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ARTICLES

Calcium-Induced Calcium Release Mediates All-or-Nothing Responses of Mesenchymal Stromal Cells to Noradrenaline

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Abstract—By using Ca^{2+} imaging and Fluo-4 dye, we examined the capability of certain agonists of G-protein coupled receptors to stimulate Ca^{2+} signaling in cultured mesenchymal stromal cells (MSC) derived from the human adipose tissue. In particular, a small subpopulation (~5%) MSC was found to respond to noradrenaline with Ca^{2+} transients. The all-or-nothing fashion was characteristic of adrenergic Ca^{2+} signaling in MSC, that is, while at low concentrations noradrenaline stimulated undetectable Ca^{2+} transients, virtually maximal responses were elicited by this agonist at any concentration above the threshold of 100–200 nM. In some experiments, MSC were loaded with the photosensitive Ca^{2+} chelator NP-EGTA to produce local or global jumps in cytosolic Ca^{2+} concentration by virtue of Ca^{2+} uncaging. Global uncaging eliciting a high enough Ca^{2+} jump triggered a Ca^{2+} transient in the MSC cytoplasm, which was similar to a noradrenaline response kinetically and by magnitude. When Ca^{2+} uncaging was produced locally, it initiated a Ca^{2+} signal that traveled along a cell with a speed that exceeded an expected one by two orders of magnitude, should Ca^{2+} signal transfer be mediated merely by passive Ca^{2+} diffusion in the presence of Ca^{2+} buffer. These findings implicated Ca^{2+}-induced Ca^{2+} release (CICR) as a mechanism amplifying local Ca^{2+} signals in MSC. Of Ca^{2+} targets involved in CICR, the ryanodine receptor and IP_3 receptor are only known. The inhibitory analysis revealed IP_3 receptors to be principally responsible for CICR in MSC, whereas a contribution of ryanodine receptors was negligible. Altogether, our results suggest that an initial noradrenaline-dependent rise in cytosolic Ca^{2+} stimulates, should it reach the threshold level, IP_3 receptors, thereby triggering an avalanche-like Ca^{2+} release from Ca^{2+} stores and underlying the all-or-nothing dependence of cellular responses on the agonist concentration.

Keywords: mesenchymal stromal cells, adrenoreceptors, Ca^{2+} signaling, ryanodine receptors, IP_3 receptors

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In studies of mesenchymal steam cells, efforts were largely aimed at the evaluation of their transplantational potential or analysis of their proliferation/differentiation and search for a mean to control these processes in vitro [1, 2], while intracellular signaling and cell-to-cell communications were basically neglected. As a result, the current knowledge on receptor systems, signaling mechanisms, paracrine and autocrine regulations in these cells and on their physiology in general are very incomplete. Although intracellular Ca^{2+} is a key mediator in intracellular signaling, as well as an important regulator of cell proliferation and differentiation, Ca^{2+} homeostasis in mesenchymal stem cells was explored in a very few studies. Most of them focused on spontaneous Ca^{2+} oscillations or analyzed effects of variable bath Ca^{2+} concentrations on growth and development of a cell population [3]. Meanwhile, more abundant information could be obtained in experiments, wherein Ca^{2+} signaling is triggered by external stimuli, including agonists of a variety of surface receptors. It is possible that some of them could turn out effective as agents stimulating differentiation of mesenchymal stem cells into distinct phenotypes. Undoubtedly, such a possibility is of practical interest.

The physiological study of individual mesenchymal stem cells in situ is quite a complicated problem, if solvable at all. As an alternative, the conventional approach for assaying mesenchymal stem cells is to isolate them from a variety of different tissues with certain methods and to maintain those in a primary culture. These cultured cells are commonly called mesenchymal stromal cells (MSCs) to distinguish them from bona fide mesenchymal stem cells operating in vivo [4].

The priority goal of our current research is the analysis of physiological processes in MSCs derived

for the human adipose tissue. By assaying responsivity of these cells to an array of agonists of different G-protein-coupled receptors (GPCR), we particularly found that noradrenaline stimulated Ca^{2+} signaling in their cytoplasm. Here we demonstrate that adrenergic MSCs respond to the agonist in an all-or-nothing manner and provide evidence that Ca^{2+}-induced Ca^{2+} release underlies this distinctive MSC feature.

MATERIALS AND METHODS

Cell isolation and culturing. Human MSCs were isolated from abdominal subcutaneous adipose tissue harvested during surgical operations from patients aged from 32 to 50 years. All donors gave informed consent for harvesting of their adipose tissue. Donors with infectious or systemic diseases and malignancies were not included in the study. Adipose tissue was washed extensively with 2 volumes of Hanks balanced salt solution (HBSS) with 1% antibiotic/antimycotic solution (HyClone, USA), cut and then digested at 37°C for 1 h with equal volumes of collagenase (66.7 U/mL; Sigma–Aldrich, USA) and dispase (10 U/mL; BD Biosciences, USA). Enzyme activity was neutralized by an equal volume of culture medium (Advance Stem basal medium for human undifferentiated mesenchymal stem cells (HyClone, USA) containing 10% of Advance stem cell growth supplement (CGS) (HyClone, USA), 1% antibiotic/antimycotic solution), and suspension was centrifuged at 200 g for 10 min. Cell pellet was resuspended in culture medium and filtered through 100-μm nylon cell strainer (BD Biosciences, USA). Erythrocytes were removed with lysis buffer. Cells were collected by centrifugation, resuspended in culture medium. MSC culture was maintained at subconfluent levels (<80% confluency) at 37°C/5% CO_2 and passaged using HyQtase (HyClone, USA). For experiments, cells of the second passage were used.

Preparation of cells for physiological experiments. Twelve hours before Ca^{2+} imaging experiments cells were maintained in the culture medium described above but without antibiotics. Just prior recordings cells were rinsed twice with the Versen solution (Sigma–Aldrich, USA). For cell isolation, the Versen solution was removed, and 200 μL HyQTase solution (HyClone) was added for 3–5 min. The enzymatic treatment was terminated by the addition of the complete culture medium (800 μL). Next, the cells were re-suspended and put into a tube for centrifugation at 0.8 g for 45 s that resulted in cell sedimentation.

Isolated cell were collected by a plastic pipette and plated onto a recording chamber of about 150 μL volume. The recording chamber was composed of a coverslip (Menzel–Glaser, Germany) with attached ellipsoidal resin wall. The chamber bottom was coated with Cell-Tak (BD Biosciences, Bedford, MA) for better cell adhesion. Attached cells were incubated at room temperature (23–25°C) in a bath solution (NaCl, 110 mM; KCl, 5.5 mM; $CaCl_2$, 2 mM; $MgSO_4$, 0.8 mM; NaH_2PO_4, 1 mM; HEPES, 10 mM, and glucose, 10 mM) containing 4 μM Fluo-4AM and 0.02% Pluronic (all from Molecular Probes, USA). After 20-min incubation the cell were rinsed several times with the bath solution and stored at 4°C for 40 min. When necessary, 2 mM $CaCl_2$ in the bath solution was replaced with 0.5 mM EGTA + 0.4 mM $CaCl_2$, thus reducing the free Ca^{2+} concentration to nearly 260 nM.

Ca^{2+} imaging and uncaging. Most of experiments were carried out using an inverted fluorescent microscope Axiovert 135 equipped with an objective Plan NeoFluar 20×/0.75 (Zeiss, Germany) and a digital ECCD camera LucaR (Andor Technology, USA). Apart from a transparent light illuminator, the microscope was equipped with a hand-made system for epi-illumination via an objective. The epi-illumination was performed using a bifurcational glass fiber. One channel, which was used for Fluo-4 excitation, transmitted 480 ± 5 nm irradiation emitted by LED controlled by a computer. Fluo-4 emission was collected at 535 ± 20 nm. Serial fluorescent images were captured every second and analyzed using Imaging Workbench 6 software (INDEC, USA). Deviations of cytosolic Ca^{2+} from the resting level were quantified by a relative change in intensity of Fluo-4 fluorescence ($\Delta F/F_0$) recorded from an individual cell. Another channel was connected to a pulsed solid laser DTL-374QT (30 mW) (Laser-Export, Russia). This unit operated in a two harmonics mode and generated not only 355-nm UV light used for Ca^{2+} uncaging but also visible light at 532 nm. The latter could penetrate into an emission channel through non-ideal optical filters and elicited optical artifacts during uncaging. For uncaging, cells were loaded with both 4 μM Fluo-4 and NP-EGTA purchased from Invitrogene (USA). Salts, buffers and inhibitors were from Sigma–Aldrich (USA) or Tocris (UK).

Confocal microscopy. In a number of experiments Ca^{2+} waves in the MSC cytoplasm were analyzed using a confocal scanning microscope Leica TCS SP5 (Leica Microsystems, Germany) equipped with an oil immersion objective Leica HCX PL APO CS 63.0 × 1.40. A day before an experiment, cells were seeded on 8-well glass-bottom plates (Nunc Lab-Tek 8 well chambered coverglass, Thermo Scientific, USA). For due loading, 1 h prior an imaging experiment the incubation media was substituted for the solution containing 9 parts of MEM with 20 mM HEPES (HyClone, USA) and 1 part of Pluronic reagent (Abcam, England) with 3 μM Fluo-8 AM (Abcam Fluo-8 No Wash Calcium Assay Kit, Abcam, England) and 3 μM NP-EGTA AM (Invitrogen, USA). Cells were incubated in the dark at 37°C for 1 h. Then cells were rinsed with imaging medium (MEM contained 20 mM HEPES) several times. Ca^{2+} indicator Fluo-8 was excited at 488 nm using an argon laser LASOS LGK 7872 ML (LASOS Lasertechnik, Germany). NP-EGTA pho-

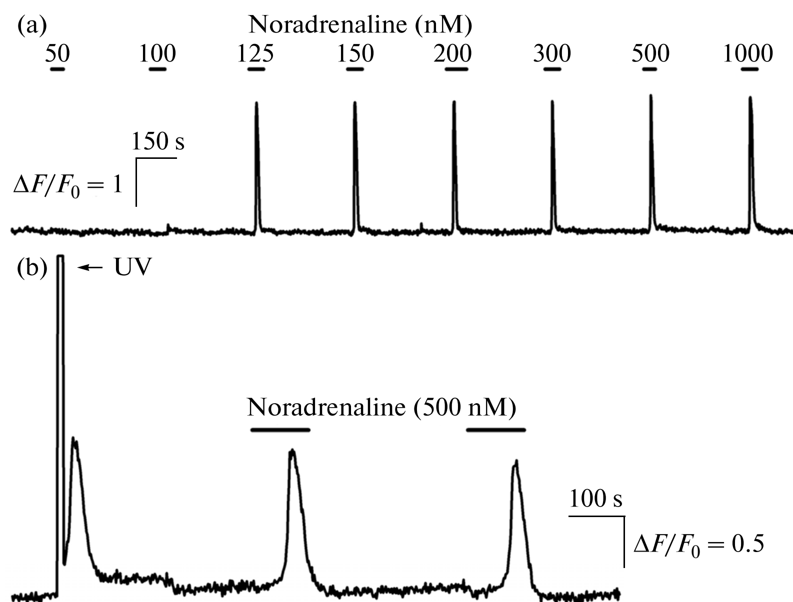

Fig. 1. MSC responses to noradrenaline. (a) Representative recording ($n = 23$) of Ca^{2+} transients in a cell stimulated by noradrenaline at different concentrations. Here and in the figures below, the moments of the compound applications are marked by horizontal lines above the recording trace. (b) Representative recording ($n = 14$) of cellular responses to NP-EGTA photolysis (8-s UV pulse at 355 nm) and noradrenaline (500 nM). Both the light and the chemical stimulus elicited Ca^{2+} transients virtually identical kinetically and by magnitude.

tolysis for local intracellular Ca^{2+} uncaging was performed using 790 nm laser for multiphoton microscopy MaiTai fs HP (Spectra-Physics, USA). Leica FRAP Wizard (Leica Microsystems) was used for intracellular Ca^{2+} monitoring and for controllable Ca^{2+} uncaging.

RESULTS AND DISCUSSION

For functional assay, MSCs were loaded with Ca^{2+} dye Fluo-4, and their responses to a variety of GPCR agonists was evaluated using the Ca^{2+} imaging approach. It turned out that a MSC population contained a small (2–5%) subpopulation of cells specifically sensitive to noradrenaline (50 nM–1 μM) that mobilized Ca^{2+} in their cytoplasm. The characteristic and unexpected feature of MSC responses to noradrenaline was that their dependence on agonist concentration was not gradual and monotonic. When noradrenaline was briefly applied in series at sequentially increased concentrations, it produced a set of Ca^{2+} transients in an all-or-nothing manner. More exactly, while low noradrenaline stimulated undetectable Ca^{2+} transients, virtually maximal responses were elicited by this substance at any concentration above the threshold of 100–200 nM (Fig. 1a).

As a likely explanation of the trigger-like responsivity, we considered a possibility that MSCs generated responses to noradrenaline in two consecutive steps. During the initial stage, the agonist produced moderate and local Ca^{2+} rise, which could not be clearly detected with non-confocal Ca^{2+} imaging. It would not be surprising if the magnitude of this initial Ca^{2+} response gradually increased with noradrenaline concentration, as is the case with Ca^{2+} signaling stimulated by diverse agonists in a variety of different cells [6–8]. Next, when this initial Ca^{2+} signal reached a threshold level, it triggered Ca^{2+}-release from Ca^{2+} stores, producing a global Ca^{2+} transient in the MSC cytoplasm. The magnitude of this secondary Ca^{2+} response should most likely be independent of noradrenaline concentration.

If this concept is true, any noradrenaline-independent stimulation, should one produce an appropriate deviation of intracellular Ca^{2+}, could elicit a noradrenaline-like response in an adrenergic cell. To verify this idea, we examined a reaction of noradrenaline-sensitive MSCs to a jump in cytosolic Ca^{2+} that was produced by Ca^{2+} uncaging. In such experiments, cells were loaded with both Fluo-4 and NP-EGTA. The latter is a photosensitive Ca^{2+} chelator with high affinity to Ca^{2+} ($K_d \sim 10^{-7}$ M), so that in a resting cell (~100 nM free Ca^{2+}), nearly half NP-EGTA molecules should be bound to Ca^{2+} ions. The short exposure of this chelator to ultraviolet light (UV) disrupts the coordination sphere responsible for Ca^{2+} binding, thereby liberating Ca^{2+} ions and producing a step-like increase in cytosolic Ca^{2+} concentration [9].

Overall, we assayed 32 MSCs loaded with NP-EGTA/Fluo-4 that were responsive to noradrenaline. Given that multiple factors could influence simultaneous loading of cells with NP-EGTA and Fluo-4, Ca^{2+} signals elicited by UV (355 nm) flashes (3–8 s)

were quite variable by kinetics and magnitude, specifically because NP-EGTA markedly increased binding capacity of intracellular Ca^{2+} buffer. Nevertheless, 14 cells were found to generate similar responses to both noradrenaline and UV pulses. The representative recording of such responses is shown in Fig. 1b. Here a cell was initially exposed to 8-s UV flash. This stimulation was accompanied by the optical artifact that was seen as a strong pulse-like signal in the experimental curve (Fig. 1b), as the used UV laser was in fact a biharmonic light source (355 nm and 532 nm). Since emission at 532 nm was too powerful and could not be completely eliminated with optical filters, it penetrated into the Fluo-4 emission channel (535 ± 20 nm) to some extent. As shown in Fig. 1b, the 8-s uncaging artifact was immediately followed by the Ca^{2+} transient that was virtually identical to the cellular response to 500 nM noradrenaline by kinetics and magnitude. Note that short (<3 s) light stimuli usually elicited a very subtle rise in intracellular Ca^{2+} (not shown), which was never accompanied by secondary huge, in fact maximal, Ca^{2+} transients observed in the case of 6–8-s UV flashes (Fig. 1b), Thus, the secondary Ca^{2+} responses generated by MSCs on light stimuli, which produced a step-like change in intracellular Ca^{2+}, also depended on stimulus intensity in an all-or-nothing manner. Taken together, the above facts convinced us that adrenergic MSCs indeed utilize the CICR mechanism to amplify an initial Ca^{2+} signal produced as intermediate step of noradrenaline transduction.

In search for independent evidence for existence of CICR in MSCs, we analyzed the spread of Ca^{2+} signals triggered by local photolysis of NP-EGTA. The rationale for such experiments was provided by the following facts. Several key factors determine the passive spread of Ca^{2+} signals within the cell cytoplasm. Firstly, Ca^{2+} ions diffuse in a geometrically complex space with multiple diffusion barriers and excluded volume due to the presence of intracellular organells and numerous macromolecules. In addition, Ca^{2+} ions bind to mobile and immobile Ca^{2+}buffer and are captured by mitochondrial Ca^{2+} uptake and removed by Ca^{2+} pumps operating in the plasma membrane and endoplasmic reticulum. These factors markedly decrease the rate of passive Ca^{2+} signals and weaken their intensity, thus making them quite local. Meanwhile, CICR seems obligatory for global Ca^{2+} signaling [10, 11]. We therefore analyzed evolution of Ca^{2+} transients elicited locally in the MSC cytoplasm, suggesting the passive diffusional mechanism to be distinguishable from the active spread of a Ca^{2+} signal reinforced by CICR. The last was expected to underlie much less delayed signals.

Indeed, in the simplest case of self-diffusion in the presence of immobile and fast Ca^{2+} buffer, the transport equation for free Ca^{2+} ions is reduced to the simple diffusion equation with the effective diffusion coefficient:

$$D_{\text{eff}} = D/(1 + \beta), \quad (1)$$

where D is the diffusion coefficient for free Ca^{2+} in a physiological solution and β is the ratio of bound to free Ca^{2+} [12, 13]. Given that for a variety of mammalian cells, β value has been estimated to exceed 100 [14], the speed of Ca^{2+} signals mediated exclusively by diffusion should be dramatically decreased in the presence of Ca^{2+} buffer due to Ca^{2+} binding. Based on the Einstein equation, one can estimate the mean square of displacement l of a diffusing particle for time t:

$$l^2 = 6Dt \quad (2)$$

As comes from this equation, free diffusion of Ca^{2+} ions ($D \approx 300\ \mu m^2/s$ [15, 16]) for 50 μm, the characteristic MSC dimension, would take nearly 1.4 s, while in the presence of intracellular Ca^{2+} buffer, the diffusion time would increase by two orders of magnitude.

Experiments were carried out using a confocal system Leica SP5 equipped with several channels for epi-illumination, including the Fluo-4 cannel (488 nm) and the channel for local photolysis at 395 nm. The representative recording is presented in Fig. 2a that shows the series of sequential confocal images of a group of cultured cells loaded with Fluo-4 and NP-EGTA. At the moment marked as $t = 0$ infrared light pulse (790 nm, 1.4 s) was produced with a powerful multiphoton laser that was aimed at a narrow slit, providing local illumination (Fig. 2a, vertical light line). The laser pulse induced NP-EGTA photolysis due to nonlinear two photon absorption at 395 nm, resulting in a local Ca^{2+} release. In the illustrated case (Fig. 2a) and for several other cells ($n = 6$), we analyzed serial images to evaluate a relative change in integral Fluo-4 fluorescence ($\Delta F/F_0$) that was recorded from a non-photobleached part of an assayed cell (region of interest, ROI) basically being opposite to the region of Ca^{2+} uncaging. The curve shown in Fig. 2b displays the time course of relative Fluo-4 fluorescence in ROI (Fig. 2a, panel 9.5 s) of the cell subjected to Ca^{2+} uncaging as shown in Fig. 2a. As illustrated, the Ca^{2+} concentration peaked within nearly 1 s after the start of NP-EGTA photolysis. Equations (1) and (2) suggest that diffusion of Ca^{2+} from the uncaging range to ROI would take 100 s or so. Thus, the passive diffusion model, wherein most of cytoplasmic factors were not included but Ca^{2+} binding, cannot account for the experimentally evaluated rate of Ca^{2+} waves initiated in MSCs by Ca^{2+} uncaging. Hence, it is necessary to suggest that the MSC cytoplasm is an active media. This inference is rather consistent with the evidence provided by the above experiments (Fig. 1) that CICR is the key event in transduction process triggered by noradrenaline in MSCs.

Multiple studies of CICR in different cells implicate solely two mechanisms, including ryanodine receptors and inositol 1,4,5-trisphosphate (IP_3)

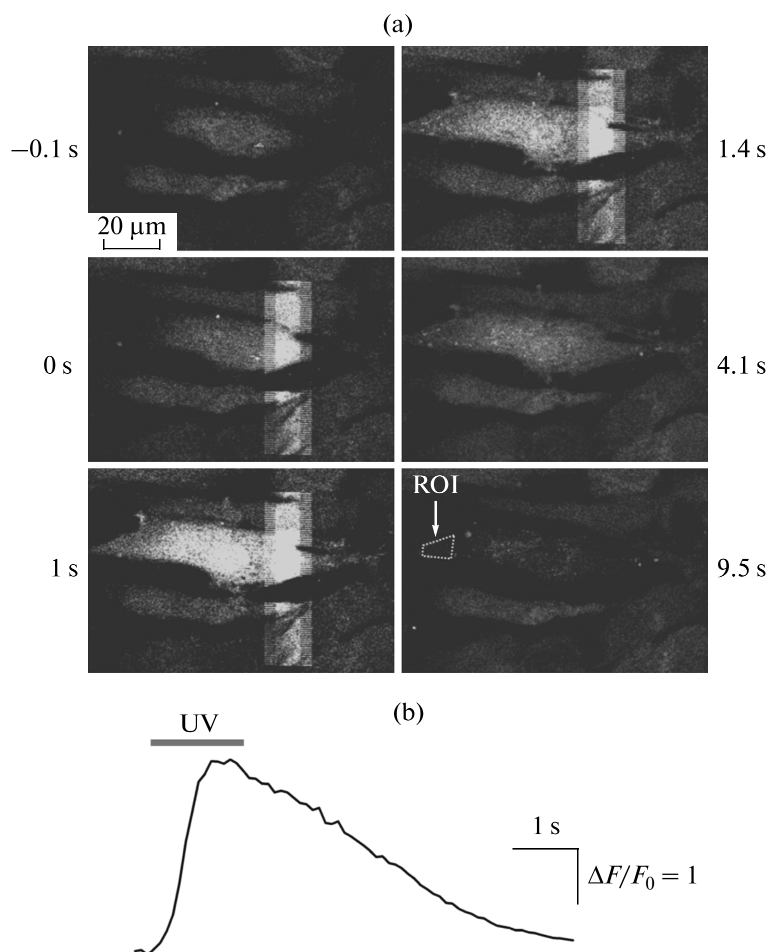

Fig. 2. Ca^{2+} wave in an individual cell. (a) Consecutive fluorescent images of a MSC group in culture loaded with the Ca^{2+} dye Fluo-4 and photosensitive Ca^{2+} chelator NP-EGTA. Fluorescence was excited at 488 nm, emission was recorded at 530 nm. Images were obtained every 100 ms. The digits at the left and right of the panels indicate the time of acquisition of the corresponding images. During the period from 0 to 1.4 s Ca^{2+} uncaging was produced due to two-photon absorption of highly intensive infrared light (790 nm). The light vertical line reflects the space distribution of the infrared illumination. In the panel 9.5 s, the trapezium marked by the arrow indicates the region of interest (ROI), wherein Fluo-4 fluorescence was captured. (b) Time course of relative fluorescence ($\Delta F/F_0$) in ROI. Here F_0 is the intensity of emission from ROI averaged over the period –0.5...–0.3 s, $F(t)$ is the intensity of emission from ROI at the moment t, $\Delta F = F(t) - F_0$.

receptors, both being Ca^{2+}-gated Ca^{2+} release channels operating in the endo/sarcoplasmic reticulum [10]. To detail a CICR mechanisms in MSCs, we employed the inhibitory analysis. In noradrenaline responsive MSCs, the initial CICR stimulating Ca^{2+} signal a priori could be produced by the agonist either by stimulating Ca^{2+} release or by initiating entry of bath Ca^{2+} through receptor-operated Ca^{2+}-permeable channels [11]. In fact, noradrenaline responses in MSCs were affected negligibly, when external Ca^{2+} was reduced from 2 mM to nearly 260 nM. Besides, the phospholipase C (PLC) inhibitor U73122 (1 μM) suppressed noradrenaline responsivity almost completely and irreversibly (Fig. 3a; $n = 31$). These findings pointed to the negligible contribution of Ca^{2+} entry and favored PLC-dependent Ca^{2+} release as the primary mechanism. When MSCs were preincubated with 50 μM ryanodine for 200–300 s, the treatment expected to completely inhibit ryanodine receptors, cell sensitivity to noradrenaline altered insignificantly (Fig. 3b; $n = 17$). In contrast, the IP_3 receptor antagonist 2-APB (50 μM) reversibly inhibited noradrenaline responses (Fig. 3b; $n = 12$). Note, however, that 2-APB can also serve as an inhibitor of Ca^{2+} entry by exerting blockage of Ca^{2+}-permeable channels, such as TRPC channels [17–19]. Given that the removal of bath Ca^{2+} influenced insufficiently adrenergic signaling in MSCs (Fig. 3a), IP_3 receptors were the main, if not sole, target for 2-APB. Thus, the above findings (Fig. 3) indicate that in adrenergic MSCs, largely IP_3 receptors mediate CICR occurring when intracellular Ca^{2+} reaches an appropriate level.

The inhibition of noradrenaline resposivity by the PLC inhibitor U73122 (Figs. 3a, 3b) indicates that in

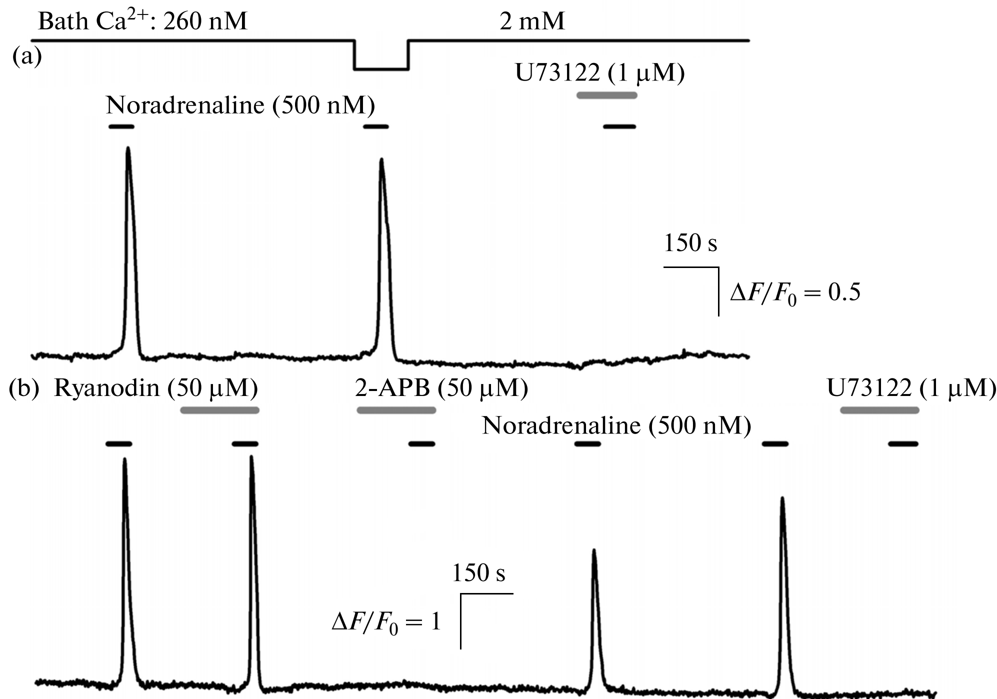

Fig. 3. Ryanodine receptors and receptor-operated Ca^{2+} entry contribute negligibly to MSC responses to noradrenaline. (a) Representative recording of noradrenaline responses with 2 mM and 260 nM Ca^{2+} in the bath and in the presence of the PLC inhibitor U73122 (1 μM). As illustrated, noradrenaline responsivity insignificantly depended on bath Ca^{2+} concentration, while U73122 completely suppressed cellular responses. (b) Representative recording demonstrating the inhibitory effects of the IP_3 receptor antagonist 2-APB (50 μM) on cell responses to noradrenaline (500 nM), while 50 μM ryanodine, a ryanodine receptors inhibitor, was ineffective.

MSCs, adrenoreceptors are coupled to PLC and IP_3 production, suggesting that the generation of initial Ca^{2+} signals involves activation of IP_3 receptors and Ca^{2+} release from Ca^{2+} store. At first glance, there is some inconsistency in this point of view, which is based on the effects of U73122 and 2-APB, since it implicates IP_3 receptors in mediating two different signaling pathway. The first is the phosphoinositide cascade that is directly stimulated by noradrenaline and responsible for small and most likely gradual Ca^{2+} transients. Another one amplifies this initial Ca^{2+} response to the maximal value by means of CICR. It should be noted however that intracellular signaling involves distinct subpopulations of IP_3 receptors that differ in their sensitivity to IP_3 and Ca^{2+} and may operate in physically separated Ca^{2+} compartments [20]. The necessary functional specialization of IP_3 receptors in the same signaling process also can be achieved due to the following amazing property: all three isoforms can be stimulated by the Ca^{2+}-binding protein CIB1 in a Ca^{2+}-dependent manner even in the absence of IP_3 [21]. This means that under certain conditions, IP_3 receptor-mediated CIRC does not require IP_3 generation (Fig. 1b).

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