Mechanisms of $[\text{Ca}^{2+}]_i$ elevation following P2X receptor activation in the guinea-pig small mesenteric artery myocytes

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Abstract:

Background: There is growing evidence suggesting involvement of L-type voltage-gated Ca$^{2+}$ channels (VGCCs) in purinergic signaling mechanisms. However, detailed interplay between VGCCs and P2X receptors in intracellular Ca$^{2+}$ mobilization is not well understood. This study examined relative contribution of the Ca$^{2+}$ entry mechanisms and induced by this entry Ca$^{2+}$ release from the intracellular stores engaged by activation of P2X receptors in smooth muscle cells (SMCs) from the guinea-pig small mesenteric arteries.

Methods: P2X receptors were stimulated by the brief local application of $\alpha\beta$-meATP and changes in $[\text{Ca}^{2+}]_i$ were monitored in fluo-3 loaded SMCs using fast x-y confocal Ca$^{2+}$ imaging. The effects of the block of L-type VGCCs and/or depletion of the intracellular Ca$^{2+}$ stores on $\alpha\beta$-meATP-induced $[\text{Ca}^{2+}]_i$ transients were analyzed.

Results: Our analysis revealed that Ca$^{2+}$ entry via L-type VGCCs is augmented by the Ca$^{2+}$-induced Ca$^{2+}$ release significantly more than Ca$^{2+}$ entry via P2X receptors, even though net Ca$^{2+}$ influxes provided by the two mechanisms are not significantly different.

Conclusions: Thus, arterial SMCs upon P2X receptor activation employ an effective mechanism of the Ca$^{2+}$ signal amplification, the major component of which is the Ca$^{2+}$ release from the SR activated by Ca$^{2+}$ influx via L-type VGCCs. This signaling pathway is engaged by depolarization of the myocyte membrane resulting from activation of P2X receptors, which, being Ca$^{2+}$ permeable, per se form less effective Ca$^{2+}$ signaling pathway. This study, therefore, rescales potential targets for therapeutic intervention in purinergic control of vascular tone.

Key words: confocal microscopy, Ca$^{2+}$ signaling, vascular smooth muscle cells, P2X receptors, voltage-gated calcium channels, Ca$^{2+}$-induced Ca$^{2+}$ release

Abbreviations: $\alpha\beta$-meATP - $\alpha\beta$-methylene-adenosine 5’-triphosphate, CICR – Ca$^{2+}$-induced Ca$^{2+}$ release, CPA – cyclopiazonic acid, IP$_3$ – inositol 1,4,5-trisphosphate, IP$_{3}$R – inositol 1,4,5-trisphosphate receptor, $[\text{Ca}^{2+}]_i$ – intracellular concentration of ionized calcium, jSR – sub-plasmalemmal (“junct- tional”) sarcoplasmic reticulum, RyR – ryanodine receptor,
SERCA – sarco-/endoplasmic reticulum Ca\(^{2+}\)-ATPase, SMC – smooth muscle cells, SPCU – sub-plasmalemmal [Ca\(^{2+}\)], upstroke, SR – sarcoplasmic reticulum, VGCC – voltage-gated Ca\(^{2+}\) channel

**Introduction**

The control of total peripheral vascular resistance, blood flow and contraction of small arteries is mediated by sympathetic nervous system via activation of postjunctional receptors in smooth muscle cell (SMC) plasma membrane by neurotransmitters released from the nerve terminals [8, 27, 52]. One of the principal excitatory neurotransmitters – ATP, released from sympathetic nerves, acts on arterial myocytes via activation of P2X purinoceptors [1, 7]. The family of P2X purinoceptors comprises seven subunits (P2X1–P2X7), each encoded by distinct gene [34]. These subunits can be assembled in various configurations to form functional homo- or heteromeric cation channels [25, 49]. In the cardiovascular system, P2X receptors are expressed predominantly on smooth muscle cells [25, 52]. In rat mesenteric arteries, the predominant P2X receptor is homomeric P2X1 [30].

Cation channels formed by P2X subunits have similar permeability for Na\(^+\) and K\(^+\), and much greater permeability for Ca\(^{2+}\) [12, 25], e.g., relative Ca\(^{2+}\) over Na\(^+\) permeability (P\(_{Ca}\)/P\(_{Na}\)) of 4.8 and 4.2 was reported for P2X1 and P2X4 receptors, respectively [11, 34]. An increase in cationic conductance upon P2X receptor activation results in depolarization of the SMC plasma membrane which, in turn, activates voltage-gated Ca\(^{2+}\) channels (VGCCs) [16, 21]. Ca\(^{2+}\) entering the cell via P2X receptors and VGCCs may potentially trigger Ca\(^{2+}\) release from intracellular calcium stores via Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR) mechanism [13] engaging Ca\(^{2+}\) sensitive Ca\(^{2+}\)-release channels in the sarcoplasmic reticulum (SR) of SMC. However, recruitment of CICR mechanism in SMCs is still an area of debates and controversy [28, 51]. Indeed, relative contribution of the SR Ca\(^{2+}\)-release to intracellular [Ca\(^{2+}\)] mobilization varies in different SMC types, and often depends on the strengths and mechanism of stimulation. Although activation of the SR Ca\(^{2+}\) release by Ca\(^{2+}\) entering the cell via VGCCs was demonstrated in voltage-clamp experiments performed on different types of visceral and vascular SMCs [4, 9, 24, 26, 44], there is a number of studies [e.g., 5, 6] demonstrating that complete depletion of the SR of Ca\(^{2+}\) does not reduce [Ca\(^{2+}\)]\(_{SR}\) transients induced by step-like depolarization of the cell membrane. The latter suggests that CICR is not recruited. An alternative explanation given by Bradley et al. [6] suggests that the SR and sarcolemma may form a passive physical barrier to Ca\(^{2+}\) influx ("Ca\(^{2+}\) trap"), which normally limits the [Ca\(^{2+}\)]\(_{SR}\) rise evoked by depolarization. The drugs, which open the SR Ca\(^{2+}\) release channels and facilitate the SR Ca\(^{2+}\) leak, diminish the influence of "Ca\(^{2+}\) trap" and may, thereby, increase amplitude of [Ca\(^{2+}\)]\(_{SR}\) transients resulting from Ca\(^{2+}\) entry via VGCCs even when the SR contains little or no Ca\(^{2+}\) [6].

Another important aspect of SMC Ca\(^{2+}\) signaling system is difference in the ability of various Ca\(^{2+}\) entry mechanisms to trigger Ca\(^{2+}\) release from the SR. This variability may arise from spatial organization and molecular composition of intracellular Ca\(^{2+}\)-release units [18, 19, 23, 32, 33]. Imaging microdomain Ca\(^{2+}\) in myocytes has reshaped our understanding of Ca\(^{2+}\) signaling and provided direct evidence validating the concept that a closed organelle system contains specialized biochemical functions ("local control concept"; [2]). Furthermore, an emerging and more revolutionary concept is that areas of the cell that are between organelles, as a consequence of their nanostructure, are also structurally specialized regions of distinct and important functions [37, 38]. We have recently demonstrated that in response to activation of P2X receptors in renal microvascular SMCs, Ca\(^{2+}\) entry via VGCCs is the major trigger of CICR, even though relative contribution of P2X receptors to Ca\(^{2+}\) entry under this conditions is greater than that of VGCCs [39]. This suggests co-localization of plasmalemmal VGCCs and the SR Ca\(^{2+}\)-release channels, and "local control" of Ca\(^{2+}\)-release mechanisms in these myocytes. The latter was also supported by the gradual dependence of [Ca\(^{2+}\)]\(_{SR}\); transients on P2X agonist concentration, despite the fact that a regenerative CICR mechanism was recruited.

Participation of P2X receptors in sympathetic control of vascular SMCs offers an attractive therapeutic target mediating substantial vasoconstrictor drive resistant to adrenoceptor antagonists [50]. As sympathetically driven splanchnic vasoconstriction is an important reflex responsible for stabilization of systemic blood pressure during exercise [29], understanding of the mechanisms linking P2X receptor activation to an increase of [Ca\(^{2+}\)]\(_{SR}\) in mesenteric ar-
tery myocytes is of great physiological relevance. In present study we evaluated relative contribution of Ca\(^{2+}\) entry via P2X receptors and VGCCs, and the SR Ca\(^{2+}\) release to elevation of \([\text{Ca}^{2+}]_i\), induced by selective stimulation of P2X purinoceptors in myocytes freshly isolated from the guinea-pig small mesenteric arteries.

A preliminary account of this study was previously published in abstract form [45].

### Materials and Methods

#### Animals

Male guinea-pig 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\(_2\) 2.5, MgCl\(_2\) 1.2, glucose 12, HEPES 10, pH was adjusted to 7.4 with NaOH.

Fluo-3 acetoxymethyl ester (fluo-3 AM) and pluronic F-127 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), \(\alpha,\beta\)-methyleneadenosine 5’-triphosphate \((\alpha\beta\text{-meATP, lithium salt})\), nicardipine, 1,3,7-trimethylxanthine (caffeine), wortmannin were obtained from Sigma Chemical Co., Poole, Dorset, UK. Cyclopiazonic acid (CPA) was from Calbiochem, USA. All other chemicals were from BDH Laboratory Supplies (AnalR grade), Pool, UK.

#### Cell preparation

Experiments were carried out on single smooth muscle cells (SMCs) freshly isolated from 3\(^{rd}\) to 7\(^{th}\) order branches of guinea-pig mesenteric artery as described previously [20]. Briefly, the mesentery was excised and the arteries were cleaned of surrounding tissue, cut out and transferred into Ca\(^{2+}\)-free PSS (see above) supplemented with (mg/ml): collagenase (Type 1A) 1, protease (Type X) 0.5, soybean trypsin inhibitor 0.5, bovine serum albumin 1 and incubated at 37°C for 22–25 min. The pieces of the tissue were then rinsed for 10 min in an enzyme-free Ca\(^{2+}\)-free PSS and gently triturated with a wide bore glass pipette. 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 \([\text{Ca}^{2+}]_i\) changes

Changes in intracellular concentration of ionized calcium (\([\text{Ca}^{2+}]_i\)) in isolated SMCs were imaged using the high affinity fluorescent Ca\(^{2+}\) indicator fluo-3. The dye was loaded by 20-min incubation of the SMCs with 5 µM fluo-3 acetoxymethyl ester (fluo-3 AM; diluted from a stock containing 2 mM fluo-3 AM and 0.025% (w/v) pluronic F-127 in dimethyl sulfoxide) followed by a 40-min wash in PSS to allow time for de-esterification. In the figures the intensity of fluo-3 fluorescence was normalized to the average fluorescence intensity in the images captured before agonist application and grey-scale coded as indicated by the bars (F/F\(_{30}\)). The temporal profiles of the agonist-induced \([\text{Ca}^{2+}]_i\); transients are illustrated by the plots showing: (1) the time course of the normalized fluo-3 fluorescence intensity (F/F\(_{30}\)) averaged within multiple sub-plasmalemmal regions where F/F\(_{30}\) changes were initiated and rose above 1.5 or (2) the time course of F/F\(_{30}\) averaged within entire confocal optical slice of smooth muscle cell [18, 39].

#### Drug application

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

Confocal microscopy

The cells 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 from the middle of the cell surface (edge) were considered to have sub-plasmalemmal origin and their temporal profiles were analyzed (see above).

Electrical recordings

The electrical recordings were performed as described previously [18]. Briefly, membrane currents were recorded using perforated-patch (200 µg/ml amphotericin B) tight seal recording using Multiclamp 700A patch-clamp amplifier (Molecular Devices Co.). Voltage protocols were generated and electrical signals were digitized at 5 kHz using a Digidata 1322A hosted by a PC running pClamp 8.2 software (Molecular Devices Co.). Voltage-gated currents were evoked by 500-ms voltage steps to 0 mV applied from a holding potential of –80 mV. In order to augment low-amplitude currents through the voltage gated L-type calcium channels, in these experiments Ba²⁺ (5 mM) was used instead of Ca²⁺ as a charge carrier. The extracellular solution was composed of (in mM): NaCl 135, CsCl 5.8, BaCl₂ 5, MgCl₂ 1.2, D-glucose 10, HEPES 10 (pH was adjusted to 7.4 with CsOH). The pipette solution contained (in mM): CsCl 126, NaCl 5, HEPES 10 (pH was adjusted to 7.4 with CsOH).

Data analysis and statistical procedures

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 the 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

The rise of intracellular concentration of ionized calcium ([Ca²⁺]ᵢ) following activation of P2X receptors is a result of combined contribution of several related processes: Ca²⁺ entry across the plasma membrane through the P2X receptor pores themselves, Ca²⁺ entry via voltage-gated Ca²⁺ channel (VGCCs) activated by P2X-receptor-mediated membrane depolarization, and Ca²⁺ release from the SR Ca²⁺ stores, triggered by Ca²⁺ entry.

Our strategy in estimation of contributions of these components to the total Ca²⁺ signal was based on elimination of Ca²⁺ entry via VGCCs using nicardipine, blocker of L-type VGCCs, and/or exclusion of the sarcoplasmic reticulum (SR) Ca²⁺ release by depletion of the intracellular Ca²⁺ stores using cyclopiazonic acid (CPA), a reversible inhibitor of the sarco/endo-plasmic reticulum Ca²⁺-ATPase (SERCA). When intracellular Ca²⁺ stores are intact, inhibition of VGCCs provides an estimate of relative contribution of the two Ca²⁺ entry mechanisms to generation of the global Ca²⁺ response. Indeed, each of the two components estimated using this approach consists of Ca²⁺ entering the cell across the plasma membrane through corresponding channel pore and Ca²⁺ released from the intracellular stores. On the other hand, when Ca²⁺ stores are depleted with CPA, only Ca²⁺ entry across the plasma membrane contributes to the total Ca²⁺ signal. Combining CPA with VGCCs antagonist allows, therefore, separating Ca²⁺ signals arising from Ca²⁺ influx through the P2X receptors and VGCCs.

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This experimental strategy involves at least three successive agonist applications and, therefore, requires a high reproducibility of αβ-meATP-induced [Ca\(^{2+}\)] transients. This was tested in separate experiments where the amplitudes of the responses to several successive applications of 10 µM αβ-meATP performed with 10 min intervals (to allow for Ca\(^{2+}\) store refilling and P2X receptor re-sensitization) were compared (Fig. 1). Three plots (Fig. 1A) illustrate the time course of the normalized intensity of fluo-3 fluorescence (F/F<sub>0</sub>) averaged at multiple sub-plasmalemmal regions of the response initiation (see Methods). Reproducibility defined as the ratio of the amplitude of succeeding response to that of the preceding one [39] was found to be 81 ± 1.39% (n = 52).

The galleries below the plots (Fig. 1B) show sequential (from left to right) images taken during the periods highlighted in the plots (Fig. 1A; i-iii, respectively). Another important feature clearly seen on the images is a remarkable constancy of sub-plasmalemmal positions of the sites where elevation of [Ca\(^{2+}\)] associated with each αβ-meATP application was initiated. This validates analysis of subplasmalemmal Ca\(^{2+}\) upstroke (SPCU) [18, 39] in all subsequent experiments.

An important aspect of our experimental strategy was to verify a method for selective and complete Ca\(^{2+}\) store depletion. It was previously reported that cyclopiazonic acid (CPA), the reversible SERCA inhibitor, exerts no direct effect on VGCCs, Ca\(^{2+}\)-dependent K\(^+\) channels or voltage-dependent K\(^+\) channels [15, 47]. Nevertheless, taking into account that any possible direct effect of CPA on VGCCs would distort fractional analysis of the αβ-meATP-induced [Ca\(^{2+}\)] transients (see below), we tested the effect of 10 min incubation with 10 µM CPA on VGCC-mediated currents in voltage-clamp experiments (Fig. 2). Stimulation of the myocytes with voltage step to 0 mV from the holding potential of –80 mV resulted in a rapidly activating inward current (Fig. 2A) with mean peak current density of –1.99 ± 0.34 pA/pF (n = 5). The properties of the current were consistent with characteristics of the current through the L-type

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**Fig. 1.** Reproducibility of [Ca\(^{2+}\)] transients induced by αβ-meATP in the guinea-pig small mesenteric artery smooth muscle cells (SMCs). The time course plot (A) reflects changes in [Ca\(^{2+}\)] induced by P2X receptor stimulation in fluo-3-loaded SMC. The 300-ms pulses of 10 µM αβ-meATP were applied to the same SMC with 10-min interval. The drug applications are denoted by black bars above the traces. The intensity fluo-3 fluorescence averaged at multiple sub-plasmalemmal regions of initiation (see insets) was normalized to the average fluorescence intensity in these regions before agonist application (F/F<sub>0</sub>). Three galleries (B) of 30 sequential images (after rotation by 90°) captured during the periods highlighted in the plot by grey background (A, i-iii, respectively) illustrate spatial pattern of [Ca\(^{2+}\)] elevation in response to each αβ-meATP application. The intensity of self-normalized fluo-3 fluorescence in the images was grey-scale coded, as indicated by the bar (F/F<sub>0</sub>).
**Fig. 2.** Ten minutes incubation with 10 µM CPA did not affect the current via L-type VGCCs. Original current traces were obtained from the same myocyte before and following 10 min incubation with 10 µM CPA (experimental protocol is shown above the traces) using perforated-patch tight seal recording in Ba²⁺-containing (see Methods) solution (A). Statistical analysis revealed no significant (p = 0.35569) changes in the peak current density following incubation with CPA (B).

**Fig. 3.** Ten minutes incubation with 10 µM CPA completely depleted intracellular Ca²⁺ stores in the guinea-pig small mesenteric artery SMCs. The Ca²⁺ store load was tested with caffeine, a well known Ca²⁺-liberating agent. Fluo-3 loaded SMCs were stimulated with 300-ms pulses of 3 mM caffeine applied with 10-min interval (A). The response to the second stimulation (Test) was related to the response to the first stimulation (Control). The Test response was obtained either in control conditions (upper panel; “Untreated” in B) or following 10-min incubation with 10 µM CPA (lower panel). The peak amplitude \((\Delta F/F_0)\) of the Test response was normalized to that of the Control response in both cases and compared (B). The αβ-meATP-induced [Ca²⁺] transient in the SMC with depleted Ca²⁺ stores (confirmed by lack of the response to caffeine) arises from Ca²⁺ influx evoked by P2X receptor activation (C). *** p < 0.001
voltage-gated Ca$^{2+}$ channels previously reported in this cell type [41]. Incubation of the cell with 10 µM CPA did not alter kinetics of the current (Fig. 2A) and had no statistically significant effect ($p = 0.35569$, paired Student’s $t$-test) on the current amplitude (Fig. 2B): the peak current density in the presence CPA was on average of $-1.90 \pm 0.31$ pA/pF ($n = 5$).

Now, when no direct effect of CPA on VGCC-mediated current was confirmed, the remaining question is to what extent Ca$^{2+}$ store in mesenteric artery SMCs is depleted by 10 min incubation with 10 µM CPA. This was tested in separate experiments (Fig. 3). We found that 10 min incubation of the SMCs with 10 µM CPA completely abolished $[\text{Ca}^{2+}]_i$ transients.

**Fig. 4.** Ca$^{2+}$ entry via voltage-gated Ca$^{2+}$ channels (VGCCs) following P2X receptor activation in the guinea-pig mesenteric artery SMCs induces Ca$^{2+}$ release from intracellular Ca$^{2+}$ stores. Effect of (A) block of VGCCs (with 5 µM nicardipine) following Ca$^{2+}$ store depletion (by 10-min incubation with 10 µM CPA) and (B) Ca$^{2+}$ store depletion following block of VGCCs on SPCU triggered by repetitive (with 10-min interval) applications of 10 µM µβ-meATP. Galleries below the traces of self-normalized fluo-3 fluorescence show grey-scale coded (F/F$_0$) confocal images sequentially captured during the periods highlighted in the traces (i-iii, respectively). (C) The amplitude of $\Delta F/F_0$ transients detected following calcium store depletion (Test) was normalized to that detected before incubation with CPA (Control), before and following block of VGCCs (left) and compared (right). ** $p < 0.0013$. Note that effect of the Ca$^{2+}$ store depletion on µβ-meATP-induced $[\text{Ca}^{2+}]_i$ transients is attenuated following block of VGCCs.
induced by 3 mM caffeine, while in the absence of CPA the response to 3 mM caffeine recovered during 10 min by 76±3% (Fig. 3 A and B; n = 12). It should be noted that our previous studies conducted on vascular SMCs [e.g., 37] demonstrated that the fraction of the calcium store depletion induced by a single application of millimolar concentrations of caffeine exceeded 90%, thus validating caffeine at millimolar concentration range as an adequate pharmacological tool to test the SR Ca\(^{2+}\) load. While application of 3 mM caffeine failed to evoke any [Ca\(^{2+}\)], response following 10 min incubation of the SMC with 10 µM CPA, subsequent stimulation of P2X receptors in the same myocyte with 10 µM αβ-meATP transient resulting from Ca\(^{2+}\) influx via both P2X receptors and VGCCs (Fig. 3C). Altogether, these results validate CPA as selective pharmacological tool for evaluation of the Ca\(^{2+}\) release contribution to the agonist-induced [Ca\(^{2+}\)], response in mesenteric artery SMCs.

As discussed above, to access contribution of P2X receptors and VGCCs to elevation of [Ca\(^{2+}\)], resulting solely from Ca\(^{2+}\) entry and relative ability of the two Ca\(^{2+}\) entry mechanisms to induce Ca\(^{2+}\) release from the SR, we related the amplitudes of the [Ca\(^{2+}\)]\(\text{transients elicited by brief pulses of 10 µM αβ-meATP (applied with 10 min interval) in the presence of 5 µM nicardipine, 10 µM CPA and their combination to that obtained in the same myocyte before incubation with the inhibitor(s). The representative results of these experiments are illustrated in Figure 4. To minimize possible effect of the VGCC block on the SR calcium load, in these experiments nicardipine was added to the bath 30 s prior to αβ-meATP application. Block of L-type VGCCs with nicardipine reduced the peak amplitude (ΔF/Φ₀) of the αβ-meATP-induced SPCU (Fig. 4A) on average by 64±2% (n = 22). The nicardipine-insensitive fraction of the response reflects Ca\(^{2+}\) entry via P2X receptors plus induced by this entry Ca\(^{2+}\) release from the SR and accounts for ~36% of the total Ca\(^{2+}\) response.

Depletion of the intracellular Ca\(^{2+}\) stores by 10 min incubation with 10 µM CPA significantly (p < 0.001) reduced the peak amplitude (ΔF/Φ₀) of the αβ-meATP-induced SPCU (Fig. 4B). The fraction of the response remaining under these conditions reflects the total Ca\(^{2+}\) entry into the SMC evoked by P2X receptor activation, which is mediated by both P2X receptors and VGCCs and accounts for 19±0.8% (n = 24) of the total Ca\(^{2+}\) response. To estimate Ca\(^{2+}\) signal arising solely from Ca\(^{2+}\) influx via P2X receptors we eliminated VGCC-mediated Ca\(^{2+}\) entry with nicardipine in the SMCs with depleted Ca\(^{2+}\) stores (Fig. 4 A and B). The remaining under these conditions fraction of the response was significantly smaller (p < 0.00001) than that remaining after Ca\(^{2+}\) store depletion (see above). This fraction reflects direct Ca\(^{2+}\) entry via P2X receptors and accounts for 11±1.4% (n = 14) of the total Ca\(^{2+}\) response. Thus, stimulation of P2XRs with αβ-meATP activates two Ca\(^{2+}\) entry pathways, both providing comparable Ca\(^{2+}\) influx into the cell: P2X-mediated (~11%) and VGCC-mediated (~8%).

To assess the ability of VGCC-mediated Ca\(^{2+}\) entry following P2X receptor activation to trigger Ca\(^{2+}\) release from the stores, we compared the effect of calcium store depletion following block of VGCCs with that observed in the absence of nicardipine (Fig. 4C). This experimental strategy (see traces; left panel), revealed that effect of the Ca\(^{2+}\) store depletion on αβ-meATP-induced [Ca\(^{2+}\)], transients is significantly (p < 0.0013) attenuated following block of VGCCs (see bar diagram plot; right panel). Indeed, in control conditions incubation with CPA reduced the response (ΔF/Φ₀) to αβ-meATP by 81±0.8% (n = 24), while when VGCCs were initially blocked, incubation with CPA decreased the response by only 58±2% (n = 8). Significant attenuation of the effect of the calcium store depletion on the SPCU by block of VGCCs implies that Ca\(^{2+}\) release from the SR following P2X receptor activation is induced mainly by Ca\(^{2+}\) entry via VGCCs.

It should be noted that Ca\(^{2+}\) store depletion (with CPA) reduced the αβ-meATP-induced response significantly (p < 0.00001) stronger than block of VGCCs, suggesting that Ca\(^{2+}\) release from the SR is partially induced by Ca\(^{2+}\) entering the cell via P2X receptors. Calcium store depletion with simultaneous block of VGCCs reduced the response significantly more (p < 0.00001) than calcium store depletion alone, suggesting that Ca\(^{2+}\) entry via VGCCs makes significant contribution to the αβ-meATP-induced Ca\(^{2+}\) mobilization. Comparison of mean amplitudes of the responses (ΔF/Φ₀) to αβ-meATP in SMCs with depleted calcium stores obtained in the absence and in the presence of nicardipine suggests that the Ca\(^{2+}\) influx via VGCCs accounts for ~8% of the total Ca\(^{2+}\) response. Taking into account reproducibility of the Ca\(^{2+}\) responses to 10 µM αβ-meATP and the effects of the pharmacological interventions described above,
we summarized contribution of several mechanisms to elevation of [Ca\textsuperscript{2+}]\textsubscript{i}, elicited by activation of P2X receptors in mesenteric artery SMCs (Fig. 5). Two major roots of the Ca\textsuperscript{2+} entry, P2X receptors and L-type VGCCs, were analyzed. The fraction of the response (ΔF/ΔF\textsubscript{0}) arising from each of these two roots (including corresponding CICR component) is plotted next to its net Ca\textsuperscript{2+} influx component and related to the total αβ-meATP-induced response. For each root, the CICR amplification factors (shown on the plot) were calculated as a ratio of the mean peak amplitudes of the corresponding fractions persisting or abolished following incubation with nicardipine and/or CPA (see above). This approach revealed that Ca\textsuperscript{2+} entry via L-type VGCCs is augmented by CICR significantly more than Ca\textsuperscript{2+} entry via P2X receptors, even though net Ca\textsuperscript{2+} influxes provided by the two mechanisms are not significantly different.

![Figure 5](image_url)  
**Fig. 5.** Comparison of the amplification of Ca\textsuperscript{2+} signals following Ca\textsuperscript{2+} entry via P2X receptors and VGCCs by Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release (CICR) mechanism. The peak amplitudes (ΔF/ΔF\textsubscript{0}) of the following fractions of the αβ-meATP-induced response observed in control conditions were analyzed: (i) fraction persisting following block of VGCCs (reflects P2X receptor-mediated Ca\textsuperscript{2+} entry plus induced by this entry Ca\textsuperscript{2+} release), (ii) fraction abolished by block of VGCCs (reflects VGCC-mediated Ca\textsuperscript{2+} entry plus induced by this entry Ca\textsuperscript{2+} release), (iii) fraction persisting following intracellular Ca\textsuperscript{2+} store depletion (reflects total Ca\textsuperscript{2+} influx via P2X receptors and VGCCs), (iv) fraction abolished by block of VGCCs in SMCs with depleted Ca\textsuperscript{2+} stores (reflects net VGCC-mediated Ca\textsuperscript{2+} entry) and (v) fraction persisting after block of VGCCs in SMCs with depleted Ca\textsuperscript{2+} stores (reflects net P2X receptor-mediated Ca\textsuperscript{2+} entry). CICR amplification coefficients (outlined by circles) were calculated as a ratio of the mean peak amplitudes of the corresponding fractions. Note that for illustration purpose the total αβ-meATP-induced response is shown as 100% (even though reproducibility of the response in control conditions was on average 81%) and all the fractions of the response are re-scaled accordingly.

### Discussion

Our results indicate that activation of P2X receptors in SMCs from the guinea-pig mesenteric artery induces parallel activation of two Ca\textsuperscript{2+} signaling pathways which differ in their efficiency in generating total intracellular [Ca\textsuperscript{2+}]. The first one is initiated by direct P2X receptor-mediated Ca\textsuperscript{2+} influx and provides 36% of the total [Ca\textsuperscript{2+}] response. Its efficiency, or amplification factor, estimated as ratio of the fraction of the resulting Ca\textsuperscript{2+} signal (ΔF/ΔF\textsubscript{0}), including corresponding CICR component, to that arising solely from the direct Ca\textsuperscript{2+} entry via P2X receptors (11%), was found to be ~3.3.

Activation of P2X receptors not only provides the pathway for the direct Ca\textsuperscript{2+} influx, but also causes depolarization of the myocyte membrane brought about by the inward cationic current and resulting in activation of VGCCs. This engages the second Ca\textsuperscript{2+} signaling pathway initiated by Ca\textsuperscript{2+} influx via VGCCs. While direct Ca\textsuperscript{2+} influx via VGCCs makes relatively small contribution (~8%) to the total intracellular [Ca\textsuperscript{2+}] response, it appears to be much more effective in triggering Ca\textsuperscript{2+} release from the SR: overall contribution of the VGCC-mediated Ca\textsuperscript{2+} influx and induced by it the SR Ca\textsuperscript{2+} release to the total [Ca\textsuperscript{2+}] response induced by P2X receptor activation was estimated as nicardipine-sensitive fraction of the αβ-meATP-inducedCa\textsuperscript{2+} signal (ΔF/ΔF\textsubscript{0}) and was found to be ~64%. Thus, Ca\textsuperscript{2+} signal initiated by VGCC-mediated Ca\textsuperscript{2+} influx is amplified by CICR ~8 times (compare with ~3.3 times for P2X-mediated Ca\textsuperscript{2+} influx).

Essential difference in the efficiency of the two Ca\textsuperscript{2+} signalling pathways suggests that they may employ different Ca\textsuperscript{2+} release mechanisms. While both Ca\textsuperscript{2+} release mechanisms should be Ca\textsuperscript{2+} dependent, they must, at least partially, discriminate between Ca\textsuperscript{2+} ions entering the cell via P2X receptors and VGCCs. Otherwise net Ca\textsuperscript{2+} influxes via P2X receptors and VGCCs, which make similar contribution to the total intracellular [Ca\textsuperscript{2+}] response (~11% and ~8%, respectively), should trigger similar Ca\textsuperscript{2+} release from the SR resulting in elevation of [Ca\textsuperscript{2+}], of the same magnitude. Differential sensing of the Ca\textsuperscript{2+} influx mechanisms by the Ca\textsuperscript{2+} release machinery suggests the existence of spatially separated regions of a localized Ca\textsuperscript{2+} increase known as microdomains [31]. Recently we have provided a line of evidences...
suggesting that L-type VGCCs in mesenteric artery SMCs are in close juxtaposition with subplasmalemmal (“junctional”) SR (jSR) elements, which were found to be enriched with inositol 1,4,5-trisphosphate receptor (IP₃R) type 1 but poor in ryanodine receptors (RyRs), while RyRs were found to be predominant at deeper central SR elements [46]. In agreement with this, [Ca²⁺]ᵢ responses to caffeine (which triggers RyR-mediated Ca²⁺ release) usually initiated at central region of the SMC, while responses to αβ-meATP started as SPCU (see also [39]). The “nanojunctions” between plasmalemma and SR elements [37, 38] may create local high [Ca²⁺]ᵢ microdomains activating IP₃R-mediated Ca²⁺ release from the SR. Indeed, it is now generally appreciated that jSR (which lies within 10–15 nm of plasmalemma), the overlying plasmalemma microdomains, and the intervening, tiny volume of cytosol may form junctional complexes that serve as the Ca²⁺ “buffer barrier”, through which Ca²⁺ can move directly between the extracellular fluid and jSR [3, 38]. This may facilitate (1) accumulation of molecules, including Ca²⁺, Na⁺ [3, 38], and IP₃, in the cytosolic microvolume from which the diffusion into the bulk cytoplasm is markedly restricted [38] and (2) local elevation of luminal [Ca²⁺] in jSR. Bearing this in mind, the following factors may favor activation of IP₃Rs by Ca²⁺ influx: (1) robust increase of [Ca²⁺]ᵢ in the junctional cytosolic microvolume [3, 38], (2) Ca²⁺ activation of type 1 IP₃Rs with positive cooperativity [14], (3) regulation of IP₃Rs by the SR luminal [Ca²⁺] [43], (4) IP₃R clustering [42] and (5) spontaneous basal activity of PLC [17, 22, 36, 40]. We have recently demonstrated that in renal microvascular myocytes activation of P2X receptors induces IP₃R-mediated Ca²⁺ release from jSR, which is activated mainly by Ca²⁺ influx through VGCCs [39]. Whether or not this mechanism is present in mesenteric artery, SMCs requires further investigation. However, it has been previously reported that arterial VGCCs, after sensing membrane depolarization, could trigger sensitive to L-type Ca²⁺ channels antagonists fast IP₃R-mediated Ca²⁺ release from the SR even in the absence of extracellular Ca²⁺ influx [10]. IP₃ production in response to depolarization-induced Ca²⁺ influx through VGCCs was demonstrated in Purkinje cells [35], intestinal smooth muscle cells and in insulin-secreting β-cells [48], thus, suggesting that this mechanism is widespread and may be involved in control of many cellular functions.

In summary, our results demonstrate 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 the Ca²⁺ release from the SR activated by Ca²⁺ influx via L-type VGCCs. This signaling pathway is engaged by depolarization of the myocyte membrane resulting from activation of P2X receptors, which, being Ca²⁺ permeable, per se form less effective Ca²⁺ signaling pathway.

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