterozygous, and Cx43-null mice, respectively. EVA factor, product of the relative values (experimental/control) obtained for the efficacy, velocity, and amplitude of the calcium waves. *$P < 0.05$ in relation to Cx43 (+/+) mice, and †$P < 0.05$ between Cx43 (+/−) and Cx43 (−/−) mice.

Table 2. *Effects of Hep on communication of slow mechanically induced intercellular Ca^{2+} signals between wild-type neonatal mouse cardiac myocytes*

Treatment	Efficacy number responding cells/cells in field	Velocity, μm/s	Amplitude, fold basal	EVA Factor
Control	0.76 ± 0.06 (19 fields)	14.77 ± 1.64 (54)	1.69 ± 0.23 (54)	
Hep (0.3 mM)	0.49 ± 0.11 (9 fields)	8.75 ± 1.81 (26)*	1.61 ± 0.08 (26)	
Hep (0.3 mM)/control	0.64	0.59	0.95	0.36
Hep (3.0 mM)	0.10 ± 0.04 (17 fields)*†	6.44 ± 0.98 (12)*	2.01 ± 0.14 (12)*	
Hep (3.0 mM)/control	0.13	0.44	1.19	0.07

All values are expressed as means ± SE; numbers in parentheses represent number of cells. Hep, heptanol. *$P < 0.05$ in relation to control myocytes, and †$P < 0.05$ between Hep treatments.

EFFECTS OF HEPTANOL ON INTERCELLULAR CA^{2+} SIGNALING. The transmission of the Ca^{2+} signal between wild-type cardiac myocytes was significantly attenuated when the cells where exposed to the gap junction channel blocker heptanol (Table 2), which rapidly and reversibly uncouples neonatal cardiac myocytes (3, 6, 51, 55). In the presence of 0.3 mM heptanol, a concentration within the range shown to reduce junctional conductance between pairs of cardiac myocytes by 20 to 30% (55), the amplitude of the mechanically triggered intercellular Ca^{2+} waves was not significantly altered, but the conduction velocity was reduced by 41% (Table 2). The inhibition of gap junction channels imposed by heptanol at this concentration markedly reduced the efficacy of Ca^{2+} signal transmission by 36%, resembling the reduction in efficacy imposed by deletion of Cx43 (Table 1 and Table 2), a condition where the junctional coupling between the myocytes is only 36% of that between wild-type cells (52). Exposure to a higher concentration of heptanol resulted in more pronounced effects. The synchronous pattern of beating of the cardiac myocytes was completely disrupted in the presence of 3 mM heptanol, and the fast component of the mechanically induced Ca^{2+} signaling disappeared (data not illustrated). At this concentration, heptanol totally abolished the communication of the slow Ca^{2+} signal in 59% of the fields analyzed (10 of 17 fields; Fig. 4) and markedly restricted the communication to only one or two cells in the remaining 41% of the cases (Table 2). The overall inhibition of the intercellular Ca^{2+} signaling observed in the presence of 3 mM heptanol was almost two times higher than that imposed by deletion of Cx43 (compare EVA values), probably reflecting the effect of the additional exclusion of the contribution of the other cardiac gap junction channels, formed by Cx40 and Cx45 (53), in the transmission of the Ca^{2+} signal.

It appears from these experiments that gap junction channels provide the main pathway for the communication of the mechanically induced intercellular Ca^{2+} waves between neonatal mouse cardiac myocytes. The disruption of this intercellular pathway either by blocking the channels with heptanol or by deleting Cx43 expression attenuated by >80% the transmission of the intercellular Ca^{2+} signal.

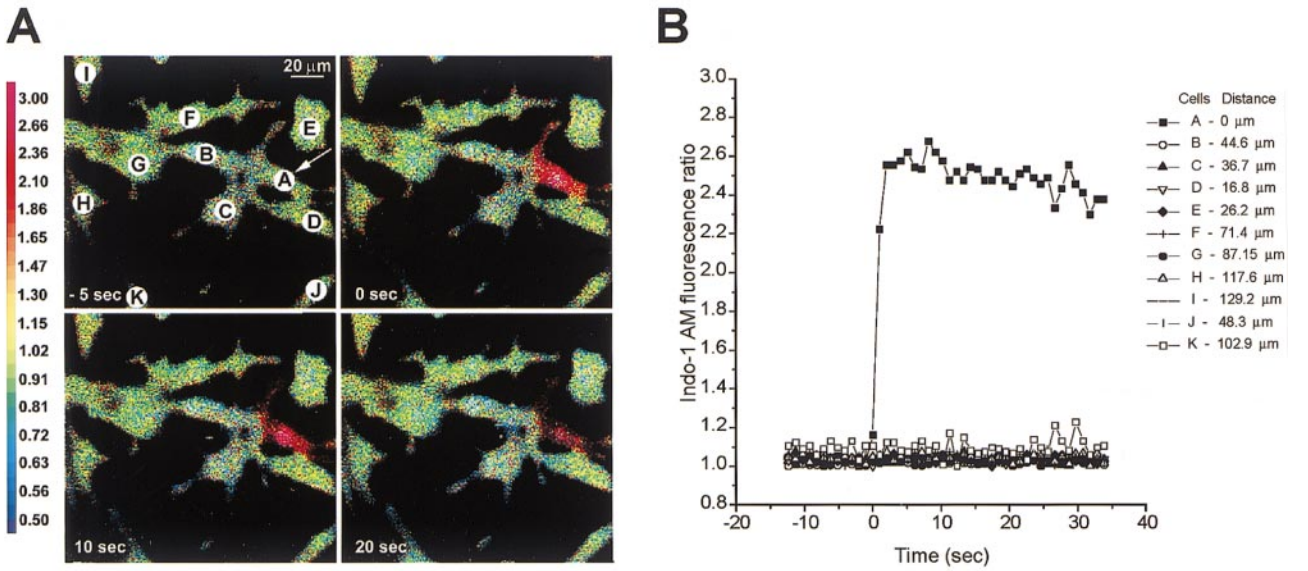

Fig. 4. Time-lapsed pseudocolored display (*A*) and graphical representation (*B*) of the changes in intracellular Ca^{2+} (indo 1-AM fluorescence ratio) induced by mechanical stimulation of *cell A* in the presence of 3 mM heptanol. Note that in the presence of this gap junction channel blocker, the mechanically induced increase in intracellular Ca^{2+} was not transmitted to the neighboring cells.

Nevertheless, the fact that limited intercellular signaling could still be observed in some heptanol-uncoupled cardiac myocytes suggests the participation of a secondary extracellular route for the transmission of the mechanically triggered Ca^{2+} signal, which would work in parallel with the intercellular gap junction-mediated route.

ATP-mediated extracellular route. The participation of mechanically released ATP in an extracellular route mediating the transmission of the slow Ca^{2+} signal via activation of purine/pyrimidine membrane P_2 receptors was investigated using suramin, a selective P_2 receptor blocker, and apyrase, an ATP/ADPase that acts as a scavenger of extracellular ATP.

EFFECTS OF SURAMIN ON THE RESPONSES INDUCED BY EXOGENOUSLY APPLIED ATP. A preliminary evaluation of the effects imposed by suramin on the responses induced by exogenously applied ATP was conducted before using this P_2 receptor blocker to study the participation of an ATP-mediated pathway in the communication of the Ca^{2+} waves. The exogenous application of ATP (10 μM) in wild-type neonatal cardiac myocytes induced either an increase or a decrease in the basal intracellular Ca^{2+} level (73 and 27% of the cells, respectively, $N = 222$). In responses of the first type, ATP induced an increase in the indo-1 fluorescence ratio, which corresponded to 1.47 ± 0.1 ($N = 161$) times the basal intracellular level, whereas in responses of the second type, ATP modestly reduced intracellular Ca^{2+} (0.87 ± 0.01 times basal indo-1 fluorescence ratio). In the presence of low concentrations of suramin (1 μM and 10 μM), the ATP-mediated increase in intracellular Ca^{2+} was significantly reduced (17.8 ± 0.02%, $N = 33$, and 24.4 ± 0.03%, $N = 26$, respectively). Curiously, at a higher concentration (100 μM), suramin not only significantly attenuated the response to ATP (20.0 ± 0.02%, $N = 45$) but, in a few cases, also significantly potentiated ATP response by 35% (35.4 ± 0.11%, $N = 6$). In the conditions where ATP induced a decrease in intracellular Ca^{2+} levels, this inhibitory action was completely blocked by suramin, and the responses to ATP were reverted to an increase in intracellular Ca^{2+}, an effect that was more pronounced in the presence of 100 μM suramin.

To evaluate whether suramin affected gap junction-mediated coupling in cardiac myocytes, we performed Lucifer Yellow injections in cultured cell clusters and measured the junctional conductance in cell pairs; neither 10 nor 100 μM suramin profoundly affected either measure of cell coupling (data not shown). A possible nonspecific action of heptanol on the responses induced by ATP-mediated activation of P_2 receptors was also investigated. Exposure to 3 mM heptanol did not significantly affect the responses of the cardiac cells to exogenously applied ATP (10 μM) (control cells 2.0 ± 0.05 times basal, heptanol-treated cells 2.1 ± 0.05, $N = 37$) contrasting with the effects of a subsequent exposure to suramin (100 μM) to the same fields, which significantly reduced the responses to ATP (1.50 ± 0.08, $N = 37$, $P < 0.05$). These observations agree with those made previously in astrocytes, which demonstrated that the responses induced by ATP activation of P_2 receptors are not altered by heptanol treatment (48).

EFFECT OF SURAMIN ON INTERCELLULAR CA^{2+} SIGNALING. The slow component of the intercellular Ca^{2+} signaling between wild-type cardiac myocytes was significantly altered in the presence of the P_2 receptor blocker suramin, whereas the fast Ca^{2+} waves were not appreciably affected (data not illustrated). In the cardiac myocyte cultures treated with 10 μM suramin, the velocity as well as the number of cells reached by the Ca^{2+} signal were markedly reduced (Table 3), imposing an overall reduction of 58% in the communication of the slow intercellular Ca^{2+} waves (EVA factor, suramin 100 μM/control). A 10-fold increase in suramin concentration did not further attenuate the propagation of the intercellular Ca^{2+} signal. On the contrary, in the presence of 100 μM suramin, the mechanically induced Ca^{2+} waves traveled faster than in the absence of this agent, although the number of responding cells was reduced in a similar way to those observed with the lower concentration of suramin (Table 3). Despite this decrease in the efficacy of the intercellular Ca^{2+} signaling, the concomitant increase in the conduction velocity forced the EVA factor to a value that is not different from 1.0 and, thus, not different from the intercellular signaling in the absence of suramin (Table 3).

EFFECTS OF APYRASE ON THE INTERCELLULAR CA^{2+} SIGNALING. The experiments involving apyrase, an ATP/ADPase, were performed at 21°C (room temperature) and 34°C to optimize the activity of this enzyme. This change in temperature from 21°C to 34°C had no significant effect on the amplitude, velocity, or efficacy of the slow intercellular Ca^{2+} wave propagation (Table 4). However, the effect of apyrase in the communication of the mechanically induced intercellular Ca^{2+} signal

Table 3. *Effects of Sur on communication of mechanically induced slow intercellular Ca^{2+} signals between wild-type neonatal mouse cardiac myocytes*

Treatment	Efficacy, number responding cells/cells in field	Velocity, μm/s	Amplitude, fold basal	EVA Factor
Control	0.76 ± 0.06 (19 fields)	14.77 ± 1.64 (54)	1.69 ± 0.23 (54)	
Sur (10 μM)	0.50 ± 0.05 (19 fields)*	8.14 ± 0.57 (77)*	1.95 ± 0.04 (77)*	
Sur (10 μM)/control	0.66	0.55	1.15	0.42
Sur (100 μM)	0.59 ± 0.04 (37 fields)	18.10 ± 0.95 (153)†	1.71 ± 0.03 (153)*†	
Sur (100 μM)/control	0.78	1.22	1.01	0.96

Values are expressed as means ± SE; numbers in parentheses represent number of cells. Sur, suramin. *$P < 0.05$ in relation to control myocytes, and †$P < 0.05$ between Sur treatments.

Table 4. *Effects of temperature and apyrase treatment on communication of slow mechanically induced intercellular Ca^{2+} waves*

Treatment	Efficacy, number responding cells/cells in field		Velocity, μm/s		Amplitude, fold basal		EVA Factor	
	21°C	34°C	21°C	34°C	21°C	34°C	21°C	34°C
Control	0.77 ± 0.04 (29 fields)	0.76 ± 0.04 (22 fields)	14.29 ± 1.17 (103)	15.39 ± 0.90 (188)	1.77 ± 0.12 (103)	1.62 ± 0.03 (188)		
Apyrase (0.45 U/ml)	0.70 ± 0.10 (3 fields)	0.74 ± 0.06 (6 fields)	11.39 ± 1.74 (14)	18.97 ± 1.69 (42)*	1.51 ± 0.09 (14)	1.83 ± 0.05 (42)*†		
Apyrase (0.45 U/ml)/control	0.91	0.97	0.80	1.23	0.85	1.13	0.62	1.35
Apyrase (4.5 U/ml)	0.84 ± 0.06 (8 fields)	0.49 ± 0.04 (8 fields)*†	10.97 ± 1.48 (37)	24.34 ± 2.50 (48)*†‡	1.96 ± 0.10 (37)	1.75 ± 0.04 (48)*†		
Apyrase (4.5 U/ml)/control	1.09	0.64	0.77	1.58	1.11	1.08	0.93	1.09

All values are expressed as means ± SE; numbers in parentheses represent number of cells. *$P < 0.05$ comparing values obtained at 21 and 34°C, †$P < 0.05$ at same temperature between control and apyrase myocytes, and ‡$P < 0.05$ between apyrase treatments.

Table 5. *Effects of Sur and combined Sur + Hep treatment on communication of slow mechanically induced intercellular Ca^{2+} waves between wild-type and Cx43-null neonatal mouse cardiac myocytes*

Treatment	Efficacy, number responding cells/cells in field		Velocity, μm/s		Amplitude, fold basal		EVA Factor	
	Cx43 (+/+)	Cx43 (−/−)	Cx43 (+/+)	Cx43 (−/−)	Cx43 (+/+)	Cx43 (−/−)	Cx43 (+/+)	Cx43 (−/−)
Control	0.76 ± 0.06 (19 fields)	0.40 ± 0.06 (28 fields)	14.29 ± 1.17 (103)	4.08 ± 0.62 (58)	1.77 ± 0.12 (103)	1.42 ± 0.04 (58)		
Sur (100 μM)	0.59 ± 0.04 (37 fields)	0.18 ± 0.04 (10 fields)*	18.10 ± 0.95 (153)*	7.14 ± 1.45 (16)	1.71 ± 0.03 (153)	1.61 ± 0.06 (16)*		
Sur (100 μM)/control	0.78	0.45	1.27	1.75	0.97	1.13	0.96	0.89
Sur (100 μM) + Hep (3 mM)	0.05 ± 0.03 (15 fields)*†	0.17 ± 0.03 (15 fields)*	3.73 ± 0.58 (6)*†	3.70 ± 0.70 (13)	1.54 ± 0.20 (6)	1.47 ± 0.08 (13)		
Sur (100 μM) + Hep (3 mM)/control	0.07	0.43	0.26	0.91	0.87	1.04	0.02	0.41
Sur (100 μM) + Hep (3 mM)/Sur (100 μM)	0.09	0.94	0.21	0.52	0.90	0.91	0.02	0.44

All values are expressed as means ± SE; numbers in parentheses represent number of cells. *$P < 0.05$ in relation to control myocytes, and †$P < 0.05$ between treatments within the same genotype.

was temperature dependent. At 21°C, the efficacy, velocity, and amplitude of the Ca^{2+} waves in the cultures treated with either 0.45 or 4.5 U/ml apyrase were not significantly different from the values obtained in the absence of this enzyme (Table 4). However, there was a tendency for the signals to propagate more slowly under these conditions. Actually, in the 0.45 U/ml apyrase-treated cultures, the combined decrease in velocity and amplitude led to a 38% reduction in the intercellular Ca^{2+} signaling (EVA factor, apyrase 0.45 U/ml per control, 21°C). At 34°C, the intercellular Ca^{2+} waves traveled faster and with higher mean amplitude in the presence of both concentrations of this ATP/ADPase. Nevertheless, in parallel with the increase in conduction velocity, the efficacy of Ca^{2+} signal communication was significantly reduced in the cultures treated with 4.5 U/ml apyrase at 34°C (Table 4), resembling the changes imposed by 100 μM suramin (see Table 3).

The alterations in the communication of the slow intercellular Ca^{2+} signaling observed when P_2 receptor activation was directly prevented by blockage with suramin or indirectly prevented by enzymatic removal of released ATP strongly suggest the participation of these receptors and ATP release in the Ca^{2+} signal propagation between neonatal mouse cardiac myocytes.

Effect of Simultaneous Blockage of ATP-Mediated and Gap Junction-Mediated Pathway on Communication of Ca^{2+} Signal

The combined contribution of P_2 receptors and gap junction channels for the propagation of the mechanically triggered intercellular Ca^{2+} waves was investigated in both wild-type and Cx43-null cardiac myocytes. Similar to what is described above for the wild-type cardiac myocytes, blockage of the ATP receptors significantly reduced the number of Cx43-null cells engaged in the communication of the Ca^{2+} signal and increased the mean conduction velocity of the intercellular Ca^{2+} wave between the responding myocytes (Table 5 and Fig. 5). Interesting, though, is the fact that, compared with control values, the reduction in efficacy imposed by removal of P_2 receptor participation in the intercellular Ca^{2+} signaling between Cx43-null myocytes was 2.5 times greater than that observed in the wild-type cultures (Table 5). When the wild-type cardiac myocytes were exposed to 3 mM heptanol still in the presence of 100 μM suramin, the communication of the Ca^{2+} signal was completely blocked in 80% of the fields analyzed. In the remaining 20% of the cases, where only 6 of 27 cells responded, the velocity of the intercellular Ca^{2+} was attenuated by 74% in relation to control values (Table 5 and Fig. 5). In contrast, the dual exposure to 3 mM heptanol and 100 μM suramin did not further reduce the efficacy of the Ca^{2+} signal transmission in the Cx43-null cultures but did attenuate the conduction velocity, which returned to values similar to those measured under control conditions, in the absence of suramin (Table 5 and Fig. 5).

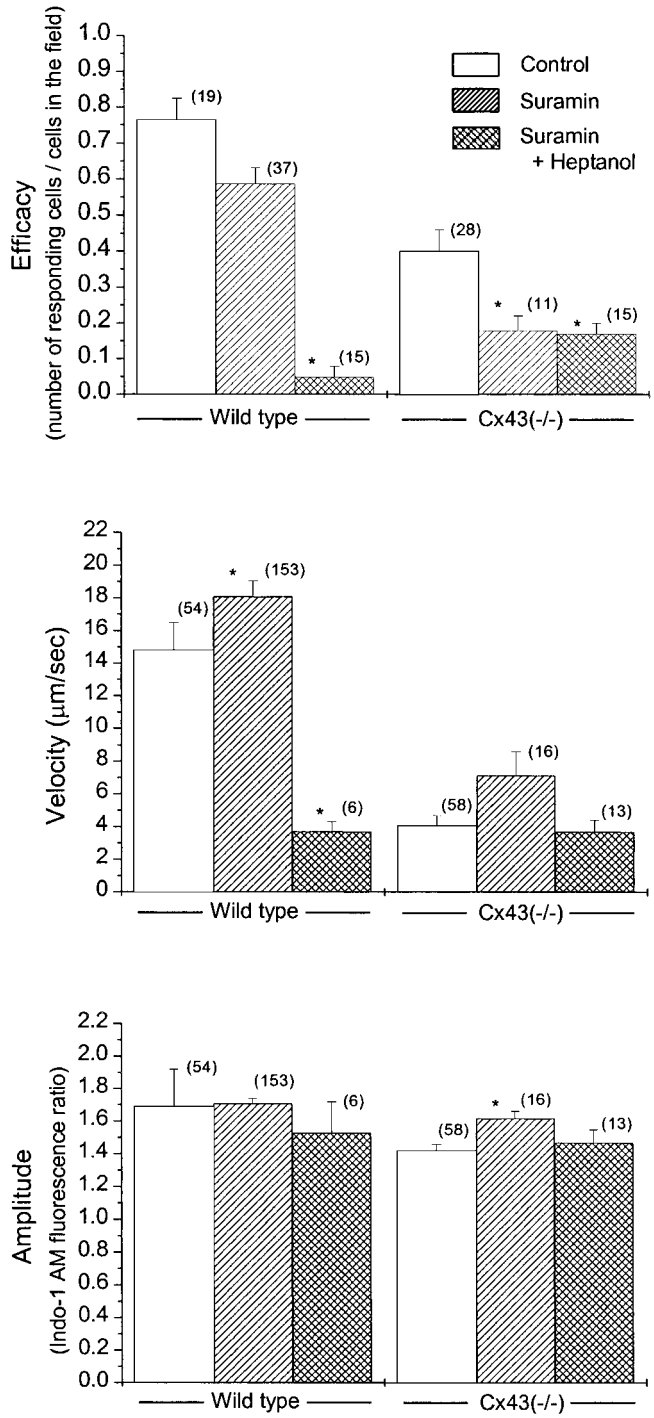

Fig. 5. Effect of 100 μM suramin and combined 100 μM suramin + 3 mM heptanol treatment on the efficacy (number of cells reached by the Ca^{2+} signal), velocity, and amplitude of the mechanically induced intercellular Ca^{2+} signaling between wild-type and Cx43-null cardiac myocytes. Bars correspond to means ± SE; number in parentheses indicated the number of cells. $^*P < 0.05$ in relation to control within the same genotype.

From the above results, it can be postulated that, in wild-type neonatal mouse cardiac myocytes in culture, the Ca^{2+} signal generated by mechanical stimulation of a single myocyte is mainly communicated to its neighbors by gap junction channels. However, an ex-

tracellular pathway generated by ATP released from the stimulated cell also contributes a minor component to the communication of the Ca^{2+} signal through the activation of cell surface membrane P_2 receptors. Interestingly, in the absence of the Cx43-mediated signaling, the participation of this paracrine purinergic pathway in the intercellular Ca^{2+} signaling seems to assume a more prominent role.

DISCUSSION

Focal mechanical stimulation of single spontaneously beating or quiescent neonatal mouse cardiac myocytes in culture can trigger the communication of intercellular Ca^{2+} waves. The Ca^{2+} signal generated in the stimulated myocyte initiates a fast, steep, and reversible increase in the intracellular Ca^{2+} level, which is transmitted to the neighboring myocytes as two temporally distinct wavefronts of intercellular Ca^{2+} waves. The first wavefront spreads with velocities more rapid than 10 mm/s and represents the fast gap junction-mediated Ca^{2+} waves associated with myocardial action potential propagation, which sweep the heart during the systolic cycle. The second wavefront, which is generated subsequent to the electrically mediated event, travels in a much slower fashion from myocyte to myocyte with mean velocities of 14 μm/s. Besides these differences in velocity and initiation time, the two wavefronts of intercellular Ca^{2+} signaling also differ in relation to their efficacy of transmission. The communication of the fast Ca^{2+} waves were confined to cells that were coupled directly or indirectly to the stimulated myocyte, whereas the slow Ca^{2+} waves were also communicated to cells that were physically separated from the group containing the stimulated cell.

The mean conduction velocity measured for the propagation of the mechanically induced intercellular Ca^{2+} waves between neonatal cardiac myocytes in culture was much slower than the intercellular Ca^{2+} waves previously reported in ventricular trabeculae (33). In this multicellular preparation, the communication of the Ca^{2+} waves is initiated at its damaged ends by Ca^{2+} release from the myofilaments followed by a sequential Ca^{2+}-induced Ca^{2+} release and diffusion along the muscle via gap junction channels, which generate the characteristic wave of local propagating contractions also termed "triggered propagated contractions" (TPCs) (11–13, 36, 56). The conduction velocities of the TPCs along the intact trabeculae vary from 1.7 to 13.4 mm/s at 19–21°C (11), with the intercellular Ca^{2+} wave that underlies the TPC traveling with velocities in the range of 0.34 to 5.47 mm/s (33). Assuming that the mechanism involved in the propagation of the intercellular Ca^{2+} waves between neonatal cardiac myocytes is similar to that proposed for the trabeculae, relying mainly on the Ca^{2+}-induced Ca^{2+} release from the SR followed by Ca^{2+} diffusion to adjacent cells through gap junction channels, at least two factors may be responsible for the lower conduction velocity observed in the neonatal cardiac myocytes in culture. The first would be the lower degree of differentiation presented by the SR in the neonatal heart (27, 50), and the second would be the absence of organized distribution of the gap junction channels in the intercalated discs at the ends of the myocytes (26, 40). Thus, in neonatal myocytes, the mobilization of Ca^{2+} from the SR and its diffusion to the adjacent cells would not be as efficient as in mature nondissociated cardiac muscle.

With regard to the pathways involved in the communication of the Ca^{2+} signal, in the majority of the cell types where mechanically induced slow intercellular Ca^{2+} waves have been observed previously, cell-to-cell transmission of the signal appears to be mainly provided by gap junction channels (44, 47). This also proved to be the case in neonatal mouse cardiac myocytes. The transmission of the intercellular Ca^{2+} waves was markedly attenuated and even completely abolished when gap junction-mediated communication was impaired by the deletion of Cx43 or by blockade of the intercellular channels with heptanol. Although it has been shown that removal of 50% of the functional Cx43-formed gap junction channels significantly slows ventricular conduction in the Cx43 (+/−) mouse heart (19, 58), the communication of the mechanically induced intercellular Ca^{2+} signal was not measurably affected. This is consistent with the lack of measurable differences in dye coupling and junctional conductance between Cx43 (+/−) and wild-type cardiac myocytes (52), which presumably reflect the existence of a high safety factor for metabolic coupling in the heart; it is also consistent with a recent optical mapping study (25) in which electrocardiogram parameters between hearts of heterozygotes did not differ from those of wild types.

Such a prominent participation of the gap junction-dependent pathway in the communication of the slow Ca^{2+} waves between cardiac myocytes is consistent with the high degree of electrical and metabolic coupling provided by gap junction channels in cardiac tissue. However, the observation that the Ca^{2+} signals could actually "jump" across cell-free areas in the cultures suggested the additional participation of an extracellular pathway. The intercellular communication of Ca^{2+} signals mediated by sequential activation of membrane receptors, induced by the release and diffusion of extracellular messengers, was first demonstrated between mast cells, with ATP as the extracellular messenger (39). In other cell types that are coupled by gap junction channels, the release of ATP from mechanically stimulated cells can provide an extracellular purinergic pathway, which can work in parallel with the intercellular gap junction route in the communication of the Ca^{2+} signal (8–10, 20–22, 28, 46). In a few cases, the purinergic pathway appears to be the primary or sole pathway involved in the transmission of the intercellular Ca^{2+} signals (7, 18, 28, 37, 39, 49). The participation of P_2 receptors and ATP as an extracellular messenger in the phenomenon of slow Ca^{2+} signaling between neonatal cardiac myocytes was first suggested by the alterations in the Ca^{2+} wave

parameters observed in the presence of suramin, a widely used P_2 receptor blocker. The dual concentration-related effects imposed by suramin could, at a first glance, be puzzling if it were not for the results obtained from the exogenous application of ATP, which both increased or decreased the intracellular Ca^{2+} levels of the neonatal cardiac myocytes. The sensitivity of both types of responses of ATP to suramin suggests the participation of distinct subtypes of P_2 receptors. The presence of a mixed and functionally antagonistic population of P_2 receptors in the neonatal cardiac myocyte culture is consistent with recent reports of the presence of an unidentified subtype of P_2 receptor in ventricular myocytes, whose activation leads to a decrease in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (24–25, 41, 59) instead of the usual P_2 receptor-mediated increase in $[Ca^{2+}]_i$ (5, 23, 45). The simultaneous activation of these receptors leading to either an increase or a decrease in the intracellular Ca^{2+} level would assume a significant modulatory role in the communication of the Ca^{2+} signal between cardiac myocytes. The alterations in velocity and efficacy of Ca^{2+} signal propagation observed upon removal of ATP participation by treatment with suramin or apyrase clearly emphasize this point. Under these conditions, the conduction velocity was markedly enhanced because the ATP receptor-independent increase in intracellular Ca^{2+} could more rapidly peak in the absence of ATP inhibitory action. However, this gain in speed was accompanied by a significant loss in the efficacy of signal transmission, because in the absence of the excitatory effects of ATP fewer cells would be engaged in the response, particularly those situated far from the stimulated myocyte or those that were not even in contact with this cell.

The importance of this extracellular ATP-mediated pathway for the communication of the Ca^{2+} signal can be further appreciated from the results obtained with the Cx43-null cardiac myocytes where the gap junction-mediated communication is impaired. The effects of ATP receptor blockage were similar to those observed for the wild-type cardiac myocytes but were much more pronounced, suggesting a markedly greater participation of the extracellular ATP-mediated pathway in the communication of the Ca^{2+} signal between Cx43-null heart cells. Whether the deletion of Cx43 in the heart is accompanied by an alteration in the population of P_2 receptors, as was demonstrated to occur in the Cx43-null astrocytes (48), remains to be investigated, but such a phenomenon could explain the increased participation of the extracellular ATP-mediated pathway in the communication of the intercellular Ca^{2+} waves between Cx43-null cardiac myocytes.

As mentioned previously, the physiological relevance of this form of slow intercellular Ca^{2+} signaling would be difficult to foresee in the normal working myocardium. However, under abnormal conditions, such as those imposed by ischemia and consequent mechanical distension of damaged cells, the intercellular communication of these slow Ca^{2+} signals would be enhanced in the damaged regions due to Ca^{2+} overload, providing an ideal circumstance for the induction of damage-induced cardiac arrhythmias. Furthermore, the increase of ATP release (17) from damaged cells would also enhance the transmission of the Ca^{2+} signals through activation of P_2 receptors. The participation of this paracrine pathway is expected to predominate 10–15 min after the beginning of the ischemic episode, when the combined effect of a sustained increase in intracellular Ca^{2+}, a decrease in pH, and an accumulation of fatty acid metabolites can lead to cellular uncoupling in the damaged region (14, 15, 29, 38, 60–62) but will not prevent the ATP-mediated activation of the P_2 receptors to continue to broadcast the Ca^{2+} signals from the damaged region to the neighboring healthy myocardium.

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