Original Paper

Extracellular Adenosine Diphosphate Ribose Mobilizes Intracellular Ca^{2+} via Purinergic-Dependent Ca^{2+} Pathways in Rat Pulmonary Artery Smooth Muscle Cells

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Key Words

Adenosine diphosphate ribose • Purinergic receptors • Pulmonary arterial smooth muscle cells • Ca^{2+} mobilization

Abstract

Background/Aims: Adenosine diphosphate ribose (ADPR), a product of β -NAD⁺ metabolism generated by the multifunctional enzyme CD38, is recognized as a novel signaling molecule. The catalytic site of CD38 orients extracellularly or intracellularly, capable of generating ADPR outside and inside the cells. CD38-dependent pathways have been characterized in pulmonary artery smooth muscle cells (PASMCs); however the physiological function of extracellular ADPR is unclear. **Methods:** Ca^{2+} mobilizing and proliferative effects of extracellular ADPR were characterized and compared with the ATP-induced responses in rat PASMCs; and the expression of purinergic receptor (P2X and P2Y) subtypes were examined in pulmonary arteries. **Results:** ADPR elicited concentration-dependent increase in $[\text{Ca}^{2+}]_i$ with a fast transient and a sustained phase in PASMCs. The sustained phase was abolished by Ca^{2+} removal and inhibited by the non-selective cation channel blocker SKF-96365, but was unaffected by TRPM2 antagonists or nifedipine. The purinergic receptor (P2X) antagonist pyridoxal-phosphate-6-azophenyl-2', 4'-disulfonate inhibited partially the transient and the sustained Ca^{2+} response, while the P2(XY) inhibitor suramin and the phospholipase C inhibitor U73122 abolished the sustained Ca^{2+} influx. The P2Y1 antagonist MRS2179 had no effect on the response. By contrast, ATP and ADP activated Ca^{2+} response exhibited a high and a low affinity component, and the pharmacological profile of ATP-induced Ca^{2+} response was distinctive from that of ADPR. BrdU incorporation assay showed that ADPR caused significant inhibition whereas ATP caused

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slight stimulation of PSMC proliferation. RT-PCR analysis found that almost all P2X and P2Y subtypes are expressed in PAs. **Conclusion:** ADPR and ATP activate Ca^{2+} responses through different combinations of multiple purinergic receptor subtypes; and extracellular ADPR may exert an autocrine/paracrine action via purinergic receptors on PSMCs.

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Introduction

Adenosine diphosphate ribose (ADPR) is increasingly recognized as a novel Ca^{2+} signaling molecule responsible for the activation of transient receptor potential melastatin 2 (TRPM2) channel [1, 2] and other Ca^{2+} pathways in several cell types [3-5]. It is produced through β -NAD⁺ and cyclic-ADP-ribose (cADPR) hydrolysis by NAD glycohydrolases in the plasma membrane, mitochondria and other subcellular compartments [6-8]. It is also generated during poly-ADPR formation and hydrolysis through the concerted actions of poly(ADPR) polymerase and poly(ADPR) glycohydrolase in response to DNA damage [9]. CD38 is a multifunctional enzyme and is the major NAD glycohydrolase in mammalian cells [10, 11]. It is best known for generating cyclic-ADPR (cADPR) through its ADP-ribosyl cyclase activity and nicotinic acid adenosine phosphate (NAADP) through a base-exchange reaction. cADPR and NAADP are the second messenger for mobilizing Ca^{2+} from the ryanodine receptor-gated sarcoplasmic reticulum (SR) and the acidic endolysosomal Ca^{2+} stores, respectively [6]. However, ADPR is the predominant catalytic product (>99%) of CD38 [12, 13]. In the plasma membrane, CD38 is generally operated as a type II ectoenzyme with the catalytic site oriented extracellularly [14, 15]. It can be also expressed in the type III orientation with the catalytic domains facing the cytosol under certain specific conditions [16, 17]. Hence, ADPR can be liberated intracellularly as a second messenger and/or extracellularly as an autocrine or paracrine signaling molecule [14]. ADPR is an endogenous messenger for the activation of the transient receptor potential melastatin 2 (TRPM2) channel [2, 18], which plays an important role in oxidative stress and immune response [19]. The autocrinal or paracrine function of ADPR is supported by studies showing extracellular application of ADPR elicited a variety of biological responses [3, 4, 20-27]. It has been proposed that ADPR and cADPR generated extracellularly by CD38 can be transported via CD38 and other transporters back into the cells to exert their biological actions [14, 28-30]. Other evidence suggests that extracellular ADPR can act as an endogenous ligand for purinergic receptor activation [3, 4, 23].

The physiological functions of CD38 and its products cADPR and NAADP have been well studied in systemic vascular tissues [31-36]. They contribute to endothelin-1, angiotensin II, and muscarinic receptor-mediated contraction and Ca^{2+} release in arterial smooth muscle [32, 33, 35-37], endothelium-dependent vasodilatation [38] and angiogenesis [39]. However, information on the roles of ADPR in vascular functions is scant. A previous study showed that electrical field stimulation releases ADPR from mesenteric arteries [40]. Extracellular ADPR can cause dilation of coronary arteries through P1-purinergic receptors due to degradation of ADPR to adenosine and adenosine monophosphate by apyrase and 5'-nucleotidase [25]. Intracellular ADPR may be also involved in the 11,12-EET-induced activation of Ca^{2+} -activated K^{+} channels in coronary arterial smooth muscle [5].

CD38-dependent signaling mechanisms have been studied in pulmonary vasculature. It was originally proposed to act as a redox-sensor in pulmonary arterial smooth muscle cells (PSMCs) for initiating hypoxic pulmonary vasoconstriction through the accumulation of cADPR [41-43]. But, this hypothesis has not yet been validated [44]. NAADP has been shown to activate "bursts" of Ca^{2+} release triggering global Ca^{2+} waves through Ca^{2+} -induced- Ca^{2+} release in PSMCs [45, 46]. We have recently characterized the expression of CD38 and the CD38-dependent Ca^{2+} response in PSMCs. We found that CD38 is highly expressed in PAs compared to other systemic arteries [47, 48]. It plays important roles in Ca^{2+} response activated by agonists, including angiotensin II, endothelin-1 and integrin-ligands through cADPR and NAADP-dependent mechanisms [47-49]. In particular, Ang II elicits CD38-

dependent Ca^{2+} release in PSMCs via PKC-dependent production of reactive oxygen species from NADPH oxidase 2 [48]. We also detected the expression of the NAADP-sensitive two-pore channels (TPC1 and TPC2) in PAs and PSMCs, and characterized the global and local Ca^{2+} events elicited by NAADP from the endolysosomal stores [49]. These studies clearly indicate that a robust CD38-dependent mechanism is present in PSMCs and it is responsible for diverse vascular functions. However, the biological effects and physiological functions of ADPR have not been examined in the PSMCs. In the present study, we sought to examine the Ca^{2+} mobilizing effects and the pharmacological properties of extracellular ADPR in PSMCs, as to expand our understanding on the physiological functions of CD38-dependent signaling pathways in the pulmonary vasculature.

Materials and Methods

Isolation and culture of PSMCs

All animal procedures were performed in accordance with the guidelines and approved by the Johns Hopkins Animal Care and Use Committee. PSMCs were enzymatically isolated and transiently cultured as previously described [50]. Briefly, male Wistar rats (150–250 g) were anesthetized with pentobarbital sodium (130 mg/kg ip). They were exsanguinated, and lungs were removed and transferred to a petri-dish filled with HEPES-buffered salt solution (HBSS) containing (in mM) 130 NaCl, 5 KCl, 1.2 MgCl_2 , 1.5 CaCl_2 , 10 HEPES, and 10 glucose, pH 7.4 (adjusted with NaOH). Second- and third-generation intrapulmonary arteries (~300–800 μm) were isolated and cleaned free of connective tissue. Endothelium was removed by gently rubbing the luminal surface with a cotton swab. Arteries were then allowed to recover for 30 min in cold (4°C) HBSS, followed by 20 min in reduced Ca^{2+} (20 μM) containing HBSS at room temperature. The tissue was digested at 37°C for 20 min in 20 μM Ca^{2+} HBSS containing collagenase (type I, 1,750 U/ml, Sigma), papain (9.5 U/ml), bovine serum albumin (2 mg/ml), and dithiothreitol (1 mM), and then removed and washed with Ca^{2+} -free HBSS to stop digestion. Single smooth muscle cells were gently dispersed by trituration with a small-bore pipette in Ca^{2+} -free HBSS at room temperature. The cell suspension was then placed on 25-mm glass coverslips and transiently (16–24 h) cultured in Ham's F-12 medium (with L-glutamine) supplemented with 0.5% fetal calf serum, 100 U/ml of streptomycin, and 0.1 mg/ml of penicillin.

Intracellular $[\text{Ca}^{2+}]$ measurement

Intracellular $[\text{Ca}^{2+}]$ ($[\text{Ca}^{2+}]_i$) in single PSMCs was measured with the membrane-permeant acetoxymethyl ester (AM) form of the Ca^{2+} -sensitive fluorescent dye fluo 3 (fluo 3-AM). PSMCs were loaded with 5 μM fluo 3-AM, which was first dissolved in dimethyl sulfoxide with 20% pluronic acid, for 45 min at room temperature ($\sim 22^\circ\text{C}$) in normal Tyrode solution containing (in mM) 137 NaCl, 5.4 KCl, 2 CaCl_2 , 1 MgCl_2 , 10 HEPES, and 10 glucose, pH 7.4 (adjusted with NaOH). Cells were then washed thoroughly with Tyrode solution to remove extracellular fluo 3-AM and rested for 15–30 min in a cell chamber to allow for complete de-esterification of cytosolic dye. Tyrode solution without MgCl_2 was used for ATP-related experiments. Indicated concentration of ADPR, ATP or ADP in Tyrode solution with 2mM Ca^{2+} or Ca^{2+} -free was directly applied to the cell chamber bath. For zero Ca^{2+} (0 Ca^{2+}) experiments, the Ca^{2+} -free Tyrode solution was replaced 100 seconds before fluorescence measurement. For antagonist experiments, antagonists were pre-incubated with PSMCs for 20 min before applying ADPR or ATP. Fluorescence measurement was performed on a Nikon Diaphot inverted microscope. The collimated light beam from a 75-W xenon arc lamp was filtered by an interference filter at 480/30 nm and focused onto the PSMCs under examination via a $\times 40$ fluorescence oil-immersion objective (Fluor 40 $\times$, Nikon). Light emitted from the cell was returned through the objective and split with a dichroic mirror, and fluorescence was detected at 535/40 nm by a photomultiplier tube. Photobleaching of fluo 3 dye was minimized using a neutral density filter (ND-3, Omega Optics) and an electronic shutter (Vincent Associates). The shutter was opened for 35 ms every second, and the fluorescence signals during the open period were integrated with a sample-and-hold circuit. The protocols were executed, and the data were collected on-line with a Digidata analog-to-digital interface (Axon Instruments, Union City, CA) and the pClamp software package. $[\text{Ca}^{2+}]_i$ was calibrated by the equation $[\text{Ca}^{2+}]_i = K_D \cdot (F - F_{bg}) / (F_{max} - F)$, where F is fluorescence, F_{bg} is background fluorescence, F_{max} is the maximum fluorescence determined in situ in cell superfused with 10 μM 4-bromo A-23187 and 20 mM Ca^{2+} , and K_D of fluo 3 is 1.1 μM .

BrdU proliferation assay

Freshly isolated rat PSMCs were cultured for two passages in SmGm with 5% FBS and growth factors, and then were trypsinized, counted and seeded at 8000 cells/well in a 96-well plate in DMEM with 0.5% FBS overnight. Next day, cells were treated with the desired drugs in appropriate medium, DMEM with either 0.5% FBS or 5% FBS. BrdU was prepared according to manufacturer's protocol (Exalpha Biologicals Inc.) and added to each well ~24 hrs after treatment. Then cells were further cultured for 24 hours before fixing. After fixing the cells, it was processed further per manufacturer's protocol and the absorbance was read using microplate reader at 450/595 nm.

Conventional RT-PCR

Deendothelialized PAs were mechanically homogenized. Subsequently, total RNA was extracted using RNeasy® Mini Kit (Qiagen, Valencia, CA) with standard procedures. Genomic DNA contamination was removed with RNase-free DNase Set (Qiagen, Valencia, CA). The amount of RNA was determined by measuring the optical density at 260 nm. Total RNA was also extracted from rat brain as positive controls. Total RNA (1 µg) was used for first-strand cDNA synthesis with random hexamer primers and Superscript III RNase H⁻ reverse transcriptase (Invitrogen) according to the manufacturer's protocol. Sense and antisense PCR primers specific to the P2X and P2Y receptors were used (see Table 1). PCR reactions were carried out using Hot-Start Taq DNA polymerase (Denville Scientific Inc., Metuchen, NJ) with the following parameters: denaturation at 94°C for 45 s, annealing at 60°C for 30 s, and extension at 72°C for 90 s. A total of 35 cycles was performed, followed by a final extension at 72°C for 10 min, and the products were then stored at 4°C. PCR products were analyzed by electrophoresis with 1.8% agarose gel and visualized by ethidium bromide staining. The PCR products were extracted and purified using QIAquick Gel Extraction Kit (Qiagen, Valencia, CA), then sequencing were performed using the Applied Biosystems 3730xl DNA Analyzer by Johns Hopkins Sanger sequencing services.

Data Analysis

Changes in [Ca²⁺]_i was measured at the peak response or 150 seconds after the application of ADPR or ATP. All data are expressed as mean ± SEM. N indicates the number of experiments as specified in the text. Concentration-response curves were fitted with the three parameter logistic model: $R = E_{max}/(1 + ([A]/EC_{50})^b)$ using the SigmaPlot software, where R is the Ca²⁺ response, E_{max} is the maximal response, [A]

Table 1. Primers for conventional RT-PCR experiments

Gene	Accession No.	Primer	Sequence (5'-3')	Nucleotide Position	Predicted Size, bp
P2X1	NM_012997.2	Sense	CTGTCAGCCTTCTTCTTTGAAT	234-255	543
		Antisense	GATGAAGAGGGTGAAGTTCTCA	755-776	
P2X2	NM_053656.2	Sense	AGGACTCTGACCCATACTGT	689-708	495
		Antisense	TCACAGGCCACTACTTGAG	1164-1183	
P2X3	NM_031075.2(V1)	Sense	AGGATTCTGTCCAGAGAATGAA	493-514	739
		Antisense	AGCCCCTTTGAGGAAATGA	1212-1231	
P2X4	NM_031594.1	Sense	CTACGAGCAGGGTCTTTCGG	1231-1250	675
		Antisense	CCCTGCCTTTGTCTACAAGGA	1886-1905	
P2X5	NM_080780.2	Sense	ATAGCCCTGAAGGGTGGTGT	926-945	483
		Antisense	ATCCTGACGAACCTCTCCA	1389-1408	
P2X6	NM_012721.2	Sense	GGCATCAACATCCACTGGGA	901-920	347
		Antisense	GGGGCTCTTGCTCTTCATA	1228-1247	
P2X7	NM_019256.1	Sense	CTTCGGCTACTCTCGGTGG	34-53	540
		Antisense	CTGCCGGTCTGGATTCTTT	554-573	
P2Y1	NM_012800.1	Sense	GTAACATGGTCACAAGAAGTCCC	1769-1791	427
		Antisense	TTGGTGTCTGTGCGCTTTT	2176-2195	
P2Y2	NM_017255.1	Sense	GGGAGATGACGGGGACCTAA	43-62	445
		Antisense	CCATTGCCCTGGACCCGAT	469-487	
P2Y4	NM_031680.1	Sense	GACTAAGGAAGCTAGGGGGC	34-53	758
		Antisense	ACCACAGCAATGGTACGGAG	772-791	
P2Y6	NM_057124.2	Sense	GGGGGAGTTTCAGGGGTAA	79-98	369
		Antisense	AACAGGCATACAGCAGGTCC	428-447	
P2Y12	NM_022800.1	Sense	ACAGAATAACCGAGCATTGGAG	107-129	859
		Antisense	GTGTTCTCGGCATTGCAGTC	946-965	
P2Y13	NM_001002853.1	Sense	GGGACACTCGGATGACACAG	516-535	282
		Antisense	GGGACGCACATTCTTCACG	778-797	
P2Y14	NM_133577.1	Sense	TGCGCTCTAAGTAACAGCC	180-199	622
		Antisense	TTAGACGCCTTGTGCCACTT	782-801	

is the concentration of the agonist, EC_{50} is the effective concentration for 50% response, and b is the slope factor. Statistical significance ($P < 0.05$) of the changes was compared with paired or unpaired Student's t -tests, or by one- or two-way ANOVA with Bonferroni post hoc test, wherever applicable.

Chemicals and drugs

2-APB and suramin were purchased from Calbiochem (Billerica, MA), ACA from Enzo life sciences (New York, NY), MRS2179 from Abcam (Cambridge, MA), and U73122 from Cayman Chemical (Ann Arbor, MI). ADPR, ATP, SKF-96365, PADS, U73343, collagenase, protease, papain, serum albumin, dithiothreitol, antibiotics and other chemicals were purchased from Sigma Chemical (St. Louis, MO). Ham's F-12 medium was purchased from Mediatech (Herndon, VA). Fluo 3-AM and pluronic acid were purchased from Molecular Probes (Eugene, OR).

Results

ADPR-induced Ca^{2+} mobilization in rat PSMCs

The effect of extracellular ADPR on global $[\text{Ca}^{2+}]_i$ was examined in transiently cultured PSMCs. ADPR elicited concentration-dependent increases in $[\text{Ca}^{2+}]_i$ with the maximal response at 1 mM (Fig. 1A, B, C). At the low concentrations (30–300 μM), ADPR elicited a brief intracellular Ca^{2+} transient that peaked in a few seconds after application and returned to the resting $[\text{Ca}^{2+}]_i$ level within 20 seconds. By contrast, ADPR at the higher concentrations (1 and 3 mM) caused a biphasic Ca^{2+} response, with a large initial transient ($2.55 \pm 0.28 \mu\text{M}$ for 1 mM and $1.51 \pm 0.14 \mu\text{M}$ for 3 mM, Fig. 1A and B), followed by a low sustain phase with a duration of several minutes ($179.5 \pm 29.0 \text{ nM}$ for 1 mM and $190.6 \pm 35.4 \text{ nM}$ for 3 mM measured after 150 s, $n = 6$, Fig. 1A and C). The EC_{50} of the initial and the sustained phase of the Ca^{2+} transient are 418 ± 49 and $436 \pm 63 \mu\text{M}$, respectively.

The Ca^{2+} response could be mediated by extracellular Ca^{2+} influx and intracellular Ca^{2+} release. To delineate the contribution of the Ca^{2+} sources, the effects of 1 mM ADPR in Ca^{2+} (2 mM)-containing and Ca^{2+} -free solution were compared. Removal of extracellular Ca^{2+} at 100 s before ADPR application attenuated the peak Ca^{2+} transients by 53% (2 mM Ca^{2+} : $1.47 \pm 0.26 \mu\text{M}$, $n = 16$; 0 Ca^{2+} : $0.70 \pm 0.13 \mu\text{M}$, $n = 12$, $P = 0.026$, Fig. 1D and E) and the sustain phase by 83% at 150 sec after ADPR application (2 Ca^{2+} : $110.1 \pm 21.7 \text{ nM}$; 0 Ca^{2+} : $19.3 \pm 8.2 \text{ nM}$, $P = 0.002$, Fig. 1D and F). These results suggest that ADPR-induced Ca^{2+} response is consisted of an intracellular Ca^{2+} release component, which contributes to the initial transient response, and a Ca^{2+} influx component, which is responsible in part for the initial transient and for the sustained Ca^{2+} response. The Ca^{2+} influx activated by ADPR was unrelated to the activation of voltage-gated Ca^{2+} channels, because nifedipine (1 μM) had no effect on both the initial and the sustained Ca^{2+} response (Fig. 1G, H, I). The ADPR-induced Ca^{2+} response was also unrelated to ryanodine receptor-dependent Ca^{2+} release. Pretreatment of PSMCs with 50 μM ryanodine had no effect on the Ca^{2+} response. In contrast, inhibition of InsP_3 receptor with 3 μM xestospongine C caused a partial reduction in both the initial transient (control: $0.93 \pm 0.07 \mu\text{M}$, $n = 9$; xestospongine C: $0.51 \pm 0.11 \mu\text{M}$, $n = 8$, $P = 0.014$) and the sustained Ca^{2+} response (control: $105.5 \pm 14.7 \text{ nM}$; xestospongine C: $58.0 \pm 15.8 \text{ nM}$, $P = 0.044$), suggesting that the Ca^{2+} response is in part mediated by the InsP_3 -receptor gated Ca^{2+} stores.

TRPM2 is not involved in the extracellular ADPR-induced Ca^{2+} influx

ADPR is the signaling molecule for the activation of TRPM2 [2, 18]. To test the possible involvement of TRPM2, ADPR-induced Ca^{2+} response was examined in PSMCs pretreated with the TRPM2 antagonists 2-Aminoethoxy diphenylborate (2-APB, 30 μM) [51, 52] or N-(p-Amylcinnamoyl) anthranilic acid (ACA, 30 μM) [53] for 20 min. 2-APB or ACA attenuated peak Ca^{2+} transient elicited by 1 mM ADPR by 32.1% or 32.0%, respectively, but both had no significant effect on the sustained Ca^{2+} response (Fig. 2A–F). The nonselective cation channel antagonist SKF-96365 was used to further examine the ADPR-induced extracellular Ca^{2+} influx. SKF-96365 at 30 μM caused an increase in the resting $[\text{Ca}^{2+}]_i$ from a control level of

Fig. 1. ADPR-induced Ca^{2+} mobilization in rat PSMCs. (A) Mean traces of Ca^{2+} response activated by ADPR (30 μM - 3 mM) in Ca^{2+} containing solution (n = 6 experiments for each group). (B and C) Concentration-response curves of Ca^{2+} responses measured at the peak and the sustained phase 150 s after ADPR application. The red line is the concentration-response relation fitted with the logistic model $R = E_{\text{max}}/(1 + ([A]/EC_{50})^b)$. (D) Mean traces of Ca^{2+} response activated by 1 mM ADPR in the absence (n = 12) or presence (n =

16) of 2 mM Ca^{2+} . (E and F) The mean values of the peak and sustained Ca^{2+} response measured at 150 s after ADPR application in the experiments shown in (D). (G) Mean traces of Ca^{2+} response activated by 1 mM ADPR in the absence (N = 9) or presence of 3 μM xestospongine C (n = 8), 50 μM ryanodine (n = 9), or 1 μM nifedipine (8). (H and I) The mean values of the peak and sustained Ca^{2+} response measured at 150 s after ADPR application in the experiments shown in (G). * indicates significant difference from the control (P < 0.05).

131.44 $\pm$ 23.19 to 297.36 $\pm$ 33.96 nM (n = 7, P < 0.005), and attenuated both the transient and the sustain phase elicited by ADPR (Fig. 2G-I). The reduction of peak Ca^{2+} transients was 52.5 $\pm$ 10.7%, and that of sustain phase was 95.6 $\pm$ 33.8%. These results suggest that the ADPR-induced sustain Ca^{2+} influx is mediated to a large extent through non-selective cation channels which are independent of TRPM2.

ADPR-induced intracellular Ca^{2+} release by activating P2 receptors in PSMCs

Extracellular nucleotides and different ribosylated nucleotide derivatives have been shown to activate Ca^{2+} signaling pathways through purinergic (P2) receptors [54]. Many P2 receptor subtypes are expressed in a variety of cells, including PSMCs [55-57]. To explore the contribution of P2 receptors in the signaling for extracellular ADPR, PSMCs were treated with the non-selective P2 receptor antagonist suramin, the P2X receptor antagonist pyridoxal-phosphate-6-azophenyl-2',4'-disulfonate (PPADS) and the specific P2Y1 receptor antagonist MRS2179 for 20 minutes before application of ADPR. Suramin at 10, 30, and 100 μM caused dramatic concentration-dependent inhibition of the ADPR-induced sustain Ca^{2+} response, with reductions of 66.0 $\pm$ 16.3%, 83.3 $\pm$ 10.1%, and 95.5 $\pm$ 3.6%, respectively. But, suramin had no significant effect on the peak response except a partial reduction of the initial

Fig. 2. Effects of TRPM2 and non-selective cation channel antagonists on ADPR-induced Ca^{2+} response in PSMCs. (A) Mean traces of Ca^{2+} response elicited by 1 mM ADPR in the absence (control) or presence of 30 μM 2-APB ($n = 7$). (B and C) The mean values of the peak and sustained Ca^{2+} response measured in the experiments shown in (A). (D) Mean traces of Ca^{2+} response activated by 1 mM ADPR in the absence (control) or presence of 30 μM ACA ($n = 9$). (E and F) The mean values of the peak and sustained Ca^{2+} response measured in experiments shown in (D). (G) Mean traces of Ca^{2+} response activated by ADPR in the absence (control, $n = 15$) or presence ($n = 7$) of 30 μM SKF-96355. (H and I) The mean values of the peak and sustained Ca^{2+} response measured in experiments shown in (G). The mean values of sustained Ca^{2+} response (C, F, I) were measured at 150 s after ADPR application. * indicates significant difference ($P < 0.05$) from the control.

Ca^{2+} transients at 100 μM (Fig. 3A, B and C). In contrast to suramin, PPADS inhibited the ADPR-induced peak $[\text{Ca}^{2+}]_i$ transients in a concentration-dependent manner and shortened the duration of the Ca^{2+} transients. The reductions were $58.9 \pm 6.6\%$, $72.3 \pm 6.7\%$, and $82.1 \pm 5.7\%$ at 1, 3 and 10 μM PPADS, respectively (Fig. 3D, E and F). The sustain phase was also partially reduced. However, 100 μM MRS2179 had no significant effect on ADPR-induced Ca^{2+} mobilization (Fig. 3G, H and I). These results suggest that the initial Ca^{2+} transient induced by ADPR is related to P2X activation, and the sustained Ca^{2+} response is mediated through P2 receptor, but both components of the response is unrelated to the P2Y1 receptors.

ADPR-induced intracellular Ca^{2+} release by PLC signaling pathway

P2Y receptors are G-protein coupled receptors, and their activation generally stimulates phospholipase C (PLC), which leads to InsP_3 production to cause sarcoplasmic Ca^{2+} release and diacylglycerol (DAG) accumulation to activate protein kinase C (PKC) and other signaling mechanisms [58]. To examine the contribution of the P2Y-PLC pathway in ADPR-induced Ca^{2+} response, PSMCs were pre-incubated with the PLC inhibitor U73122 (3 μM) or its inactive analog U73343 (3 μM) for 20 min. U73122 significantly attenuated both the peak and the sustain response by $48.0 \pm 12.9\%$ and $66.6 \pm 7.9\%$, respectively, whereas U73343 had no effect on the Ca^{2+} response (Fig. 3J, K and L). Co-incubation of U73122 and PPADS almost completely obliterated the peak Ca^{2+} response. These results suggested that the PLC pathway contributes significantly to the ADPR-induced Ca^{2+} response; and activation of PLC together with the P2X receptors can account for most of the ADPR-induced peak Ca^{2+} response.

Fig. 3. Effects of P2 receptor and PLC antagonists on ADPR-induced Ca^{2+} response in PSMCs. (A) Mean traces of Ca^{2+} response elicited by 1 mM ADPR in the absence (control, $n = 16$) or presence of 10 ($n = 7$), 30 ($n = 9$) and 100 μM ($n = 5$) of the P2(XY) antagonist suramin. (B and C) The mean values of the peak and sustained Ca^{2+} response measured in experiments shown in (A). (D, E, F) Mean traces, peak and sustained ADPR response activated by ADPR in the absence (control, $n = 9$) or presence of 1 ($n = 4$), 3 ($n = 7$) or 10 μM ($n = 5$) of the P2X inhibitor PPADS. (G, H, I) Mean traces, peak and sustained Ca^{2+} response activated by ADPR in the absence (control, $n = 9$) or presence ($n = 4$) of 100 μM of the P2Y1 antagonist MRS2179. (J, K, L) Mean traces, peak and sustained Ca^{2+} response activated by 1 mM ADPR in the absence (control, $n = 7$) or presence of 3 μM U73122 ($n = 7$), 3 μM U73343 ($n = 7$) or U73122 + PPADS ($n = 4$). * indicates significant difference ($P < 0.05$) from the control.

ATP and ADP-mediated Ca^{2+} mobilization in rat PSMCs

To elucidate the P2 receptor-dependent Ca^{2+} signals, we examined the Ca^{2+} response activated by ATP, a typical endogenous P2 agonist, in PSMCs for comparison. Application of ATP at 10 nM to 1 mM activated robust Ca^{2+} responses with distinctive kinetics and apparent sensitivities. At sub-micromolar concentrations, ATP elicited a fast transient elevation in $[\text{Ca}^{2+}]_i$ that declined almost completely to the baseline within 20–40 seconds (Fig. 4A, C). Maximal peak Ca^{2+} response of $0.98 \pm 0.07 \mu\text{M}$ ($n = 8$) was reached at 100 nM ATP. Further increase in ATP concentration (1 μM –1 mM) elicited a much larger transient peak response followed by a sustained elevation in $[\text{Ca}^{2+}]_i$ with a duration exceeding 5 min (Fig. 4B, D). The peak and the sustain response reached a second maxima at 100 μM with $\Delta[\text{Ca}^{2+}]_i$ of 3.63 ± 0.38 and $0.30 \pm 0.03 \mu\text{M}$ ($n = 6$), respectively. The concentration-dependent peak Ca^{2+} response curve was best-fitted with two logistic functions with a high affinity response (EC_{50} : 20.8 ± 4.4 nM) and a low affinity response (EC_{50} : $1.20 \pm 1.62 \mu\text{M}$). The EC_{50} s for the high and low affinity sustained responses were 12.9 ± 11.2 nM and $6.03 \pm 0.41 \mu\text{M}$, respectively. These results suggest that ATP activates at least two populations of receptors with different affinities.

We further evaluated the effects of another endogenous P2 agonist ADP on Ca^{2+} response. Similar to the ATP, application of ADP induced biphasic Ca^{2+} response which exhibited both the high affinity and low affinity components (Fig. 4E and F). The high affinity component

Fig. 4. ATP and ADP-induced Ca^{2+} mobilization in rat PSMCs. (A and B) Mean traces of Ca^{2+} response activated by ATP at 10 - 300 nM and at 1 μM - 1 mM in PSMCs ($n = 6-8$). (C and D) The concentration-dependent ATP-induced peak and sustained Ca^{2+} response fitted with two logistic functions with a high and a low affinity EC_{50} . (E and F) Mean traces of Ca^{2+} response activated by ADP at 30 nM - 3 μM , and at 10 μM - 10 mM in PSMCs ($n = 5-8$). (G and H) The concentration-dependent ADP-induced peak and sustained Ca^{2+} response fitted with two logistic functions with a high and a low affinity EC_{50} .

Fig. 5. The contributions of P2 receptors and PLC signaling pathway to the Ca^{2+} response activated by a low concentration of ATP in PSMCs. (A) Mean traces of Ca^{2+} response activated by 100 nM ATP in the absence (control, $n = 7$) or presence of 100 μM suramin ($n = 7$), 3 μM PPADS ($n = 7$), or 100 μM MRS2179 ($n = 5$). (B) The mean values of peak Ca^{2+} response activated by ATP in experiments shown in (A). (C) Mean traces of Ca^{2+} response activated by 100 nM ATP in the absence (control, $n = 7$) or presence of 3 μM U73122 ($n = 5$) or 3 μM U73343 ($n = 6$). (D) The mean values of peak Ca^{2+} response activated by ATP in experiments shown in (C). * indicates significant difference ($P < 0.05$) from the control.

reached the maximum at 10 μM ADP with a peak Ca^{2+} response of $1.72 \pm 0.31 \mu\text{M}$ and a sustained Ca^{2+} response of $63.8 \pm 11.7 \text{ nM}$ ($n = 8$); and the low affinity component reached the maximum at 1 mM with a peak response of $3.45 \pm 0.37 \mu\text{M}$ and a sustained response of $332.5 \pm 34.4 \text{ nM}$ ($n = 7$) (Fig. 4G and H). Further increase in ADP concentration caused a significant reduction in both peak and sustained Ca^{2+} response. The EC_{50} for the peak and sustained Ca^{2+}

Fig. 6. The contributions of P2 receptors and PLC signaling pathway to the Ca^{2+} response activated by a high concentration of ATP in PSMCs. (A) Mean traces of Ca^{2+} response activated by 100 μM ATP in the absence ($n = 6$) or presence ($n = 8$) of 2 mM Ca^{2+} . (B and C) The mean values of the peak and sustained Ca^{2+} response activated by ATP in experiments shown in (A). (D) Mean traces of Ca^{2+} response activated by 100 μM ATP in the absence (control, $n = 8$) or presence of 100 μM suramin ($n = 6$), 100 μM MRS2179 ($n = 5$) or 10 μM PPADS ($n = 4$). (E and F) The mean values of the peak and sustained Ca^{2+} response activated by ATP in experiments shown in (D). (G) Mean traces of Ca^{2+} response activated by 100 μM ATP in the absence (control, $n = 8$) or presence of 3 μM U73122 ($n = 7$) or 3 μM U73343 ($n = 4$). (H and I) The mean values of the peak and sustained Ca^{2+} response activated by ATP in experiments shown in (G). * indicates significant difference ($P < 0.05$) from the control.

responses were 0.81 ± 0.11 and 0.96 ± 0.25 μM , respectively, for the high affinity component; and 117.01 ± 72.71 and 134.14 ± 6.02 μM , respectively, for the low affinity Ca^{2+} response (Fig. 4G and H). The ED_{50} s for both the high and low affinity components of the ADP-induced response were one or two orders of magnitude higher than those of ATP indicating that ATP is more potent than ADP for activating Ca^{2+} response in PSMCs.

The pharmacological property of ATP-induced Ca^{2+} response was further examined using the purinergic receptor antagonists. The transient Ca^{2+} response elicited by a low concentration of ATP (100 nM) was partially inhibited by PPADS and U73122, but not by suramin, MRS2179 or U73343 (Fig. 5A-D). The reduction was $74.1 \pm 8.3\%$ for PPADS (3 μM , $n = 7$, $P < 0.05$) and $63.9 \pm 17.0\%$ for U73122 (3 μM , $n = 5$, $P < 0.05$). ATP at a high concentration (100 μM) elicited a biphasic response. Removal of extracellular Ca^{2+} had no effect on the peak but completely abolished the sustained Ca^{2+} response (Fig. 6A-C). The peak Ca^{2+} response was unaffected by suramin (100 μM), PPADS (10 μM) or MRS2179 (100 μM), but the sustained phase was partially inhibited by suramin with a reduction of $42.8 \pm 8.0\%$ ($n = 6$, $P < 0.05$) (Fig. 6D-F). By contrast, both the peak and sustained Ca^{2+} response were almost completely abolished by U73122 with $89.2 \pm 3.0\%$ reduction ($n = 7$, $P < 0.05$) in the peak transients and $85.8 \pm 4.6\%$ reduction ($n = 7$, $P < 0.05$) in the sustained Ca^{2+} response (Fig. 6G-I). These results show that both the P2X receptors and the P2Y-PLC pathway contribute to the transient Ca^{2+} response of ATP at the low concentration, whereas the P2Y-PLC pathway is almost exclusively responsible for the Ca^{2+} response induced by ATP at the high concentration. Furthermore, the pharmacological profiles of ATP- and ADPR-activated

Fig. 7. The effects of ADPR and ATP on PSMCs proliferation determined by BrdU incorporation assay. (A) ADPR-induced concentration-dependent inhibition of proliferation of PSMCs cultured under serum-starved condition (0.5% FBS) or in the presence of 5% FBS ($n = 5$ experiments each). (B) Concentration-dependent proliferative response induced by ATP in PSMCs cultured under serum-starved condition (0.5% FBS) or in the presence of 5% FBS ($n = 5$ experiments each).

Ca^{2+} responses are different, suggesting that they are mediated via different combinations of P2 receptor subtypes.

Comparison of the effects of ADPR and ATP in PSMC proliferation

To further compare the effects of ADPR with ATP on cellular functions, we examine the effects of the two agonists on PSMC proliferation determined by BrdU incorporation assay. ADPR at 0.03 to 1 mM caused concentration-dependent inhibition of PSMC proliferation under serum-starved condition (0.5% FBS) or in the presence of 5% FBS. The anti-proliferative effect of ADPR at 1 mM was significantly stronger under serum-starved condition with a maximum inhibition of $64.8 \pm 4.6\%$ ($n = 5$), compared to $21.5 \pm 4.5\%$ ($n = 5$, $P < 0.001$) (Fig. 7A) in PSMCs cultured with 5% FBS. By contrast, ATP at concentration between 0.01 to 100 μM caused slight stimulation of PSMC proliferation in the presence of 5% FBS, and significant inhibition of proliferation ($29.4 \pm 12.6\%$, $n = 5$, $P < 0.05$) at 100 μM in serum-starved PSMCs (Fig. 7B). The clear differences in the effects of ADPR and ATP on PSMC proliferation further suggested that the two endogenous agonists may exert different physiological actions on PSMCs through different combinations of P2 receptor subtypes.

Expression of P2 receptors in rat PAs

The expression of P2 receptor subtypes in PAs was examined using conventional RT-PCR. Figure 8 shows the amplified PCR products generated from endothelium-denuded PAs. PCR products of all P2X subtypes (P2X1, P2X2, P2X3, P2X4, P2X5, P2X6 and P2X7) and P2Y receptor subtypes (P2Y1, P2Y2, P2Y4, P2Y6, P2Y12, P2Y13 and P2Y14) were obtained in PAs. All these RT-PCR amplified products had sizes corresponding to the predicted values. They were verified by sequencing and matched with positive controls generated from brain tissue under identical amplification conditions (Fig. 8A and B). Among the P2X subtypes, P2X1 and P2X5 signals were stronger in PAs than in brain and the P2X6 signal was the weakest in PAs (Fig. 8A and C). For the P2Y subtypes, P2Y1, P2Y2 and P2Y4 products were most abundant while P2Y6 and P2Y12 were the least expressed in the PA tissues (Fig. 8D). These results clearly indicate that multiple P2X and P2Y receptor subtypes are expressed in PSMCs.

Discussion

The present study examined the Ca^{2+} mobilizing effects and the pharmacological properties of extracellular ADPR in PSMCs. We found that extracellular ADPR elicits robust biphasic Ca^{2+} response through extracellular Ca^{2+} influx and intracellular Ca^{2+} release in PSMCs. The ADPR-induced Ca^{2+} influx is unrelated to voltage-gated Ca^{2+} channels or TRPM2 activation, but is mediated by at least two populations of P2 receptors, one of which is suramin-sensitive PLC-coupled P2 receptors and the other is PPADS-sensitive P2X receptors. The pharmacological profile of ADPR-induced Ca^{2+} response is different from that of the endogenous P2 agonists ATP and ADP, which exhibit two components of high affinity and low affinity Ca^{2+} responses. The high affinity Ca^{2+} response activated by ATP is sensitive to

Fig. 8. RT-PCR analysis of purinergic receptor subtypes in endothelium-denuded intralobar pulmonary arteries (PA). (A) RT-PCR products of P2X1, P2X2, P2X3, P2X4, P2X5, P2X6 and P2X7 in rat PA and brain. (B) RT-PCR products of P2Y1, P2Y2, P2Y4, P2Y6, P2Y12, P2Y13 and P2Y14 in rat PA and brain. (C and D) Comparison of P2X subtypes (P2X1, P2X2, P2X3, P2X4, P2X5, P2X6 and P2X7) and P2Y subtypes (P2Y1, P2Y2, P2Y4, P2Y6, P2Y12, P2Y13 and P2Y14) in rat PA.

PPADS and the low affinity Ca²⁺ responses is mediated predominantly by P2Y-PLC pathways. ADPR also caused significant inhibition of PASM cell proliferation, in contrast to the slight enhancement of proliferation stimulated by ATP. Moreover, transcripts of a wide spectrum of P2X and P2Y receptor subtypes are expressed in PASM cells. These results suggest that ADPR may exert its autocrine/paracrine actions in PASM cells through activation of multiple P2 receptors distinctive to those activated by ATP (and ADP) to mobilize Ca²⁺ and elicit specific physiological responses.

Previous studies suggested that ADPR may act as an autocrine/paracrine signaling molecule for mediating physiological responses [3, 14, 27]. Extracellular ADPR can be produced locally by the ectoenzyme CD38 from β -NAD⁺ effluxes through connexin-43 hemichannels [59-61] or released during cell injury [62] and nerve stimulation [21, 40, 63]. It has been shown that extracellular ADPR causes inhibition of T-cell and NK cell proliferation [20, 44], alterations in gluconeogenesis, pyruvate production and oxygen uptake in liver cells [22], relaxation of colon [28, 64] and coronary arteries [25], and increase in [Ca²⁺]_i of human monocytes [26] and insulin secreting cells [3, 4, 27]. The present study shows for the first time that extracellular ADPR can activate Ca²⁺ response in PASM cells. The ADPR-induced Ca²⁺ influx in PASM cells is not due to TRPM2 activation because the sustained Ca²⁺ response was unaffected by TRPM2 antagonists 2-APB and ACA [51-53]. Hence, translocation of ADPR into the cytoplasm to activate TRPM2 is not a major mechanism for extracellular ADPR-induced Ca²⁺ response. This is consistent with a previous study in β -cells showing ACA is ineffective

for inhibiting ADPR-induced Ca^{2+} response [24], but is in contrast to the complete inhibition of the Ca^{2+} response by 2-APB in β -cells and insulinoma RIN-5F cells [3, 27]. Since 2-APB is also an InsP_3 -receptors antagonist [65], the inhibitory effect of 2-APB in these studies was presumably related to the inhibition of InsP_3 -receptors [3, 27]. Our observation of a partial inhibition of the transient Ca^{2+} response by 2-APB, and significant inhibition of the Ca^{2+} response by the InsP_3 -receptor antagonist xestospongin C suggest that ADPR can elicit Ca^{2+} release via the InsP_3 -receptors in PSMCs.

ADPR-induced Ca^{2+} response in PSMCs is mediated by more than one purinergic receptor subtype, including P2X and P2Y receptors. P2X-receptors are non-cation channels permeable to Na^+ , K^+ and Ca^{2+} ions; and activation of these channels causes membrane depolarization and increase in $[\text{Ca}^{2+}]_i$ [54]. P2Y-receptors are G_q or G_i protein-coupled receptors [58]. Their activation generally enhances phospholipase C (PLC_β) activity to generate InsP_3 for sarcoplasmic Ca^{2+} release and subsequent store-operated Ca^{2+} entry (SOCE); and produce diacylglycerol (DAG) to activate protein kinase C (PKC) and receptor-operated cation channels (ROCC). The partial inhibition of the peak and the sustained Ca^{2+} response by PPADS indicates that P2X receptor mediated Ca^{2+} response is a significant component of the ADPR-induced Ca^{2+} response in PSMCs. Effective inhibition of the sustained phase by the P2(XY) inhibitor suramin, the PLC inhibitor U73122, and xestospongin C suggests that the sustained Ca^{2+} response is mediated to a large extent by the P2Y-dependent mechanisms, most likely through the activation of SOCE and ROCE. This is supported by the marked inhibition of the ADPR-induced sustained Ca^{2+} influx by the SOCE and ROCE antagonist SKF-96365. Previous studies showed that extracellular ADPR acts as an endogenous ligand of P2Y1 receptor for activating Ca^{2+} response in β -cells [3] and membrane hyperpolarization in colon smooth muscle [64, 66]. The ADPR-induced Ca^{2+} response in PSMCs, however, is unrelated to P2Y1 receptor activation, because the P2Y1 specific antagonist MRS2179 failed to inhibit the transient or the sustained Ca^{2+} response even at an extremely high concentration (100 μM). Hence, ADPR is capable of stimulating different purinergic receptor subtypes in a cell-specific manner.

Previous studies in human monocytes and HEK293 cells showed that prior application of ATP desensitized the ADPR-induced Ca^{2+} response, suggesting that ADPR and ATP activate Ca^{2+} response through a common mechanism [4, 26]. ATP is a natural ligand for both P2X and P2Y receptors and is released as a co-transmitter from sympathetic and sensory neurons, and from endothelial cells to cause constriction or relaxation of systemic arteries [67]. There is little information regarding ATP-induced Ca^{2+} response in PSMCs [68-70]. A previous study showed that ATP activates small Ca^{2+} influx similar to the P2X agonists 2-methylthio-ATP and α,β -methylene-ATP, and triggers Ca^{2+} oscillations which could be mimicked by the P2Y agonist UTP in PSMCs [70]. ATP also activates Ca^{2+} -activated Cl^- currents sensitive to suramin but not to PPADS [71]. Our results show that the ATP-induced Ca^{2+} response in PSMCs is complex with distinctive high (K_D of 10-20 nM) and low affinity (K_D of 1-6 μM) for both the transient and sustained Ca^{2+} response. The high affinity and low affinity responses have different Ca^{2+} kinetics and sensitivities to the purinergic receptor antagonists. For example, PPADS was effective for inhibiting the transient Ca^{2+} response elicited by ATP at a low concentration ATP, but not at a higher concentration. Suramin inhibited partially the sustained Ca^{2+} influx but failed to block the transient Ca^{2+} response elicited by a high concentration of ATP. Nevertheless, both the high and low affinity Ca^{2+} response to ATP are sensitive to U73122, indicating that P2Y-dependent pathways are the major mechanism of ATP-induced Ca^{2+} response. ADP, an endogenous agonist of P2Y receptors, elicited Ca^{2+} response similar to ATP but with a lower potency. This further suggest the involvement of P2Y receptors, other than the ADP-selective P2Y1, P2Y12 and P2Y13 [72]. The complexity of the ATP-induced Ca^{2+} response suggests multiple purinergic-receptors are activated, and most likely involves many more P2 receptor subtypes compared to the ADPR-induced Ca^{2+} response.

The expression of multiple P2X and P2Y receptors in PSMCs have been reported previously [55-57], and is substantiated by the complete analysis of P2X and P2Y mRNA

expression in the present study. Except the low levels of P2X6, P2Y6 and P2Y12 mRNA, transcripts of all P2X and P2Y subtypes were clearly expressed in PA smooth muscle. This is congruent with immunohistological detection of P2X1, P2X4 and P2X7 proteins [56, 73, 74], and pharmacological identification of P2X1, P2Y1, P2Y6, and P2Y12-dependent contraction in pulmonary arteries [57, 75]. The large repertoire of P2X and P2Y receptors in PSMCs suggest that ADPR and ATP may utilize different combinations of purinergic receptors to mediate their specific physiological functions in pulmonary vasculature. This notion is supported by our observation that ADPR elicited an inhibitory effect on PSMC proliferation similar to the reports in T-lymphocytes and natural killer cells [20, 24], as in contrast to the stimulatory effect of ATP on PSMC proliferation [76]. Since various purinergic receptors are known to modulate pulmonary vasoreactivity and PSMC proliferation [57, 75, 76], extracellular ADPR may provide a specific mechanism for paracrine/autocrine regulation of pulmonary circulation. In conclusion, we have for the first time characterized the Ca²⁺ mobilizing effect of extracellular ADPR in PSMCs; this may underlie a novel mechanism for the regulation of pulmonary vascular functions.

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Disclosure Statement

None.

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