Mechanisms of the sarcoplasmic reticulum Ca\textsuperscript{2+} release induced by P2X receptor activation in mesenteric artery myocytes

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\textbf{Abstract}

Background: ATP is one of the principal sympathetic neurotransmitters which contracts vascular smooth muscle cells (SMCs) via activation of ionotropic P2X receptors (P2XRs). We have recently demonstrated that contraction of the guinea pig small mesenteric arteries evoked by stimulation of P2XRs is sensitive to inhibitors of IP\textsubscript{3} receptors (IP\textsubscript{3}Rs). Here we analyzed contribution of IP\textsubscript{3}Rs and ryanodine receptors (RyRs) to [Ca\textsuperscript{2+}], transients induced by P2XR agonist αβ-meATP (10 μM) in single SMCs from these vessels.

Methods: The effects of inhibition of L-type Ca\textsuperscript{2+} channels (VGCCs), RyRs and IP\textsubscript{3}Rs (5 μM nicardipine, 100 μM tetracaine and 30 μM 2-APB, respectively) on αβ-meATP-induced [Ca\textsuperscript{2+}], transients were analyzed using fast x-y confocal Ca\textsuperscript{2+} imaging.

Results: The effect of IP\textsubscript{3}R inhibition on the [Ca\textsuperscript{2+}] transient was significantly stronger (67 ± 7%) than that of RyR inhibition (40 ± 5%) and was attenuated by block of VGCCs. The latter indicates that activation of VGCCs is linked to IP\textsubscript{3}R-mediated Ca\textsuperscript{2+} release. Immunostaining of RyRs and IP\textsubscript{3}Rs revealed that RyRs are located mainly in deeper sarcoplasmic reticulum (SR) while sub-plasma membrane (PM) SR elements are enriched with type 1 IP\textsubscript{3}Rs. This structural peculiarity makes IP\textsubscript{3}Rs more accessible to Ca\textsuperscript{2+} entering the cell via VGCCs. Thus, IP\textsubscript{3}Rs may serve as an “intermediate amplifier” between voltage-gated Ca\textsuperscript{2+} entry and RyR-mediated Ca\textsuperscript{2+} release.

Conclusions: P2X receptor activation in mesenteric artery SMCs recruits IP\textsubscript{3}R, IP\textsubscript{3}R-mediated Ca\textsuperscript{2+} release from sub-PM SR, which is facilitated by activation of VGCCs. Sensitivity of IP\textsubscript{3}R, IP\textsubscript{3}R-mediated release to VGCC antagonists in vascular SMCs makes this mechanism of special therapeutic significance.

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\textbf{Introduction}

Sympathetic nerve activity controls total peripheral vascular resistance and, hence, systemic blood pressure via regulation of the contractile activity of smooth muscle cells (SMCs) in the wall of small arteries. One of the principal sympathetic neurotransmitters, ATP, triggers contraction of arterial SMCs via activation of ionotropic P2X receptors (P2XRs) resulting in a robust increase of [Ca\textsuperscript{2+}].

Both Ca\textsuperscript{2+} entry across the plasma membrane through P2XRs themselves and Ca\textsuperscript{2+} entry via voltage-gated Ca\textsuperscript{2+} channels (VGCCs) activated by P2X-receptor-mediated membrane depolarization were found to contribute in the increase of [Ca\textsuperscript{2+}]. We have recently demonstrated that net contribution of these two Ca\textsuperscript{2+} entry pathways is small, and that the major contribution of Ca\textsuperscript{2+} to SMC contractility is generated by IP\textsubscript{3}R-mediated Ca\textsuperscript{2+} release.
influxes to the total [Ca\textsuperscript{2+}] response, elicited by P2X receptor activation in mesenteric artery SMCs, is only 20%, while the remaining 80% of the Ca\textsuperscript{2+} signal is brought about by the sarcoplasmic reticulum (SR) Ca\textsuperscript{2+} release, which is induced by these two Ca\textsuperscript{2+} influxes [1]. Being similar in size, these two fluxes of Ca\textsuperscript{2+} into the cell, however, differ considerably in their ability to trigger Ca\textsuperscript{2+} release from intracellular stores. Indeed, we found that rise of [Ca\textsuperscript{2+}], initiated by VGCC-mediated Ca\textsuperscript{2+} entry is amplified 8 fold by subsequent Ca\textsuperscript{2+} release, while in the case of P2X-mediated Ca\textsuperscript{2+} influx the Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release (CICR) amplification factor is only 3.3 [1]. In our previous study on arterial rings we have demonstrated that phasic contractions of the guinea-pig small mesenteric arteries induced by P2X receptor stimulation are sensitive to the inositol 1,4,5-trisphosphate receptor (IP\textsubscript{3}R) inhibitor 2-aminoethoxydiphenyl borate (2-APB) [2]. This unexpected finding suggests possible involvement of IP\textsubscript{3} in intracellular Ca\textsuperscript{2+} signaling engaged by activation of ionotropic P2XRs, which are not coupled to the G\textsubscript{q11}-GTP/phospholipase C (PLC)/IP\textsubscript{3} system [3]. It should be noted however, that in multicellular preparations, such as vascular rings, superfusion of the P2X receptor agonist to experimental chamber could activate P2XRs not only in the SMCs, but also in the extrasynaptic dendritic regions of sympathetic nerves. As a result, Ca\textsuperscript{2+} that enters through P2XRs into the nerve endings may induce the release of noradrenaline, which acting upon metabolotropic \alpha\textsubscript{1}-adrenoreceptors of SMCs would stimulate PLC-\beta via G\textsubscript{q11} protein leading to an increase of [IP\textsubscript{3}], and IP\textsubscript{3}-mediated Ca\textsuperscript{2+} release [4,5]. To overcome these complications, here we used single freshly isolated mesenteric artery SMCs and fast x-y confocal Ca\textsuperscript{2+} imaging and analyzed relative contribution of IP\textsubscript{3}Rs and ryanodine receptors (RyRs) to elevation of [Ca\textsuperscript{2+}], in response to P2X receptor activation. Similarly to that, what we have previously reported in renal microvascular SMCs [6], we found that P2X receptor activation induces IP\textsubscript{3}R-mediated Ca\textsuperscript{2+} release from sub-plasmalemmal (“junctional”) sarcoplasmic reticulum (jSR) enriched with IP\textsubscript{3}Rs but poor in RyRs. This IP\textsubscript{3}R-mediated Ca\textsuperscript{2+} release seems to be tightly coupled to activation of VGCCs, which occur due to depolarization caused by P2X-mediated cationic current. Thus, our results further support the notion that, in contrast to cardiac muscle, excitation–contraction coupling in vascular smooth muscle may occur by Ca\textsuperscript{2+} entry through VGCCs which evokes an initial IP\textsubscript{3}R-mediated Ca\textsuperscript{2+} release activated via a CICR mechanism.

A preliminary account of this study was previously published in abstract form [7].

Materials and methods

Animals

Male guinea-pigs 250–350 g were humanely killed by decapitation after cervical dislocation as approved under Schedule 1 of the UK Animals (Scientific Procedures) Act 1986 and by the Animal Care Committee of Bogomoletz Institute of Physiology.

Experimental solutions and reagents

Physiological salt solution (PSS) was composed of (mM): NaCl 120, KCl 6, CaCl\textsubscript{2} 2.5, MgCl\textsubscript{2} 1.2, glucose 12, HEPES 10, pH was adjusted to 7.4 with NaOH.

Fluo-3 acetoxymethyl ester (fluo-3 AM), fluo-4 acetoxymethyl ester (fluo-4 AM), pluronic F-127 and CellMask\textsuperscript{TM}Orange stain were obtained from Invitrogen Ltd. (Paisley, UK). Protease (Type X), collagenase (Type 1A), soybean trypsin inhibitor (Type II-S), bovine serum albumin, N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES), dimethyl sulfoxide (DMSO), \alpha\textsubscript{B}-methylene-adenosine-5-triphosphate (\alpha\textsubscript{B}-meATP, lithium salt), nicardipine, 1,3,7-trimethylxanthine (caffeine), wortmannin, 1-[6-((17b)-3-methoxyestra-1, 3,5(10)-trien-17-yl][amino]hexyl]-1H-pyrorrole-2,5-dione (U-73122), 1-[6-((17b)-3-methoxyestra-1,3,5(10)-trien-17-yl)[amino]hexyl]-2,5-pyridilinedione (U-73343) were obtained from Sigma Chemical Co., Poole, Dorset, UK. Cyclopiazonic acid (CPA), ryanodine was from Calbiochem, USA. All other chemicals were from BDH Laboratory Supplies (AnalaR grade), Pool, UK.

Cell preparation

Experiments were carried out on single smooth muscle cells (SMCs) freshly isolated using enzymic dispersion of 3rd to 7th order branches of the guinea-pig mesenteric artery, as described previously [1,8]. Small aliquots of the cell suspension were then placed in experimental chambers, diluted with PSS and left at 4 °C for 25–40 min to attach to glass cover slips forming the bottom of the experimental chamber. To minimize SMC contraction, 40 μM of wortmannin was added to the bathing solution 10 min before imaging was commenced. All experiments were performed at room temperature (20–25 °C) within 8 h of cell isolation.

Visualization of [Ca\textsuperscript{2+}], changes

Changes in intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{i}) in isolated SMCs were imaged using the high affinity fluorescent Ca\textsuperscript{2+} indicators fluo-3 or fluo-4. The dyes were loaded by 20-min incubation of the SMCs with 5 μM acetoxymethyl ester (AM) of the dye as previously described [1,8–12]. The intensity of fluo-3 or fluo-4 fluorescence was normalized to the averaged fluorescence intensity in the images captured before agonist application and gray-scale coded as indicated by the bars (F/F\textsubscript{0}). The temporal profiles of the agonist-induced [Ca\textsuperscript{2+}], transients are illustrated by the plots showing the time course of the normalized intensity of fluo-3 or fluo-4 fluorescence (F/F\textsubscript{0}) averaged either (1) within multiple sub-plasmalemmal regions where F/F\textsubscript{0} changes were initiated and rose above 1.5 or (2) within entire confocal optical slice of smooth muscle cell, or (3) within 4 neighboring pixels along the scan line at the sites depicted by arrows on the line-scan image [1,6,8–12].

Drug application

10 μM \alpha\textsubscript{B}-methylene-adenosine 5’-triphosphate (\alpha\textsubscript{B}-meATP) or 3 mM caffeine was applied to the SMCs as a brief (<300 ms) pulse through a glass micropipette (located within 100–200 μm of the cell) attached to the outlet of pressure ejector Picospritzer III (Intracel Ltd., UK). Similar application of the control solution (without agonist) had no effect on [Ca\textsuperscript{2+}]. In the experiments where the same SMC was stimulated with \alpha\textsubscript{B}-meATP or caffeine, the agonist containing micropipette was replaced in between successive agonist applications. Antagonists were superfused through the experimental bath.

Confocal microscopy

The SMCs were imaged using a LSM 5 PASCAL laser scanning confocal microscope (Carl Zeiss, Jena, Germany). The x–y confocal images were acquired at 20–40 Hz using a Zeiss plan-Apochromat 40 × 1.4 N.A. oil-immersion objective. The excitation beam was produced by the 488 nm line of a 200 mW argon ion laser and illumination intensity was attenuated to 0.6–0.7%. Fluo-3 fluorescence was captured at wavelengths above 505 nm. To optimize signal quality the pinhole was set to provide a confocal optical section below 1.2 μm (measured with 0.2 μm fluorescent beads). The focus was adjusted to acquire the images from the middle of
the myocyte depth. In the images acquired in this way, the events initiated within 1 μm of the cell surface (edge) were considered to have sub-plasmalemmal origin and their temporal profiles were analyzed (see Fig. 1). To improve temporal resolution and to relate the dynamics of [Ca^{2+}] changes to the position of the cell plasma membrane (PM), in some experiments [Ca^{2+}] imaging was performed using the line-scan mode of the confocal scanner. The scan line was set to cross PM in parallel with long axis of the myocyte and scanned at 0.38 ms/line (confocal optical section < 0.9 μm). In these experiments, the PM was visualized with CellMask™Orange by incubating the SMCs for 30 s with 2.5 μg/ml of the dye. The fluo-4 fluorescence was excited at 488 nm and captured at 505–530 nm; the CellMask™Orange fluorescence was excited at 543 nm and captured above 560 nm. The line-scan images were formed by aligning (from left to right) the successive fluorescence images of the scan line so that the horizontal dimension of the images is time (increasing from left to right) and the vertical dimension is the position along the scan line [8–12].

Immunostaining of RyRs and IP3Rs

The sarcoplasmic reticulum (SR) in isolated SMCs was stained by 30-min incubation of the cells with 2 μM Brefeldin A BODIPY 558/568 [6], and IP3Rs and RyRs were immunostained as previously described [6,11]. Briefly, to visualize type a IP3Rs, specific antibody developed in rabbit were used at 1:300 dilution (Sigma–Aldrich Co., RBI, Natick, MA, USA), while RyRs were detected with a monoclonal mouse anti–RyR antibody at 1:480 dilution (Sigma–Aldrich Co., RBI, Natick, MA, USA). The primary antibody binding was visualized by incubating the SMCs with

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**Fig. 1.** The αβ-meATP-induced [Ca^{2+}] responses in mesenteric artery SMCs were visualized with x–y (A) and line-scan (B) confocal Ca^{2+} imaging. The plot (A) shows the time course of self-normalized fluo-3 fluorescence averaged (gray trace) within sub-PM regions of initiation (inset left) and (black trace) within total confocal optical slice of the SMC. The difference in the kinetics of the response is highlighted on the expanded time scale (inset right). The gallery (below) shows confocal images captured during the period highlighted in the plot by gray background. Line-scan images (B) of CellMask™Orange (top) and fluo-4 (middle) fluorescence and their overlay (bottom) illustrate that αβ-meATP-induced [Ca^{2+}] wave originates at sub-PM region and propagates toward the cell center. The time course of fluo-4 fluorescence (averaged over 4 pixels) at the sites, depicted by black and gray arrows on the line-scan image (middle), is shown on the plot (right) in corresponding color.
Alexa Fluor 633 conjugated to chicken anti-rabbit IgG (1:300 dilution, Invitrogen Ltd., UK) and Alexa Fluor 488 conjugated to goat anti-mouse IgG (1:300 dilution, Invitrogen Ltd., UK). In controls, primary antibodies were omitted from the experimental media. Before imaging, the SMCs were embedded in mounting medium containing DAPI to visualize the cell nucleus.

Experimental chambers were placed on the stage of an Axiovert 200M inverted microscope attached to a LSM 510 META laser-scanning unit (Zeiss, Oberkochen, Germany). The x-y confocal images were acquired using a Zeiss plan-Apochromat 63 × 1.4 N.A. oil-immersion objective. The pinhole was set to provide a confocal optical slice below 0.6 μm. To avoid any bleed-through in immunofluorescence experiments, SMCs were labeled using fluorophores with extremely well separated emission spectra: DAPI (λ(em) = 460 nm), Alexa Fluor 488 (λ(em) = 519 nm), Brefeldin A BODIPY 558/568 (λ(em) = 568 nm) and Alexa Fluor 633 (λ(em) = 647 nm). The multitrack mode of an LSM 510 META confocal microscope was used. The adequacy of the imaging protocol was confirmed by control experiments on the single-labeled SMCs.

Data analysis and statistical procedures

In all the experiments αβ-meATP or caffeine were applied to the same SMC at least twice with a 10-min interval to allow for Ca\(^{2+}\) store refilling and P2XR re-sensitization. The peak amplitude of the response was assessed from relative change in the intensity of fluo-3 fluorescence (ΔF/Δt). The response to the succeeding application of the agonist (Test) was related to the response to the first application (Control) in each case. The test response was obtained either in control conditions (to evaluate reproducibility of the agonist-induced [Ca\(^{2+}\)]\(_i\) transients) or following a 10-min incubation with a drug or combination of several drugs [6,11]. Image processing was carried out using custom routines written in IDL (Research Systems Inc., Boulder, CO, USA). The final figures were produced using MicroCal Origin (MicroCal Software Inc., Northampton, MA, USA) and CorelDraw 7.0 (Corel Corporation, Canada). Where appropriate, the data are expressed as mean values ± SEM for the number of cells (n) analyzed. Comparative analysis of the data groups was performed using Student’s t-test for paired or unpaired samples, as appropriate, with the threshold for statistical significance set at the 0.05 level.

Results

Both fast x-y (Fig. 1A) and line-scan (Fig. 1B) confocal Ca\(^{2+}\) imaging revealed that the response to stimulation of P2XRs in the guinea-pig mesenteric artery SMCs with 10 μM αβ-meATP was initiated as an abrupt increase of [Ca\(^{2+}\)]\(_i\) at multiple sub-plasmalemmal regions, a sub-PM [Ca\(^{2+}\)] upstroke (SPCU) [11], not unlike the responses to stimulation of muscarinic cholinoreceptors or P2X receptors that we have previously reported in intestinal SMCs [11,12] and renal microvascular myocytes [6], respectively.

Reproducibility of the amplitude of SPCUs elicited by repetitive (with a 10-min interval) stimulation of mesenteric artery SMCs with 10 μM αβ-meATP, was found to be (see Methods)

![Fig. 2. 2-APB (30 μM) exerts no effect on (A) the Ca\(^{2+}\) entry mechanisms induced by P2X receptor activation and (B) the sarcoplasmic reticulum (SR) calcium load. Left panels (A, B) illustrate experimental protocols. Depleting the calcium store with 10 μM CPA unmasked transmembrane Ca\(^{2+}\) entry induced by P2X receptor stimulation (A). The peak amplitude of the response to caffeine (ΔF/Δt) reflects the SR calcium load (B). Note that 2-APB had no statistically significant effect on mean amplitude of the αβ-meATP-(after CPA) and caffeine-induced responses: p = 0.53 (A) and p = 0.37 (B).]
81 ± 1.4% (n = 52) [1]. Bearing this in mind, we tested the effects of inhibition of RyRs, IP$_3$Rs, phospholipase C (PLC) and VGCCs on αβ-meATP-induced [Ca$^{2+}$]$\text{C6}$ responses in these SMCs.

**Contribution of RyRs and IP$_3$Rs to the SR Ca$^{2+}$ release following P2X receptor activation**

To access contributions of RyRs and possible involvement of IP$_3$Rs to the SR Ca$^{2+}$ release following P2X receptor activation, tetracaine [11,13,14] or ryanodine [6,9] and 2-aminoethoxydiphenyl borate (2-APB) [6,11] were used as pharmacological tools to block RyRs or IP$_3$Rs, respectively. In some experiments cyclopiazonic acid (CPA) [1,6,11] was used to deplete the calcium store by inhibition of sarco-/endoplasmic reticulum Ca$^{2+}$-ATPase (SERCA) [1,6,11,15–17].

Since it was previously reported that 2-APB may affect mechanisms of intracellular Ca$^{2+}$ homeostasis other than those involving IP$_3$Rs, such as inhibition of Ca$^{2+}$-permeable cation channels and SERCA [18,19], an important aspect of our experimental strategy was to test the effects of 30 μM 2-APB on the SR calcium load and Ca$^{2+}$ entry mechanisms engaged by P2X receptor activation in mesenteric artery SMCs (Fig. 2).

To examine whether 2-APB influences the Ca$^{2+}$ entry mechanisms mobilized by P2X receptor stimulation, the intracellular calcium stores were depleted by 10 min incubation with 10 μM CPA prior to stimulation with αβ-meATP [6]. With depleted calcium stores, αβ-meATP-induced [Ca$^{2+}$]$\text{C6}$ transients result solely from transmembrane Ca$^{2+}$ influx and, as illustrated in Fig. 2A, were insensitive to 30 μM 2-APB (n = 7). This observation is in agreement with our previous demonstrations that 2-APB does not inhibit currents through either VGCCs [11] or P2XRs [6]. Possible sensitivity of the SR calcium load mechanisms to 2-APB was assessed with 3 μM caffeine applied in control conditions and after 10 min incubation with 30 μM 2-APB (Fig. 2B). Both application of caffeine evoked similar [Ca$^{2+}$]$\text{C6}$ transients (n = 8) suggesting that 30 μM 2-APB does not affect the SR calcium load in our preparation, not unlike we have previously reported in renal microvascular SMCs [6]. Taken together the above observations validate 30 μM 2APB as selective probe for IP$_3$R-mediated Ca$^{2+}$ release in mesenteric artery SMCs.

To analyze contribution of IP$_3$Rs and RyRs to the SR Ca$^{2+}$ release following P2X receptor activation we related the amplitude of [Ca$^{2+}$]$\text{C6}$ transients elicited by 10 μM αβ-meATP in the presence of 30 μM 2-APB (Fig. 3A) or 100 μM tetracaine (Fig. 3B) to that obtained in the same myocyte in control conditions. The effect of IP$_3$R inhibition with 30 μM 2-APB was found to be significantly (p < 0.001) stronger than that of RyR inhibition with 100 μM tetracaine: the SPCU amplitude in the presence of 2-APB (when RyRs are unaffected) was reduced by 67 ± 7% (n = 16), while in the presence of tetracaine (when IP$_3$Rs are unaffected) was reduced by 40 ± 5% (n = 26) of that in control (Fig. 3C).

**Functional coupling between VGCCs and the SR Ca$^{2+}$-release channels**

To assess the link between Ca$^{2+}$ entry and Ca$^{2+}$ release mechanisms that take a place after P2X receptor activation we

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**Fig. 3.** The effects of IP$_3$R (A) and RyR (B) inhibition on αβ-meATP-induced SPCU are compared. The plots show the time course of normalized fluo-3 fluorescence (F/F$_0$) averaged at multiple sub-PM regions of initiation. The images taken during the periods highlighted on the plots by gray background are presented below the plots, (i) and (ii), respectively. Normalized fluo-3 fluorescence in the images is gray scale coded (bar: F/F$_0$). The bar diagram plot (C) compares 2-APB-sensitive and tetracaine-sensitive fractions of the αβ-meATP-induced response. ***p < 0.001.
evaluated how relative contribution of IP$_3$Rs and RyRs to the αβ-meATP-induced [Ca$^{2+}$]$_i$ transients is altered by block of VGCCs. The results are presented in Fig. 4. In the presence of 5 μM nicardipine intracellular Ca$^{2+}$ signaling is triggered by Ca$^{2+}$ that enters the SMC solely via P2XRs [1]. Under these conditions, relative contribution of IP$_3$R-mediated Ca$^{2+}$ release to the αβ-meATP-induced [Ca$^{2+}$]$_i$ transient became lesser (37 ± 3%, n = 9) than contribution of RyRs (63 ± 3%, n = 7). Significant attenuation (p < 0.001) of the IP$_3$R contribution to the SPCU in the presence of nicardipine implies that IP$_3$R-mediated Ca$^{2+}$ release is mainly linked to activation of VGCCs.

Summary of the effects of the SR Ca$^{2+}$-release channel antagonists in control solution and following block of VGCCs is presented in Fig. 5A and B, which compares amplitudes of SPCU induced by P2X receptor activation under conditions of (1) preserved VGCC-mediated Ca$^{2+}$ entry component (Fig. 5A) and (2) Ca$^{2+}$ entry mediated solely via P2XRs (Fig. 5B). Inhibition of VGCCs resulted in substantial decrease of IP$_3$R contribution to the αβ-meATP-induced [Ca$^{2+}$]$_i$ transient. Indeed, the tetracaine/ryanodine-resistant fraction (the main contributor of which is IP$_3$R-mediated Ca$^{2+}$ release; see also [1,6]) decreased after block of VGCCs more than 4 times: from 60 ± 5% (n = 26)/62 ± 6% (n = 3) to 16.6 ± 2% (n = 7)/18 ± 3 (n = 3), respectively. At the same time 2-APB-resistant fraction (which arises mainly from RyR-mediated Ca$^{2+}$ release) decreased only by 10%; from 33 ± 7% (n = 16) to 22.7 ± 3% (n = 9). It should be noted, however, that fraction of the αβ-meATP-induced fluo-3 response persisting in the presence of nicardipine (36 ± 3%; n = 22) was similar to that remaining after block of IP$_3$Rs with 2-APB (33 ± 7%; n = 16). This suggests that: (1) direct Ca$^{2+}$ influx via VGCCs per se makes rather small contribution to the αβ-meATP-induced response, but is considerably amplified by IP$_3$R-mediated Ca$^{2+}$ release (see also [1]), and (2) activation of VGCCs is not the only mechanism which couples P2X receptor activation to the IP$_3$R-mediated Ca$^{2+}$ release.

While P2XRs do not signal via phospholipase C (PLC)-linked pathway, basal levels of [IP$_3$]$_i$, produced by spontaneous activity of PLC [6,9,20–22] may play a permissive role in activation of IP$_3$R-mediated Ca$^{2+}$ release by voltage-gated Ca$^{2+}$ entry. We, therefore, tested the effect of commercially available PLC inhibitors on the amplitude of the αβ-meATP-induced [Ca$^{2+}$]$_i$ transients. The aminosteroid compound U-73122 and its relatively inactive analog U-73343 [23] both significantly reduced the response (Fig. 5C). However, the effect of 2.5 mM U-73122 (the reduction of the amplitude by 65 ± 7%; n = 5) was significantly stronger than that of 2.5 mM U-73343 (the reduction of the amplitude by 42 ± 5%; n = 4). Although these compounds are known to have many side effects attributable to alkylation of various proteins [20], the difference between the effect of U-73122 and that of U-73343 suggests that IP$_3$R-mediated Ca$^{2+}$ release following P2XR activation depends on PLC activity.

The functional coupling between VGCCs and IP$_3$Rs suggests the expression of IP$_3$Rs in sub-plasmalemmal "junctional" SR (jSR) elements. The structural basis for this coupling could be spatial co-localization of VGCCs in the plasma membrane and IP$_3$Rs in sub-plasmalemmal SR elements. Spatial co-localization between IP$_3$Rs and VGCCs was previously suggested in glomerular cells [24].
Fig. 5. Summary of the effects of VGCC/IP3R/RyRs/P2XRs inhibitors on αβ-meATP-induced SPCU. Bar diagram plots compare the peak amplitude of the αβ-meATP-induced fluo-3 response with its tetracaine/ryanodine- or 2-APB-resistant fractions before (A) and after (B) block of VGCCs. Note that block of VGCCs have much stronger effect on the tetracaine-resistant fraction than on 2-APB-resistant one. "Untreated" refers to the Ca2+-release channel blockers. Involvement of IP3R in activation of IP3, R-mediated Ca2+ release following P2X receptor stimulation was assessed by PLC inhibition (C). The bar diagram plot compares the peak amplitude of the αβ-meATP-induced fluo-3 response with its U-73122- and U-73343-resistant fractions. ***p < 0.001.

their functional coupling as well as predominant expression of type 1 IP3R in jSR was recently demonstrated in SMCs from the guinea-pig ileum [11] and renal microvascular smooth muscle cells [6].

Sub-plasmalemmal SR is enriched with IP3Rs

Intracellular Ca2+ stores, visualized in mesenteric artery SMCs with the low-affinity Ca2+ indicator fluo-3FF, coincided with SR elements, visualized with Brefeldin A BODIPY (n = 12), and consisted of some central elements and a sub-plasmalemmal SR network (Fig. 6A). Similar organization of the SR was previously reported in other SMC types [8,10,11]. Immunostaining of RyRs and type 1 IP3Rs in the guinea-pig mesenteric artery SMCs, in which the SR was stained with Brefeldin A BODIPY and nucleus was stained with DAPI revealed (Fig. 6B) that IP3Rs are predominantly expressed in sub-plasmalemmal SR elements, while RyR are mainly located in the central deeper SR (n = 15), particularly in the perinuclear region of the myocyte, not unlike in renal microvascular myocytes [6]. This peculiarity of IP3R distribution favors VGCC-IP3R coupling, which could be facilitated by accumulation of molecules, including Ca2+ [16,25] and IP3 in the cytosolic microvolume between jSR and plasmalemma microdomains from which the diffusion into the bulk cytoplasm is markedly limited [16].

Discussion

Previously we have demonstrated that upon P2X receptor activation, SMCs from the guinea-pig small mesenteric arteries employ an effective mechanism of the Ca2+ signal amplification, the major component of which is Ca2+ release from the SR, which is activated mainly by Ca2+ influx via L-type VGCCs [1]. Signaling via VGCC pathway, which is engaged by depolarization of the myocyte membrane elicited by P2X receptor-mediated inward cationic current, was found to be more profound than signaling via Ca2+ permeable P2XRs per se. Here we demonstrate a significant attenuation of the P2X-linked [Ca2+], responses by inhibition of either IP3Rs or RyRs. This decrease could not be attributed to possible non-specific effects of the Ca2+-release channel inhibitors on either Ca2+ influx mechanisms or SERCA activity, thus suggesting that both RyRs and IP3R are involved in purinergic regulation of circulation [2]. Relative contribution of IP3Rs to Ca2+ release was found to be much stronger than that of RyRs, even though it is unlikely that stimulation of vascular myocytes with 10 μM αβ-meATP recruits any metabotropic receptors coupled to a Gq/11/PLC/IP3 signaling pathway [6]. Although activation of PLC-B by Gq is accelerated at [Ca2+], levels achieved during cell signaling, direct activation of PLC-B by Ca2+ is also unlikely [20,26]. Thus, our finding raises the question about source of IP3 recruited following P2X receptor activation. One possibility could be continuous presence of basal IP3 in the cytosol resulting from spontaneous background activity of PLC [9,20–22]. Because of diffusional limitations, the background IP3, within narrow gaps between membrane and jSR [16,25] could be essentially higher than in the bulk cytosol. IP3 activates the IP3Rs mainly via decrease of their sensitivity to an inhibitory effect of elevated [Ca2+], without altering the activation properties [27,28]. Under these conditions IP3-R could serve as CICR-channel [11] even without concurrent increase of [IP3], especially in the presence of other factors that increase sensitivity of IP3-R to Ca2+. One of these factors could be ATP, which is known at physiological intracellular concentrations to increase sensitivity of IP3-Rs to Ca2+. It was demonstrated that IP3 and ATP may act as allostERIC modulators of IP3-Rs, either decreasing deactivation of IP3-R by elevated [Ca2+], or increasing its Ca2+ sensitivity upon activation, respectively [28]. Other factors that are known to be involved in regulation of IP3-R, and perhaps its Ca2+ sensitivity, are calmodulin [29], the family of Ca2+-binding proteins, CaBP [30] and the whole range of proteins, which is engaged in intracellular Ca2+ signaling [9,28]. Thus, basal level of cytoplasmic IP3 together with possible modulators of IP3-Rs could play a permissive role in activation of IP3-R-mediated Ca2+ release by P2XRs and voltage-gated Ca2+ entry.

Suggested above mechanisms of IP3-R recruitment to purinergic regulation of circulation should not discriminate between Ca2+ entering via P2XRs or VGCCs. However, our experiments with nicardipine revealed that inhibition of VGCCs affects the contribution of IP3-Rs to purinergic Ca2+ signaling significantly more than that of RyRs. If IP3-Rs are equally accessible for Ca2+ entering the cell through either P2XRs or VGCCs, then block of one of these Ca2+ entry pathways should not alter relative contribution of the RyR-mediated and IP3-R-mediated Ca2+ release to resulting intracellular Ca2+ signal. Our results indicate that this is not the case. Indeed, following the block of VGCCs, relative contribution of IP3-R-mediated Ca2+-release to the αβ-meATP-induced [Ca2+], response
(which was dominating in nicardipine-free solution) became lesser then contribution of RyR-mediated Ca$^{2+}$-release. This observation strongly suggests that IP$_3$Rs activation is linked to activation of VGCCs.

Additional support for this hypothesis comes from the observation that nicardpine, VGCCs blocker, inhibits approximately the same fraction of the $\alpha\beta$-meATP-induced [Ca$^{2+}$], response as 2-APB, the IP$_3$R blocker (Fig. 5). Since 2-APB does not affect Ca$^{2+}$ entry mechanisms per se (Fig. 2) this finding suggests that Ca$^{2+}$-release via IP$_3$Rs depends mainly on activation of VGCCs. At least two mechanisms are consistent with observed recruitment of IP$_3$ to purinergic signaling pathways. Many recent reports demonstrated that G protein/PLC/InsP$_3$ pathway could be triggered by activation of VGCCs. The ability of VGCC activation, either by membrane depolarization or agonist, to trigger activation of G protein-mediated IP$_3$ synthesis and Ca$^{2+}$ release via IP$_3$Rs was
demonstrated in variety of vascular SMCs. This mechanism has been referred to as calcium channel-induced calcium release (CCICR) [reviewed in 31]. If such metabotropic action of L-type VGCCs takes place in our preparation it could account for both, the mechanism of IP₃ involvement in purinergic signaling and much more effective amplification of Ca²⁺ signaling via VGCCs than via P2XRs. However, it does not explain residual 2-APB sensitivity of the αβ3-meATP-induced [Ca²⁺]ᵢ response, which persists following block of VGCCs with nicardipine (Fig. 5). This, however, could be readily explained in terms of spontaneous background activity of PLC serving to maintain [IP₃]ᵢ at a level, sufficient to permit activation of IP₃Rs by voltage-gated Ca²⁺ entry (see Fig. 5C). Indeed, it is now well appreciated that JSR (which lies within 12–15 nm of plasmalemma), the overlying plasmalemma microdomains, and the intervening, tiny volume of cytosol form junctional complexes that serve as the Ca²⁺ “buffer barrier”, through which Ca²⁺ can move directly between the extracellular fluid and JSR [16,25]. This may facilitate (1) local elevation of luminal [Ca²⁺]ᵢ in JSR and (2) accumulation of molecules, including Ca²⁺, Na⁺ [16,25], and IP₃, in the cytosolic microvolume from which the diffusion into the bulk cytoplasm is markedly limited [16]. Taking this into account, the following factors may favor activation of IP₃Rs by Ca²⁺ influx: (1) robust increase of [Ca²⁺]ᵢ in the junctional cytosolic microvolume [16,25], (2) spontaneous basal activity of PLC [9,20–22], (3) Ca²⁺ activation of type 1 IP₃Rs with positive cooperatively [28], (4) regulation of IP₃Rs by the SR luminal [Ca²⁺] [32] and (5) IP₃R clustering [33].

Predominant expression of IP₃Rs in JSR, may not only facilitate recruitment of IP₃ in purinergic signaling, but also justify IP₃R-mediated Ca²⁺ release as a major contributor to elevation of [Ca²⁺]ᵢ, following P2X receptor activation. Immunodetection of RyRs and type 1 IP₃Rs in mesenteric artery SMCs with stained SR and nucleus (Fig. 6) revealed that IP₃Rs are predominantly expressed in subplasmalemmal JSR elements, while RyR are mainly located in the central deeper SR in the perinuclear region of the myocyte. This structural peculiarity makes IP₃Rs more accessible to Ca²⁺ entering the cell through the membrane. Although co-localization VGCCs and IP₃Rs has not been directly demonstrated in this study by means of immunocytochemistry or electron microscopy, subplasmalemmal location of IP₃Rs suggests them as primary target for Ca²⁺ entering the cell, while RyRs in deeper SR elements could be activated at a later stage by Ca²⁺ released via IP₃Rs, thus providing further amplification of IP₃-R-mediated Ca²⁺ signals. Furthermore, extracellular ATP has been recently shown to produce selective facilitation of L-type VGCC activation and strong potentiation of IP₃-R-mediated Ca²⁺ release in arterial myocytes [34]. Thus, IP₃Rs may serve as a functional link (“intermediate amplifier”) between voltage-gated Ca²⁺ entry and RyR-mediated Ca²⁺ release [9,11].

In conclusion, the results of this study demonstrate that in mesenteric artery SMCs apart from RyR-mediated Ca²⁺ release activation of P2XR5s induces robust Ca²⁺ release via IP₃Rs, which appears to be a major contributor to elevation of [Ca²⁺]ᵢ and depends mainly on activation of dihydropyridine sensitive VGCCs. Because IP₃-R-mediated purinergic signaling in vascular SMC is sensitive to Ca²⁺ channels antagonists, our observations might have special pathophysiological and pharmacological significance.

Precise mechanisms of the recruitment of IP₃Rs to P2X-mediated Ca²⁺ signaling in vascular myocytes await further investigation.

Conflict of interest

There are no known conflicts of interest associated with this publication.

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References


